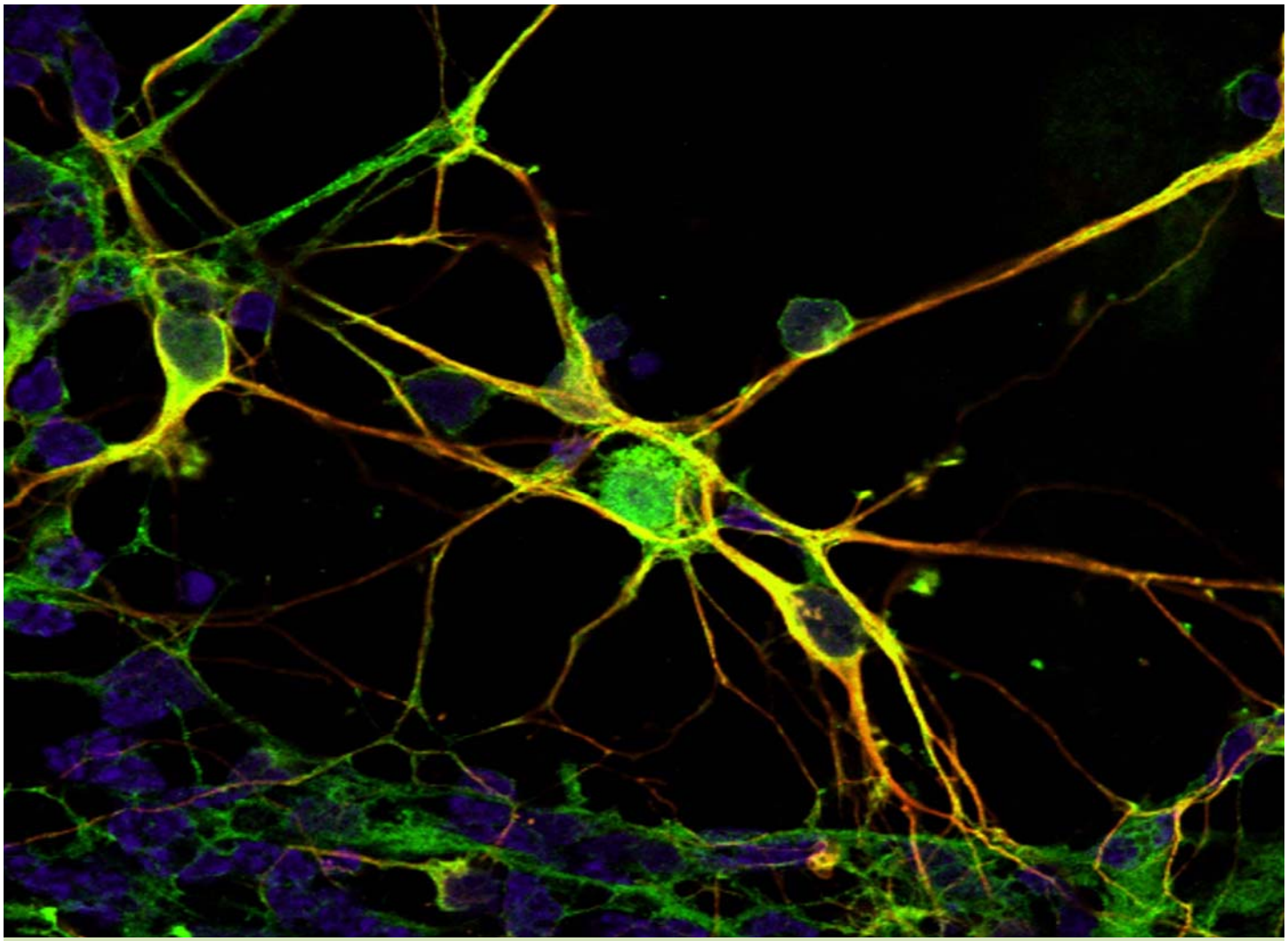

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Impact of diabetes on exocytosis/neurotransmitter release in the retina

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Universidade de Coimbra

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Cover:

Image showing the immunolabeling of vesicular GABA transporter (green) and β-III tubulin (red) in cultured retinal neural cells, obtained by confocal microscopy. Nuclei are stained with DAPI (blue). 630 x magnification.

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Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para prestação de provas de Doutoramento em Biociências, na especialidade de Biologia Celular e Molecular.

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Publications

The results presented in this dissertation were published or submitted for publication in peer-reviewed scientific journals, as follows:

1. Joana M. Gaspar*, Filipa I. Baptista*, Joana Galvão, Áurea F. Castilho, Rodrigo A. Cunha and António F. Ambrósio. Diabetes differentially affects the content of exocytotic proteins in hippocampal and retinal nerve terminals. Neuroscience. 2010 Sep 15; 169(4):1589-600. (* both authors contributed equally to this work).

2. Filipa I. Baptista*, Joana M. Gaspar*, Armando Cristovão, Paulo F. Santos, Attila Kofalvi and António F. Ambrósio. Diabetes induces early transient changes in the content of vesicular transporters and no major effects in neurotransmitter release in hippocampus and retina. Brain Res. 2011, 1383:257-269. (*both authors contributed equally to this work).

3. Filipa I. Baptista, Áurea F. Castilho, Joana M. Gaspar, Joana Liberal, Célia A. Aveleira and António F. Ambrósio. High glucose does not affect the content and distribution of exocytotic proteins and vesicular glutamate and GABA transporters in cultured retinal neural cells. Submitted for publication.

4. Filipa I. Baptista*, Célia A. Aveleira*, Áurea F. Castilho, Carolina Fernandes, Núria Simões and António F. Ambrósio. High glucose upregulates interleukin-1β: cell specific effects of high glucose and interleukin-1β in retinal neural cultures. Submitted for publication. (*both authors contributed equally to this work).

5. Filipa I. Baptista, Maria J. Pinto, Filipe Elvas, Ramiro D. Almeida and António F. Ambrósio. Diabetes induces alterations in the content and distribution of kinesin motor proteins in the rat retina. Submitted for publication.

6. Filipa I. Baptista, Maria J. Pinto, Ramiro D. Almeida and António F. Ambrósio. Diabetes affects anterograde axonal transport motor proteins in the hippocampus. Under review in PLoS One.

Note: The results presented in this dissertation, included in Chapters 2-7, are formatted according to the style of the journal where the manuscripts were submitted for publication, with minor modifications.

Table of contents

Abbreviations

AD	Alzheimer´s disease
AGEs	Advanced glycation end-products
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	One-way analysis of variance
AR	Aldose reductase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BRB	Blood-retinal barrier
BSA	Bovine serum albumine
CA	*Cornu Ammon*
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAST	CAZ-associated protein
CAZ	Cytomatrix assembled at the active zone
CNS	Central nervous system
COX-2	Cyclooxigenase 2
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCCT	Diabetes Control and Complications Trial
DG	Dentate gyrus
DIV	Days *in vitro*
DPM	Desintegrations per minute
DG	Dentate gyrus
DOC	Deoxycholate
DTT	Dithiothreitol

ECF	Enhanced chemifluorescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EPSCs	Excitatory postsynaptic currents
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GFAT	glutamine:fructose-6-phosphate amidotransferase
GlcNac	N-acetylglucosamine
GMP	Guanosine monophosphate
GLUT	Glucose transporter
GSH	Glutathione
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
ICAM-1	Intercellular adhesion molecule 1
IKK	IκB kinase
ICE	Interleukin-1ß-converting enzyme
IGF	Insulin-like growth factor
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-IR	Interleukin-1 receptor
INL	Inner nuclear layer
iNOS	Inducible nitric oxide synthase
INSR	Insulin receptor
IPL	Inner plexiform layer
IRAK	Interleukin-1receptor associated kinase
JNK	c-Jun N-terminal kinases
KIFs	Kinesin superfamily proteins

KO	Knockout
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MAP-2	Microtubule-associated protein 2
mGluR	Metabotropic glutamate receptor
mRNA	messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF	Neurofilament
NFL	Nerve fiber layer
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSF	N-ethylmaleimide sensitive factor
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS/IS	Outer segment/inner segment
PaS	Parasubiculum
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PrS	Presubiculum
PSD	Postsynaptic density
RIM	Rab3-interacting molecules
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPE	Retinal pigment epithelium
SCa	Slow component a
SCb	Slow component b
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SM	Sec1/Munc18
SNAP-25	Synaptosomal-associated protein-25
SNAREs	Soluble N-ethylmaleimide-sensitve-factor attachment protein receptor
STZ	Streptozotocin
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween 20
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
TFAF6	Tumour necrosis factor receptor-associated factor 6
TLR	Toll like receptor
TIR	Toll/Interleukin 1 receptor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor
V-ATPase	Vacuolar-type H+-ATPase
VGlut	Vesicular glutamate transporters
VGAT	Vesicular GABA transporter
VAMP-2	Vesicle-associated membrane protein 2
ZO	*Zonula Occludens*

Resumo

A retinopatia diabética é uma das principais causas de perda de visão e cegueira no mundo. Esta patologia é considerada uma doença vascular. No entanto, tem sido demonstrado que a parte neural da retina também é afetada, mesmo antes de serem detetadas lesões microvasculares. As alterações registadas em eletroretinogramas de pacientes e animais diabéticos, e a perda de sensibilidade à cor e ao contraste são sinais precoces de disfunção neuronal na retina. Contudo, as alterações moleculares e celulares causadas pela diabetes na retina não estão completamente esclarecidas. Assim, o objetivo principal deste trabalho consistiu em clarificar o impacto da diabetes, e de condições que mimetizam hiperglicemia e processos inflamatórios, na retina neural, a nível molecular, tendo-se dado uma atenção particular a alterações a nível pré-sináptico, e a nível celular, nomeadamente ao impacto nos diferentes tipos de células, neurónios, células da glia e microglia.

Inicialmente, avaliou-se o efeito da diabetes no conteúdo de proteínas sinápticas envolvidas em processos de exocitose e libertação de neurotransmissores. A diabetes foi induzida com uma única injeção de estreptozotocina, em ratos Wistar. Os níveis proteicos das *SNAREs* (sintaxina-1, VAMP-2 e SNAP-25), sinapsina-1, sinaptotagmina-1, sinaptofisina e rabfilina 3a, foram analisados em terminais nervosos purificados e em extratos totais de retina, durante fases iniciais da diabetes (duas, quatro e oito semanas). Nos terminais nervosos, os níveis de VAMP-2 diminuíram às duas semanas, aumentaram às quatro semanas e foram semelhantes ao controlo às oito semanas. Os níveis de sintaxina-1 e sinaptofisina diminuíram nos terminais nervosos após duas semanas, mas às quatro e oito semanas de diabetes recuperaram, sugerindo que a retina é capaz de reagir/recuperar do insulto inicial causado pela diabetes. O conteúdo da sinapsina-1 diminuiu significativamente em terminais nervosos de retina em todos os tempos de estudo. Por sua vez, os níveis de SNAP-25, sinaptotagmina-1e rabfilina 3a não foram alterados pela diabetes. Em extratos totais de retina, não se detetaram alterações nos níveis das proteínas sinápticas, demonstrando que as alterações estavam a ocorrer especificamente a nível pré-sináptico. Estes resultados indicam que a diabetes afeta diferencialmente o conteúdo de proteínas exocitóticas em terminais nervosos de neurónios da retina.

Tendo em conta que alterações na exocitose podem contribuir para alterações na libertação de neurotransmissores, avaliou-se também o impacto da diabetes (duas e oito semanas) na libertação basal e evocada de [^{14}C]glutamato e [^{3}H]GABA, em terminais nervosos de retina. Os níveis proteicos de VGluT-1, VGluT-2, VGAT e da subunidade α1A dos canais de cálcio P/Q também foram analisados. Após duas semanas de diabetes, o conteúdo proteico de VGluT-1, VGluT-2, VGAT e da subunidade α1A dos canais de cálcio P/Q diminuiu nos terminais nervosos de retina, enquanto que às oito semanas de diabetes não foram detetadas alterações, com a exceção do VGAT, cujos níveis de encontravam aumentados. Uma vez mais, não foram detetadas alterações nos níveis das proteínas sinápticas em extratos totais de retina. Relativamente à libertação de neurotransmissores, os níveis de libertação do glutamato foram demasiado baixos para se poder inferir alguma conclusão. A libertação evocada de GABA diminuiu após 8 semanas

de diabetes. Estes resultados indicam que a diabetes pode afetar o terminal pré-sináptico, o que poderá levar a alterações na transmissão sináptica.

Dado que a hiperglicemia é considerada o principal fator causador das complicações associadas à diabetes, investigou-se se a exposição prolongada a glucose elevada por si só, procurando mimetizar uma situação de hiperglicemia, alteraria o conteúdo proteico e a localização de proteínas sinápticas envolvidas no processo de exocitose (sintaxina-1, VAMP-2, SNAP-25, synapsina-1, sinaptotagmina-1, sinaptofisina, rabfilina, VGLUT e VGAT), em culturas primárias de células de retina. As células foram expostas durante quatro ou sete dias, a glucose elevada (30 mM) ou manitol (25 mM; + 5 mM de D-glucose), que foi usado como controlo osmótico. A exposição a glucose elevada não alterou a morfologia neuronal. Adicionalmente, o conteúdo proteico total e distribuição celular das proteínas estudadas envolvidas na exocitose mantiveram-se inalterados, sugerindo que a hiperglicemia poderá não ser o fator principal que contribui para as alterações neuronais causadas pela diabetes, ocorrendo possivelmente uma combinação da hiperglicemia com outros fatores, nomeadamente a falta de insulina e a inflamação.

Existem cada vez mais evidências que sugerem que a inflamação será um importante fator que contribui para o desenvolvimento de retinopatia diabética. No entanto, tem sido dada pouca atenção aos efeitos de mediadores pró-inflamatórios, e em particular da IL-1β, nos diferentes tipos celulares da retina, e à forma como os diferentes tipos de células interagem numa situação de inflamação. Neste trabalho, avaliou-se se a glucose elevada *per se* seria capaz de alterar a expressão de IL-1β em culturais de células de retina. Adicionalmente, procurou-se identificar o tipo de células que expressam IL-1β e IL-1R1, e investigou-se o efeito da exposição a glucose elevada e IL-1β em cada tipo celular, de modo a elucidar qual o tipo celular mais afetado nas culturas primárias de retina. A exposição das culturas a glucose elevada causou um aumento da expressão de IL-1β. A glucose elevada e a IL-1β também afetaram diferencialmente a proliferação das células da microglia e da macroglia. Enquanto a exposição a elevada glucose diminui a proliferação das células da macroglia e microglia, a IL-1β aumentou a proliferação destes dois tipos de células. A exposição a IL-1β, durante 24 horas, não causou morte celular, nem induziu alterações nos níveis de proteínas sinápticas e marcadores neuronais.

As alterações detetadas, em sinaptossomas de retina, nos níveis de proteínas sinápticas envolvidas na exocitose, sem se terem registado alterações nos extratos totais de retina, juntamente com evidências de estudos anteriores, sugerem que o transporte axonal na retina pode estar a ser afetado pela diabetes. Desta forma, analisou-se o efeito da diabetes e da exposição a glucose elevada no conteúdo proteico e distribuição das proteínas motoras KIF1A, KIF1B e dineína, que são responsáveis pelo transporte axonal nos neurónios. As proteínas motoras KIF1A e KIF5B foram particularmente afetadas pela diabetes na retina, tendo-se detetado uma diminuição dos níveis proteicos de KIF1A e da imunoreatividade da KIF1A e KIF5B nas várias camadas da retina, o que pode contribuir para alterações no transporte anterógrado, e consequentemente para a disfunção neuronal na retina. Contudo, não foram detetadas alterações no conteúdo proteico e distribuição celular das proteínas motoras após exposição a glucose elevada durante 7 dias, sugerindo que as alterações observadas numa condição de diabetes,

poderão resultar de outros fatores, como por exemplo a falta de insulina ou processos inflamatórios, e não propriamente devido à hiperglicemia *per se*. Por outro lado, efeitos sinérgicos destes fatores poderão ser mais relevantes do que os potenciais efeitos de cada um dos fatores por si só.

A diabetes também tem sido associada a alterações cognitivas e de memória, sugerindo que o hipocampo é afetado por esta patologia. As alterações induzidas pela diabetes, detetadas ao nível de proteínas exocitóticas, em sinaptossomas de hipocampo, assim como as alterações detetadas em culturas primárias de hipocampo expostas a glucose elevada, nas quais se registou uma acumulação de algumas proteínas sinápticas no corpo celular, sugerem que o transporte destas proteínas para a sinapse pode estar afetado. Assim, investigou-se se a diabetes poderia afetar os níveis proteicos e a distribuição de algumas proteínas motoras no hipocampo. Em culturas primárias de hipocampo, também se avaliou se a glucose elevada poderia afetar os níveis e distribuição de proteínas motoras e sinápticas, e de mitocôndrias, dando particular atenção a eventuais alterações nos axónios. Detetaram-se alterações nas proteínas motoras KIF1A e KIF5B no hipocampo de rato, 8 semanas após indução da diabetes. Adicionalmente, em axónios de neurónios de hipocampo em cultura, a exposição a glucose elevada causou alterações em proteínas motoras responsáveis pelo transporte anterógrado de proteínas sinápticas importantes para a exocitose, nomeadamente KIF1A e KIF5B. A exposição a glucose elevada também aumentou o número de acumulações de KIF-1A e sinaptotagmina-1, e causou uma diminuição da imunoreactividade de KIF5B, SNAP-25 e sinaptofisina, especificamente nos axónios de neurónios de hipocampo. Estas alterações sugerem que o transporte anterógrado mediado por estas cinesinas pode estar alterado em neurónios de hipocampo, o que poderá contribuir para as alterações já detetadas na neurotransmissão no hipocampo de pacientes e animais diabéticos.

Como conclusão, este estudo sugere que a diabetes induz alterações nos terminais pré-sinápticos na retina, nomeadamente ao nível do conteúdo proteico de várias proteínas sinápticas envolvidas na exocitose. A libertação de neurotransmissores na retina também poderá ser afetada pela diabetes. As proteínas motoras responsáveis pelo transporte axonal anterógrado também são afetadas pela diabetes quer na retina quer no hipocampo. No entanto, a exposição prolongada a glucose elevada não induziu alterações significativas tanto ao nível das proteínas sinápticas como das proteínas motoras em culturais de células de retina, sugerindo que outros fatores, tais como a falta de insulina e inflamação, provavelmente atuando sinergicamente com a hiperglicemia, poderão contribuir para alterações neurais na retina e hipocampo. Embora cada fator isolado possa ter alguns efeitos, os resultados apresentados neste trabalho sugerem que é a combinação de fatores, que agindo simultaneamente, leva à disfunção neuronal. No seu conjunto, os efeitos da diabetes na retina e hipocampo poderão contribuir para as alterações visuais e cognitivas detetadas em animais e pacientes com diabetes.

Abstract

Diabetic retinopathy is a leading cause of vision loss and blindness worldwide. Although it is considered a microvascular disease, increasing evidence has shown that the neural components of the retina are also affected, even before the detection of microvascular dysfunction. Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina. Nevertheless, the molecular and cellular alterations caused by diabetes in the retina are not fully understood. Thus, our main goal was to further clarify the effects of diabetes, hyperglycemic like and inflammatory like conditions, in the neural retina, at molecular and cellular level. We gave a particular attention to changes occurring at the presynaptic level, as well as the impact on different cell types, namely neurons, glial and microglial cells.

Firstly, we evaluated the effect of diabetes, on the content of synaptic proteins involved in exocytosis and on neurotransmitter release. Diabetes was induced in Wistar rats with a single intraperitoneal injection of streptozotocin. The protein levels of SNAREs (syntaxin-1, VAMP-2 and SNAP-25), synapsin-1, synaptotagmin-1, synaptophysin, and rabphilin 3a were evaluated in purified nerve terminals and in total extracts of retina, during the early stages of diabetes (two, four and eight weeks). In retinal nerve terminals, VAMP-2 levels decreased at two and increased at four weeks of diabetes, and were similar to those found in control animals at eight weeks of diabetes. Syntaxin-1 and synaptophysin levels decreased at two weeks of diabetes, but at four and eight weeks were similar to controls, suggesting that the retina is able to recover from, or react to, the initial insult caused by diabetes. Synapsin-1 content decreased in retinal nerve terminals at all time points studied. SNAP-25, synaptotagmin-1 and rabphilin 3a levels remained unchanged in retinal nerve terminals at all time points. No changes were observed in the levels of exocitotic proteins in total extracts, showing that the changes detected were specifically occurring at presynaptic level. These results indicate that diabetes differentially affects the content of exocytotic proteins in retinal nerve terminals.

Since the impairment in exocytosis may contribute to changes in neurotransmitter release, we also evaluated the effect of diabetes (two and eight weeks) on both basal and evoked release of [^{14}C]glutamate and [^{3}H]GABA in retinal nerve terminals. The protein levels of VGluT-1, VGluT-2, VGAT and α1A subunit of P/Q calcium channels were also analyzed.

At two weeks of diabetes, the content of VGluT-1, VGluT-2, VGAT and α1A subunit decreased in retinal nerve terminal. At eight weeks of diabetes no changes were detected, with the exception of VGAT levels, which were increased. Again, no changes were detected in the content of these proteins in retinal total extracts. Regarding neurotransmitter release, the levels of glutamate released were too low to draw ant conclusion. However, the evoked release of GABA decreased at eight weeks in retinal nerve terminals. These results further indicate that diabetes can affect the pre-synaptic terminal, which may cause impairments in synaptic transmission.

Given that hyperglycemia is considered the main trigger of diabetic complications, we analyzed whether long-term high glucose, to mimic a prolonged hyperglycemic condition, could

changes the content and localization of synaptic proteins involved in exocytosis (syntaxin-1, VAMP-2, SNAP-25, synapsin-1, synaptotagmin-1, synaptophysin, rabphilin 3a, VGluT-1 and VGAT) in primary retinal cultures. Neurons were exposed for four or seven days to high glucose (30 mM) or mannitol (25 mM; plus 5 mM D-glucose), which was used as osmotic control.
Neuronal morphology was not significantly affected by high glucose. Moreover, prolonged elevated glucose did not alter both the total content and cellular distribution of proteins involved in exocytosis, suggesting that hyperglycemia may not be the primordial factor contributing for neuronal changes caused by diabetes, but rather a combination of hyperglycemia with other factors, such as the lack of insulin and inflammation.

Increasing evidence indicates that inflammation is an important player in the pathogenesis of diabetic retinopathy. Increased levels of cytokines, as for example IL-1β, have been found in the retina of diabetic animals. Little attention has been given to the effect of high glucose and IL-1β on different retinal cell types and how these cell types interact under an inflammatory condition. We evaluated if high glucose *per se* was capable of changing the expression of IL-1β in retinal neural cells. Moreover, we identified which cell types produce IL-1β and express IL-1RI and studied the cell-specific effects of high glucose and IL-1β in retinal neural cultures, to elucidate which cell types are mostly affected. Cell proliferation, viability and death and expression of specific cell markers will be evaluated in primary retinal cultures. We showed that high glucose *per se* upregulates the levels of IL-1β in retinal neural cells. Additionally, high glucose and IL-1β differently affected microglial and glial cells, changing their proliferation in retinal neural cultures. High glucose, decreased glial and microglial cell proliferation, whereas under IL-1β their proliferation increased. Moreover, IL-1β did not induced changes in cell death and in the levels of synaptic proteins and neuronal markers, suggesting that activated microglia is not having a deleterious effect in neuronal cells, at least for 24h of exposure to IL-1β.

The changes detected in the levels of exocytotic synaptic proteins in retinal synaptosomes, with no changes in total extracts, together with other evidences reported by previous studies, suggest that axonal transport in the retina may be impaired in diabetes. We analyzed the effect of diabetes and high glucose on the content and distribution of KIF1A, KIF5B and dynein motor proteins that are responsible for axonal transport in neurons. KIF1A and KIF5B motor proteins were affected by diabetes in the retina, namely in the content of KIF1A and distribution of KIF1A and KIF5B in the retinal layers decreased, which may contribute to impaired anterograde axonal transport and consequently to neuronal dysfunction in the retina. No changes were detected in the content and cellular distribution of motor proteins in primary retinal cells after exposure to high glucose for 7 days, suggesting that the changes observed in motor proteins under diabetes, may be probably due to insulin deficiency rather than hyperglycemia.

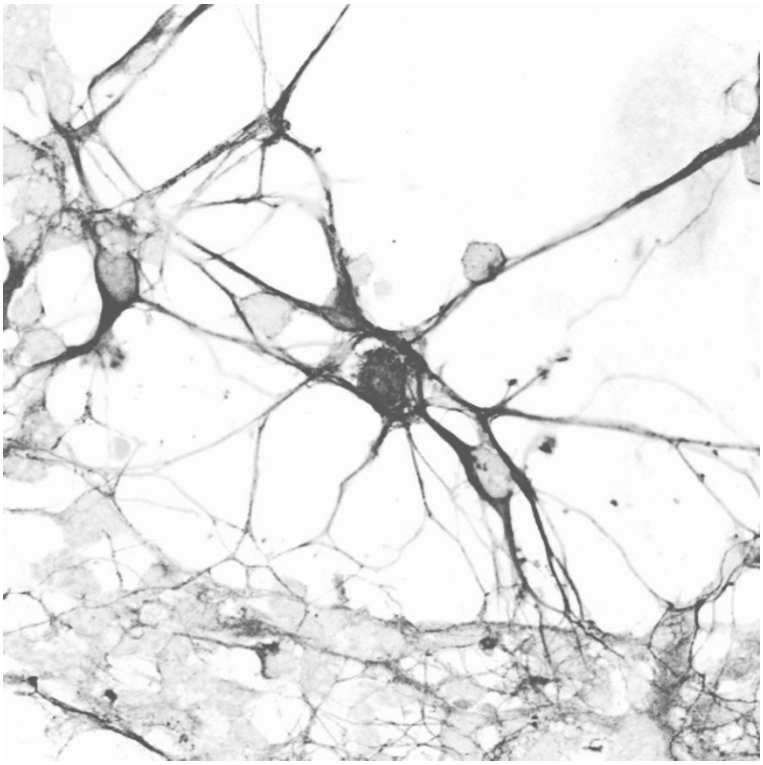
Diabetes has also been associated with cognitive and memory impairments, suggesting that hippocampus is affected by this disease. Previous detected changes in the levels of exocytotic proteins in hippocampal synaptosomes induced by diabetes and in hippocampal cell cultures exposed to elevated glucose, where an accumulation of some of these proteins was found at the cell body, suggests that the axonal transport of these proteins to the synapse may be affected. We

evaluated the effect of early diabetes and high glucose on the content and distribution of KIF1A, KIF5B and dynein motor proteins in the hippocampus and hippocampal neurons, respectively. We also evaluated whether high glucose *per se* in cultured hippocampal neurons, could affect the levels and distribution of motor proteins, synaptic proteins and mitochondria, giving particular attention to changes in axons. KIF1A and KIF5B motor proteins were altered in the hippocampus of diabetic rats at 8 weeks of diabetes. Moreover, in the axons of hippocampal neurons, high glucose leads to changes in KIF1A and KIF5B, motor proteins responsible for the anterograde axonal transport of synaptic proteins important for exocytosis. High glucose increased the number of fluorescent accumulations of KIF1A and synaptotagmin-1 and decreased KIF5B, SNAP-25 and synaptophysin immunoreactivity specifically in axons of hippocampal neurons. These changes suggest that anterograde axonal transport mediated by these kinesins may be impaired in hippocampal neurons, which may lead to deficts in the anterograde transport of synaptic proteins, thus contributing to previously detected changes in neurotransmission in the hippocampus of diabetic humans and animal models.

In conclusion, diabetes induces changes in the presynaptic compartment in the retina, namely in the content of several synaptic proteins involved in exocytosis. Neurotransmitter release can also be affected. Anterograde axonal transport motor proteins are affected by diabetes in the retina and hippocampus of diabetic animals. Nevertheless, a prolonged exposure of retinal cell cultures to elevated glucose did not induce significant changes in both synaptic and motor proteins, suggesting that other factors, such as the lack of insulin and inflammation, likely acting synergistically with hyperglycemia, might contribute to neural changes in the retina, and hippocampus. Although each factor isolated may have some effects, the results presented in this work suggest that it's the combinatory range of factors acting simultaneously that result in neuronal dysfunction. Altogether, these alterations might contribute to visual and cognitive impairments detected in diabetic animals and humans.

Chapter 1

Introduction



1.1 The retina

The retina is a thin multi-layered sensory tissue that lines the back of the eye. It comprises complex neural circuitry that converts the graded electrical activity of photoreceptors into action potentials that travel to the brain via axons in the optic nerve. The optic nerve is formed by ganglion cell axons and, additionally, incoming blood vessels that open into the retina to vascularize the inner retinal layers. The ganglion cells lie in the inner part of the retina closest to the vitreous, and the photoreceptors lie in the outermost part followed by the pigment epithelium and choroid. Light must travel through the thickness of the retina before activating photoreceptors. The absorption of photons by the visual pigment of the photoreceptors is translated into a biochemical message, and then into an electrical message that can stimulate the succeeding neurons of the retina until being transmitted to the brain from the spiking discharge pattern of ganglion cells (Purves et al., 2004).

The retina is constituted mainly by neurons, but several other cell types also exist which include glial cells (Müller cells, astrocytes, and microglial cells), endothelial cells and pericytes, and epithelial cells. Retinal cells are arranged in a highly organized manner, consisting of alternate layers of neuron cell bodies and processes.

The retinal pigment epithelium (RPE) is the outermost layer of the retina and is formed by a continuous monolayer of cuboidal epithelial cells. Next, there is the photoreceptor layer constituted by the outer and inner segments (OS/IS) of photoreceptors, and the outer nuclear layer (ONL) constituted by the nuclei of the photoreceptors. The following layer is called the outer plexiform layer (OPL) and is constituted by the photoreceptor axons and terminal endings that synapse with the dendrites of bipolar and horizontal cells. The inner nuclear layer (INL) contains the nuclei of bipolar, horizontal, amacrine and Müller cells. The bipolar cells have their axon terminals in the inner plexiform layer (IPL), where they synapse with the ganglion cell dendrites and the processes of amacrine cells. Finally, there is the ganglion cell layer (GCL), which contains the nuclei of ganglion cells and some displaced amacrine cells. The axons of ganglion cells converge to form the nerve fiber layer (NFL) that leaves the eye as the optic nerve (Figure 1.1).

1.1.1 Retinal cell types

1.1.1.1 The neuronal retina

There are five types of neurons in the retina: photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells (Figure 1.1). The processes and synaptic contacts of these cells are located in the inner and outer plexiform layers, whereas cell bodies are located in the inner nuclear, outer nuclear, and ganglion cell layers.

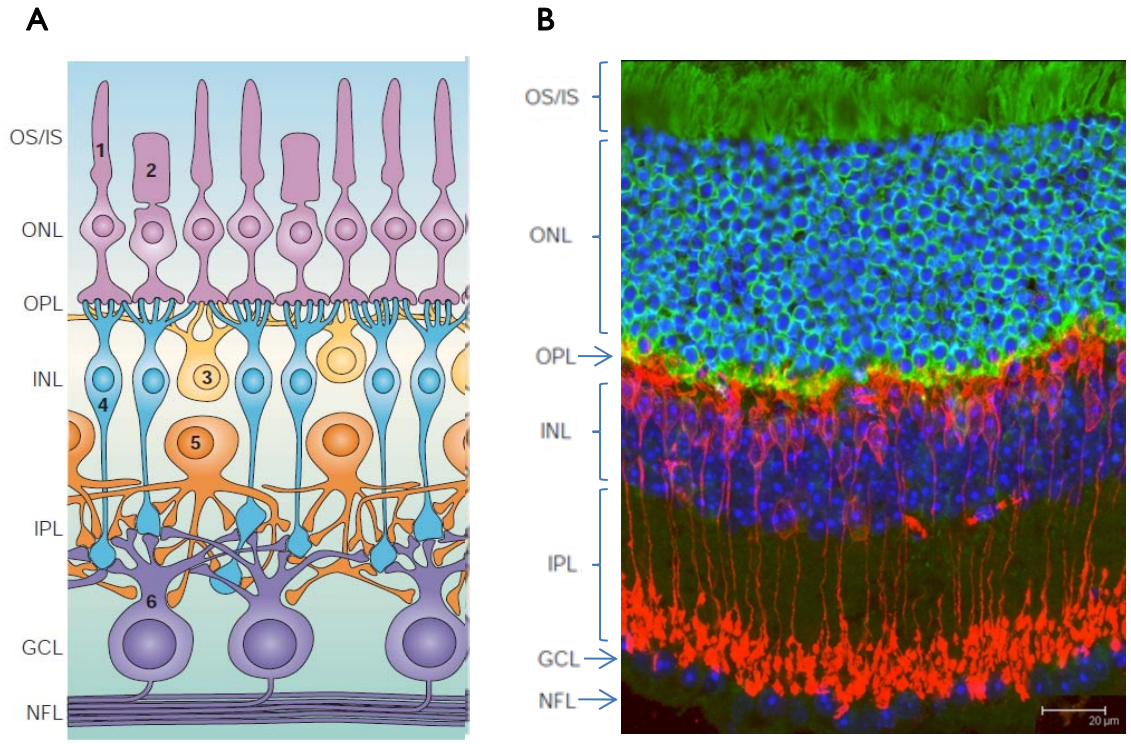


Figure 1.1. Scheme of retinal neuronal cells and layers. (A) Five types of neurons of the retina: rod (1) and cone (2) photoreceptors, horizontal cells (3), bipolar cells (4), amacrine cells (5) and ganglion cells (6); (A and B) Retinal layers: OS/IS, outer and inner segments of rods and cones; ONL, outer nuclear layer; OPL, outer plexiform layer; INL; inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, optic nerve fiber layer (adapted from Wassle, 2004 and www.retinalmicroscopy.com).

Photoreceptors

Rods and cones are the two types of photoreceptors in the retina. Both have an outer segment composed of membranous disks that contain light-sensitive photopigments, an inner segment full of mitochondria, a cell body with the nucleus and the synaptic terminals, which contact with bipolar or horizontal cells. The rods are narrower than the cones and are distributed differently across the retina, but the chemical process that supports phototransduction is similar in both. Absorption of light by the photopigment in the outer segment of the photoreceptors initiates a cascade of events that changes the membrane potential, and therefore the amount of neurotransmitter released by the photoreceptor synapses, onto the cells they contact.

Bipolar cells

Bipolar cells are the second-order neurons in the retina that receive light-elicited signals from rod and cone photoreceptors and transmit them to amacrine and ganglion cells in the inner retina. Bipolar cells receive synaptic input from either rods or cones, being designated rod bipolar or cone bipolar cells, respectively. Ten different types of cone bipolar cells and only one type of rod bipolar cell were identified in the mammalian retinas.

The visual pathway can be classified in a vertical signal pathway and a lateral inhibition pathway. There are two parallel vertical pathways, the cone and rod pathways. In the cone pathway, cones make direct synapses onto cone bipolar cells which in turn directly synapse with ganglion cells. This pathway can be further divided into the ON- and OFF- pathway, based on how cone bipolar cells react to glutamate released by cones. The differential response to glutamate is based on the presence of metabotropic or ionotropic glutamate receptors that make the synapse sign-inverting (Figure 1.2 *red* arrows) and sign-conserving (Figure 1.2 *green* arrows), respectively.

In the mammalian rod pathway, rods make synaptic contacts with only one type of bipolar cell (ON pathway), the rod depolarizing bipolar cell, whereas cones make synaptic contacts with either cone depolarizing (ON) or hyperpolarizing (OFF) bipolar cells. Rod depolarizing bipolar cells do not make output synapses directly on ganglion cells (indirect signal pathway), but on AII amacrine cells, which make electrical synapses with the cone depolarizing bipolar cells (ON) that then send signals to ON ganglion cells. AII amacrine cells also make inhibitory glycinergic synapses with hyperpolarizing bipolar cells (OFF) and OFF-ganglion cells. So, in addition to direct cone synaptic inputs, cone depolarizing bipolar cells receive rod-mediated signals from AII amacrine cell electrical synapses, and cone hyperpolarizing bipolar cells receive rod-mediated signals from AII amacrine cells chemical synapses (Pang et al., 2010; Wassle, 2004).

Ganglion cells

Ganglion cells collect visual information in their dendrites from bipolar cells and amacrine cells (retinal interneurons) and transmit it through their axons to the brain. Ganglion cells are also divided into two major subgroups: ON-center and OFF-center, thus preserving both ON and OFF pathways initiated with bipolar cells. Ganglion cells are able to form action potentials which travel over long distances, in this case through the optic nerve to brain visual centers.

Horizontal cells

Horizontal cells are the interneurons of distal vertebrate retina. They are characterized by their extensive horizontal processes and their cell bodies are located in the outer part of the INL. Their processes enable lateral interactions between photoreceptors and bipolar cells at the OPL that maintain the visual system sensitivity to luminance contrast over a wide range of light intensities.

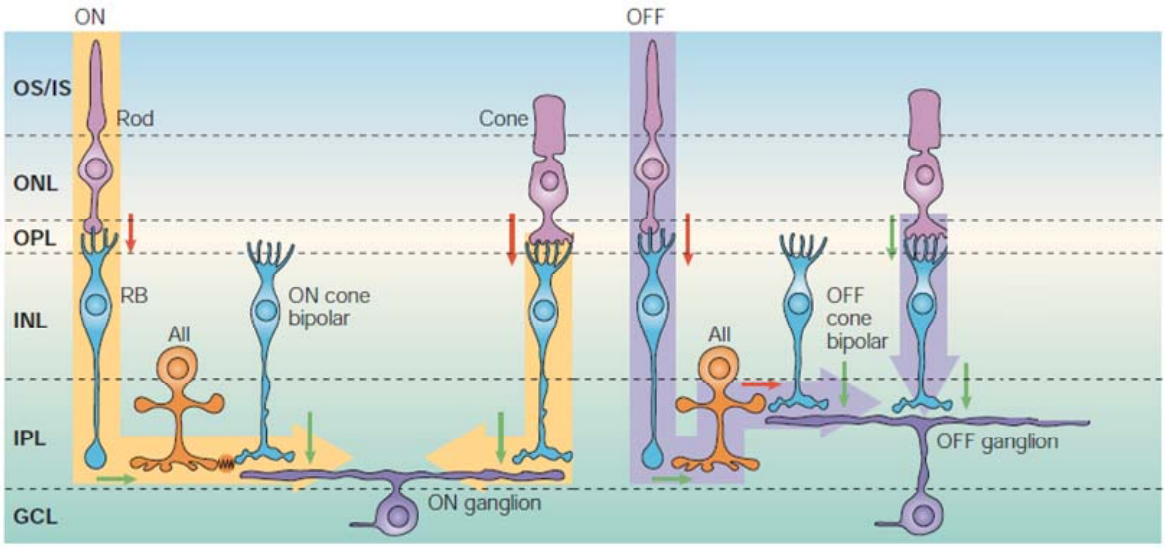


Figure 1.2. Classical visual pathways of the mammalian retina. In the ON pathway, cones and rods are hyperpolarized by light and transfer their signals onto the invaginating dendrites of cone or rod bipolar (RB) cells, causing a sign inversion at the synapse (*red* arrow). ON cone bipolar cells directly synapse with ON ganglion cells. RB cells transfer their signal through a synapse (*green* arrow) onto AII amacrine cells. AII amacrine cells make electrical synapses with the axons of ON cone bipolar cells, which in turn synapse (*green* arrow) with ON ganglion cells. In the cone OFF pathway, OFF cone bipolar cells directly synapse with OFF ganglion cells. In the rod OFF pathway, AII cells make inverting, glycinergic synapses (*red* arrow) with the axons of OFF cone bipolar cells, which in turn synapse (*green* arrow) with OFF ganglion cells. (adapted from Wassle, 2004).

Horizontal cells mediate inhibitory feedforward and lateral feedback interactions in the outer retina at photoreceptor terminals and bipolar cell dendrites. The actions of horizontal cells propagate forward in the retina so they can be identified in the physiology of retinal ganglion cells, and in visual perception.

Amacrine cells

Amacrine cells are interneurons that interact at the second synaptic level of the retina.

In the human retina, around 30 types of amacrine cells can be distinguished, depending on their dendritic size, stratification and neurotransmitter released. Most amacrine cells are inhibitory neurons in the mammalian retina, containing the common inhibitory neurotransmitters GABA or glycine. Approximately half of the amacrine cells are glycinergic. Their cell bodies are located in the INL and their dendrites are more vertically oriented and provide local interactions between the different strata of the IPL, such as between the ON- and the OFF-sublaminas. Many amacrine cells connect bipolar cells and retinal ganglion cells forming an alternative, indirect route between them. The other half of the amacrine cells are GABAergic. Their cell bodies are also found in the inner half of the INL, however, they are sometimes displaced into the ganglion cell

layer (GCL). Acetylcholine, serotonin, dopamine and substance P are other neurotransmitters released by some amacrine cells (Kolb, 1997). Different subclasses of amacrine cells are thought to make distinct contributions to visual function.

1.1.1.2 Glial cells of the retina

Müller cells

Müller cells are the principal glial cell of the retina. They form architectural support structures stretching radially across the thickness of the retina. Through the extensive arborization of their processes, Müller cells constitute an anatomic and functional link between neurons and blood vessels.

Müller cells possess a range of functions all of which are vital to the health of the retinal neurons having a symbiotic relationship with them (Newman and Reichenbach, 1996). Thus, Müller cell functions include supply endproducts of anaerobic metabolism (breakdown of glycogen) to fuel aerobic metabolism in the nerve cells; protection of neurons from exposure to excess neurotransmitters such as glutamate using uptake mechanisms to recycle this transmitter; phagocytosis of neuronal debris; control homeostasis and protection of neurons from harmful changes in their ionic environment by taking up extracellular potassium and redistributing it.

Astrocytes

Astrocytes are mostly located in the ganglion cell layer and nerve fiber layer but can also be found in the inner plexiform and nuclear layers. They have a distinctive morphology of a flattened cell body and radiating processes filled with intermediate filaments consisting of glial fibrillary acid protein (GFAP). Their morphology changes from extremely elongated near the optic nerve to a symmetrical stellate form in the far peripheral retina.

Although astrocytes have a close relationship with retinal neurons, they are mainly associated with retinal blood vessels. Astrocytes, like Müller cells, envelop retinal blood vessels regulating the blood-retinal barrier (BRB) properties. Moreover, astrocytes contain glycogen, which they degrade to provide glucose to neurons, remove neural waste products, and regulate ionic homeostasis by maintaining the extracellular levels of potassium.

Microglia

Microglial cells are widely regarded as the resident immunological cells of the central nervous system and they are responsible for the immunomodulatory function in the retina.

Microglial cells are distributed through the retina and serve as sensors of the local environment by interacting with neurons, glia and endothelium. Under normal conditions, microglial cells are characterized by a highly ramified morphology called resting microglia. Upon any insult, these cells rapidly transform into an activated state characterized by ameboid

morphology. Activated microglial cells can migrate to the site of injury, proliferate, and release a variety of factors such as cytokines, nitric oxide (NO) and reactive oxygen species (ROS).

Also, these cells transform themselves into phagocytic cells capable of presenting antigen to circulating immune cells, participating in the immune responses. Activated microglia participates in the resolution of local injury, but chronic activation may lead to exaggerated microglia responses, leading to retinal damage and neuronal apoptosis.

1.1.1.3 The vascular retina

Besides neuronal and glial cells, the retina is also constituted by epithelial and vascular cells (endothelial cells and pericytes). The retinal pigmented epithelium (RPE) is the outermost layer of the retina and is composed by a monolayer of polarized epithelial cells. RPE is critical for the normal function and support of the outer retina and it is specially important for the photoreceptors, since its layer of pigmented cells absorbs the spread light focused by the lens on the retina, phagocytes the outer segments of photoreceptors and recycles the light-sensitive pigments, essential for the visual cycle. Additionally, RPE serves as a selective permeable barrier between the retina and the choroid blood vessels due to the existence of tight junctions between epithelial cells, know as the outer BRB.

The endothelial cells of the retinal capillaries constitute the inner component of the BRB due to the tight junctions that confer highly selective barrier properties to the capillaries. Specifically, the inner BRB controls the flow of ions, water, nutrients, and potential toxins into and out of the retinal parenchyma in order to maintain the specialized environment proper to neural function. The pericytes are smooth-muscle like cells that cover the endothelial cells in the retinal capillaries. Pericytes regulate the vascular tone, support the capillary wall, secrete extracellular material and exhibit phagocytic activity.

1.1.2 Principal neurotransmitters in the retina

1.1.2.1 Glutamate

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Glutamate acts via two classes of receptors, the ligand-gated ion channel (ionotropic receptors) or G-protein coupled receptors (metabotropic receptors).

The ionotropic glutamate receptors are multimeric assemblies of four subunits and are subdivided into three groups, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl D-aspartate (NMDA) and kainate receptors, which have similar pharmacological properties and were named based on their main agonist. NMDA receptors can be formed by the combination of four of the seven subunits (GluN1, GluN2A-2D and GluN3A-3B) of the class of

NMDA receptors. Likewise, AMPA receptors are assemblies of GluA1-4 subunits. Kainate receptor subunits are named GluK1-5 (Collingridge et al., 2009).

The metabotropic glutamate receptors are subdivided into three groups, based on sequence similarity, pharmacology and intracellular signalling mechanisms. Group I mGlu receptors (mGlu1 and mGlu5) are coupled to phospholipase C (PLC) and intracellular calcium signalling, while group II (mGlu2 and mGlu3) and group III receptors (mGlu4, mGlu6, mGlu7 and mGlu8) are negatively coupled to adenylyl cyclase (Niswender and Conn, 2010).

The excitatory retinal neurons of the vertical pathway are glutamatergic. Photoreceptors, bipolar cells, and the ganglion cells exhibit "strong" glutamate immunoreactivity (Crooks and Kolb, 1992). Photoreceptors use the excitatory amino acid glutamate to transmit signals to the next order neurons in the chain. The action of glutamate upon bipolar cells occurs through two different types of postsynaptic receptors: the metabotropic glutamate receptor 6 (mGluR6), which, as mentioned previously, is a group III G-protein coupled receptor involving a second messenger cascade and cyclic guanosine monophosphate (cGMP) for activation of the receptor-associated channel (in the ON-bipolar cell), and the ionotropic AMPA/kainate receptors (OFF-bipolar and horizontal cells) (Nawy and Jahr, 1990; Slaughter and Miller, 1983a, b).

1.1.2.2 Gamma aminobutyric acid

The classical inhibitory neurotransmitter in the retina is gamma aminobutyric acid (GABA). GABA can be found in different types of amacrine cells, and in one or more classes of horizontal cells in most vertebrate retinas. In human peripheral retina, GABA is present in the inner plexiform layer and in about half of the amacrine cell bodies in the lower row of amacrine cells in the inner nuclear layer (Crooks and Kolb, 1992). The amacrine cells which contain GABA belong to medium to large-field types of amacrine cells, and most of these cells also colocalize with another neurotransmitter like serotonin or acetylcholine (starburst amacrine cells). Neuropetides are also commonly colocalized with GABA. The inhibitory action of GABA is mediated by the receptors present on the cell membrane, and results in a reduction of neuronal excitability. At least three types of GABA receptors have been characterized in the retina, $GABA_A$ and $GABA_C$ receptors which are ligand-gated ion channels (ionotropic receptors) and $GABA_B$ receptors, a type of G protein-coupled receptors (metabotropic receptors).

Besides glutamate and GABA, other classic neurotransmitters are also present in the retina. The classic inhibitory neurotransmitter glycine, accounts for most of the small-field types of amacrine cells. In addition, some types of bipolar cells contain glycine in mammalian retinas including monkey and human retinas. The neuromodulator dopamine is found in some types of amacrine cell in the mammalian retina, whereas acetylcholine (excitatory neurotransmitter) is found in mirror symmetric amacrine cells in the vertebrate retina (starburst cells).

1.2 The hippocampus

1.2.1 Basic circuits in hippocampus and principal neurons

The hippocampus is a brain region that is involved in memory forming and associative learning. It is a limbic system structure that is particularly important in forming new memories and connecting emotions and senses to memories. The hippocampus is a seahorse shaped paired structure, with one hippocampus located in the left brain hemisphere and the other in the right hemisphere. It can be distinguished as a zone where the cortex narrows into a single layer of densely packed pyramidal neurons which curl into a tight "U" shape. One edge of the "U" is embedded into a backward facing strongly flexed "V"-shaped cortex, the dentate gyrus (DG). The DG is a tightly packed layer of small granule cells wrapped around the end of the hippocampus proper (refers to the four areas of *Cornu Ammonis*; CA). Next there are several CA areas: first the CA4 (which underlies the DG), followed by CA3, then a very small zone called CA2 and finally by the CA1. The CA areas are all filled with densely packed pyramidal cells similar to those found in the neocortex. After CA1 comes an area called the *subiculum* and next comes a pair of poorly-defined areas called the *presubiculum* (PrS) and *parasubiculum* (PaS), followed by a transition to the cortex proper (mostly the entorhinal area of the cortex). (Figure 1.3)

The hippocampus is structurally organized into different cell layers composed by nucleus of different neuronal types, called principal neurons. The DG subregion consists of three cell layers (Figure 1.4): the principal or granular cell layer (g), the molecular cell layer (iml and oml) and the polymorphic cell layer also known as hilus (h) (Amaral et al., 2007). CA1 and CA3 have only a principal cell layer, called pyramidal cell layer. Besides these principal neurons, hippocampus also has interneurons and glial cells (astrocytes, oligodendrocytes and microglial cells). The principal neurons of DG are granular cells, which have a small cell body and are arranged into the granular cell layer. The dendrites of granular cells extend perpendicularly to the granular cell layer, into the overlying molecular layer, where they form synapses with several neurons from different regions (Anderson et al., 2007). The axons of granular cells (called mossy fibers) extend into the polymorphic cell layer and then enter in CA3 *stratum lucidum* (Anderson et al., 2007). In the CA subregions, the principal neurons are pyramidal cells, which divide hippocampus in CA1 and CA3 subregions, based on the organization and morphology of these neurons. The pyramidal cell layer is composed by cell bodies of pyramidal neurons, which have complex dendritic trees extending perpendicularly to the cell layer in both directions (multipolar neurons). The apical dendrites of CA3 neurons receive different types of synaptic contacts. The dendrites of pyramidal neurons of CA3 subregion are covered with spines onto which most excitatory synapses terminate.

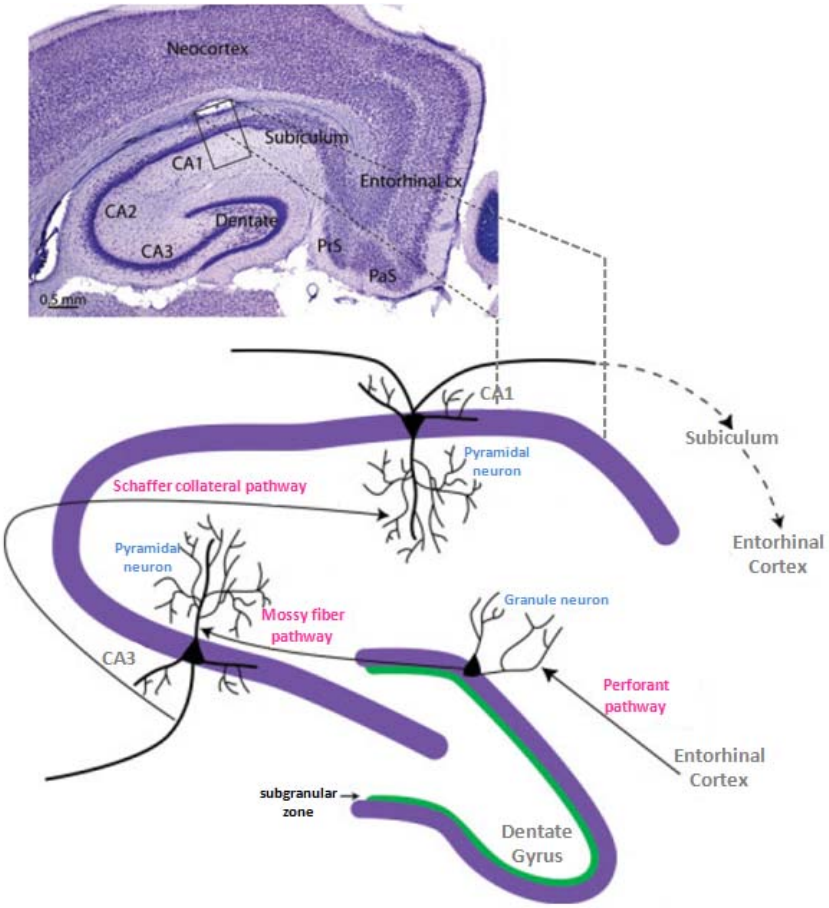


Figure 1.3. Neurons and circuits of the hippocampus. Structural organization of neurons in the hippocampal CA1, CA3 and dentate gyrus subregions. The pathway crucial to memory formation comes from the association areas of the cortex passing via the entorhinal cortex to the hippocampus and returning to the entorhinal cortex via the subiculum. In the hippocampus, three steps within this pathway compose the tri-synaptic circuit (labeled in *pink*: the perforant, mossy fiber, and Schaeffer collateral pathways) (adapted from Eid et al., 2008; McCaffery et al., 2006).

The major pathways of signal flow through the hippocampus combine to form a loop. Neurons located in the entorhinal cortex project their axons through the *subiculum* and terminate in the DG, giving rise to the perforant pathway. Electrical stimulation of this pathway with brief electrical pulses evokes a typical response in the DG, the monosynaptic excitatory postsynaptic potentials (EPSP) and a population spike. Granule cells of DG project through distinctive mossy fibers making synapses with dendrites of pyramidal cells of CA3 subregion.

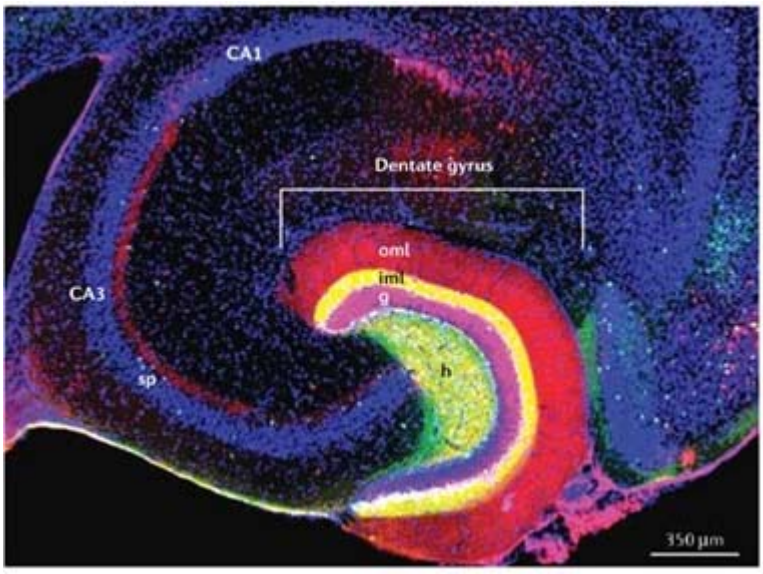


Figure 1.4. Structural organization of the DG. The axons of the mossy fiber cells in the hilar region (h) of the hippocampus, give rise to a laminated projection in the inner molecular layer (iml). The outer molecular layer (oml) is the termination zone of fibers from the entorhinal cortex. The pyramidal layer (sp) in the areas CA1 and CA3, and the granule cell layer (g) of the DG are stained with Dapi (*blue*) (Forster et al., 2006).

Stimulation of mossy fibers evokes an excitatory postsynaptic potential (EPSP) and a population spike in CA3 pyramidal neurons, followed by inhibition in the appropriate target neurons. Granular cells can also make synapses with cells in the polymorphic layer, providing connections to other levels of DG. The CA3 pyramidal cells project to CA1, a projection called Schaffer collateral pathway, and also to other cells of CA3 subregion. The perforant path from DG to CA3 to CA1 is called the trisynaptic circuit (Figure 1.4).

1.3 Diabetes *mellitus*

Diabetes *mellitus* is a group of metabolic diseases characterized by chronic hyperglycemia that result from defects in the body's ability to produce and/or use insulin. Insulin is produced by the pancreas and helps glucose enter cells from the bloodstream in order to be converted into energy. In uncontrolled diabetes, there is an inadequate supply of glucose to the cells and a build-up of glucose in the bloodstream. This leads to abnormalities in almost the entire metabolic system, including carbohydrate, lipid and protein metabolism consequently affecting the heart, kidneys, brain, eyes, and other parts of the body.

Diabetes incidence is increasing drastically and it is estimated that in 2030, 552 million people will have diabetes (Whiting et al., 2011).

1.3.1 Classification of diabetes *mellitus*

According to the etiological classification, Diabetes *mellitus* is classified in type I diabetes *mellitus*, type 2 diabetes *mellitus*, diabetes *mellitus* due to other specific mechanisms or diseases and gestational diabetes *mellitus*. Nevertheless, the vast majority of cases of diabetes fall into type 1 and type 2 diabetes which are the two major types of diabetes.

Type I diabetes *mellitus*

Type I diabetes *mellitus* is caused by insulin deficiency due to destruction of pancreatic β-cells principally via an autoimmune reaction, which is itself triggered by different factors. The destruction of pancreatic β-cells usually progresses until the stage of absolute insulin deficiency. Type I diabetes develops in association with certain hereditary factors plus inducements/environmental factors, such as virus infections. This pathology is typically regarded as developing rapidly in young people, usually under 30 years of age, affecting 5 to 10% of diabetic population (ADA, 2012), but it can also occur in any age group.

Type 2 diabetes *mellitus*

Accounting for approximately 90% of all cases of diabetes, type 2 diabetes onset is commonly regarded to be in middle age or later, however this type of diabetes *mellitus* has been shown to be increasing in children and young people (Bloomgarden, 2004).

Type 2 diabetes *mellitus* develops in association with multiple genetic factors that lead to decreased insulin secretion or insulin resistance. The risk for disease progression is augmented by lifestyle habits such as overeating and lack of exercise leading to obesity, and also by environmental factors. Decreased insulin secretion and decreased insulin sensitivity are both involved in the onset of type 2 diabetes *mellitus*, but its contribution vary among patients (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes et al., 2010).

Other types of diabetes due to specific causes

Other types of diabetes are (A) Diabetes *mellitus* with identified genetic abnormalities, where several single genetic abnormalities have been indicated as the cause of this pathology. These are subdivided into genetic abnormalities related to pancreatic β-cell function and genetic abnormalities relevant to mechanisms of insulin action. Each group can be further divided according to the type of genetic abnormality; (B) Various types of diabetes associated with other disorders and conditions which include diabetes associated with pancreatic disease, endocrine disease, liver disease, drug use, exposure to chemical substances, viral infections, and various genetic syndromes (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes et al., 2010).

Gestational diabetes *mellitus*

Glucose metabolism disorder that develops and is usually diagnosed during pregnancy. The prevalence may range from 1% to 14% of pregnancies. Most women classified with gestational

diabetes *mellitus* have normal glucose homeostasis during the first half of the pregnancy and develop a relative insulin deficiency during the last half, leading to hyperglycemia. Glucose metabolism disorders during pregnancy often return to normal after delivery, but the risk of developing diabetes in the future is increased in women who have disorders of glucose metabolism during pregnancy (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes et al., 2010).

1.3.2 Diabetic retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes *mellitus* and is the leading cause of vision loss and blindness among working-age adults in Western countries. Diabetic retinopathy is a clinically well-defined, sight-threatening, chronic microvascular complication (Hammes et al., 2011). The clinical hallmarks of diabetic retinopathy include increased vascular permeability, leading to edema, and endothelial cell proliferation.

During the first two decades of disease, nearly all patients with type 1 diabetes and more than 60% of patients with type 2 diabetes develop retinopathy. In the Wisconsin Epidemiologic Study of diabetic Retinopathy (WESDR), 3.6% of patients with type 1 diabetes and 1.6% of patients with type 2 diabetes were legally blind. In the group of patients with type 1 diabetes, 86% of blindness was attributable to diabetic retinopathy. In type 2 diabetes patients, with other common eye diseases, one-third of the cases of legal blindness were due to diabetic retinopathy (Klein et al., 1984).

The duration of diabetes is probably the strongest predictor for the development and progression of retinopathy. Among patients with type 1 diabetes in the WESDR, the prevalence of any retinopathy was 8% at 3 years, 25% at 5 years, 60% at 10 years, and 80% at 15 years.

Much of the research effort has been focused on vascular changes, but increasing evidence shows that other degenerative changes occur beyond the vascular cells of the retina. These include increased apoptosis, glial cell reactivity, microglial activation, and altered glutamate metabolism. When occurring together, these changes may be considered as neurodegenerative and could explain some of the functional deficits in vision that begin soon after the onset of diabetes (Barber, 2003). Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). Moreover, it was reported increased apoptosis in retinal neurons of diabetic animals (Barber et al., 1998) and patients (Abu-El-Asrar et al., 2004; Barber et al., 1998; Lieth et al., 2000). In addition, glutamate, the main neurotransmitter in the retina, may be involved in retinal neurodegeneration in diabetic retinopathy. In the retinas of diabetic animals, the release of [^{3}H]D-aspartate evoked by KCl is increased after 4 weeks of diabetes, comparing to the retinas of age-matched control animals (Santiago et al., 2006a). It was also reported that high glucose levels alter the subunit composition of glutamate receptors, which may contribute to impaired glutamatergic neurotransmission, and ultimately cell death (Santiago et al., 2006b).

1.3.2.1 Classification and treatment of diabetic retinopathy

Several efforts have been made to classify the retinal lesions observed during the progression of diabetic retinopathy. The classification of diabetic retinopathy into stages is based on the presence of visible ophthalmologic changes and the manifestation of retinal neovascularization (ETDRS, 1991). This classification, was constructed on the basis that diabetic retinopathy uniformly progresses to proliferative retinopathy, assuming that retinal neovascularization and proliferative retinopathy are direct consequences of diabetes. However, some evidence support the thesis that proliferative retinopathy occurs in diabetes as a result of the extensive capillary closure and ischemia and is, consequently, relatively independent of the diabetic general metabolic status (Cunha-Vaz, 1978). Taking into account the classification proposed by Cunha-Vaz, diabetic retinopathy can be divided in: preclinical retinopathy, nonproliferative retinopathy, diabetic macular edema, preproliferative retinopathy, and proliferative retinopathy (Cunha-Vaz, 2006).

In mild cases, treatment for diabetic retinopathy is not necessary. Nevertheless, regular eye exams are critical to monitor any progression. Strict control of blood glucose levels and blood pressure levels can greatly reduce or prevent diabetic retinopathy. In more advanced cases, treatment is recommended to stop the damage of diabetic retinopathy and prevent vision loss. The current treatment for diabetic retinopathy include photocoagulation, in which laser flashes are used to burn the areas of retina containing leaking blood vessels, intraocular steroid injection (that helps reducing the amount of fluid leaking into the retina), surgical removal of the vitreous to help improve vision if the retina has not been severely damaged, and injections of anti-VEGF (vascular endothelial growth factor) that helps preventing the formation of new blood vessels in proliferative diabetic retinopathy.

1.3.3 Diabetic encephalopathy

Diabetic encephalopathy is one of the complications of diabetes, characterized by impaired cognitive functions, and electrophysiological, neurochemical, and structural abnormalities. It may involve direct neuronal damage caused by dysregulation of intracellular glucose. Still, the pathogenesis of this disease is complex and its diagnosis is not very clear. Recent data suggest that insulin/C-peptide deficiency may exert a primary and key effect in diabetic encephalopathy. Administration of C-peptide partially prevents the impairment in the insulin growth factor (IGF) system in the brain and prevents neuronal apoptosis in the hippocampus of diabetic patients. Those findings provide a basis for application of C-peptide as a potentially effective therapy for diabetes and diabetic encephalopathy (Cai et al., 2011b). The cognitive decline occurs in type 1 and type 2 diabetes, but the degree of cognitive dysfunction and the way abnormalities are manifested is different in both types (Gispen and Biessels, 2000; Sima, 2010; Wrighten et al., 2009). Type 1 diabetic patients have impairments in learning and memory, problem solving, and mental and motor speed (Ryan, 1988) and type 1 diabetic young adults have mild central brain atrophy which is associated with changes in intellectual performances (Ferguson et al., 2005). In type 2 diabetic patients moderate impairments have been reported, mainly in tasks involving

verbal memory or complex information processing. However, other cognitive skills such as problem solving appear to be relatively unaffected (Ryan et al., 2006).

In diabetic animal models, it was detected impaired performance in complex tasks, such as Morris water maze or spatial-object learning task (Biessels and Gispen, 1996; Biessels et al., 1998; Popovic et al., 2001). Moreover, the development of behavioral deficits is dependent on diabetes duration (Gispen and Biessels, 2000; Sima et al., 2004), and the deficits can be prevented with intensive insulin treatment (Biessels et al., 1998) or by lowering corticosteroid levels (Stranahan et al., 2008a; Stranahan et al., 2008b). In the streptozotocin-induced diabetic rats, dendritic morphological changes also occur in pyramidal neurons located in structures related to cognitive processes, such as prefrontal cortex, occipital cortex and hippocampus (Martinez-Tellez et al., 2005). The authors have suggested that NO, glucocorticoids, stress, astrogliosis, and glutamate may participate in the dendritic morphological changes. It is nowadays accepted that several factors underlie the development of complications in the central nervous system (CNS) as a consequence of diabetes. It is possible that different factors act in additive or synergistic ways to impair neuronal homeostasis and increase neuronal vulnerability, therefore contributing to cognitive decline.

1.3.4 Contributors to diabetic retinopathy and encephalopathy

Several factors can contribute to diabetes-induced complications. Hyperglycemia and decreased insulin secretion or insulin activity are certainly central factors that contribute to the complex pathophysiological characteristics of diabetes.

1.3.4.1 Hyperglycemia

Hyperglycemia is considered the primary pathogenic factor for the development of diabetic complications. Clinical trials have shown that improved glycemic control in diabetic patients is associated with decreased development and progression of retinopathy either in type 1 (DCCT, 1995) or type 2 diabetes (UKPDS, 1998). Early tight glycemic control can prevent or delay the progression of diabetic retinopathy. Moreover, it has been suggested that hyperglycemia can also contribute to cognitive impairments and dementia in diabetic patients since a better control of blood glucose levels leads to a better cognitive performance in type 2 diabetic patients (Ryan et al., 2006). Elevated glucose levels trigger various processes and activate several biochemical pathways that ultimately induce cell dysfunction and eventually cell death. The normal fate of intracellular glucose is to be phosphorylated by hexokinase into the glycolytic pathway. However, under elevated glucose levels, hexokinase saturates and glucose is derived into other metabolic pathways (Figure 1.5).

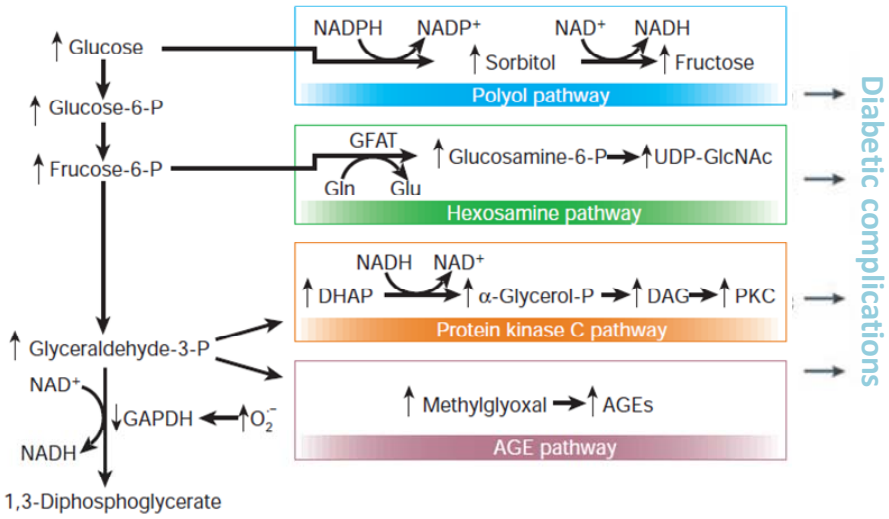


Figure 1.5. There are four potential mechanisms by which hyperglycemia induces diabetic complications: increasing polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (adapted from Brownlee, 2001).

Polyol pathway

In the polyol pathway, glucose is reduced to sorbitol by the enzyme aldose reductase (AR). Sorbitol is then oxidized to fructose by sorbitol dehydrogenase. In this second enzymatic reaction, nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH, which increases the ratio NADH/NAD^+. Under hyperglycemia, the levels of intracellular glucose increase and considerable amounts of glucose are shuttled to the polyol pathway which leads to accumulated sorbitol in the cell. As a result, toxicity increases either due to the increase in the intracellular osmolarity or to the reduction of the cellular redox state, making cells more susceptible to oxidative damage (Tomlinson and Gardiner, 2008). All these changes contribute to tissue damage and alterations within the retinal vasculature. In experimental diabetes, the accumulation of sorbitol in the retinas is associated with basement membrane thickening, pericyte loss and microaneurysms formation (Fong et al., 2004). In the diabetic retina, the increase in the NADH/NAD^+ ratio may contribute to increased ROS production and diacylglycerol (DAG), decreased NO production, as well as to abnormal growth factor expression and vascular permeability (Pugliese et al., 1991; Van den Enden et al., 1995). In the hippocampus of streptozotocin (STZ)-induced diabetic rats, it was reported a significant increase in the activity of AR (Aragno et al., 2005). The inhibition of AR protects against cell damage in the retina and brain of diabetic mice (Yeung et al., 2010).

Advanced glycation end-products

Advanced glycation end products (AGEs) are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The initial products of this reaction are called Schiff bases, which spontaneously rearrange themselves into Amadori products. With time, these intermediate products are transformed into irreversibly bound and chemically reactive adducts, the AGEs. This pathway can destabilize several biological processes and it has been suggested to have an important role in the pathogenesis of diabetic complications and in neurodegenerative disorders (Wang et al., 2009; Yamagishi et al., 2005). As a consequence of chronic hyperglycemia, endogenous AGEs are capable of inducing a wide range of abnormal responses *in vivo*, such as aberrant expression and secretion of growth factors and proinflammatory cytokines, enhancement of pro-oxidant states, alteration of extracellular matrix, and cell apoptosis. In diabetic retinopathy, AGEs may affect retinal cells by receptor-dependent and/or receptor independent pathways (Zong et al., 2011). Activation of the AGEs receptor (RAGE) triggers intracellular signals and destabilizes biological processes. AGEs may bind to cells such as endothelial cells, pericytes, glia, and microglia through their receptors, to induce intracellular signaling in a receptor-dependent manner. RAGE is ubiquitously expressed in various retinal cells and is upregulated in the retinas of diabetic patients, resulting in activation of pro-oxidant and proinflammatory signaling pathways. Intracellular AGEs cause cell damage in a receptor-independent manner by activating mitochondrial superoxide dismutase and signaling intermediates such as protein kinase C (PKC) (Stitt, 2010). This AGE-RAGE axis appears to play a central role in the sustained inflammation, neurodegeneration, and retinal microvascular dysfunction occurring in diabetic retinopathy. High levels of AGEs have been detected in brain and spinal cord of diabetic animals, being responsible for the glycosylation of cytoskeletal proteins, such as tubulin and actin (Pekiner et al., 1993; Williams et al., 1982). As a consequence of their modification by glycation, structural and functional properties of the axon may be altered, slowing the axonal transport, and contributing to axonal atrophy and degeneration (Sugimoto et al., 2008).

PKC activation

The PKC family is composed of at least eleven isoforms of serine/threonine kinases that are widely distributed in mammalian tissues. The PKC family is classified into three subgroups depending on the organization of their catalytic and regulatory domains and second messenger requirement (Jaken, 1996; Newton, 1997): classical PKCs (cPKCs: α, βI, βII, γ), novel PKCs (nPKCs: δ, ε, η, θ, μ) and atypical PKCs (aPKCs: ζ, ι/λ).

Hyperglycemia leads to *de novo* synthesis of DAG which directly activates PKC in vascular endothelial cells. Among the different eleven PKC isoforms, the beta isoform (PKCβ) is the predominant isozyme activated in vascular tissue during hyperglycemia (Aiello, 2002) and an inhibitor of PKCβ (ruboxistaurin) has been shown to be efficient in the treatment of the complications induced by hyperglycemia in the retina in both preclinical and human trials (reviewed by Danis and Sheetz, 2009; Pathak et al., 2012). Although, DAG is the main activator of

PKC, enhanced activity of PKC isoforms can also result from the interaction between AGEs and their receptors and increased flux of the polyol pathway by increasing ROS (Keogh et al., 1997; Kowluru, 2001; Portilla et al., 2000). Activation of PKCδ by hyperglycemia has been associated with the apoptosis of retinal pericytes and the increase of acellular capillaries in the retina (Geraldes et al., 2009). Moreover, recently, it was reported that tumor necrosis factor-alpha (TNF-α) signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability, which may also contribute to diabetic retinopathy complications (Aveleira et al., 2010b). Additionally, evidences indicate that PKC underlies at least some of the neuronal alterations induced by diabetes. PKCδ activation contributes to neuro-retinal apoptosis in diabetic rats by inhibiting Akt-mediated signaling pathways (Kim et al., 2008). Activation of PKC also results in an increase in synaptic transmission in diabetic rats although to a much lesser extent than in control rats, and those diabetic animals present a reduction in long-term potentiation (LTP) (Kamal et al., 2003).

Hexosamine pathway activation

It has been suggested that the hexosamine pathway also plays a role in the pathogenesis of diabetic retinopathy. Under normal physiological conditions, only a small part of the intracellular glucose follows through hexosamine pathway. Glucose enters the glycolysis pathway by conversion to glucose-6-phosphate, and then to fructose-6-phosphate. However, hyperglycemia increases the flux of fructose-6-phosphate into the hexosamine pathway. In this pathway, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT), which is then rapidly metabolized to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Madsen-Bouterse and Kowluru, 2008). UDP-GlcNAc is a substrate for the addition, of a single N-acetylglucosamine to serine or threonine residues of nuclear and cytoplasmic proteins. This form of protein glycosylation modifies proteins involved in transcription, signaling, and metabolism. It was also reported that excessive glucose flux through the hexosamine pathway may direct retinal neurons to undergo apoptosis in a bimodal fashion, for example, via perturbation of the neuroprotective effect of insulin mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins (Nakamura et al., 2001). Moreover, hyperglycemia induces the activation of the hexosamine pathway, increasing N-acetylglucosamine acylation (GlcNAcylation) of proteins, which activates the expression of genes that contribute to the pathogenesis of diabetic complications (Dias and Hart, 2007).

Oxidative stress

Oxidative stress is defined as an increase in the steady-state levels of reactive ROS (Kowluru and Chan, 2007). Increased oxidative stress in diabetes is thought to promote the development of several diabetic complications. However, the exact mechanism by which oxidative stress could contribute to the development of diabetic complications still remains to be clarified. Diabetes may cause ROS production through glucose auto-oxidation, increased flux through the polyol pathway, and increases in protein glycation (Giugliano et al., 1996). ROS may also activate

AR and PKC and increase AGEs production and DAG formation. Superoxide (O^{-2}) levels are elevated in diabetic rat retinas and in Müller and endothelial cells exposed to elevated glucose. Hydrogen peroxide (H_2O_2) levels are also increased in the retinas of diabetic rats (Ellis et al., 2000). Moreover, high glucose and oxidative stress increased apoptosis in retinal endothelial cells (Leal et al., 2009). In the brain of diabetic animals there is also an increase in oxidative and nitrosative stress, which can contribute to neuronal damage and cognitive and behavioral deficits (Fukui et al., 2001). Treatment with antioxidants prevents or ameliorates neuronal changes in the retina and brain of diabetic animals (Comin et al., 2010; Leal et al., 2005; Wang et al., 2010).

1.3.4.2 Insulin and Insulin Growth Factor-1

Insulin is a hormone synthesized by pancreatic β-cells and its main role is to stimulate glucose uptake from the blood by cells in peripheral tissues, leading to a reduction of glucose levels in the circulation. Glucose transporter type 4 (GLUT4) is responsible for insulin-regulated glucose translocation into the cells. There are some tissues that do not require insulin for efficient uptake of glucose like the brain and the liver.

IGF-I is structurally related to proinsulin and when administered to human subjects it enhances insulin sensitivity. However because of its growth promoting properties and its relationship to growth hormone, it has been proposed to be related with the development of diabetic complications (Whitmire et al., 2011).

Retinal neurons depend on insulin receptor activity for survival (Barber et al., 2001). Long-term instability in retinal insulin signalling may impair insulin-dependent anabolic activities such as protein synthesis in the retina (Chihara, 1981) and increase cell death (Reiter and Gardner, 2003). It was found that exogenous insulin given systemically reduced the number of neuronal apoptotic cells in the retina of diabetic rats, which suggested a neurotrophic action of insulin (Barber et al., 1998). Later it was demonstrated anti-apoptotic effects of insulin on neonatal rat retinal neurons via activation of the phosphatidylinositol 3-kinase/Akt (PI 3-kinase/Akt) pathway and inhibition of caspase-3 (Barber et al., 2001).

Several evidences suggest that the impairment in insulin signaling may be an important factor for the development of cognitive impairments under diabetes. In diabetic animals, the expression of insulin receptor is decreased in hippocampus (Dou et al., 2005). Insulin treatment prevents morphological changes in hippocampal CA3 subregion induced by diabetes (Magarinos et al., 2001). In type 1 diabetic rats, signaling prevents cognitive deficits and protects against hippocampal damage (Biessels et al., 1998). Moreover, insulin prevented the decrease in glutamate uptake in astrocytes and the decrease in glial fibrillary acidic protein (GFAP) levels caused by diabetes in the cerebral cortex, hippocampus, and cerebellum (Coleman et al., 2010). Insulin or proinsulin C-peptide can also prevent apoptotic neuronal cell death (Jafari Anarkooli et al., 2009; Sima and Li, 2005) and improves cognitive deficits (Biessels et al., 1998). Administration of C-peptide partially improves the condition of the IGF system in the brain and prevents neuronal apoptosis in the

hippocampus of diabetic patients (Cai et al., 2011b). Recently, it was reported that diabetes during pregnancy strongly influences the regulation of IGF-1R and INSR (insulin receptor) in the right/left developing hippocampi and the rigid control of maternal glycemia by insulin administration normalized these effects (Hami et al., 2012).

IGF-1 was one of the first growth factors to be directly linked with diabetic retinopathy (Hyer et al., 1989). Initial reports demonstrated that an acute increase in serum levels of IGF-1 preceded the onset of neovascularization in diabetic animals (Grant et al., 1993; Hyer et al., 1988) whereas reduction of serum IGF-I levels inhibits retinal neovascularization in a murine ischemic model (Smith et al., 1997). More recently, it was demonstrated that IGF-1 induces upregulation of VEGF and intercellular adhesion molecule-1 (ICAM-1) and increased BRB permeability by altering the tight junction complex (Haurigot et al., 2009). Moreover, it was proposed that hyperglycemia induces a signaling switch in vascular endothelial cells that results in enhanced sensitivity to the growth promoting effects of IGF-I (Clemmons et al., 2011).

1.4 Inflammation in diabetic retinopathy

Several evidences indicate that diabetic retinopathy has characteristics of a low-grade chronic inflammatory disease, including increased production of cytokines, such as interleukin-1 beta (IL-1β) and TNF-α, up-regulation of cyclooxygenase-2 (COX-2), increased expression of adhesion molecules and increased leukocyte adhesion and vascular permeability (Carmo et al., 2000; Kowluru et al., 2003; Leal et al., 2007; Miyamoto et al., 1999).

Increased levels of cytokines, such as IL-1β, IL-6 (interleukin-6) and TNF-α, have been found in the vitreous fluid of diabetic patients (Abu el Asrar et al., 1992; Patel et al., 2008; Yuuki et al., 2001). Moreover, the levels of IL-Iβ are increased in the retinas of STZ-induced diabetic rats (Carmo et al., 1999; Gerhardinger et al., 2005; Kowluru and Odenbach, 2004a; Krady et al., 2005), and this increase was correlated, with an increase in BRB permeability (Carmo et al., 1999, 2000). Furthermore, treatment with cyclosporine A, an anti-inflammatory drug, decreased the levels of IL-1β in the retinas of diabetic rats (Carmo et al., 1999, 2000). Retinal TNF-α levels are increased in diabetic patients, especially in patients with proliferative diabetic retinopathy (Demircan et al., 2006; Gustavsson et al., 2008; Schram et al., 2005) and elevated levels of TNF-α were also detected in the retinas of diabetic rats (Behl et al., 2008; Joussen et al., 2002; Krady et al., 2005). Recently, it was reported that TNF-α decreased the protein and mRNA content of the tight junction proteins *zonula occludens-1* (ZO-1) and claudin-5 and altered the cellular localization of these proteins in bovine retinal endothelial cells. Dexamethasone prevented TNF-α-induced cell permeability through glucocorticoid receptor transactivation and nuclear factor-kappaB (NF-κB) transrepression (Aveleira et al., 2010b).

It has been shown that Müller glial cells isolated from diabetic rats acquire a complex reactive phenotype in response to diabetes, increasing the expression of inflammation-related genes (Gerhardinger et al., 2005). Additionally, it was demonstrated that $[Ca^{2+}]_i$ changes triggered by purinergic receptor activation, both in retinal neurons and microglial cells, are potentiated in

cultured retinal cells exposed to high glucose, which may lead to increased release of inflammatory mediators in diabetic retinas (Pereira et al., 2010). Recently, it was showed that activated microglia are able to influence Müller cells directly, and initiate a program of bidirectional microglia-Müller cell signaling that can mediate adaptive responses following injury. Müller cells exposed to activated microglia, exhibit marked alterations in cell morphology and gene expression and increased proinflammatory factor production, which in turn increased microglial activation in a positive feedback loop (Wang et al., 2011). However, still little attention has been given to the effect of proinflammatory cytokines on the neural retina.

1.4.1 Interleukin-1β and diabetic retinopathy

IL-1β is a proinflammatory cytokine that upregulates several inflammatory mediators, including IL-1β itself, TNF-α, COX-2, prostaglandins, inducible nitric oxide synthase (iNOS) and chemokines (Chai et al., 1996; Chung and Benveniste, 1990; Rothwell and Luheshi, 2000; Sparacio et al., 1992).

Experimental studies showed that intravitreal administration of IL-1β increased vascular permeability which appears to be mediated by leukocyte adhesion, NF-κB activation and retinal capillary cell death (Bamforth et al., 1997; Kowluru and Odenbach, 2004a). Inhibition of caspase-1 (IL-1β converting enzyme), by minocycline inhibited the diabetes-induced increase in IL-1β and decreased the degeneration of retinal capillaries in diabetic and galactosemic mice (Vincent and Mohr, 2007).

IL-1β is primarily synthesized as an immature 31 kDa precursor called pro-IL-1β. This pro-IL-1β is processed to IL-1β (17.5 kDa) by proteolytic cleavage by the interleukin-1 converting enzyme (ICE/caspase-1). Within the retina, endothelial cells, glial and microglial cells constitute the major source of IL-1β (Gerhardinger et al., 2005; Kowluru and Odenbach, 2004b). IL-1β elicits responses in cells only through the activation of IL-1 type I (IL-1RI) receptor, although it can also bind to IL-1 type II receptors (IL-1RII) and to the soluble forms of IL-1RI and IL-1RII. In the retina, IL-1RI is mainly expressed in the inner retina, especially in retinal ganglion cells and Müller cells (Diem et al., 2003; Namekata et al., 2008). IL-1β is also synthesized in the brain by glial cells and certain neurons (Schneider et al., 1998). Moreover, IL-1 receptors were found in different regions of the CNS with the highest abundance in the hippocampus (Rothwell and Hopkins, 1995). Recently, it was demonstrated that IL-1RI is differently distributed in the hippocampus and in the subcellular compartments of primary hippocampal neurons, being enriched at synaptic sites (Gardoni et al., 2011). IL-1 receptors belong to the interleukin-1 receptor/Toll-like receptor (TLR) superfamily, characterized by the presence of an intracellular Toll/IL-1R (TIR) domain. This domain is also shared by the downstream adapter molecule MyD88 (myeloid differentiation primary-response protein 88). Upon receptor activation, it is believed that a TIR domain signaling complex is formed between the receptor and the adapter TIR domains. (Figure 1.6A) (Burns et al., 1998; Wesche et al., 1997a).

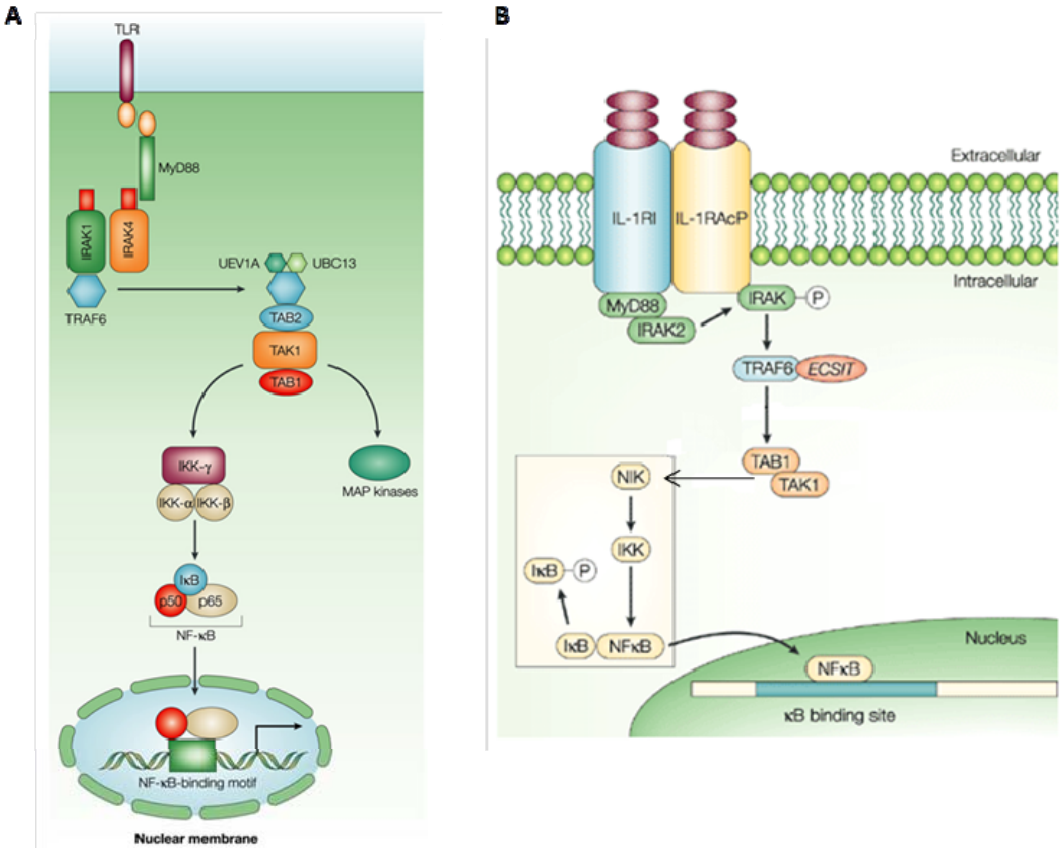


Figure 1.6. Toll/interleukin-1 (IL-1)-receptor. (A) The adaptor molecule MyD88 mediates the Toll-like receptor (TLR)-signalling pathway that activates IRAKs (IL-1receptor associated kinases) and TRAF6 (tumour necrosis factor receptor-associated factor 6), and leads to the activation of the IKK (IκB kinase) complex. The IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes (adapted from Akira and Takeda, 2004). (B) IL-1β signals through IL-1RI, which acts in concert with the IL-1 receptor accessory protein (IL-1RAcP) to initiate signal transduction (Allan and Rothwell, 2001).

The IL-1R accessory protein (IL-1RAcP) is a key element on the activation of the signal transduction cascade (Wesche et al., 1997b). The IL-1β binding to IL-1RI causes the recruitment of IL-1RAcP, and this receptor complex then recruits the intracellular TIR domain-containing adapter protein MyD88 which then recruits the serine/threonine kinases IL-1R-associated kinase (IRAK). IRAK interacts with tumor necrosis receptor-associated factor-6 (TRAF6) to activate several downstream protein kinases that ultimately lead to NF-κB and c-Jun N-terminal kinase (JNK) activation, which are involved in survival and inflammatory responses (Figure 1.6B). IL-1RI also activates other mitogen-activated protein kinases (MAPKs), such as p38 and extracellular

signal-regulated activated kinase 1 and 2 (ERK1/2), as well as PI3K (O'Neill and Greene, 1998; Subramaniam et al., 2004).

Recently, it was shown that high glucose and IL-1β downregulate interleukin-1 type I receptor (IL-1RI) in retinal endothelial cells by enhancing its degradation by a lysosome-dependent mechanism (Aveleira et al., 2010a). These findings suggest that under high glucose or inflammatory conditions, in retinal endothelial cells, IL-1RI receptors are activated by IL-1β and a fraction of these receptors is internalized and goes into the nucleus where they might control gene expression. Other receptors are directed for lysosomal degradation, which is the way cells use to regulate the processes linked to IL-1RI activation, preventing an overactivation of IL-1β-triggered signaling pathways.

1.5 Synaptic transmission

Chemical transmission is the major mean by which neurons communicate with one another in the CNS. The pre- and postsynaptic events are highly regulated and subject to use-dependent changes that are the basis for plasticity and learning in the CNS. Although direct electrical connections also occur, these account for transmission of information between nerves only in specialized cases. Neurons send out a large number of chemical signals (neurotransmitters) enabling synaptic communication. Synaptic transmission is characterized by fast and localized transfer of a signal from presynaptic neurons that activates specific receptor molecules in postsynaptic neurons producing a current flow (Sudhof, 2008). Chemical transmission requires first, the synthesis of the neurotransmitter in the presynaptic nerve terminal and its storage in secretory vesicles. Regulated release of neurotransmitter into the synaptic cleft between the pre- and postsynaptic neurons is also required. The secretion of neurotransmitters is triggered by the influx of Ca^{2+} through voltage-gated channels, giving rise to a transient and local increase in Ca^{2+} concentration within the presynaptic terminal that induces fusion of synaptic vesicles with plasma membrane, and consequently neurotransmitter release into the synaptic cleft (Augustine, 2001). The presence of specific receptors for the neurotransmitter on the postsynaptic membrane is needed. At the postsynaptic side, specific neurotransmitter receptors are concentrated at the postsynaptic density (PSD), where they receive the signal from presynaptic terminal and propagate it by changing the postsynaptic potential (Figure 1.7).

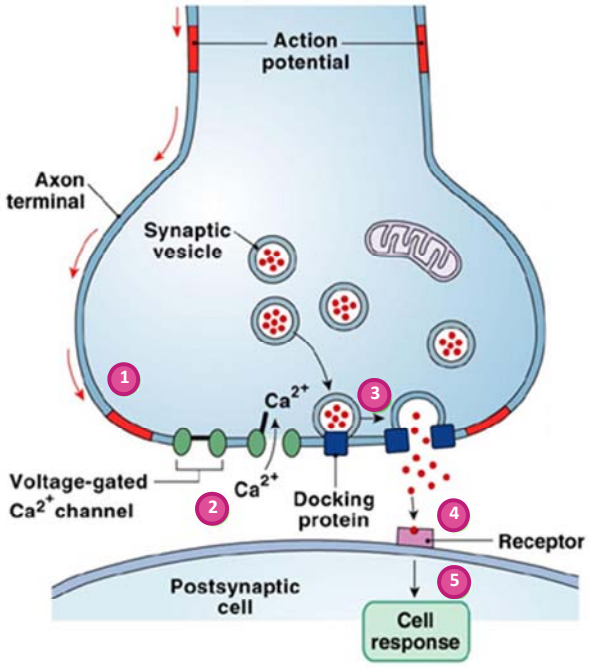


Figure 1.7. Synaptic transmission. First, an axon potential depolarizes the axon terminal (1); The depolarization opens voltage-gated Ca^{2+} channels and Ca^{2+} enters the cell (2); Calcium entry triggers exocytosis of synaptic vesicle contents (3); Neurotransmitter diffuses across the synaptic cleft and binds receptors on the postsynaptic cell (4); Neurotransmitter binding initiates a response in the postsynaptic cell (5) (adapted from www.colorado.edu/intphys/Class/IPHY3430-200/006neurons.htm).

1.5.1 Synaptic vesicles

Synaptic vesicles are small (approximately 40 nm diameter) and abundant organelles whose function is to take up and release neurotransmitters at the synaptic cleft (Figure 1.8). They are relatively simple organelles and only a limited number of proteins fit into a sphere of such diameter. Synaptic vesicles contain two classes of obligatory components: transport proteins (involved in neurotransmitter uptake) and trafficking proteins (that participate in synaptic vesicle exocytosis, endocytosis, and recycling). Transport proteins are composed of proton pumps that generate electrochemical gradients, which fuels neurotransmitter uptake and neurotransmitter transporters that regulate the actual uptake of neurotransmitters from the cytoplasm into the synaptic vesicles. Vesicular glutamate and GABA transporters, for example, transport glutamate and GABA respectively into vesicles by this process.

Trafficking proteins are more complex and include intrinsic membrane proteins, peripherally bound proteins, and proteins such as soluble N-ethylmaleimide-sensitve-factor (NSF) attachment protein receptor (SNAREs) proteins. Little is known about how these proteins are specifically deposited into synaptic vesicles.

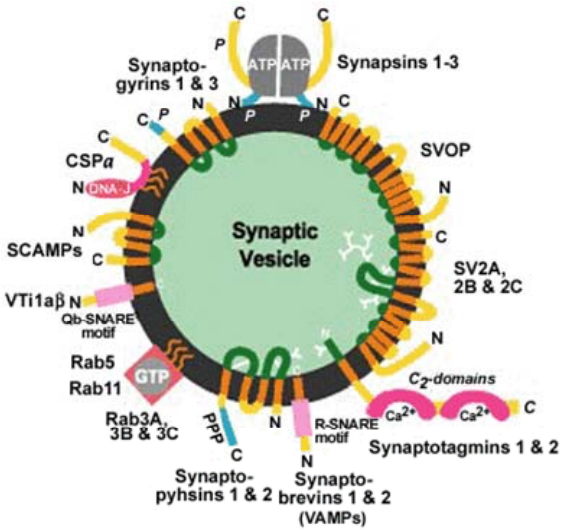


Figure 1.8. Synaptic vesicle. Synaptic vesicle showing the different synaptic vesicle trafficking proteins. Proteins are shown schematically (*green*, intravesicular sequences; *orange*, transmembrane regions; *blue*, phosphorylation domains; *yellow*, other sequences). The white connecting lines in the intravesicular space identify disulfide bonds, and the branched white lines indicate sugar residues (adapted from Sudhof, 2004).

1.5.1.1 Synaptic vesicles pools

Three major synaptic vesicle pools have been proposed: a readily releasable pool, a recycling pool and a reserve pool (Rizzoli and Betz, 2005). The recycling pool consists of the synaptic vesicles which recycle upon moderate (physiological) stimulation, typically about 10–20% of all vesicles. The readily releasable pool consists of recycling pool vesicles which find themselves docked and primed for release and these are the vesicles released immediately upon stimulation. Finally, the reserve pool hosts vesicles which are reluctant to release, and which are therefore only recruited upon high-frequency stimulation, after depletion of the recycling pool (Figure 1.9A and B). Recently, it was proposed a further refinement of the pool model. In this model, a recycling pool of vesicles does exist, and the recycling pool vesicles docked at active zones form the readily releasable pool. However, the differentiation between the recycling and reserve vesicles is not permanent; the recycling vesicles may re-release several times, maintaining the recycling pool status, but they will eventually "mature" into reserve vesicles, over a timescale of minutes to hours (Figure 1.9C) (Denker and Rizzoli, 2010).

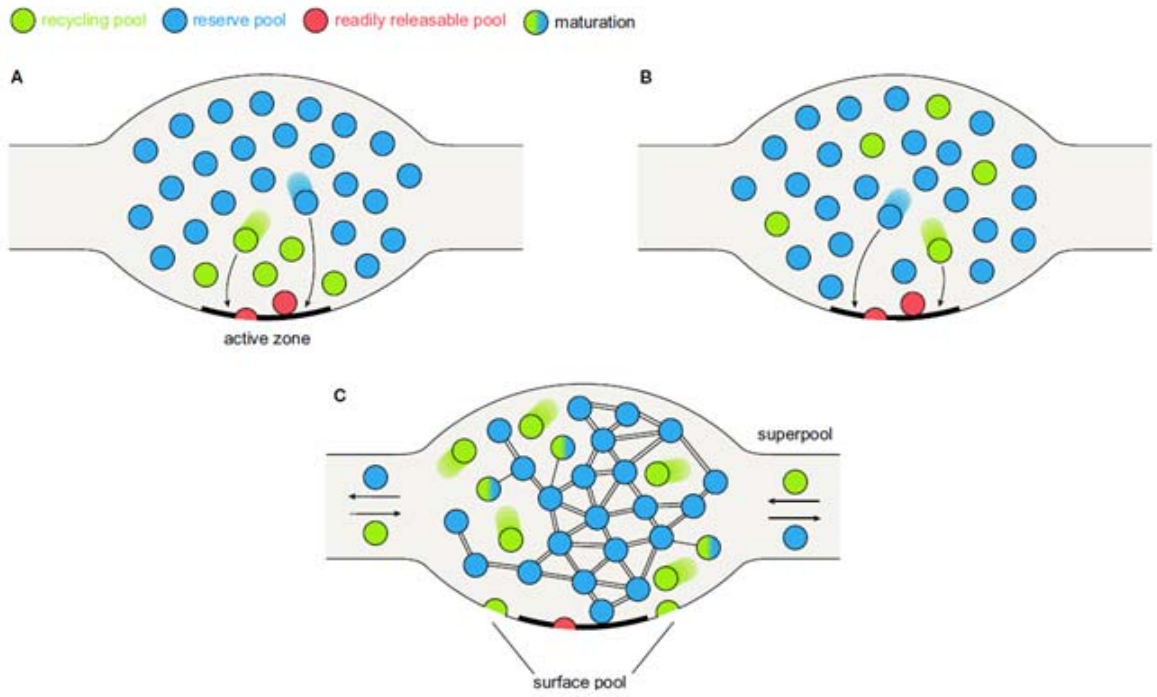


Figure 1.9. Synaptic vesicle pool models. (A) After depletion of the readily releasable pool (*red*), the recycling pool vesicles, located directly behind the readily releasable pool, come into play (*green*). Under moderate stimulation, they are recruited to the active zone (left arrow) and released. Very high stimulation causes the depletion of the recycling pool and recruits the reserve pool vesicles (*blue*) from areas even further away from the active zone (right arrow). (B) Upon arrival of an action potential, readily releasable pool vesicles (which are in this model only the recycling pool vesicles finding themselves docked and primed at the active zone) are released first, followed by release of recycling pool vesicles (right arrow). Likewise, continuous stimulation at high-frequency results in recycling pool depletion and recruitment of reserve pool vesicles (left arrow) (C) As above, recycling and reserve pool vesicles are spatially intermixed, but recycling pool vesicles are highly mobile and the movement of reserve pool vesicles is restricted by binding to some scaffolding molecule. With time, recycling pool vesicles can "mature" into reserve pool vesicles, by binding the scaffolding molecules and integrating into the vesicle cluster, as indicated by the *green-blue* intermediate forms. Recycling pool vesicles reach the active zone, due to their permanent mobility; stimulation does not "move" them toward the active zone, it just allows them to fuse. The frequent exchange of both recycling and reserve vesicles between synapses forms a super-pool (adapted from Denker and Rizzoli, 2010).

1.5.2 Presynaptic active zone

The active zone is located at the presynaptic plasma membrane precisely opposite the synaptic cleft and is composed of electron-dense aggregates of proteins that mediate and regulate neurotransmitter release (Dresbach et al., 2001; Zhai and Bellen, 2004). Ultrastructural studies have revealed that the presynaptic active zone is close and precisely aligned with the PSD and is associated with an electron-dense cytoskeletal matrix, referred as cytomatrix assembled at the active zone (CAZ) (Figure 1.10A). CAZ-specific proteins include Munc13s, which are essential for neurotransmitter release, the Rab3-interacting molecules (RIM1 and RIM2), which are scaffolding

proteins regulating presynaptic events and Bassoon and Piccolo, also scaffold proteins for vesicle interactions that may link exocytosis and endocytosis of vesicles at the active zone (Figure 1.10B). Within the CAZ, a network of microfilaments and proteins are also present including synaptic vesicle fusion proteins, cytoskeletal proteins, such as actin, tubulin and spectrin, and voltage-gated calcium channels. All of these CAZ-associated proteins are implicated in the functional and spatial organization of the individual steps of the synaptic vesicle cycle. The CAZ is thought to mediate crucial events of synapse formation and function, including spatial restriction of neurotransmitter release to active zones and local recruitment of proteins and organelles.

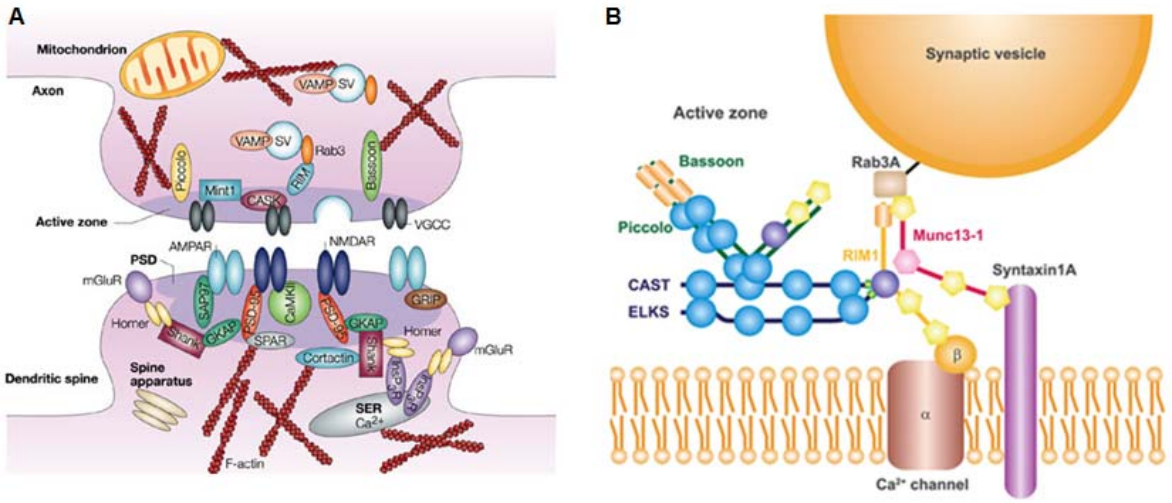


Figure 1.10. Active zone. (A) Vesicle fusion occurs at the active zone, which is characterized by specific proteins such as RIM, Bassoon and Piccolo. The active zone is directly opposed to the postsynaptic density (PSD) (adapted from Li and Sheng, 2003). (B) Protein–protein interactions at the active zone that mediate attachment of the vesicle to the target membrane (adapted from Hida and Ohtsuka, 2010).

1.5.3 Neurotransmitter release by exocytosis / Synaptic vesicle exocytosis

Neurotransmitter release is mediated by exocytosis of synaptic vesicles at the presynaptic active zone of nerve terminals. When an action potential depolarizes the presynaptic plasma membrane, Ca^{2+}-channels open, and Ca^{2+} flows into the nerve terminal to trigger the exocytosis of synaptic vesicles, thereby releasing their neurotransmitters into the synaptic cleft.

Synaptic vesicles constitute the central organelle for neurotransmitter release. Each presynaptic nerve terminal contains hundreds of synaptic vesicles that are filled with neurotransmitters. Synaptic vesicles undergo a trafficking cycle in the nerve terminal that can be divided into sequential steps (Figure 1.11). First, neurotransmitters are actively transported into synaptic vesicles (step 1), and synaptic vesicles cluster in front of the active zone (step 2). Then synaptic vesicles dock at the active zone (step 3), where the vesicles are primed (step 4) to convert them into a state of competence for Ca^{2+}-triggered fusion-pore opening (step 5). Ultimately the fusion with the plasma membrane leads to the consequent release of the

neurotransmitter into the synaptic cleft. After fusion-pore opening, synaptic vesicles endocytose and recycle (step 6). Exocytosis is a highly regulated process and so, each step is regulated by several important proteins, such as synapsins in vesicle trafficking to nerve terminal, Rab3a in docking, SNARE proteins in priming and fusion, and synaptotagminin-1 in fusion. Moreover, recent studies have given new insight into how Munc18-1 collaborates with SNARE proteins in fusion, how the vesicular Ca^{2+} sensor synaptotagmin 1 triggers fast release, and how the vesicular Rab3a protein regulates release by binding to the active zone proteins RIM1α and RIM2α (Sudhof and Rizo, 2011). All these evidences contribute to the understanding of neurotransmitter release.

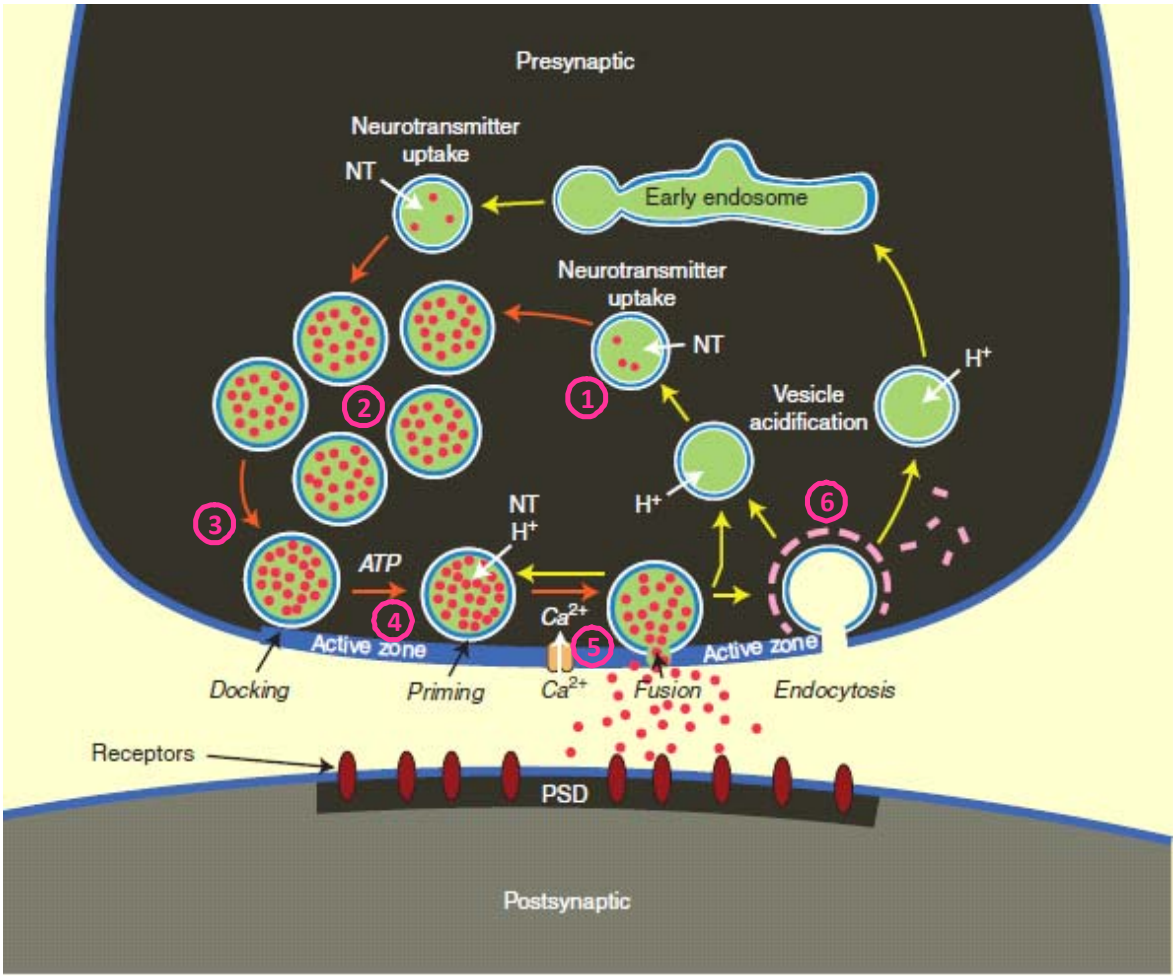


Figure 1.11. Synaptic vesicle cycle. Exocytosis steps are indicated with red arrows. Synaptic vesicles are filled with neurotransmitters by active transport (step 1) and form the vesicle cluster that may represent the reserve pool (step 2). Vesicles dock at the active zone (step 3), where they undergo a priming (step 4) that makes them competent for Ca^{2+} triggered fusion (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via several routes (step 6) indicated by the yellow arrows (Sudhof and Rizo, 2011).

1.5.3.1 Neurotransmitter uptake into synaptic vesicles

Synaptic vesicles accumulate and store neurotransmitters by active transport, driven by a vacuolar proton pump (V-ATPase) whose activity generates an electrochemical gradient across the vesicle membrane (Maycox et al., 1988). Most vesicles contain only a single V-ATPase molecule and it is the single largest vesicle component that extends from the vesicle more than half of the vesicle radius. The V-ATPase is composed of a larger peripheral complex called V1, which includes ATPase activity, and an integral membrane complex called V0, which mediates proton translocation (Perin et al., 1991). Glutamate is taken up into synaptic vesicles by three differentially expressed vesicular glutamate transporters (VGlut1-VGlut3) (Fremeau et al., 2002). In the case of VGluts the main driving force for vesicular uptake is the membrane potential, whereas for other transporters, e.g., vesicular GABA transporter (VGAT), the membrane potential and the proton gradient both contribute to uptake (Figure 1.12) (Fykse and Fonnum, 1996).

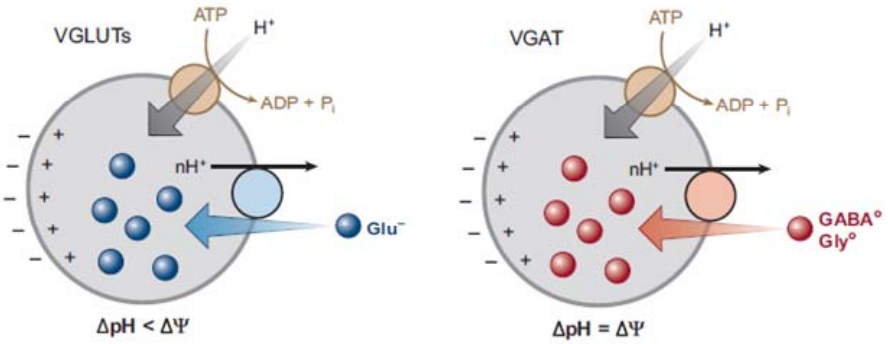


Figure 1.12. Vesicular transporters. Vesicular neurotransmitter transporters depend differentially on the two components of the electrochemical gradient of H^+ ($\Delta\mu H^+$). A V-ATPase generates a $\Delta\mu H^+$ across the vesicle membranes. The vesicular transporters use this gradient to drive the transport of neurotransmitters into synaptic vesicles by coupling the translocation of neurotransmitter to H^+ running down $\Delta\mu H^+$. GABA and glycine are transported as neutral zwitterions by VGAT, which depends equally on both the chemical and the electrical component of $\Delta\mu H^+$. VGLUTs transport the negatively charged glutamate and thus rely more on membrane potential ($\Delta\Psi$) than chemical gradient for H^+ (ΔpH) (adapted from Chaudhry et al., 2008).

1.5.3.2 Synaptic vesicle mobilization - Synapsin role

Synaptic vesicles within nerve terminals are organized in clusters and this organization is thought to promote coordinated vesicle translocation, preparation for release, and recycling. As previously mentioned, vesicles in the releasable pool are docked to the synaptic membrane and represent a subpopulation of the recycling pool, which is comprised out of all the vesicles that are involved in the exo/endocytic pathway at mild stimulation paradigms, whereas the reserve pool includes the vesicles that are not typically involved in release and recycling, but become mobilized upon intense stimulation. Synapsin has been identified as a major molecular player regulating the

transition between these functional pools. Synapsins coat the vesicle surface and regulate the size and dynamics of a reserve pool of vesicles that can be mobilized during elevated synaptic activity (Fdez and Hilfiker, 2006). It has been proposed that synapsin in its dephoshorylated state attaches synaptic vesicles to the cytoskeleton. Studies demonstrated that Ca^{2+} entry stimulates synapsin phosphorylation at Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII)- and protein kinase A (PKA)-dependent sites, whereas dephosphorylation occurs at MAPK/calcineurin-dependent sites (Jovanovic et al., 2001; Yamagata et al., 2002). Synapsin interaction with vesicles and cytoskeleton was found to be promoted by activity-dependent synapsin phosphorylation via the tyrosine kinase Src (Messa et al., 2010), and this process may be opposing to serine phosphorylation by CaMK, PKA, and MAPK. Thus, synapsin interaction with vesicles, synapsin dispersion, and vesicle mobilization are controlled by a complex, dynamic, and activity-dependent synapsin phosphorylation/dephosphorylation cycle which is highly regulated at multiple sites. Additionally, a molecular interaction with Rab3a, a small cytosolic protein that is thought to target vesicles to active zones via interaction with vesicles and active zone machinery, suggests that vesicle targeting to active zones may be mediated via synapsin/Rab3a interaction (Bykhovskaia, 2011).

1.5.3.3 Vesicle docking

Synaptic vesicles are mobilized and docked at the active zone of the plasma membrane. These vesicles are preferentially located around sites of Ca^{2+} entry and are characterized by their close apposition to the plasma membrane. Docking ensures that synaptic vesicles are anchored to the active zone of the synapse.

Proteins that mediate the docking process need to bind to several proteins, forming a complex that links plasma and vesicle membranes, and should guarantee the specificity of vesicle targeting (Becherer and Rettig, 2006). The better candidates for proteins involved in docking are proteins that form the cytomatrix in the active zone. The presynaptic active zone of synapses consists of a dense accumulation of cytomatrix proteins, some of which, like Munc13, ELKS/CAZ-associated protein (CAST), and RIMs, are localized highly specifically at active zones (Schoch and Gundelfinger, 2006). RIM interacts simultaneously with Rab3a, Munc-13, synaptossomal-associated protein 25 (SNAP-25) and Ca^{2+} channels. The interaction of Rab3a with RIM may have a role in the recruitment of vesicles to the active zone, thus mediating the docking process (Sudhof, 2008). Rab3a knock out mice have a decreased pool of docked vesicles and consequently spontaneous neurotransmitter release is diminished (Coleman et al., 2007). Recently, it was found that RIM proteins co-ordinately regulate key functions for fast transmitter release, enabling a high presynaptic Ca^{2+} channel density and vesicle docking at the active zone (Han et al., 2011).

1.5.3.4 Vesicle priming

Once in the active zone of presynaptic terminals, synaptic vesicles undergo a priming process in which they become fusion competent. Priming activates synaptic vesicles for exocytosis, thereby creating the readily releasable pool of vesicles. It has been proposed that the formation of SNARE complex occurs partially during this step and that the SNARE complex

completes the assembly only when Ca^{2+}-triggered fusion occurs (Sorensen et al., 2006). During priming, there are molecular rearrangements and lipid modifications, as well as the interactions between synaptic vesicle proteins. The synaptic vesicle membrane fusion machinery is thought to be composed of SNARE and Sec1/Munc18 (SM)-like proteins and constitutes a central element of priming. It has been demonstrated that Munc13s interact with SNARE proteins and also binds to RIM proteins (Guan et al., 2008; Huang et al., 2011). Recently it was described that RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13 (Deng et al., 2011).

1.5.3.5 SNARE complex and interacting proteins

Synaptic vesicle fusion involves the assembly of SNARE complex, which is composed by the vesicle-associated membrane protein 2 (VAMP-2) or synaptobrevin, synaptosomal-associated protein 25 (SNAP-25) and syntaxin-1. The SNARE complex brings the synaptic vesicle membrane close to the plasma membrane promoting the vesicle fusion (Lin and Scheller, 2000). SNARE proteins are present on plasma and vesicle membranes and associate into tight core complexes during fusion. The core complex is formed when four SNARE motifs assemble into a parallel four-helical bundle, with the transmembrane regions of the SNAREs emerging on the C-terminus. Four different classes of SNARE motifs exist (R, Qa, Qb, and Qc SNARE motifs), and stable SNARE complexes only form when the four helical bundle contains one SNARE motif from each class (Jahn et al., 2003). Synaptic exocytosis is mediated by three SNARE proteins, VAMP-2 (vesicle-associated membrane protein), which is on synaptic vesicles, and syntaxin 1 and SNAP-25, present on the presynaptic plasma membrane. The synaptic core complex is formed by the R-SNARE motif from VAMP-2, the Qa-SNARE motif from syntaxin 1, and the Qb- and Qc-SNARE motifs from SNAP-25 (which contains two SNARE motifs). A member of the SM proteins appears to be essential for every intracellular fusion reaction and to act in conjunction with SNAREs (Figure 1.13) (Rizo and Sudhof, 2002). It has been suggested that the assembly of the SNARE complex depends on the dissociation of syntaxin-1 and Munc-18 (Burkhardt et al., 2008). The phosphorylation of Munc-18 by PKC may provide a mechanism for its dissociation from syntaxin-1 (Barclay et al., 2003). The conformational change of syntaxin-1 to an opening position is mediated by Munc-13 acting in concert with Munc-18 to form the syntaxin-1/SNAP-25 heterodimer and promote the priming of vesicles (Richmond et al., 2001).

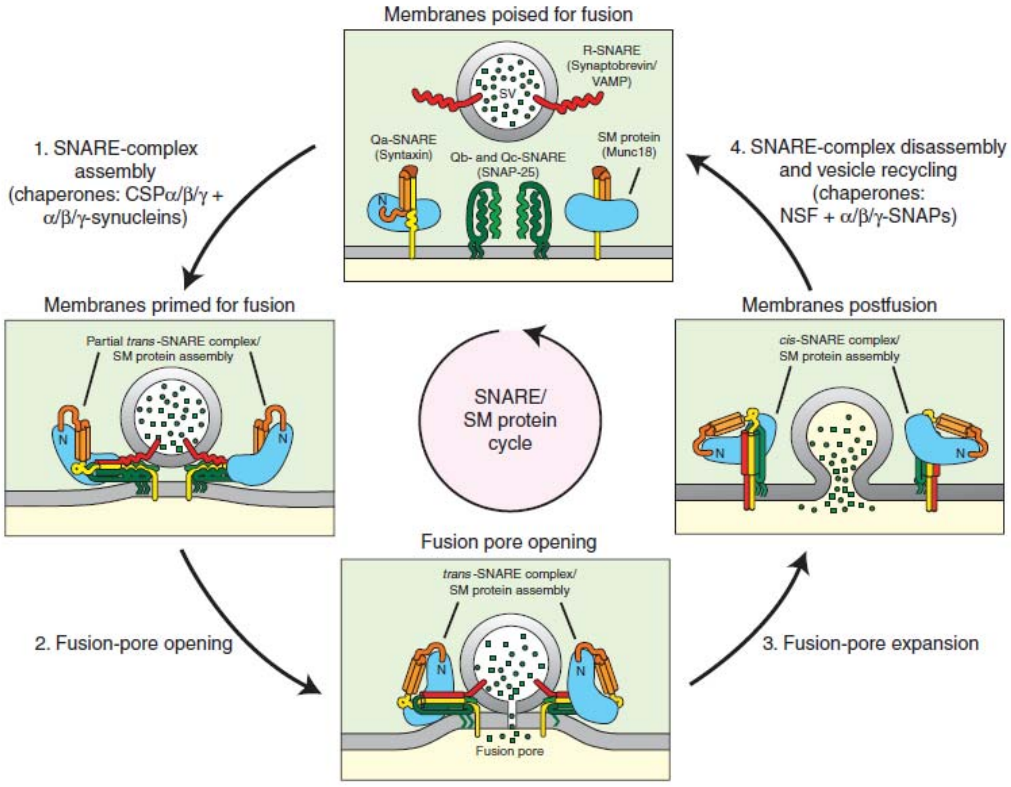


Figure 1.13. The SNARE/SM protein cycle. SNARE and SM proteins prior to fusion when they are localized to the membranes either as natively unfolded proteins or as proteins folded in complexes distinct from canonical SNARE complexes. During priming (1) SNARE proteins partially zipper up into trans-complexes, and the SM protein associates with the trans-complexes by binding to the syntaxin amino terminus. Full SNARE-complex assembly then pulls the membranes apart, opening the fusion pore (2), which expands such that the vesicle membrane collapses into the target membrane, and the trans-SNARE complexes are converted into cis-SNARE complexes (3). Afterward, cis-SNARE complexes are dissociated by the ATPase NSF and vesicles recycle to start another round of the cycle (4) (adapted from Sudhof and Rizo, 2011).

Besides, the interaction of proteins (Figure 1.14) such as Rab3a (Fischer von Mollard et al., 1994), RIM and rabphilin, other proteins are thought to also regulate SNARE function at the synapse (synaptophysins and complexins). Synaptophysins are abundant synaptic vesicle proteins that bind directly to VAMP-2 (Washbourne et al., 1995). VAMP-2 cannot simultaneously bind to synaptophysins and participate in the SNARE complex, which suggests that binding of VAMP-2 to synaptophysin restricts the availability of VAMP-2 for fusion. Complexins are small neuronal proteins that bind to assembled synaptic core complexes (McMahon et al., 1995). Studies suggest a model where the primary function of complexins is to freeze a primed state that involves a fully assembled SNARE complex that is crucial for fast Ca^{2+}-triggered neurotransmitter release (Rizo and Sudhof, 2002).

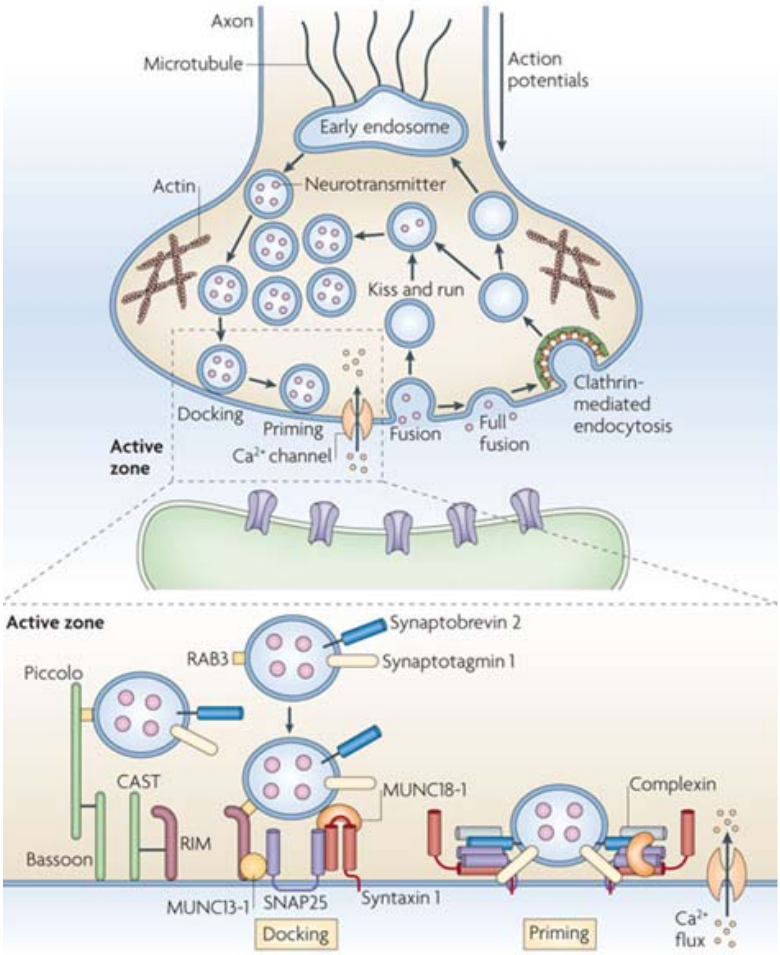


Figure 1.14. SNARE complex and interacting proteins. Protein–protein interactions at the active zone mediate attachment of the vesicle to the target membrane. Rab3 in the vesicle binds to RIM, which also interacts with Munc13-1. In addition, vesicles interact with a docking complex comprising Munc18-1 and syntaxin 1 in the closed conformation. Then, Munc13-1 induces the opening of syntaxin 1 to promote the formation of the synaptobrevin 2–syntaxin 1–Munc18-1–SNAP25 SNARE complex. Synaptotagmin 1, participates in this complex, as well as complexin, which maintains the complex in an activated but 'off' state, until Ca^{2+} enters and binds to synaptotagmin 1. Munc18-1 also binds to syntaxin 1 in the open conformation, contributing to specificity and stimulating fusion of the trans-SNARE complex (de Saint Basile et al., 2010).

1.5.3.6 Vesicle fusion - The role of calcium channels and Synaptotagmin -1

Vesicle fusion is the last step of exocytosis, which culminates with the delivery of the neurotransmitter into the synaptic cleft. In preparation for fusion, synaptic vesicles dock at the active zone and are primed to become Ca^{2+} responsive. When an action potential invades the nerve terminal, voltage-gated Ca^{2+} channels open, and the resulting pulse of intracellular Ca^{2+} triggers fusion-pore opening of ready releasable vesicles. In neurons, Ca^{2+} influx is made mainly

through P/Q- (CaV2.1) or N-type Ca^{2+} channels (CaV2.2). The fusion of synaptic vesicles requires relatively high intracellular Ca^{2+} concentrations (50 - 100 μM). Voltage-sensitive Ca^{2+} channels are heteromeric proteins composed by an α1 subunit that forms the ion-conducting pore. This subunit is associated with two auxiliary subunits, α2δ and β, that regulate the properties of the channel pore. The α1 subunit also associates with synaptic proteins, such as syntaxin-1, SNAP-25, and synaptotagmin-1. This binding is critically dependent on the concentration of the intracellular Ca^{2+} (Wiser et al., 1996). Ca^{2+} influx triggers at least two components of release that are probably mechanistically distinct: a fast, synchronous phasic component is induced rapidly, in as little as 50 μs after a Ca^{2+} entry, and a slower asynchronous component continues for more than 1 s as an increase in the rate of spontaneous release after the action potential. Both components of release are strictly Ca^{2+} dependent but change differentially upon repetitive stimulation.

Synaptotagmin-1 is a protein localized at synaptic vesicles and an essential Ca^{2+} sensor for fast exocytosis (Chapman, 2008). This protein has a large cytoplasmic domain, consisting of tandem C2 domains which serve as Ca^{2+} sensors that transduce the Ca^{2+} signal into an activation of the membrane fusion machinery. This activation is mediated by the Ca^{2+}-dependent interaction of the synaptotagmin C2-domains with phospholipids and SNARE proteins. In triggering exocytosis, synaptotagmins do not act alone, but require the cofactor complexin, which binds to SNARE complexes and simultaneously activates and clamps the SNARE complexes, thereby positioning the SNARE complexes for subsequent synaptotagmin action (Figure 1.14) (Sudhof, 2012). When synaptotagmin-1 does not have Ca^{2+} bound, it inhibits the vesicle fusion catalyzed by the SNARE complex. Upon the binding of Ca^{2+}, synaptotagmin-1 accelerates SNARE-catalyzed fusion via simultaneous interactions with membrane and SNARE proteins (Geppert et al., 1994).

1.5.4 Synaptic vesicle endocytosis and recycling

After fusion-pore opening and the release of neurotransmitter, synaptic vesicles undergo endocytosis and are recycled by alternative pathways: two fast pathways in which the vesicles either remain at the active zone for refilling (Figure 1.15A) (kiss-and-stay) or are recycled locally without clathrin-mediated endocytosis (Figure 1.15B) (kiss-and-run), and a slower pathway that involves clathrin-mediated endocytosis (Figure 1.15C). The fast pathway is used preferentially to recycle vesicles rapidly into the readily releasable pool at low stimulation frequencies, whereas the slow clathrin-dependent pathway kicks in at higher stimulation frequencies. Clathrin-mediated endocytosis involves invaginating membrane, recruiting and assembling the clathrin coat, pinching off and finally disassembling the coat.

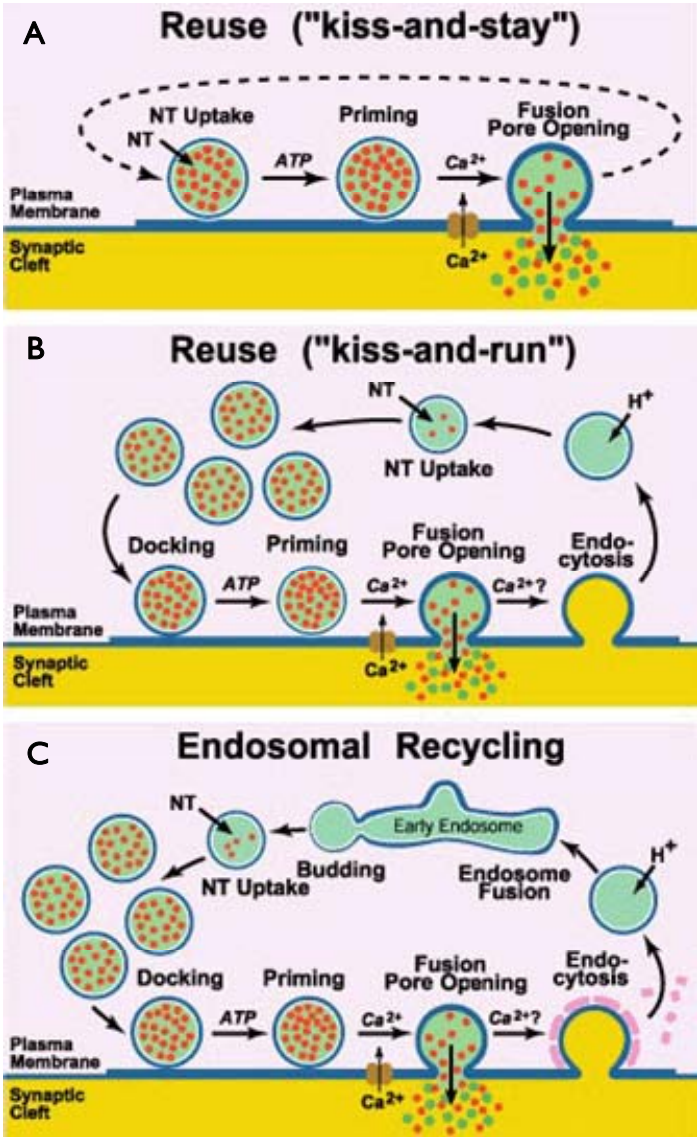


Figure 1.15. Vesicular endocytosis and recycling. After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via several routes: local reuse (A), fast recycling without an endosomal intermediate (B), or clathrin-mediated endocytosis with recycling via endosomes (C) (adapted from Sudhof, 2004).

1.5.5 Calcium-independent release of neurotransmitters

In many synapses, specific neurotransmitter transporters on neurons and glia contribute to inactivate postsynaptic receptors and to terminate synaptic responses by clearing neurotransmitter from the synaptic cleft. These neurotransmitter transporters are members of a large superfamily with four major branches: Na and Cl-dependent symporters (e.g., for GABA,

monoamines and glycine), Na and K-dependent antiporters (for example, for glutamate), vesicular transporters, and amino acid transport systems. Each of these transporters utilizes the energy available from the transmembrane ion gradients and membrane potential to provide the energy to drive their neurotransmitter substrate up a steep concentration gradient.

Under several conditions the release of amino acid neurotransmitters correlates with changes in membrane potential and/or intracellular Na^+ concentration, rather than changes in intracellular Ca^{2+} concentration. Those changes lead to a reversion of membrane transporters and neurotransmitter release independently of Ca^{2+} (Richerson and Wu, 2003).

It has been suggested that this may be the case for synaptic transmission between photoreceptors and horizontal cells of the vertebrate retina. Experiments on synaptic transmission from photoreceptors to horizontal cells showed that lowering extracellular Ca^{2+} can actually promote Ca^{2+} infux through voltage-activated Ca^{2+} channels via a modification of surface potential of plasma membranes (reviewed by Piccolino et al., 1999).

1.6 Axonal transport

1.6.1 Cellular cytoskeleton

Axonal transport occurs along the cellular cytoskeleton. The cytoskeleton provides structural support to the neuron, but also allows the cell to grow or change in size and shape over time. There are three major components of the neuronal cytoskeleton: microtubules, intermediate filaments and microfilaments.

1.6.1.1 Microtubules

Microtubules are formed from the polymerization of α,β-tubulin dimmers forming rigid hollow rods with approximately 25 nm in diameter. In developing neurons, this polymerization is very dynamic and required for the normal outgrowth of the axon and growth cone. However, as neurons mature, their microtubules become less dynamic, primarily through interactions with microtubule-associated proteins (MAPs) that stabilize the polymer. The high concentration of MAPs such as tau in the axon of mature motor neurons makes these microtubules significantly more stable.

Microtubules are the main elements responsible for the polarity of the axon. The α-tubulin side of the microtubule defines the minus end which is located closer to the cell body whereas the β-tubulin side defines the plus end which is located closer to the synapse. This orientation not only gives polarity to the microtubule but also to the axon (Conde and Caceres, 2009). The polarity of the microtubule directs motors of axonal transport to undergo anterograde (toward the plus end) or retrograde (toward the minus end) transport. In contrast, in dendrites microtubules are found in mixed polarity.

1.6.1.2 Neurofilaments

Neurofilaments are the 10 nm intermediate filaments found specifically in neurons and assemble from three subunit polypeptides based upon the apparent molecular weight: NF-L (70 kDa), NF-M (150kDa), and NF-H (200kDa) (Sharp et al., 1982). Once assembled, these filaments lack overall polarity, and do not undergo the remodeling characteristic of actin and microtubules. Neurofilaments serve primarily to provide structural stabilization to the cell, and to regulate the radial growth of axons. Aggregation of neurofilaments is a common marker of neurodegenerative disease (Liu et al., 2011a).

1.6.1. Microfilaments

Microfilaments (or actin filaments) are the thinnest filaments of the cytoskeleton, having 6 nm in diameter. The actin cytoskeleton provides both dynamics and stability. Actin monomers assemble into a flexible helical polymer with two distinct ends, one fast growing and one slower growing. In the cell, actin filaments are often bundled into networks and can be stabilized by interacting proteins. Actin plays a role in the formation of new spines as well as stabilizing spine volume increase (Dillon and Goda, 2005) and the dynamic of actin leads to the formation of new synapses as well as increased cell communication.

1.6.2 Motor proteins

Intracellular transport is powered by three sets of molecular motors: myosins, kinesins and dyneins. Myosins move on microfilaments and are thought to be responsible for short range transport, whereas kinesin and dynein proteins use microtubule as track for long distance transport and are capable of recognizing the microtubule polarity (Langford, 1995). A number of studies have shown that most kinesin-family motors move towards the plus-end of the microtubules that are usually used to deliver cargos towards the cell periphery. In contrast, dynein moves in the oposite direction for transport toward the cell center (Hirokawa and Takemura, 2005). Kinesins, dyneins and myosins have the so-called "motor domains" that move along the microtubules or actin filaments in a specific direction by using the energy derived from ATP hydrolysis.

1.6.2.1 Kinesins

Kinesins are microtubule based anterograde intracellular transport motors (Figure 1.16). Conventional kinesins usually form a protein dimer of two identical heavy chains and each of them binds to a kinesin light chain. The heavy chain contains a highly conserved globular head called motor domain, which includes a microtubule-binding site and an ATP-binding/hydrolysis site, a short, flexible neck linker, a stalk domain which has a long, central coiled-coil region for dimerization and a tail domain for light chain and cargo binding (Verhey et al., 2011). The two heads of kinesin move in a hand-over-hand mechanism along the microtubule. This movement is highly processive, meaning that once bound to the microtubule the motor will move over long

distances prior to detaching. This processivity makes kinesin a very efficient motor for the transport of cargo over the long distances of the axon.

According to Miki and colleagues (Miki et al., 2001) kinesin superfamily proteins (KIFs) constitute 15 kinesin families, which are termed kinesin 1 to kinesin 14B according to the results of phylogenetic analyses. According to the distinctive localization of their motor domains, they are classified into 3 major types: NH2 terminal motor (N-kinesin); middle motor (M-kinesin) and C-terminal motor (C-kinesin). In general, N-kinesins and C-kinesins drive microtubule plus end and minus end directed motilities, respectively, and M-kinesins depolymerize microtubules. In 2004, the KIFs were renamed by a standardized kinesin nomenclature based on 14 family designations that unifies all previous phylogenies and nomenclature proposals, while allowing individual sequence names to remain the same, and for expansion to occur as new sequences are discovered. (Lawrence et al., 2004).

1.6.2.2 Dyneins

Cytoplasmic dynein is the major motor protein driving retrograde transport (Figure 1.16). Based on both functional and structural criteria dyneins are divided in only two classes, axonemal and cytoplasmic dyneins (Hook and Vallee, 2006).

About five forms of axonemal dynein have been identified and they are mainly involved in bending of cilia and flagella of eukaryotic cells (Hook and Vallee, 2006). Only two cytoplasmic dyneins have been discovered and they play important roles in vesicle trafficking, cell division, cell polarization and cell movement (Levy and Holzbaur, 2006; Mallik and Gross, 2004; Vale, 2003).

Cytoplasmic dynein is a multisubunit complex that contains two heavy chains that are associated with intermediate chains, light intermediate chains, and light chains. The heavy chains harbor ATPase activity and bind microtubules, whereas the other chains are involved in cargo binding and binding to dynactin.

While dynein is sufficient to generate force along microtubules, the accessory or activator complex, dynactin, is required for most dynein functions *in vivo*. The overall bias is toward minus end-directed motility, but the ability of dynein to change directions may allow the motor to avoid obstacles encountered during active transport along the axon. Studies on dynein have shown that the dynein motor wanders across the surface of the microtubule, and takes frequent backward steps (Wang et al., 1995). Also, it was shown that individual fluorescently labeled dynein-dynactin complexes exhibit bidirectional motility towards both the plus and minus ends of microtubules, reflecting the flexibility of the dynein structure that leads to an enhanced ability to navigate around obstacles in the cell. (Ross et al., 2006).

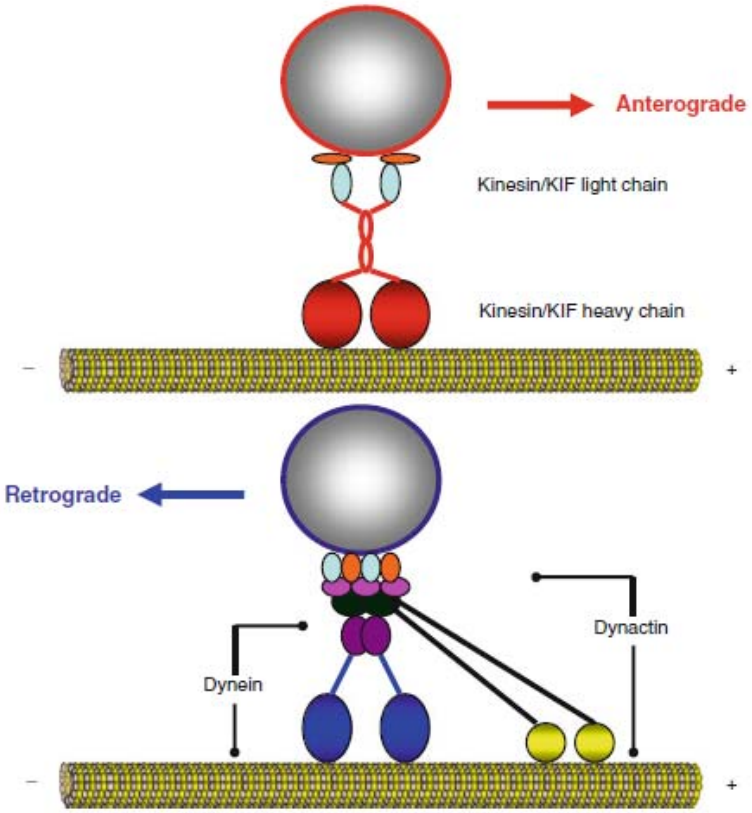


Figure 1.16. Motor proteins. The motor molecules for anterograde transport are kinesins, which consist of two heavy chains and two light chains. Each heavy chain consists of a globular motor domain, a coiled-coil stalk domain, and a C-terminal tail domain. The motor for the retrograde transport is dynein. Dynein consists of two globular heads, the motor domains, which are connected to a basal structure by two stalks (Nixon and Yuan, 2011).

1.6.3 Intracelular transport in neurons

Intracellular transport of protein and organelle cargoes is an essential requirement for neurons. Neurons are polarized with axons and dendrites and their architecture makes them particularly dependent on intracellular transport since most neuronal proteins are synthesized in cell bodies and mechanisms are required to direct axonal and dendritic transport.

Cargoes have to move long distances and must be targeted to specific compartments (e.g. synaptic proteins are targeted to the axon terminal). The main mechanism to deliver cellular components to their action site is long-range microtubule-based transport in which the molecular motors (kinesins and dynein) run in the microtubules. In axons, microtubule orientation is nearly uniform, with the plus ends pointing toward the synapse and the minus ends facing the cell body. As most molecular motors of the kinesin family unidirectionally move toward the microtubule plus end, they mostly mediate transport toward the synapse (anterograde). In the opposite

direction, the molecular motor cytoplasmic dynein moves toward the microtubule minus end and mediates transport of most cargoes toward the cell body (retrograde) (Figure 1.17).

Classically, axonal transport is divided into fast and slow axonal transport based on the bulk speeds of cargo movement. Cargoes such as vesicles and mitochondria move by fast axonal transport at speeds of 1 μm/s, whereas cytoskeleton components move in slow axonal transport at speeds of 1 mm/day. The net rate appears to be largely determined by size with the smallest cargoes in almost constant motion, while mitochondria and larger structures frequently pause giving a lower average rate. It is clear that both fast and slow axonal transport are mediated by the same "fast" molecular motors kinesin and cytoplasmic dynein and that the reduced rate in slow axonal transport is caused by multiple prolonged pauses during the process of transport down the axon (Miller and Heidemann, 2008; Roy et al., 2000; Wang et al., 2000).

1.6.3.1 Fast and slow axonal transport

Axonal transport is bidirectional, many proteins that are distributed by fast anterograde transport also return in the retrograde direction. However, proteins transported at slow rates are degraded when they reach their destination and are not detected in the retrograde component. Fast anterograde transport powered by motor proteins is vital for the growth and survival of axon and synapse.

Predominantly, the membrane-associated proteins move in one of the fast rate components, while cytoplasmatic proteins move as part of the slow components. The various organelles transported anterogradely are moved along the axon by one or more motor proteins. The differing rates of fast anterograde transport appear to result from the varying sizes of organelles, with increased drag on larger structures resulting in a slower net movement.

Fast axonal transport is essential for both anterograde and retrograde transport and functions via distinct carriers. In contrast, slow axonal transport only moves towards the positive end of the microtubules (anterograde transport) (Nixon, 1992). Slow axonal transport carries cytoskeletal components, including microtubule and neurofilament elements (Nixon, 1998). Cytoplasmic and cytoskeletal elements in axonal transport move with rates at least two orders of magnitude slower than fast transport.

Slow component a (SCa) consist of the cytoskeletal proteins that form neurofilaments and microtubules. Rates of transport for SCa proteins in mammalian nerve range from 0.2–0.5 mm/day in optic axons to 1 mm/day in motor neurons of the sciatic nerve. Slow component b (SCb) represents a complex and heterogeneous rate component, including hundreds of distinct polypeptides ranging from cytoskeletal proteins such as actin (and tubulin) to soluble enzymes of intermediary metabolism (such as the glycolytic enzymes).

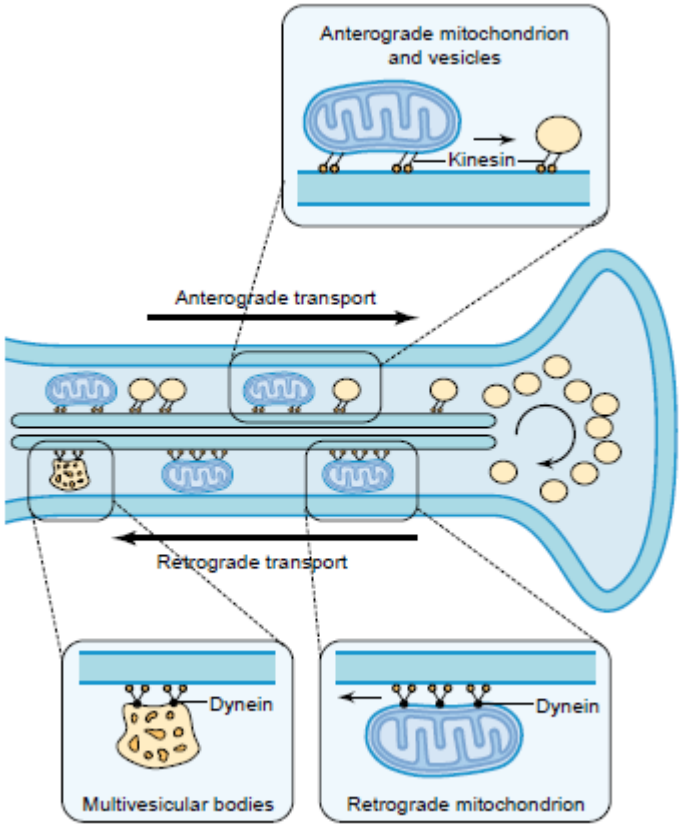


Figure 1.17. Fast axonal transport. Movement of membrane-bounded organelles along axonal microtubules in both the anterograde and retrograde directions (adapted from Seigel et al., 2006).

1.6.3.2 Anterograde transport

Anterograde transport provides newly synthesized components essential for neuronal function and maintenance. Ultrastructural studies have demonstrated that the material moving in fast anterograde transport includes many small vesicles and tubulovesicular structures as well as mitochondria and dense-core vesicles (Tsukita and Ishikawa, 1980). Material in fast anterograde transport is needed for supply and turnover of intracellular membrane compartments (i.e. mitochondria and endoplasmic reticulum) and for the supply of proteins required for the maintenance of axonal metabolism.

A variety of materials move in fast anterograde transport including membrane-associated enzymes, neurotransmitters, neuropeptides and membrane lipids. Most are synthesized in the cell body and transported intact, but some processing events occur in transit. The population of small organelles is particularly varied in function and composition. Some correspond to synaptic vesicle precursors and contain neurotransmitters and associated proteins, while others may contain channel proteins or other materials destined for the axolemma.

1.6.3.3 Retrograde transport

Retrograde fast axonal transport primarily transports endosomes generated from endocytosis at the nerve terminals. Some of these endosomes contain ligand-bound growth-factor receptors, and thus deliver the associated signals to the cell body.

Cargoes moving in retrograde transport are structurally heterogeneous and, on average, larger than the structures observed in anterograde transport. Once retrogradely transported material reaches the cell body, the cargo may be delivered to the lysosomal system for degradation, to nuclear compartments for regulation of gene expression, or to the Golgi complex for repackaging. Retrograde transport mainly carries vesicular cargoes involved in signaling from postsynaptic terminals on, for example, signaling endosomes (Cui et al., 2007). Dynein mediated retrograde transport is essential for the delivery of neurotrophic factors from the axon terminal to the cell body (Chao, 2003; Reynolds et al., 2000). In addition, proteins and membranes captured by endocytosis or autophagy are transported retrogradely back to the cell body for processing or degradation (Hollenbeck, 1993; Tsukita and Ishikawa, 1980).

1.6.3.4. Motor coordination in bidirectional transport

A functional transport system not only needs the activity of individual motors, but also requires the concerted action of multiple motors. There are evidences that bi-directional motility occurs in the cell via direct or indirect interactions between oppositely oriented motors. The cargo can move for long distances in each direction before undergoing a switching event to generate movement in the opposite direction. Two models (Figure 1.18) have been proposed to explain reversals, regulated switching of motor activity or tug-of-war between opposite motors (Gross, 2004; Verhey et al., 2011; Welte, 2004). In the regulated switching model, minus end- and plus-end-directed motors alternate their activities due to regulatory mechanisms that rapidly activate and inactivate motors in a mutually exclusive manner. In some cases of bidirectional transport, inhibition or removal of one motor abolishes transport in both directions rather than enhances transport in the opposite direction. This indicates that the motors are not competing with each other (only one type of motor is active at any time) and that both motors are required for the regulatory systems to operate. The regulatory systems, such as the dynactin complex, have been envisioned to involve specific cargo proteins that regulate individual motors. The tug-of-war model requires that motors of opposite directionality pull against each other and that one class of motor wins to effect a reversal in its favor. In this scenario, the cargo finds itself in the position of having both a fully functional plus-end transport system and a fully functional minus-end system, each intent on going its own way. The cargo is caught in the middle of the tug-of-war between the two, and moves back and forth as a result. It moves in the direction of whichever transport system is instantaneously providing more force, thus temporarily winning the tug-of-war.

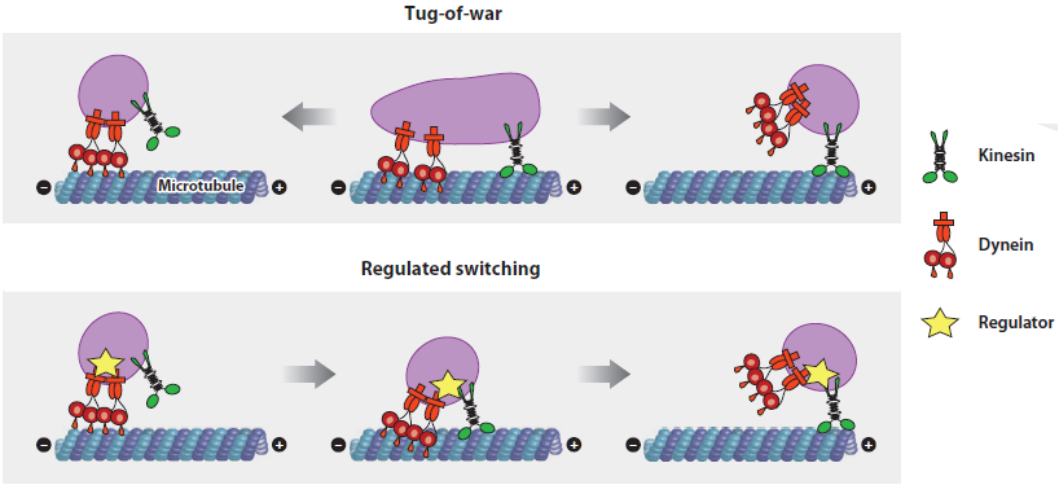


Figure 1.18. Models for bidirectional axonal transport. In the model of tug-of-war, dynein and kinesin generate force against each other in way that one motor wins to ensure reversal in its favor. In the regulated switching model, the activity of motors is regulated to ensure that one type of motor is active at any time (adapted from Verhey et al., 2011).

1.6.3.5 Mitochondria and axonal transport

The supply of appropriate levels of ATP, which is mainly produced by mitochondria via oxidative phosphorylation, is required for proper neuronal function and survival. The majority of mitochondria are produced in the cell body and transported along axonal microtubules by protein motors to reach areas with high ATP and calcium buffering requirements. Distal cellular compartments such as synapses depend upon the efficient delivery of mitochondria through active transport to provide local sources of ATP. Generally, kinesin motors drive anterograde mitochondrial transport, while dyneins are responsible for retrograde transport, nevertheless, single mitochondrion rarely move in only one direction. Their transport along microtubules typically involves pauses of short and long duration and abrupt changes in direction, which suggests that individual mitochondrion are simultaneously coupled to kinesins, dyneins, and anchoring machinery whose actions compete or oppose one another (Figure 1.19). Averaging the bidirectional and saltatory components yields a net mitochondrial velocity that falls between fast moving vesicles and slow-moving cytoskeletal proteins: 0.3–2.0 μm/s (Cai et al., 2011a).

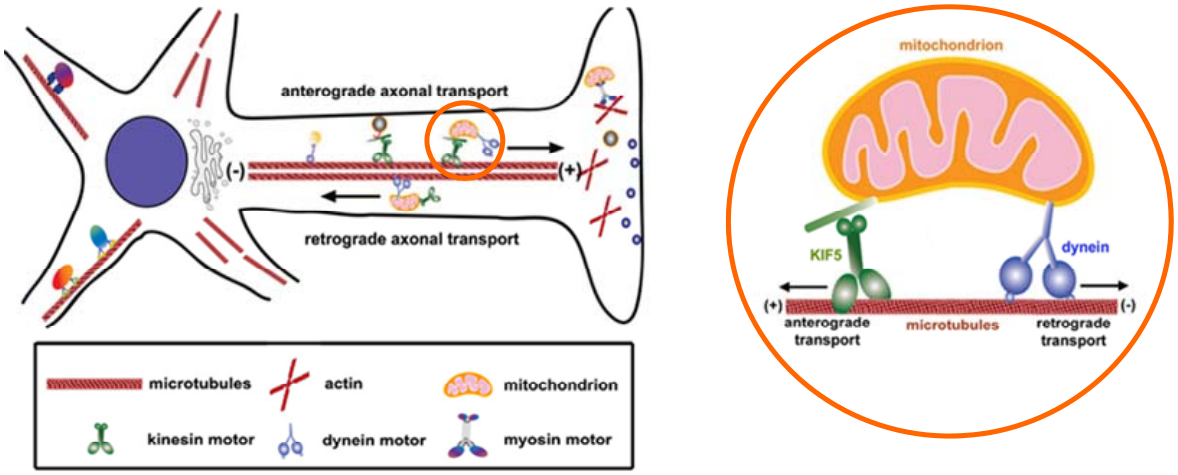


Figure 1.19. Axonal mitochondrial transport. In axons, microtubules are uniformly organized with the plus (+) ends facing toward the axonal terminals and the minus (−) ends toward the cell body. While kinesin motors are mostly plus-end directed, dyneins travel toward the minus ends of microtubules. Kinesin motors generally mediate anterograde axonal transport of mitochondria and dynein drives their retrograde axonal transport (adapted from Cai et al., 2011a).

1.6.4 Axonal transport defects in neurodegenerative diseases and diabetes

As it was previously mentioned, neurons are highly polarized cells, which make them particularly dependent on active intracellular transport. Deficits in this transport contribute to the pathogenesis of multiple neurodegenerative diseases.

Inhibition of axonal transport leads rapidly to loss of function in the distal axon and to "dying back" axonal degeneration. Recent studies have revealed that defects of the cytoskeleton and axonal transport are associated with several types of peripheral neuropathy and some central neurological diseases (Cheng et al., 2011). Direct evidence from genetic studies demonstrates that mutations in major components of the cytoskeleton and axonal transport result in axonal defects in several types of Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis, Alzheimer's disease, and other types of genetic neurological disorders (De Vos et al., 2008). In addition, post-translational modifications of cytoskeleton proteins also result in axonal defects in metabolic diseases like diabetic neuropathy. Defects in mitochondria transport lead to altered distribution of mitochondria along the axon, consequently leading to an inability to meet local ATP demands. Indeed, defects in mitochondrial transport along the axon have been implicated in several disease models (Zhao et al., 2010). Although mitochondrial transport and distribution have been quantified only in selected cases, some evidence shows that similar depletion of mitochondria numbers/ function within axons occurs in neurodegenerative diseases (Brownlees et al., 2002; Stamer et al., 2002). In Huntington's disease, Alzheimer's disease and amyotrophic lateral

sclerosis, it has been reported defects in mitochondrial function and transport For example, in Alzheimer's disease, and Aβ disrupts mitochondrial transport (Manczak et al., 2006).

Axonal transport is known to be affected in experimental models of diabetes. Retrograde fast axonal transport is reduced in diabetic animals (Jakobsen et al., 1981; Lee et al., 2002; Lee et al., 2001). In addition, the slow component of anterograde axonal transport for actin, tubulin, and the two lightest subunits of the neurofilament triplet are also impaired in diabetic mice with a leptin receptor mutation (Vitadello et al., 1985). Similarly, in STZ-induced diabetic animals, slow axonal transport of neurofilament and microtubule components is also reduced, resulting in decreased axonal caliber (Medori et al., 1988).

Interestingly, the impairment of axonal transport in diabetic nerves is more significant in sensory than in motor fibers (Macioce et al., 1989), which is consistent with the sensory predominant neuropathy observed in patients with diabetes. Potential mechanisms underlying the potential effects of hyperglycemia on axonal transport could include: (1) the induction of metabolic abnormalities in the neuronal cell body that affect the synthesis of elements necessary for axonal transport; (2) increased glycation or phosphorylation of axonal transport elements, which leads to deterioration in the efficiency of axonal transport (McLean, 1997); and/or (3) deleterious glycation in Schwann cells, affecting the myelin sheath stability and inducing demyelinating neuropathy (Toth et al., 2008).

Additionally, studies using Fluoro-Gold labelling showed that diabetes affects the retrograde axonal transport in retinal ganglion cells (Ino-Ue et al., 2000; Zhang et al., 2000), and very recently, a deficit in anterograde transport from the retina to the superior colliculus was reported at 6 weeks after diabetes induction with STZ (Fernandez et al., 2012). These studies demonstrate that diabetes can also impair axonal transport in CNS neurons.

Further studies to better understand the mechanisms underlying the cytoskeletal and axonal transport defects in axonal neuropathies will be important in order to provide new treatments for these diseases.

1.7 Objectives

Diabetic retinopathy is a leading cause of vision loss and blindness worldwide. Increasing evidence has shown that the neuronal components of the retina are affected even before the detection of vascular lesions, but the mechanisms underlying neuronal dysfunction remain unclear. Several evidences strongly suggest that diabetes may impair neurotransmission at presynaptic level, leading to changes in neurotransmitter release. However, the presynaptic mechanisms underlying these changes are not fully explored and clarified. Therefore, the main goal of this work was to give further insight into the molecular and cellular alterations in the retina induced by diabetes, giving a particular attention to changes occurring in nerve terminals and to changes occurring in neural cells.

Since the impairment of exocytosis might affect neurotransmission, likely contributing to alterations previously found in retinal physiology detected under diabetes, and recent evidences point to alterations in some exocytotic proteins in central nervous system induced by diabetes, in Chapter 2 we investigated the impact of diabetes (type 1 animal model, STZ-induced diabetes) on the content of several exocytotic proteins, at different time points, during the early stages of the disease. Since exocytosis depends on several specialized proteins that control and regulate neurotransmitter release, we analyzed the levels of SNARE complex proteins (VAMP-2, syntaxin-1 and SNAP-25), which are critical for exocytosis, and also synapsin-1, synaptophysin, rabphilin 3a and synaptotagmin-1.

Release of neurotransmitters is a highly regulated process. Changes detected in the exocytotic machinery in the retina may affect, at least partially, synaptic transmission, thus contributing to the alterations detected in electroretinograms of diabetic animals and patients. In Chapter 3, we aimed to evaluate the effect of diabetes on basal and evoked release of glutamate and GABA in retinal nerve terminals. The impact of diabetes on the protein content of vesicular glutamate and GABA transporters was also analyzed.

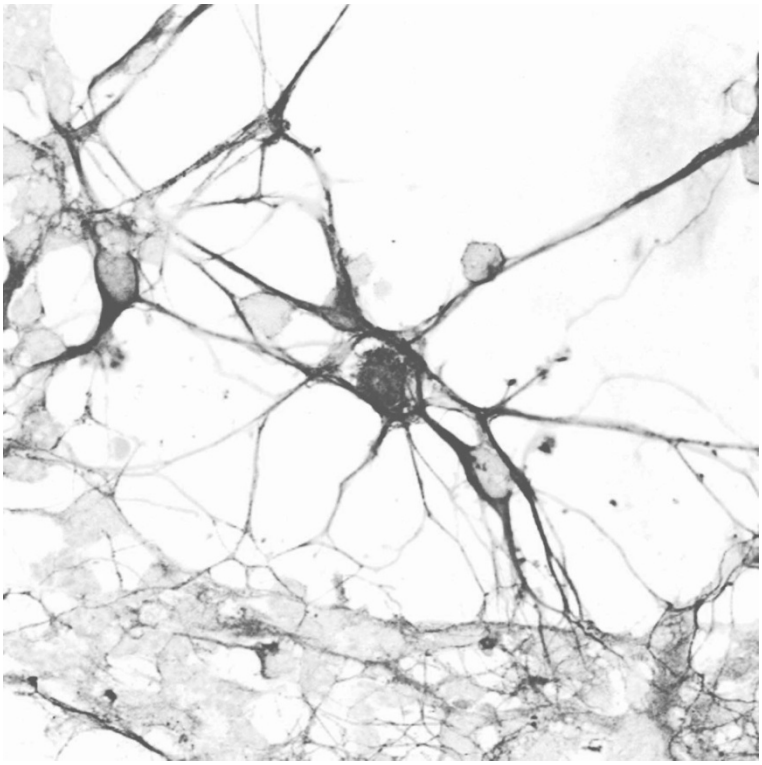
Hyperglycemia is considered the main trigger of diabetes complications. Hyperglycemia triggers various processes that induce cell dysfunction and consequently cell death in the retina. In Chapter 4, we evaluated whether prolonged exposure to elevated glucose *per se* (in order to mimic chronic hyperglycemia) contributes to changes in the content and localization of synaptic proteins involved in exocytosis, and of the vesicular transporters for glutamate and GABA, in primary retinal cultures. Several evidences also indicate that diabetic retinopathy has characteristics of a low-grade chronic inflammatory disease. However, little attention has been given to the effect of IL-1β on the different retinal cell types. So, in Chapter 5, we aimed to evaluate if prolonged high glucose *per se* is capable of changing the expression of IL-1β in retinal neural cells. Moreover, we aimed to identify which cell types produce IL-1β and express IL-1RI, as well as to evaluate the cell-specific effects of high glucose and IL-1β in retinal neural cell cultures.

The changes detected in the levels of exocytotic synaptic proteins in retinal nerve terminals (Chapter 2), with no changes in total extracts, together with other evidences reported by previous studies, suggest that axonal transport in the retina may be impaired in diabetes. In Chapter 6, we analyzed the impact of diabetes in axonal transport in the retina, evaluating the effect of early diabetes on the content and distribution of KIF1A, KIF5B and dynein motor proteins that are responsible for axonal transport of organelles, protein complexes and mRNAs. A growing body of evidence demonstrates that cognitive and behavioral deficits can occur as a late complication of diabetes. Although several brain areas can contribute to cognitive impairments, hippocampus seems to be particularly susceptible to diabetes. Previous detected changes in the levels of exocytotic proteins in hippocampal nerve terminals induced by diabetes, as well as in hippocampal cell cultures induced by exposure to elevated glucose, where an accumulation of some of those proteins at the cell body was detected, suggests that the axonal transport of these proteins to the synapse may be affected. To our knowledge no studies exist on the impact of diabetes on axonal transport in the hippocampus. Therefore, in Chapter 7, we

evaluated the impact of early diabetes on the content and distribution of KIF1A, KIF5B and dynein motor proteins in the hippocampus. Moreover, we aimed to evaluate whether high glucose *per se* could affect the levels and distribution of motor proteins, synaptic proteins and mitochondria in hippocampal neurons, giving particular attention to changes in axons.

Chapter 2

Diabetes differentially affects the content of exocytotic proteins in retinal nerve terminals



This chapter is part of an original research manuscript, published in Neuroscience (2010 Sep 15; 169(4): 1589-1600) by Filipa I. Baptista, Joana M. Gaspar, Joana Galvão, Áurea F. Castilho, Rodrigo A. Cunha and António F. Ambrósio The manuscript compares the effect of diabetes on exocytotic proteins in the retina and hippocampus. The results obtained in the hippocampus were part of the PhD thesis of Joana M. Gaspar

Abstract

Diabetic retinopathy has been considered a microvascular disease. However, increasing evidence, including alterations in color and contrast perception, suggests that the neural retina is also affected by this disease. A few studies have shown that diabetes differentially affects neurotransmitter release in retina, and induces structural and molecular changes in nerve terminals. In the present work, we detailed the impact over time of diabetes (2, 4 and 8 weeks of diabetes) on a large array of exocytotic proteins in the retina.

The exocytotic proteins density was evaluated by immunoblotting in purified synaptosomes and in total extracts of retina from streptozotocin-induced diabetic and age-matched control animals.

Diabetes affected differentially the content of synaptic proteins (VAMP-2, SNAP-25, syntaxin-1, synapsin-1 and synaptophysin) in retinal nerve terminals. In general, the alterations in retina occurred early, but were transitory, with the exception of synapsin-1, since its content decreased at all time points studied. The content of synaptotagmin-1 and rabphilin 3a in nerve terminals was not affected. In total extracts, no changes were detected in the retina of diabetic rats.

These results show that diabetes affects the content of several exocytotic proteins in the retina at the presynaptic level. These changes might influence neurotransmission and underlie, at least partially, previously detected physiological changes in diabetic humans and animal models. Since diabetes differentially affects exocytotic proteins, according to tissue and insult duration, functional studies will be required to assess the physiological impairment induced by diabetes on the exocytosis in central synapses.

Keywords: Diabetes; Retina; Nerve Terminals; Synaptic Proteins; Exocytosis.

Introduction

Diabetes *mellitus* is the most common metabolic disorder in humans, and it has been associated with several complications, such as diabetic retinopathy and diabetic encephalopathy (Mellitus., 2003).

Diabetes-induced cognitive impairments have been associated with changes in the integrity and function of hippocampus (Stranahan et al., 2008a; Trudeau et al., 2004), impairing long-term potentiation (LTP) and facilitating the induction of long-term depression (LTD) (Artola et al., 2005; Kamal et al., 1999), although it is not clear yet if these effects are mainly due to pre- or postsynaptic changes (Kamal et al., 2006). Diabetes also induces morphological changes in neurons, including synaptic vesicle depletion in mossy fiber nerve terminals (Magarinos and McEwen, 2000), dendritic atrophy of CA3 pyramidal neurons (Reagan et al., 1999), and increases the expression of the presynaptic marker synaptophysin (Grillo et al., 2005). Several evidences have also demonstrated that diabetes induces changes in neurotransmitter release in different brain regions. Neurotransmitters and brain regions appear to be differentially affected, and the effects also depend on duration and severity of diabetes (Guyot et al., 2001; Misumi et al., 2008; Miyata et al., 2007; Morris and Pavia, 2004; Satoh and Takahashi, 2008; Yamato et al., 2004).

Emerging evidence has indicated that retinal neurons may also be affected by diabetes, even before the detection of microvascular dysfunction, the hallmark of diabetic retinopathy (Antonetti et al., 2006). Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). Diabetes increases apoptosis in neural cells in human and rat retina early in the course of the disease (Barber et al., 1998; Gastinger et al., 2006). In primary retinal neural cell cultures, we showed that elevated glucose increases neural cell death (Santiago et al., 2007). Diabetes and high glucose also increase the evoked release of [^{3}H]D-aspartate in retina and retinal cell cultures, respectively (Santiago et al., 2006a).

The alterations in neurotransmitter release caused by diabetes may result, at least partially, from changes in the exocytotic machinery. Recently, it was reported that diabetes induces a decrease in the content or expression of some exocytotic proteins in hippocampal and retinal nerve terminals (Duarte et al., 2009; VanGuilder et al., 2008). Elevated glucose and diabetes also downregulate the expression of several genes coding for exocytotic proteins in pancreatic β-cells (Abderrahmani et al., 2006b; Ostenson et al., 2006; Zhang et al., 2002), whereas in pancreatic α-cells high glucose increases the expression of exocytotic proteins (McGirr et al., 2005). Several evidences have also demonstrated that diabetes induces changes in neurotransmitter release in different brain regions (Guyot et al., 2001; Misumi et al., 2008; Miyata et al., 2007; Morris and Pavia, 2004; Satoh and Takahashi, 2008; Yamato et al., 2004).

Since the impairment of exocytosis might affect neurotransmission, contributing to alterations in retinal physiology detected under diabetes, and recent evidences point to alterations in some synaptic proteins, here we further investigated the impact of diabetes on the protein

content of several exocytotic proteins, focusing on different time points during the early stages of the disease, analyzing a broader array of proteins associated with the vesicular release machinery.

Methods

Animals

Male Wistar rats (Charles River Laboratories), eight weeks-old, were randomly assigned to control or diabetic groups. All animals were handled according with the EU guidelines for the use of experimental animals (86/609/EEC). Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, freshly dissolved in 10 mM sodium citrate buffer, pH 4.5) (Sigma, St. Louis, MO, USA). Hyperglycemic status (blood glucose levels exceeding 250 mg/dl) was confirmed two days later with a glucometer (Elite, Bayer, Portugal). Before sacrifice, rats were weighted, and blood samples were collected for measurement of glucose levels and to determine the percentage of glycated hemoglobin. Diabetic rats and age-matched controls were anesthetized with halothane and then sacrificed, two, four and eight weeks after the onset of diabetes.

Determination of glycated hemoglobin (HbA1c) levels

The percentage of glycated hemoglobin was determined using the DCA2000 Kit (Bayer), according to the manufacturer instructions. The animals that presented glycated hemoglobin levels exceeding 5% were considered diabetic.

Preparation of retinal synaptosomal extracts

Retinas of diabetic and age-matched control rats were used for preparation of synaptosomes, as previously described (VanGuilder et al., 2008), with minor alterations. Immediately after animal sacrifice, both eyes were enucleated, retinas were dissected and merged in 10 ml of ice-cold sucrose-HEPES solution. Retinas were washed three times combining gentle vortexing and buffer replacement in order to remove photoreceptor outer segments. Then, the retinas were homogenized in 5 ml of fresh sucrose buffer and the homogenate was centrifuged at 200 x *g* for 10 min at 4◦C to pellet nuclear fraction. The supernatant was centrifuged at 800 x *g* for 12 min at 4°C. The resulting supernatant was centrifuged at 16,100 x *g* for 20 min at 4°C to obtain the synaptosomal fraction. The synaptosomal pellet was rinsed in a detergent-based extraction buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.5% SDS, supplemented with complete miniprotease inhibitor cocktail tablets, pH 7.2, at 4°C) and stored at -80°C until use.

Preparation of total retinal extracts

The eyes of diabetic and age-matched control animals were enucleated and placed in cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at

4°C). The retinas were lysed in RIPA buffer supplemented with complete mini protease inhibitor cocktail tablets and 1 mM DTT, pH 7.2, at 4°C. Then, the lysates were sonicated and centrifuged at 16,100 x *g* for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95◦C. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 8%-12% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. The membranes were incubated overnight at 4°C with the primary antibody directed against the respective protein (listed in Table 1). After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature with the respective alkaline phosphatase-linked secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK), prepared in TBS-T with 1% low-fat milk. The membranes were processed for protein detection using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare) and a Storm device (Molecular Dynamics, GE Healthcare). Digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

The membranes were then reprobed and tested for β-actin (Sigma, St.Louis, MO, USA) immunoreactivity (1:5,000) to prove that similar amounts of protein were applied in the gels.

Statistical analysis

Results are presented as mean ± SEM. Statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Student's *t* test (variance analysis was not undertaken since the effect of age on the content of exocytotic proteins was not the aim of this study). Thus, gels were always loaded with samples from age-matched animals and not from animals with different ages. A Kruskal-Wallis analysis of variance was performed to evaluate the effect of diabetes duration on the content of exocytotic proteins. Differences were considered significant for $p<0.05$.

Table 2.1 List of primary antibodies

Primary Antibody	Sample	Dilution	Protein (µg)	Source
Mouse anti-SNAP-25	Synaptosomes	1:40,000	10	Sigma
	Total Extracts	1:1,000	20	
Rabbit anti-VAMP-2	Synaptosomes	1:2,000	10	Chemicon
	Total Extracts	1:2,000	10	
Mouse anti-Syntaxin-1	Synaptosomes	1:20,000	10	Synaptic Systems
	Total Extracts	1:20,000	10	
Mouse anti-Synapsin-1	Synaptosomes	1:10,000	10	Synaptic Systems
	Total Extracts	1:5,000	10	
Mouse anti-Synaptophysin	Synaptosomes	1:20,000	10	Sigma
	Total Extracts	1:10,000	10	
Rabbit anti-Synaptotagmin-1	Synaptosomes	1:40,000	10	Synaptic Systems
	Total Extracts	1:40,000	10	
Mouse anti-Rabphilin 3a	Synaptosomes	1:1,000	20	Synaptic Systems
	Total Extracts	1:1,000	20	

Results

Animals

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (243.9±8.0 g for control animals and 256.1±4.6 g for diabetic animals). The glucose levels were also similar in both groups (92.9±4.5 mg/dl for controls and 95.1±5.9 mg/dl for diabetic animals). Average weight, blood glucose levels and the percentage of glycated hemoglobin for both diabetic and aged-matched control rats at the time of death are given in Table 2. A marked impairment in weight gain occurred in diabetic rats comparing with age-matched controls in all time points analyzed. Diabetic animals also presented significantly higher blood glucose levels and elevated percentage of glycated hemoglobin comparing to age-matched controls.

Effect of diabetes on the content of SNARE complex proteins

The SNARE complex is a key structure for synaptic vesicle exocytosis. It is formed by the synaptic vesicle-associated membrane protein 2 (VAMP-2), which interacts with two membrane-associated proteins, SNAP-25 (synaptosome-associated protein with 25 kDa) and syntaxin-1 (Leenders and Sheng, 2005). The effect of diabetes on the content of VAMP-2, SNAP-25 and syntaxin-1 was analyzed in retinal nerve terminals by Western blotting. Diabetes induced changes in the content of VAMP-2 in retinal nerve terminals. Two weeks after the onset of diabetes VAMP2 levels decreased significantly to 61.3 ± 9.3% of the control, but after four weeks VAMP-2

Table 2.2 Average weight, blood glucose levels and percentage of glycated hemoglobin of diabetic and aged-matched control rats.

Diabetes duration		Weight (g)	Blood Glucose (mg/dL)	%HbAc
2 Weeks	Control (n=13)	330.6±14.0	98.8±3.2	3.5±0.1
	Diabetic (n=13)	268.5±9.6**	484.4±24.2***	6.3±0.2***
4 Weeks	Control (n=12)	326.3±9.4	98.3±5.2	3.3±0.9
	Diabetic (n=15)	231.0±8.5**	513.7±31.2***	9.6±0.4***
8 Weeks	Control (n=14)	414.6±10.6	88.9±4.1	3.2±0.1
	Diabetic (n=14)	229.9±8.8***	552.1±20.1***	8.7±0.5***

Measurements were made immediately before the sacrifice of the animals. ** p <0.01, *** p <0.001.

levels increased to 156.8 ± 14.6% of the control. However, at eight weeks after diabetes induction, the protein levels returned to values similar to those found in age-matched control animals (84.7 ± 9.3% of the control; Figure 1.A).To test whether the potential changes in the content of SNARE proteins at the nerve terminal level were a consequence of changes in the total content of those proteins, the effect of diabetes in retinal total extracts was also evaluated. In this case, no changes were found in the protein levels of VAMP-2 in retinal total extracts (Figures 1.B).

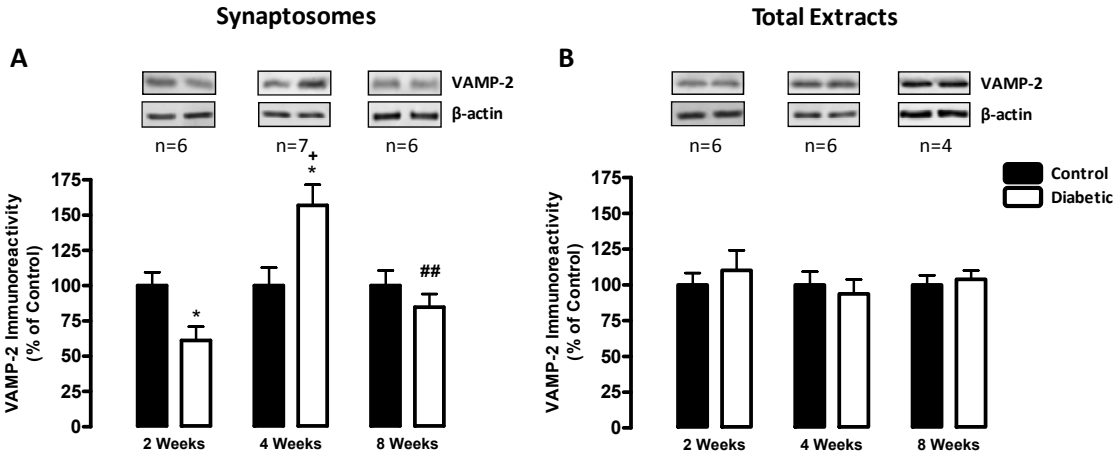


Figure 1. Diabetes induces changes in the protein content of VAMP-2 in retinal nerve terminals. The protein levels of VAMP-2 were analyzed by immunoblotting in extracts of retinal nerve terminals (A) and also in total extracts of retina (B), isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-7 animals. * p < 0.05 compared to age-matched control using Student's t test; $^{+}$ p < 0.05 compared to two

weeks of diabetes; ## $p < 0.01$ compared to four weeks of diabetes using a Kruskal-Wallis analysis of variance.

In retinal synaptosomes obtained from diabetic rats, SNAP-25 protein levels were similar to those detected in age-matched controls at all time points (Figure 2.A). In total extracts of the retina, no significant differences were found in the protein levels of SNAP-25 between diabetic and age-matched control animals (Figure 2.B).

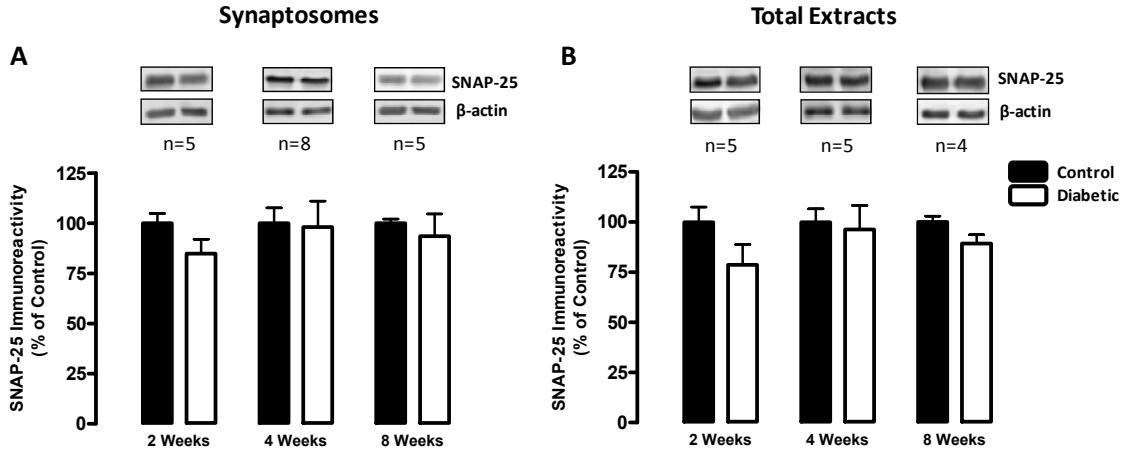


Figure 2. Diabetes does not induce changes in the protein content of SNAP-25 in the retina. The protein levels of SNAP-25 were analyzed by Western blotting in extracts of synaptosomes (A), and also in total retinal extracts (B) from diabetic or age-matched control animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that the same amount of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-8 animals compared to age-matched control.

In which concerns to the content of syntaxin-1 in retinal synaptosomes, it significantly decreased two weeks after the onset of diabetes (reduction to 49.3 ± 13.4% of the control), but there was a recovery to control levels at four and eight weeks of diabetes (Figure 3.A). In retinal total extracts from diabetic animals no significant alterations were detected in the levels of this protein comparing to the extracts from age-matched control animals (Figure 3.B).

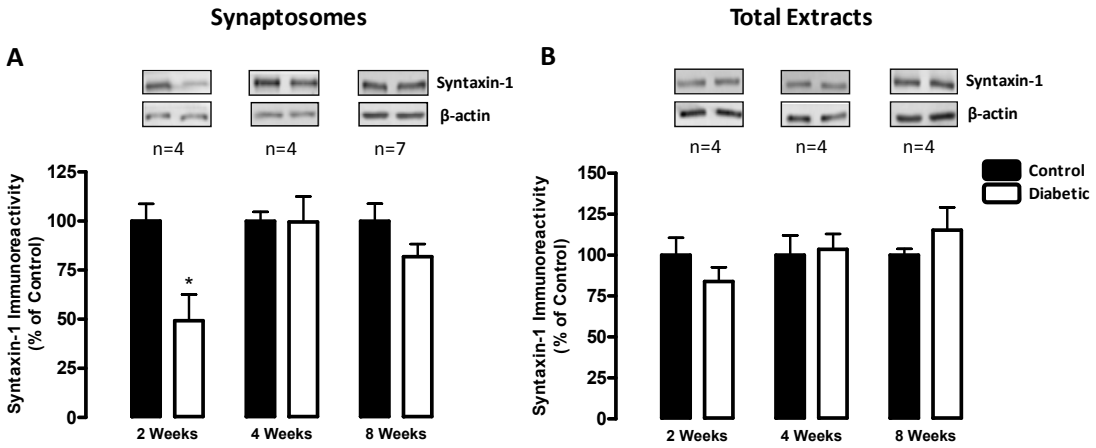


Figure 3. Diabetes induces changes in the protein content of syntaxin-1 in retinal nerve terminals. The protein levels of syntaxin-1 were analyzed by immunoblotting in extracts of purified nerve terminals of retina (A), and also in total extracts (B), obtained from control or diabetic animals. Representative Western blots are presented above graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM. * $p < 0.05$.

Diabetes decreases the content of synapsin-1 in retinal nerve terminals

Synapsins are abundant synaptic vesicle proteins that anchor synaptic vesicles to the cytoskeleton (Ceccaldi et al., 1995). In retinal nerve terminals, a significant decrease in the content of synapsin-1 was found in diabetic samples for all time points (Figure 4.A). This decrease was more pronounced at two weeks of diabetes (reduction to 47.9 ± 10.1% of the control). In total extracts from retina, no significant differences were detected in synapsin-1 content between diabetic and age-matched control animals (Figure 4.B).

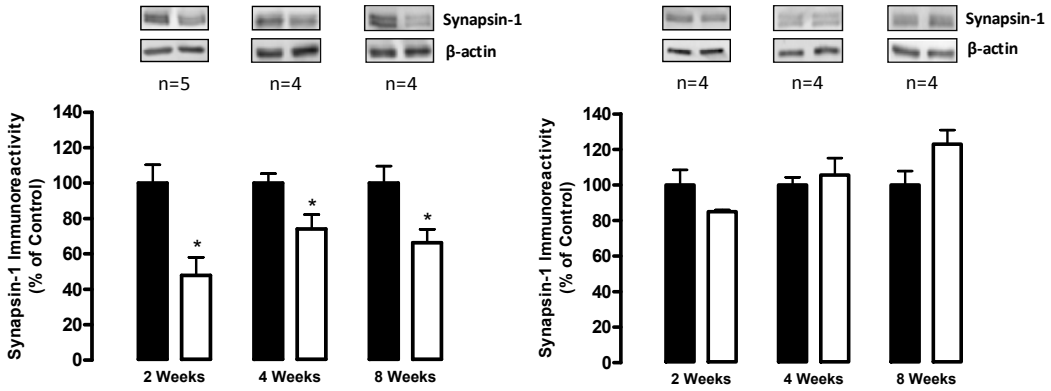


Figure 4. Diabetes induces changes in the protein content of synapsin-1 in retinal nerve terminals. The protein levels of synapsin-1 were analyzed by immunoblotting in purified synaptosomes and total extracts obtained from the retina (A and B, respectively), from both diabetic or age-matched control animals. Representative Western blots are presented above graphs, with the respective loading controls (β-actin), in order to confirm that identical amounts of protein from control and diabetic samples were loaded in the

gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-5 animals.* $p < 0.05$ compared to age-matched control using Student's *t* test.

The content of synaptophysin is altered by diabetes in retinal nerve terminals

Synaptophysin is a synaptic vesicle protein that is ubiquitously expressed in neurons, being a widely used marker for nerve terminals. In retinal nerve terminals, the protein levels of synaptophysin decreased, but only after two weeks of diabetes, to 70.2 ± 9.0% of the control (Figure 5.A). In total retinal extracts, no differences were detected between diabetic and age-matched control animals (Figure 5.B).

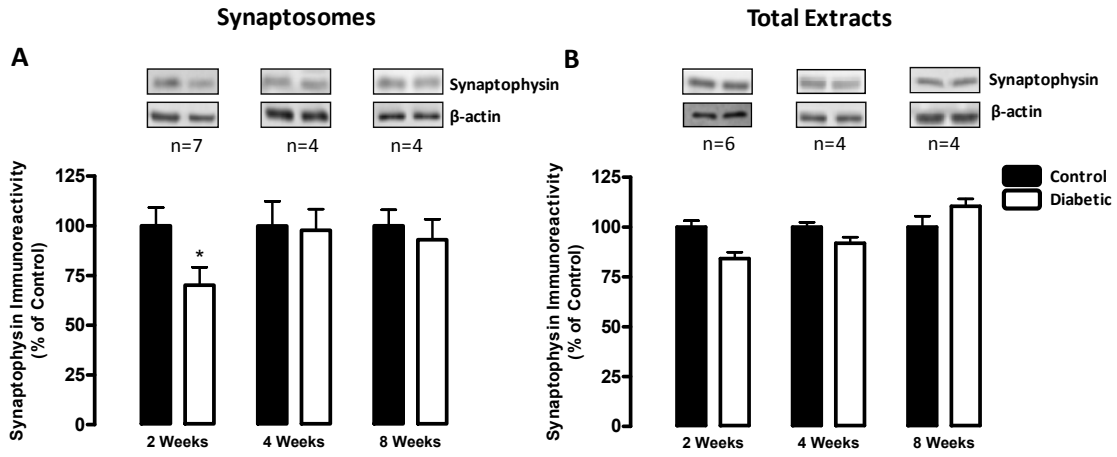


Figure 5. Diabetes induces changes in the protein content of synaptophysin in retinal nerve terminals. The protein levels of synaptophysin were analyzed by Western blotting in purified retinal synaptosomes (A) and total extracts of retina (B), from control or STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-7 animals. * $p < 0.05$ compared to age-matched control animals using Student's *t* test.

Diabetes does not affect the content of synaptotagmin-1 and rabphilin 3a

Synaptotagmin-1 is localized in synaptic vesicles and acts as a calcium sensor that regulates exocytosis (Chapman, 2008). The protein content of synaptotagmin-1 was not significantly altered by diabetes in retinal nerve terminals (Figure 6.A). Similar results were obtained for total extracts (Figure 6.B).

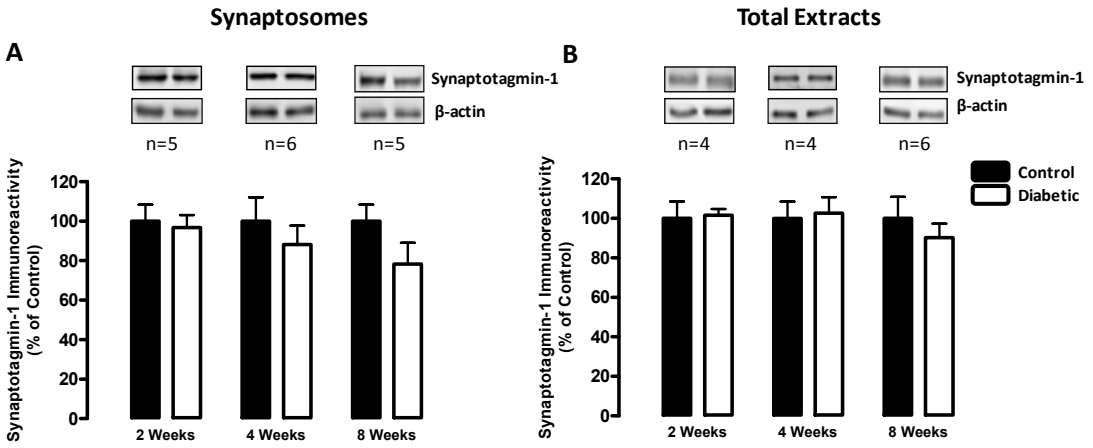


Figure 6. Diabetes does not induce changes in synaptotagmin-1 protein levels. The protein levels of synaptotagmin-1 were analyzed by immunoblotting in purified synaptosomes or total extracts from retina (A and B, respectively). Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-6 animals compared to age-matched control.

Rabphilin 3a is a downstream target protein for the small GTPase binding protein Rab3a that regulates the SNARE complex assembly (Deak et al., 2006). As for synaptotagmin-1, diabetes did not induce changes in the content of rabphilin 3a in retinal nerve terminals (Figure 7.A) and in total extracts of retina (Figure 7.B).

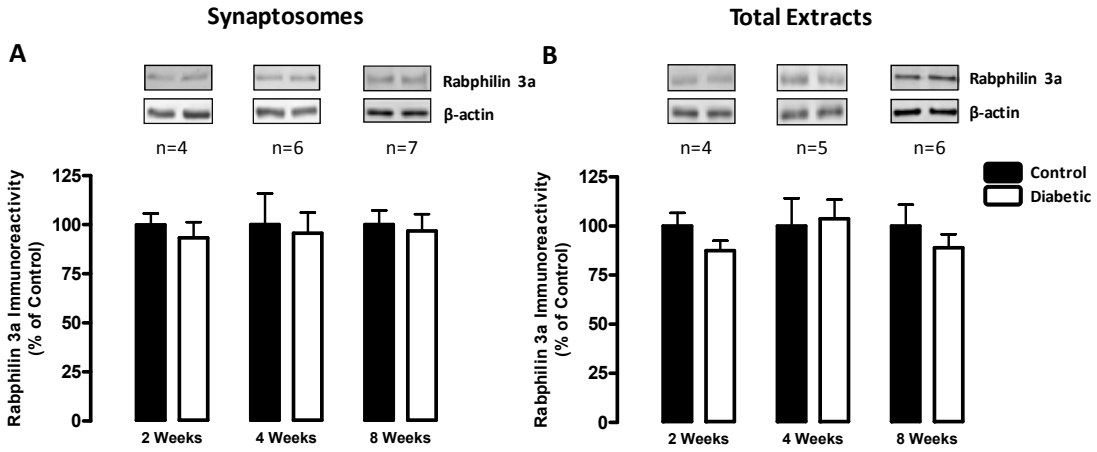


Figure 7. Rabphilin 3a protein levels are not affected by diabetes in the retina. The protein levels of rabphilin 3a were analyzed by immunoblotting in purified retinal synaptosomes (A), and also in total extracts of retina (B), from either diabetic animals or aged-matched controls. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage

of age-matched controls, and data are presented as mean ± SEM of 4-7 animals compared to age-matched control.

Discussion

In this study, we demonstrate that diabetes alters the content of several synaptic proteins involved in exocytosis in retinal nerve terminals. The alterations occur early in retina but most of them where not persistent along the time points of the study.

Alterations in contrast sensitivity and colour perception (Daley et al., 1987; Roy et al., 1986) induced by diabetes might be due, at least in part, to changes in neurotransmission, at both pre- and post-synaptic levels. Diabetes and high glucose increase the release of [^{3}H]-D-aspartate in retina and retinal cell cultures (Santiago et al., 2006a), and elevated glucose also increases ATP release in retinal cell cultures (Costa et al., 2009). Moreover, diabetes affects neurotransmitter release in various brain regions, and basal or stimulated neurotransmitter release were found to be increased or decreased (Guyot et al., 2001; Misumi et al., 2008; Morris and Pavia, 2004; Satoh and Takahashi, 2008; Yamato et al., 2004). These evidences strongly suggest that diabetes induces alterations in nerve terminals. Indeed, a reduction in the density of synaptic proteins was found recently in retinal and hippocampal nerve terminals after four weeks of diabetes (Duarte et al., 2009; VanGuilder et al., 2008).

In this work, we further explored the effect of diabetes, particularly during the early stages of the disease (two to eight weeks), on proteins involved in exocytosis in retinal nerve terminals. For that, we analyzed more proteins than previous studies, and evaluated the profile of changes in the retina according to the duration of diabetes. All proteins analyzed in this study have an important role in exocytosis, and changes in their content can have a negative impact on synaptic function.

The content of SNARE proteins, VAMP-2 and syntaxin-1 were altered in retinal synaptosomes, but SNAP-25 protein levels were not. In retinal nerve terminals the alterations in VAMP-2 protein levels were peculiar, since there was a decrease at two weeks, an increase at four weeks and no effect at eight weeks. The decrease in VAMP-2 levels may affect vesicle fusion. Consequently, neurons may overexpress VAMP-2, which may lead to an elongation of individual neurites (Shirasu et al., 2000), as a compensatory mechanism. However, this process is transitory since at eight weeks of diabetes the protein levels of VAMP-2 recover to values similar to controls.

Several studies using knockout (KO) animals have shown the importance of the SNARE complex as a key structure for synaptic vesicle exocytosis. SNAP-25 KO mice leads to lethality at birth and secretion is severely impaired (Sorensen et al., 2003), and in SNAP-25 KO neuronal cultures neurotransmitter release is almost abolished (Bronk et al., 2007). Moreover, HPC-1/syntaxin 1A KO mice show impaired LTP in hippocampal slices and impaired memory consolidation (Fujiwara et al., 2006). In retinal nerve terminals no changes in SNAP-25 content

were detected, however Syntaxin-1 content was decreased in retinal nerve terminals after two weeks of diabetes and returned to levels similar to control at four and eight weeks.

Synapsin-1 binds synaptic vesicles to cytoskeleton components preventing their migration to the presynaptic membrane (Ceccaldi et al., 1995). The loss of synapsin-1 decreases the number of vesicles and might also induce structural changes in nerve terminals (Takei et al., 1995). The content of synapsin-1 significantly decreased in retinal nerve terminals of diabetic animals. In fact, this protein was the most affected in the retina, since its content was altered at all time points studied. Diabetes decreases synapsin-1 phosphorylation in retinal synapses (VanGuilder et al., 2008), that may lead to the immobilization of vesicles within the presynaptic terminal and to the prevention of vesicle fusion. Furthermore, synapsin-1- and synapsin-2-deficient mice exhibit decreased number of synaptic vesicles and synaptic depression upon high-frequency stimulation. They also display impairment in memory performance during senescence (Corradi et al., 2008).

The content of synaptophysin decreased in retinal synaptosomes, but only after two weeks of diabetes. In the retina of diabetic animals, VanGuilder and colleagues (2008) also showed a reduction of synaptophysin levels in retinal synaptosomes and suggested that this reduction may change neurotransmitter vesicle pool by decreasing the vesicle recycling (Daly and Ziff, 2002). The reduction of synaptophysin in retinal nerve terminals might be explained by the degradation of this protein by the ubiquitin-proteosome pathway, which is enhanced by the activation of angiotensin II type 1 receptors under diabetic conditions (Kurihara et al., 2008).

Our results also suggest that a possible impairment of the exocytotic process caused by diabetes does not appear to be due to changes in proteins acting as calcium sensors in nerve terminals. The content of synaptotagmin-1 and rabphilin3a was not affected by diabetes in the retina. Synaptotagmin-1 has two domains that bind Ca^{2+} (Geppert et al., 1994). Synaptic vesicle fusion is also regulated by rab3, a synaptic vesicle GTP-binding protein that binds rabphilin 3a, which has calcium binding domains and the ability to bind the cytoskeleton, participating in synaptic vesicle trafficking (Sudhof, 1997).

In general, the effects observed were not aggravated with increased duration of diabetes, at least for the time points studied. In fact, for a few proteins there was a recovery or a tendency to recover. Moreover, no alterations were detected in the total extracts protein content, whereas the content of several proteins was reduced in nerve terminals; these observations suggest that the alterations detected at the level of nerve terminals may also reflect an impairment of the trafficking of these proteins to the synapse. Regarding this fact, our results differ from those obtained by VanGuilder and colleagues (2008), since they found reduced protein levels in total extracts, and changes were not transitory. These differences might be explained because different rats strains were used, which might respond differently to diabetes. For example, a recent study reported that an inflammatory response is detected in streptozotocin-induced diabetic Sprague-Dawley rats, but not in other rat strains (Kirwin et al., 2009).

As mentioned before, in this work we also investigated the effect of diabetes on the content of exocytotic proteins in the hippocampus, which leaded us to conclude that retina and hippocampus are differently affected by diabetes. The content of SNARE proteins, was not

changed in the same way in these two tissues. VAMP-2 levels were not affected in the hippocampus, whereas in retinal nerve terminals, VAMP-2 levels were changed at all time points studied. Conversely, no changes in SNAP-25 content were detected in retinal nerve terminals, but a significant decrease was detected in hippocampal nerve terminals. Only syntaxin-1 content was decreased in both hippocampal and retinal nerve terminals. Moreover, in hippocampal nerve terminals the changes persisted, whereas in retinal nerve terminals they were transitory, suggesting that the retina is able to recover from or react to the initial insult caused by diabetes. Synapsin-1 content significantly decreased in hippocampal and retinal nerve terminals of diabetic animals. In fact, this protein was the most affected in the retina, since its content was altered at all time points studied. The hippocampus and retina also behaved differently in response to diabetes also for synaptophysin. In hippocampal nerve terminals, the content of synaptophysin increased at all time points, whereas it decreased in the retina, but only after two weeks of diabetes.

Altogether, our results suggest that hippocampal nerve terminals appear to be more affected by diabetes than retina. In fact, in addition to more pronounced and persistent changes in hippocampal nerve terminals, only in hippocampal total extracts did we find a reduction in the content of two proteins, SNAP-25 and syntaxin. Changes were detected early in the retina, but, with the exception of synapsin-1, the protein content in retinal nerve terminals was re-established. Since the retina is known to be early affected by diabetes (Barber, 2003), and memory and cognitive deficits appear later in diabetic patients (Gispenand Biessels, 2000), we would expect more pronounced changes in the retina. Nevertheless, these results demonstrate that the alterations are more prominent and persistent in the hippocampus (Gaspar et al., 2010).

In conclusion, the changes detected in retinal nerve terminals might contribute to synaptic dysfunction and consequently to visual impairments detected in diabetic animals and humans. However, functional studies will be required to assess the effect of diabetes on exocytosis in central synapses.

Acknowledgments

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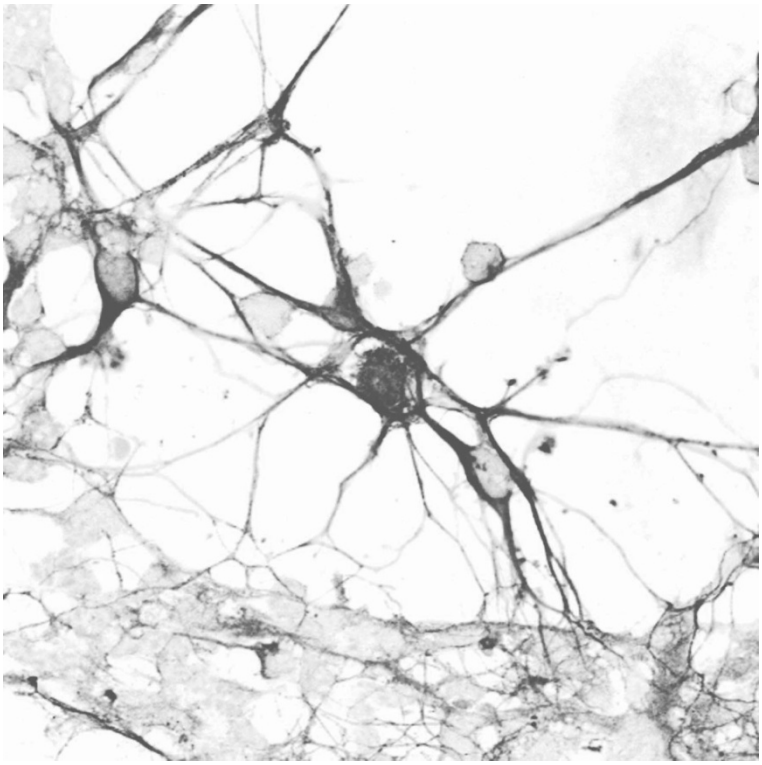
Chapter 3

Diabetes induces early transient changes in the content of vesicular transporters and no major effect in GABA release in retina



This chapter is part of an original research manuscript, published in Brain Research 2011 Apr 6;1383:257-69 by Filipa I. Baptista, Joana M. Gaspar, Armando Cristóvão, Paulo F. Santos, Attila Köfalvi and António F. Ambrósio. The manuscript is a comparative study related with the effect of diabetes on the protein content of vesicular glutamate and GABA transporters and on neurotransmitter release in retina and hippocampus. The results obtained in hippocampus were part of the PhD thesis of Joana M. Gaspar.

Abstract

Diabetes induces changes in neurotransmitter release in central nervous system, which depend on the type of neurotransmitter and region studied. In this study, we wanted to evaluated the effect of diabetes (two and eight weeks duration) on basal and evoked release of [^{14}C]glutamate and [^{3}H]GABA in retinal synaptosomes. We also analyzed the effect of diabetes on the protein content of vesicular glutamate and GABA transporters, VGluT-1, VGluT-2 and VGAT, and on the α_{1A} subunit of P/Q type calcium channels, which are abundant in nerve terminals.

The protein content of vesicular glutamate and GABA transporters, and of the α_{1A} subunit, was affected in retinal synaptosomes. Changes occurred early, at two weeks of diabetes, but after eight weeks almost no changes were detected, with the exception of VGAT in the retina. Regarding neurotransmitter release, no major changes were detected. After two weeks of diabetes, neurotransmitter release was similar to controls. After eight weeks of diabetes, the evoked GABA release decreased in retina. Since the radioactive levels obtained for [^{14}C]glutamate release experiments were too low we were not able to study glutamate release.

In conclusion, diabetes induces early transient changes in the content of glutamate and/or GABA vesicular transporters, and on calcium channels subunit, in retinal synaptosomes, but only minor changes in the release of GABA. These results point to the importance of diabetes-induced changes in neural tissues at the presynaptic level, which may underlie alterations in synaptic transmission, particularly if they become permanent during the later stages of the disease.

Keywords: Diabetes, Retina, Neurotransmitter release, Glutamate, GABA.

Introduction

Diabetes *mellitus* is a metabolic disease resulting from impairment in insulin secretion and/or insulin resistance leading to hyperglycemia. Diabetes is associated with several diseases, such as diabetic retinopathy and encephalopathy (ADA, 2010), which are characterized by functional and structural alterations in retina and brain, respectively.

In the retina, the presynaptic component is affected under diabetic conditions, since a decrease in the levels of several exocytotic proteins in retinal nerve terminals was recently reported (Gaspar et al., 2010a; VanGuilder et al., 2008). In the retina of diabetic animals and in retinal neural cell cultures exposed to elevated glucose, we found an increase in the evoked release of [^{3}H]D-aspartate (marker of the glutamate transmitter pool) (Santiago et al., 2006a) and ATP (Costa et al., 2009). Moreover, elevated levels of GABA and glutamate were detected in the vitreous of patients with proliferative diabetic retinopathy (Ambati et al., 1997). We also reported that diabetes changes the content of ionotropic glutamate receptor subunits in the retina, which may account to retinal dysfunction (Santiago et al., 2009). In addition, it has been suggested that changes in GABA signalling may underlie the alterations in electroretinogram (ERG) responses in streptozotocin (STZ)-induced diabetic rats (Ramsey et al., 2006) and diabetic patients (Ambati et al., 1997). Diabetes modulates the properties of $GABA_C$ receptors in retinal bipolar cells, probably through alterations in the gene expression of GABA receptor subunits, which might therefore underlie changes in ERG of diabetic patients (Ramsey et al., 2007). In the retina of diabetic animals, changes in the content and localization of GABA are evident, with both amacrine and Müller cells accumulating high concentration of GABA (Ishikawa et al., 1996; Takeo-Goto et al., 2002). Moreover, diabetes leads to a generalized attenuation in the content of free amino acids in the retina and to a decrease in the uptake of aspartate, while GABA uptake is enhanced (Vilchis and Salceda, 1996). However, by nuclear magnetic resonance spectroscopy, we recently showed that the levels of the majority of the intermediate metabolites and amino acids present in retina is not affected by diabetes (Santiago et al., 2010).

Importantly, the synaptic level of GABA and glutamate is determined by two important factors: the integrity of the release and re-uptake systems. In nerve terminals, specific vesicular transporters (VGluT1-3 for glutamate, and VGAT for GABA) allow the incorporation of neurotransmitters into synaptic vesicles. These transporters have an essential role in transmitter recycling and homeostasis in the Central Nervous System, and several studies evidence their role in neurological disorders (Benarroch, 2010; Vemuganti, 2005).

Other key elements involved in neurotransmitter release are voltage-dependent calcium channels which mediate the entry of Ca^{2+} ions into nerve terminals. Calcium channels are multisubunit complexes composed of α_1, β, α_2/δ, and γ subunits. The channel activity is directed by the pore-forming α_1 subunit, whereas the others act as auxiliary subunits regulating this activity. The α_{1A} subunit is predominantly expressed in neuronal tissues (Evans and Zamponi, 2006).

Thus, our previous observations showing that diabetes changes the protein content of several exocytotic proteins in rat retinal and hippocampal nerve terminals (Gaspar et al., 2010a) has prompted us to detect further alterations that occur at the presynaptic level and mapping the effect of diabetes on basal and evoked glutamate and GABA release in the retina. Moreover, since the release of glutamate and GABA depend on the transport of the amino acids into synaptic vesicles, we also evaluated the content of VGluT-1, VGluT-2 and VGAT, searching for possible impairments in these proteins under diabetic conditions, and aiming to correlate possible changes with alterations in neurotransmitter release.

Experimental Procedures

Materials

Reagents were acquired from Sigma, St. Louis, MO, USA, with the exception of those described along the text.

Animals

Male Wistar rats (Charles River Laboratories, Barcelona, Spain), eight weeks-old, were randomly assigned to control or diabetic groups. All animals were handled according with the EU guidelines for the use of experimental animals (86/609/EEC). Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, freshly dissolved in 10 mM sodium citrate buffer, pH 4.5). Hyperglycemic status (blood glucose levels exceeding 250 mg/dl) was confirmed two days later with a glucometer (Elite, Bayer, Portugal). Before sacrifice under halothane anesthesia, rats were weighted, and blood samples were collected to measure glucose levels.

Preparation of retinal synaptosomal extracts

The two retinas of each diabetic and age-matched control rats were used for preparation of synaptosomes, as previously described (VanGuilder et al., 2008), with minor alterations. Immediately after animal sacrifice, both eyes were enucleated, and the two retinas were dissected and merged in 10 ml of ice-cold sucrose-HEPES solution. Retinas were washed three times combining gentle vortexing and buffer replacement to remove photoreceptor outer segments. Then, the retinas were homogenized in 4 ml of fresh sucrose buffer and then the homogenate was centrifuged at 200 x *g* for 10 min at 4°C to pellet nuclear fraction. The supernatant was centrifuged at 800 x *g* for 12 min at 4°C. The resulting supernatant was centrifuged at 16,100 x *g* for 20 min at 4°C to obtain the synaptosomal fraction used in release experiments. For synaptosomal extracts the pellet was rinsed in a detergent-based extraction buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.5% SDS, supplemented with complete miniprotease inhibitor cocktail tablets, pH 7.2), at 4°C and stored at -80°C until use.

Preparation of retinal total extracts

The eyes of diabetic and age-matched control animals were enucleated and placed in cold phosphate-buffered saline [(PBS), in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4 at 4°C]. The retinas were lysed in RIPA buffer supplemented with complete mini protease inhibitor cocktail tablets and 1 mM DTT, pH 7.2, at 4°C. Then, the lysates were sonicated and centrifuged at 16,100 x *g* for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

[^{3}H]GABA release assay for retinal synaptosomes

Release experiments were performed as previously described by (Köfalvi et al., 2007), with some modifications. The synaptosomal pellet was resuspended in 1 ml of Krebs solution. The radioalabeled compound [4.2 μCi/ml [^{3}H]GABA (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] was added to the synaptosomes for 10 min at 37°C. All solutions contained the GABA transaminase/ glutamate decarboxylase inhibitor, aminooxyacetic acid (100 mM). Aliquots (200 μl) of the preloaded synaptosomes were transferred to 1 ml of oxygenated Krebs solution and then to perfusion chambers, being trapped in Whatman GF/C filters and superfused continuously at a rate of 0.75 ml/min until the end of the experiment. After a washout period (15 min), samples (2 min perfusion) were collected for liquid scintillation assay. The volume of sample (perfusate) that was collected in the vial for scintillation counting was 1.5 ml. All experimental procedures were performed at 37°C. At the 4th and the 12th min of the sample collection period, release of transmitters was evoked with 30 mM KCl for 1 min each time. In these release experiments, a 30 mM KCl stimulus was chosen because the evoked response triggered with 20 mM KCl in retinal synaptosomes was weak.

Western blot analysis

The protein concentration of each sample was determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 4%-8% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. The membranes were incubated with the primary antibody VGluT-1 1:10,000 and VGluT-2 1:5,000 (Sigma Aldrich, St.Louis, MO, USA), VGAT (1:2,000; Synaptic Systems, Goettingen, Germany) and α_{1A} P/Q Type calcium channel (1:200; Alomone Labs, Jerusalem, Israel) overnight at 4°C. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature with the respective alkaline phosphatase-linked secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK), prepared in TBS-T with 1% low-fat milk. The membranes were processed for protein detection using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare, Buckinghamshire, UK) and

a Storm device (Molecular Dynamics, GE Healthcare, Buckinghamshire, UK). Digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed and tested for β-actin (1:20,000; Sigma, St. Louis, MO, USA) immunoreactivity to prove that similar amounts of protein were applied in the gels.

Radioactivity assay and calculations

The radioactivity released from the synaptosomal preparations was measured with a Packard 2900 Tricarb (Canberra, Australia) liquid scintillation spectrometer, equipped with Dynamic Color Corrected DPM Option providing absolute activity (disintegrations per minute, DPM) calculation and correction for different color quenching. The release of the transmitters was calculated as the percentage of the amount of radioactivity in the tissue at the sample collection time point. We expressed the radioactivity value from each sample (perfusate) as the % of the filter content at the period of time corresponding to the release of each sample, which is called as fractional release (FR%).

Statistical Analysis

Results are presented as mean ± SEM. Statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Student's *t*-test. Differences were considered significant for $p<0.05$.

Results

Animal weight and glycemia

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (289.9 ± 5.1 g for control and 284.6 ± 7.8 g for diabetic animals). The glucose levels were also similar in both groups (91.5 ± 2.4 mg/dl for controls and 86.0 ± 3.5 mg/dl for diabetic animals). Average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table 1. A marked impairment in weight gain occurred in diabetic rats comparing with age-matched controls in both time points analyzed. Diabetic animals also presented significantly higher blood glucose levels comparing to age-matched controls (Table 1).

Table 1: Average weight and blood glucose levels of diabetic and aged-matched control rats.

Diabetes duration		Weight (g)	Blood Glucose (mg/dl)
2 Weeks	Control (n=15)	312.3±13.6	97.0±6.6
	Diabetic (n=15)	240.1±8.2***	467.8±26.9***
8 Weeks	Control (n=15)	401.1±8.1	94.1±2.7
	Diabetic (n=15)	278.5±7.9***	431.0±19.9***

Measurements were made immediately before the sacrifice of the animals. ***p <0.001

Diabetes changes the protein content of vesicular glutamate transporters in retinal synaptosomes after two weeks of diabetes

The protein levels of vesicular glutamate transporters were evaluated by immunoblotting in synaptosomes and total extracts of retina from both diabetic and age-matched control animals. In retinal synaptosomes, the protein content of VGluT-1 and VGluT-2 significantly decreased after two weeks of diabetes (reduction to 69.5 ± 8.8% and 77.5 ± 8.2%, respectively, compared to age-matched controls; Figures 1A and 1B). However, after eight weeks of diabetes no significant changes were observed in retinal synaptosomes. In total extracts (Figures 1C and 1D) no significant differences were detected in the protein content of both vesicular transporters between diabetic and age-matched control animals.

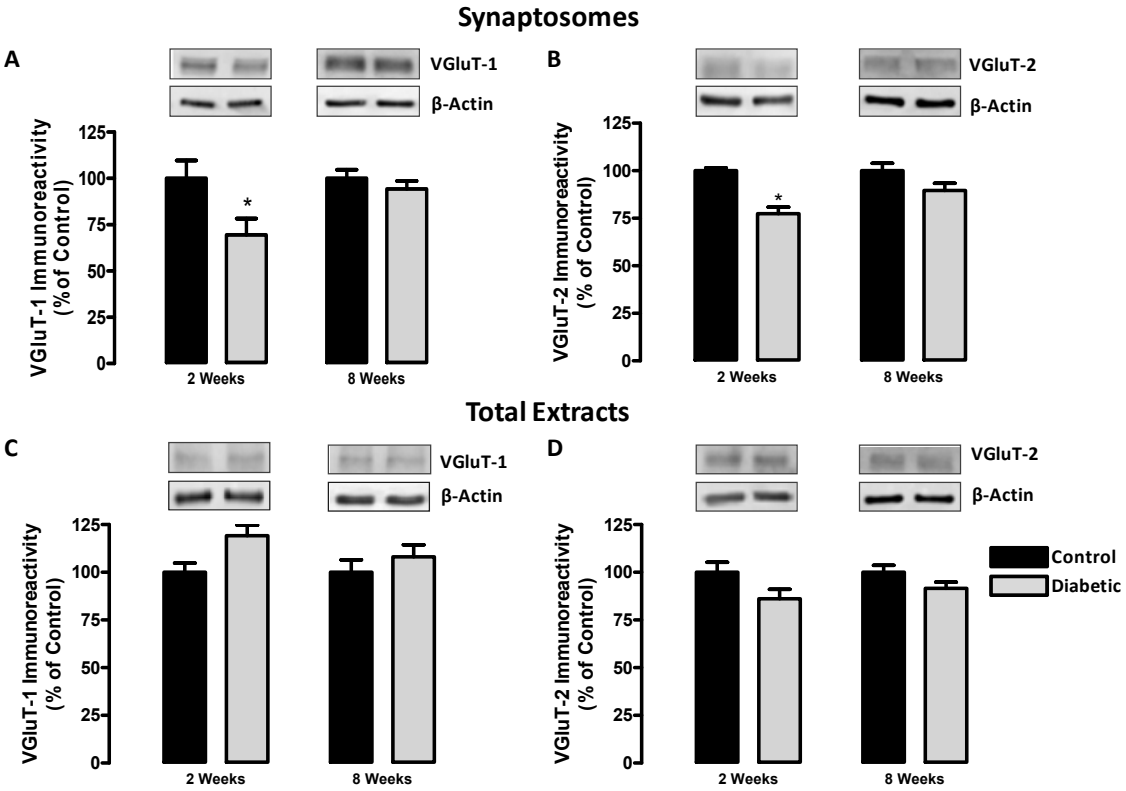


Figure 1: Diabetes induces changes in the protein content of vesicular glutamate transporters in retinal synaptosomes after two weeks of diabetes. The protein levels of VGluT-1 and VGluT-2 were analyzed by immunoblotting in extracts of retinal synaptosomes (A and B) and in retinal total extracts (C and D) isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 5 animals. * $p < 0.05$, compared to age-matched control animals.

Diabetes changes the protein content of vesicular GABA transporter in retinal synaptosomes

The protein content of VGAT significantly decreased after two weeks of diabetes in retinal synaptosomes (reduction to 44.7 ± 3.3%, Figure 2A). However, eight weeks after the onset of diabetes, the protein levels of VGAT were significantly increased in retinal synaptosomes (143.2 ± 3.9%; Figure 2A). In total extracts, no significant differences were observed in the protein levels of VGAT between diabetic animals and age-matched controls (Figures 2B).

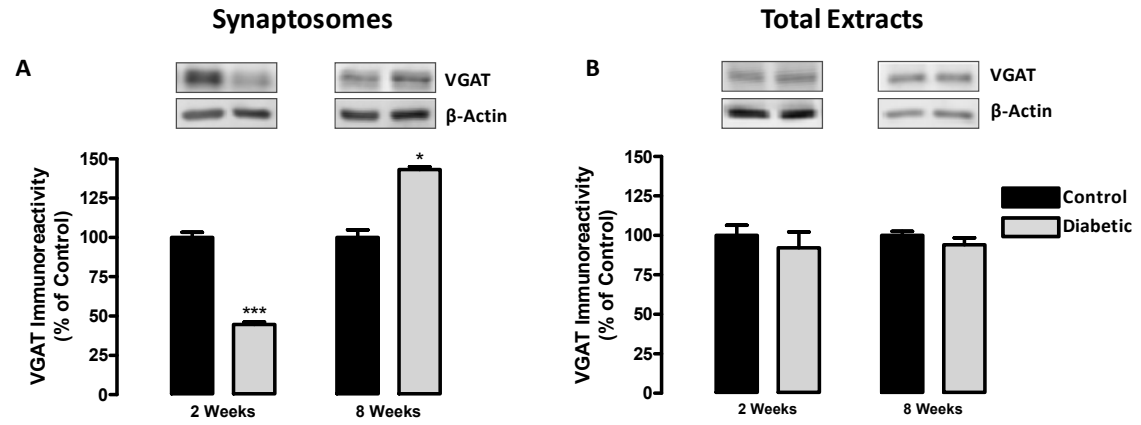


Figure 2: Diabetes induces changes in the protein content of vesicular GABA transporter in retinal synaptosomes. The protein levels of VGAT were analyzed by immunoblotting in retinal synaptosomes (A), and also in retinal total extracts (B), from diabetic animals and aged-matched controls. Representative western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 4 animals. * $p < 0.05$, *** $p < 0.001$, compared to age-matched control animals.

The protein content of the subunit α_{1A} of P/Q calcium channels decreased after two weeks of diabetes in retinal synaptosomes

The protein content of α_{1A} subunit of P/Q type calcium channels significantly decreased in retinal synaptosomes (reduction to 55.2 ± 3.0% of age-matched controls) after two weeks of diabetes (Figures 3A). Conversely, eight weeks after the onset of diabetes, the protein levels of α_{1A} subunit in synaptosomes were similar to those found in controls. In total extracts from retina, no significant changes were observed in the protein levels of this subunit (Figures 3B).

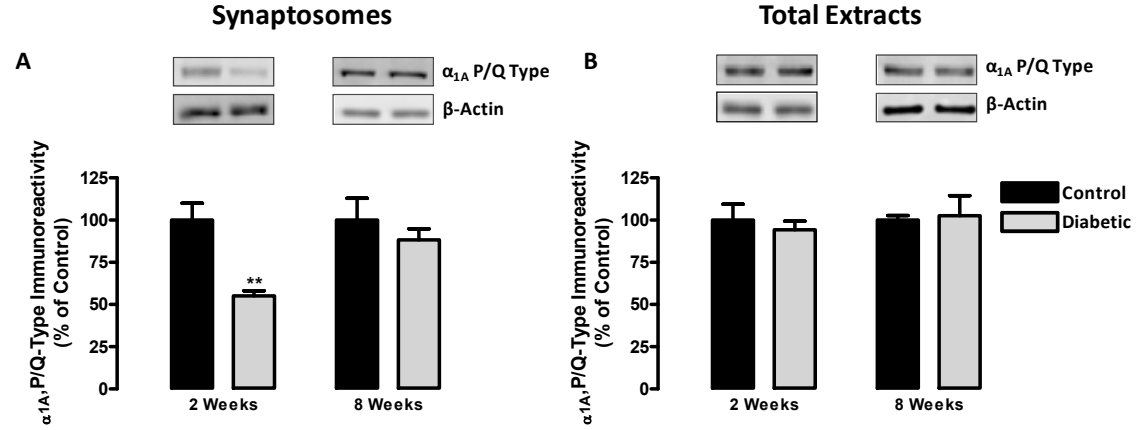


Figure 3: The content of α_{1A} subunit of P/Q type calcium channels decreases at two weeks of diabetes in retinal synaptosomes. The protein levels of α_{1A} P/Q type calcium channels were analyzed by immunoblotting

in retinal synaptosomes (A), and also in retinal total extracts (B), from diabetic animals and aged-matched controls. Representative western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 4 animals. * $p < 0.05$, ** $p < 0.01$, compared to age-matched control animals.

[³H]GABA release decreased after eight weeks of diabetes in retinal synaptosomes

In retinal synaptosomes, we only measured the release of [³H]GABA because the radioactive levels obtained for [^{14}C]glutamate release experiments were too low. The basal release of [³H]GABA before the first stimulus was similar in both control and diabetic animals after two and eight weeks of diabetes (Figures 4A and 4B). After two weeks of diabetes, the KCl-evoked [³H]GABA release (S1 and S2) was similar to the one observed in age-matched control animals (Figure 4A and 4C). However, after eight weeks of diabetes there was a significant decrease in [³H]GABA release after the second stimulus (4.68 ± 0.26 FR% for control and 3.96 ± 0.31 FR% for diabetic animals, respectively; Figures 4B and 4D). Consequently, the S2/S1 ratio at eight weeks of diabetes, but not at two weeks of diabetes, was significantly decreased compared to control (Figure 4E).

Discussion

In the present study, we demonstrated that diabetes induces transient changes in the protein content of vesicular glutamate and GABA transporters and also in the α_{1A} subunit of P/Q type calcium channels in retinal nerve terminals but no changes were observed in total extracts. Moreover, our results show a slight decrease in the evoked release of GABA in retinal synaptosomes. A fine balance between GABA and glutamate is essential for a proper brain and retinal function and any imbalance may lead to physiological alterations. Although these changes, particularly those related with neurotransmitter release, are only slight changes, this study shows that diabetes can affect the pre-synaptic compartment in neuronal issues, and also that the balance between glutamate and GABA might be affected early under diabetic conditions.

Alterations in contrast sensitivity and color perception (Daley et al., 1987; Roy et al., 1986), induced by diabetes, might be due, at least in part, to changes in neurotransmission, at pre- and/or post-synaptic level. Recently, we demonstrated that diabetes induces changes in the content of several synaptic proteins involved in exocytosis in both retinal and hippocampal synaptosomes (Gaspar et al., 2010a), suggesting that diabetes might impair neurotransmitter release early in the course of the disease. In the retina, there are a few studies where the effect of diabetes or hyperglycemic conditions on neurotransmitter release was analyzed. In the retina of diabetic animals and in high glucose-treated retinal neural cell cultures, we showed that the release of [³H]D-aspartate is increased (Santiago et al., 2006a). Likewise, we found that the release of ATP in retinal cell cultures exposed to high glucose is also increased (Costa et al., 2009).

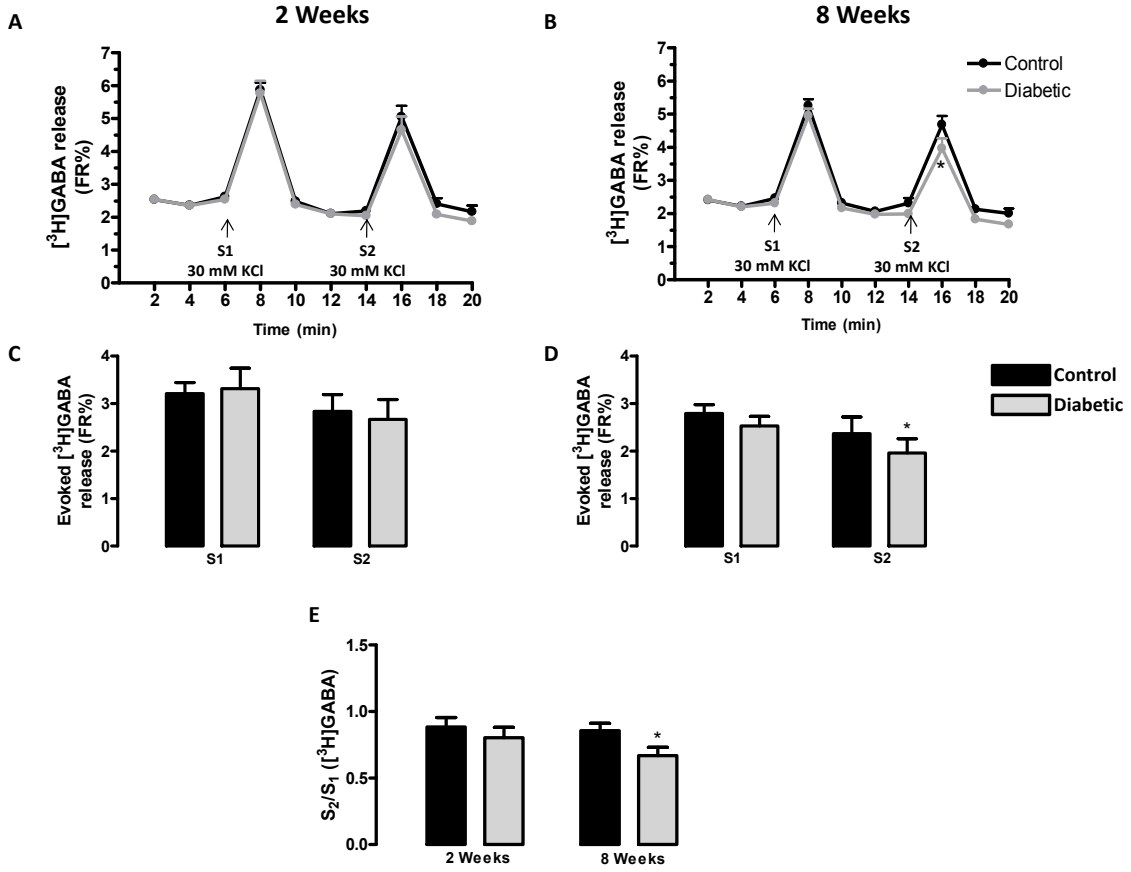


Figure 4: Diabetes affects the release of GABA in retinal synaptosomes. Synaptosomes were loaded with [^{3}H]GABA, and after 15 min of washout, 2 min perfusate samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 30 mM KCl for 1 min each. (A) GABA release at two weeks of diabetes; (B) GABA release at eight weeks of diabetes; (C-D) S1 and S2 at two and eight weeks time points for diabetic and age-matched control animals; (E) S2/S1 ratio at two and eight weeks time points for diabetic and age-matched control animals. All data points represent the mean ± SEM of 11 animals. * $p < 0.05$ compared to age-matched control animals.

VGluT-1 and -2 are specific markers for glutamatergic neurons, and changes in their content may underlie changes in glutamatergic transmission (Benarroch, 2010; Phillips et al., 2010). A significant decrease in the content of both transporters was observed in retinal synaptosomes after two weeks of diabetes. The content of VGAT also decreased after two weeks of diabetes in retinal synaptosomes. This decrease in VGAT, VGluT-1 and -2 content in retinal synaptosomes after two weeks of diabetes, could lead to a slower packaging of GABA and glutamate which can contribute to changes in synaptic transmission. However, our results suggest that although the protein content of VGAT is affected at two weeks, the loading capacity of vesicular transporters appears not to be changed by diabetes, since the uptake of [^{3}H]GABA into synaptosomes was not

affected. However, the decrease in the levels of the vesicular transporters was transient, at least in the early stages of diabetes, suggesting that after the initial stress conditions induced by diabetes both tissues are somehow able to react against diabetes-induced stress. In a previous work, we found that the content of synaptic proteins in retinal synaptosomes is affected mainly after two weeks of diabetes, recovering to control levels for longer periods (Gaspar et al., 2010), suggesting that changes can be plastic and reversible, and neural tissues are able to react against stress conditions, at least temporarily.

The P/Q type voltage-gated calcium channels are abundant in nerve terminals and play a predominant role in neurotransmitter release at central synapses (Ambrosio et al., 1997). P/Q type calcium channels co-localize densely with syntaxin-1 at the presynaptic nerve terminals (Westenbroek et al., 1995) and can be isolated as a complex with SNARE proteins (Bennett et al., 1992; Leveque et al., 1994). As for vesicular transporters, retina recovered the levels of α_{1A} subunit after eight weeks of diabetes, supporting the fact that retina is able to recover from changes occurring in synaptic proteins induced by diabetes, at least temporarily. Impaired Ca^{2+} regulation may result in synaptic dysfunction, impaired plasticity and neuronal degeneration (Mattson, 2007). Elevated glucose impairs calcium homeostasis in retinal neural cells, which may have implications for the mechanisms of vision loss in diabetic retinopathy (Santiago et al., 2006c). The observed decrease in the levels of α_{1A} subunit in diabetic animals might be considered a protective strategy against Ca^{2+} overload.

We found no changes in the release of GABA at two weeks of diabetes in the retina. However, at eight weeks of diabetes we found a small, but significant, impairment in the evoked release of GABA, during the second stimulus. Previously, we demonstrated that the evoked release of [^{3}H]D-aspartate from the retina increases at four weeks of diabetes, increasing also in retinal neural cultures exposed to high glucose (Santiago et al., 2006a). These observations, namely the decrease in the evoked [^{3}H]GABA release and the increase in the evoked release of [^{3}H]D-aspartate (marker of glutamate transmitter pool) in the diabetic rat retinas suggests that diabetes can lead to the impairment of neurotransmission. Moreover, if these alterations in transmitter release persist for longer periods, they can somehow contribute to neuronal apoptosis detected in the diabetic retina (Barber et al., 1998; Park et al., 2003), which may be correlated to the hypothesis of glutamate-induced retinal neurodegeneration in diabetic retinopathy.

As previously mentioned, this work was a comparative study related with the effect of diabetes on the protein content of vesicular glutamate and GABA transporters and on neurotransmitter release in retina and hippocampus. The protein content of vesicular glutamate and GABA transporters, and of the α_{1A} subunit, was differently affected by diabetes in hippocampal and retinal synaptosomes. The changes were more pronounced in the retina than in hippocampus. VGluT-1 and VGluT-2 content was not affected in hippocampus. Moreover, changes occurred early, at two weeks of diabetes, but after eight weeks almost no changes were detected, with the exception of VGAT in the retina. Regarding neurotransmitter release, no major changes were detected. In hippocampal synaptosomes, we were able to evaluate the release of glutamate.

After two weeks of diabetes, neurotransmitter release of both GABA and glutamate was similar to controls in retina and hippocampal synaptosomes. After eight weeks of diabetes, the basal release of glutamate slightly increased in hippocampus and the evoked GABA release decreased in retina.

Taken together, these results demonstrate that diabetes induces alterations in retinal nerve terminals, namely after two weeks of diabetes in the density of vesicular GABA and glutamate transporters and α1A subunit of P/Q type calcium channels. Changes in transmitter release, which were not very pronounced, were only detected at eight weeks of diabetes. If these changes persist or become more prominent, an imbalance between excitation and inhibition might occur, which may lead to neuronal dysfunction, and ultimately to visual impairments detected in diabetic animals and humans.

Acknowledgments

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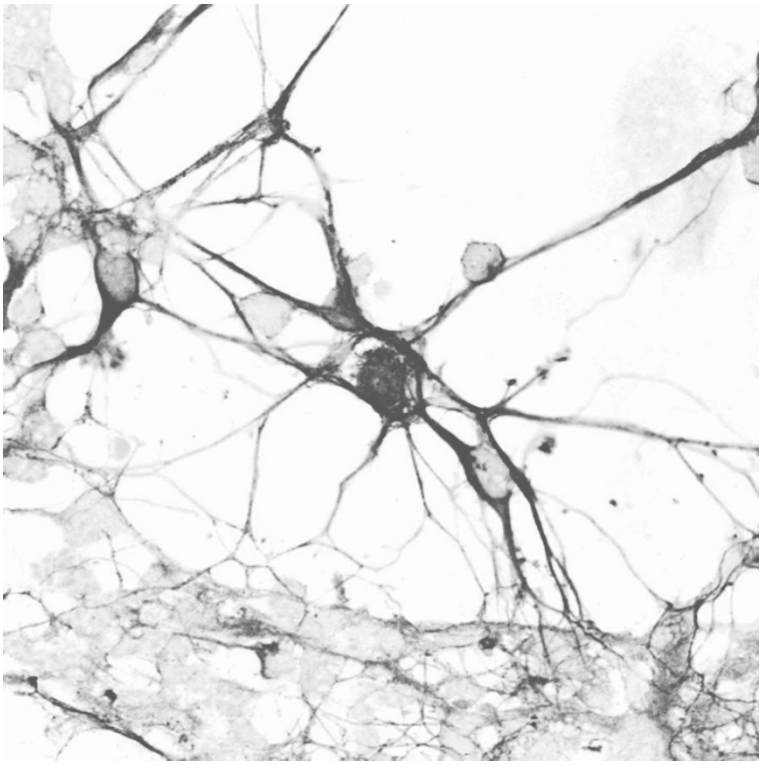
Chapter 4

High glucose does not affect the content and distribution of exocytotic proteins and vesicular glutamate and GABA transporters in cultured retinal neural cells



The results presented in this chapter were submitted for publication by Filipa I. Baptista, Áurea F. Castilho, Joana M. Gaspar, Joana Liberal, Célia A. Aveleira and António F. Ambrósio.

Abstract

Diabetic retinopathy is a leading cause of vision loss and blindness. Increasing evidence has shown that the neuronal components of the retina are affected even before the detection of vascular lesions. Recently, we demonstrated that diabetes decreases the content of synaptic proteins involved in exocytosis in retinal synaptosomes. Hyperglycemia is considered the main factor for the development of diabetic complications, although other factors might contribute for neural changes. To clarify if hyperglycemia can be the main trigger of synaptic changes, we evaluated whether prolonged elevated glucose *per se*, changes the content and distribution of several synaptic proteins in retinal neurons.

Rat retinal neural cells were cultured for 9 days. Cells were exposed to elevated D-glucose or D-mannitol, for 4 or 7 days. The protein content and distribution of SNARE proteins (SNAP-25, syntaxin-1, VAMP-2), synapsin-1, synaptophysin, synaptotagmin-1, rabphilin 3a, VGluT-1 and VGAT, were evaluated by Western blotting and immunocytochemistry.

Prolonged elevated glucose did not alter the total content and cellular distribution of synaptic proteins in both time points studied, suggesting that hyperglycemia may not be the primordial factor contributing for neuronal changes caused by diabetes, but rather a combination of hyperglycemia with other factors, such as the lack of insulin, increased level of glucocorticoids and inflammation.

Keywords: Diabetic retinopathy; hyperglycemia; retinal cells; synaptic proteins; exocytosis.

Introduction

Diabetic retinopathy, a leading cause of vision loss and blindness worldwide, is considered a microvascular disease. However, increasing evidence has shown that the neural components of the retina are also affected, even before the detection of microvascular changes (Antonetti et al., 2006). Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). Moreover, it has been shown that diabetes increases apoptosis in neural cells in human and rat retina early in the course of the disease (Barber et al., 1998; Gastinger et al., 2006). We also showed that long-term exposure (7 days) to elevated glucose increases cell death in primary retinal neural cell cultures (Santiago et al., 2007). Nevertheless, Gaddini and colleagues (2009) demonstrated that no signs of neuronal damage are evident when retinal explants are exposed to high glucose for 48 h.

Hyperglycemia is the hallmark of diabetes and is considered to play a central role in vascular cell dysfunction and degeneration, underlying the progression of diabetic vascular complications (King, 1986; Klein, 1995; Roy et al.). Taking this into account, in previous works, we aimed to evaluate whether hyperglycemia *per se* could affect neural retina, and particularly neurotransmitter release. We showed that prolonged exposure to high glucose increases [^{3}H]D-aspartate release in retinal neural cell cultures (Santiago et al., 2006a), increases the accumulation of extracellular ATP and decreases ATP degradation (Costa et al., 2009), clearly demonstrating that elevated glucose affects the release of neurotransmitters in retinal cells. Moreover, in high glucose-treated cells, the increase in $[Ca^{2+}]_i$ triggered by KCl, kainate or purinergic P2 receptors activation is enhanced and the recovery to basal Ca^{2+} levels is delayed (Pereira et al., 2010; Santiago et al., 2006b), which may account for the increase in neurotransmitter release from retinal neurons. In the retinas of diabetic animals, the release of [^{3}H]D-aspartate evoked by KCl is increased after 4 weeks of diabetes, comparing to the retinas of age-matched control animals (Santiago et al., 2006a). More recently, we also found that after 8 weeks of diabetes the basal release of glutamate is slightly increased in hippocampal synaptosomes and the evoked GABA release is decreased in retinal synaptosomes, showing that diabetes might affect neurotransmitter release (Baptista et al., 2011).

The alterations in neurotransmitter release caused by diabetes/hyperglycemia may result, at least in part, from changes in the exocytotic machinery. Diabetes and elevated glucose downregulate the expression of several genes coding for exocytotic proteins in pancreatic β-cells (Abderrahmani et al., 2006b; Zhang et al., 2002). Additionally, it has been demonstrated that diabetes induces a decrease in the content of some exocytotic proteins in hippocampal and retinal nerve terminals (Duarte et al., 2009; Gaspar et al., 2010a; Ostenson et al., 2006; VanGuilder et al., 2008). Since an impairment of exocytosis might affect neurotransmission, which in turn could contribute to alterations in retinal physiology observed in diabetes, in previous works we further investigated the effects of diabetes/hyperglycemia on several proteins involved in exocytosis in retinal (and hippocampal) neurons. We analyzed more proteins than previous works (Duarte et

al., 2009; Gaspar et al., 2010a; Ostenson et al., 2006; VanGuilder et al., 2008), at different time points of diabetes duration. We demonstrated that the content of several proteins involved in exocytosis, as well as glutamate and GABA vesicular transporters, and the α(1A) subunit of P/Q type calcium channels, can be affected in the retina (and hippocampus), particularly in nerve terminals (Baptista et al., 2011). Taking into account these results, and since hyperglycemia is considered the main trigger of diabetes complications, despite the involvement of other factors, we also aimed to analyze whether prolonged elevated glucose *per se*, which mimics chronic hyperglycemic conditions, could affect the content of exocytotic/synaptic proteins and/or their cellular distribution in both cultured hippocampal and retinal neurons. In hippocampal neurons, a long-term exposure to elevated glucose induces changes in the protein content of SNAP-25, synaptotagmin-1 and VGluT-1, as well as in the cellular localization of syntaxin-1, synaptotagmin-1 and VGluT-1, occurring an accumulation of these proteins in the cell bodies of hippocampal neurons (Gaspar et al., 2010b). In the present work, to check whether hyperglycemia *per se* might be a prevalent feature leading to the molecular changes detected in nerve terminals in the retinas of diabetic animals, we analyzed, in retinal neural cell cultures, the effect of elevated glucose on the content and cellular distribution of several proteins that control and regulate neurotransmitter release: the SNARE complex proteins (VAMP-2, SNAP-25 and syntaxin-1), which are critical for exocytosis, synapsin-1 (binds synaptic vesicles to the cytoskeleton), synaptophysin (presynaptic marker), rabphilin 3a (vesicle-associated protein that is a target of rab 3A small GTP-binding protein), synaptotagmin-1 (Ca^{2+} sensor), and vesicular glutamate and GABA transporters.

Materials and Methods

Primary cultures of rat retinal neural cells

Procedures involving animals were conducted in accordance with the EU Directive 2010/63/EU for animal experiments. Retinal cell cultures were obtained from the retinas of 3–5 days-old Wistar rats, as previously described (Santiago et al., 2006b). Briefly, rat pups were decapitated, and the retinas were dissected under sterile Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4), with a dissecting microscope. The retinas were digested with trypsin (0.1%, 15 min, at 37°C; Gibco Invitrogen Corporation; Carlsbad, CA, USA), in Ca^{2+}- and Mg^{2+}-free Hank's solution. After digestion, the cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated FBS (Gibco Invitrogen Corporation; Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were plated at a density of $2.0x10^6$ cells per cm^2 on poly-D-lysine substrate (0.1 mg/ml). The cells were maintained at 37°C in a humidified incubator with 5% CO_2/air. After 2 or 5 days in culture, cells were incubated with 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for

further 4 or 7 days in culture. The concentration of glucose in control conditions was 5 mM. The cells were used for experimentation on the ninth day of culture.

Preparation of extracts of cultured retinal cells

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at 4°C) and then lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4). The lysates were incubated on ice for 30 min and then centrifuged at 16,100 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 8%-12% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were then incubated with primary antibodies (listed in Table 1) overnight at 4°C. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated with anti-mouse or anti-rabbit alkaline phosphatase-linked IgG secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK) in TBS-T with 1% low-fat milk for 1 h at room temperature. The membranes were processed for protein detection using the enhanced chemifluorescence substrate (ECF; GE Healthcare). Fluorescence was detected on an imaging system (Storm 860 gel and blot image system, Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden) and the digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed and tested for β-actin immunoreactivity (1:5,000) to prove that similar amounts of protein were applied in the gels. The samples corresponding to 4 days exposure to high glucose experiments were applied in different gels of those corresponding to 7 days exposure experiments, which might account for different band intensities, but does not necessarily mean that there was a decrease or an increase in the content of the studied protein. We never analyzed the effect of time of exposure to high glucose on a specific protein.

Table 1. List of primary antibodies

Antibody	Western Blot	Immunocytochemistry	Company
Rabbit anti-Tuj-1	-	1:1,000	Covance
Mouse anti-SNAP-25	1:1,000	1:100	Synaptic Systems
Rabbit anti-VAMP-2	1:2,000	1:100	Chemicon
Mouse anti-Syntaxin-1	1:5,000	1:50	Synaptic Systems
Mouse anti-Synaptotagmin-1	1:5,000	1:100	Synaptic Systems
Mouse anti-Synapsin-1	1:5,000	1:500	Synaptic Systems
Mouse anti-Synaptophysin	1:5,000	1:50	Chemicon
Rabbit anti-Rabphilin 3a	1:1,000	1:100	Synaptic Systems
Rabbit anti-VGAT	1:1,000	1:100	Synaptic Systems
Rabbit anti-VGluT-1	1:5,000	1:100	Synaptic Systems

Immunocytochemistry

Retinal cell cultures were washed three times with PBS and fixed with 4% paraformaldehyde and 4% sucrose for 10 min at room temperature. Cells were then washed three times with PBS and then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. Non-specific binding was prevented incubating cells with with 10% goat serum/0.2% Tween-20 in PBS for 20 min. Cells were then incubated with the primary antibodies (listed in Table 1) for 2 h at room temperature. After incubation, cells were rinsed three times with PBS and incubated with the secondary antibodies Alexa Fluor 594-conjugated anti-mouse IgG (1:250) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:250) for 1 h at room temperature in the dark. The nuclei were stained with DAPI (1:5,000). Upon rinsing three times with PBS, the coverslips were mounted on glass slides using Dako Fluorescent mounting medium (Dako, Denmark). The preparations were visualized in a laser scanning confocal microscope LSM 510 META (Zeiss, Germany).

Statistical analysis

Results are presented as mean ± SEM. Statistical significance was determined by using ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p<0.05$.

Results

High glucose does not affect the content and localization of SNARE proteins

Before evaluating the effects of elevated glucose on the content and localization of exocytotic proteins and vesicular transporter proteins, we analyzed the morphology of retinal neurons by immunocytochemistry using a Tuj 1 (neuron-specific class III beta-tubulin) antibody.

High glucose does not induce changes in exocytotic proteins in retinal neural cells

The exposure of cultured retinal cells to elevated concentrations of D-glucose (30 mM) or D-mannitol (24.5 mM + 5.5 mM glucose), for 4 or 7 days, did not induce any alteration in the neuronal morphology (Fig. 1).

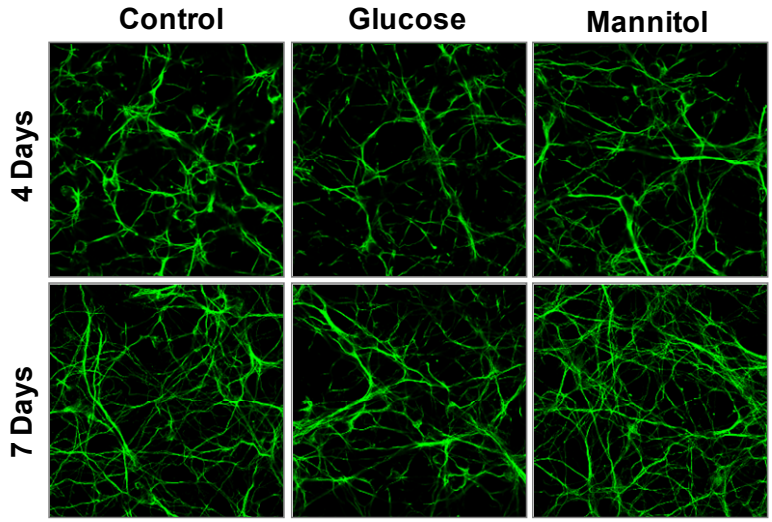
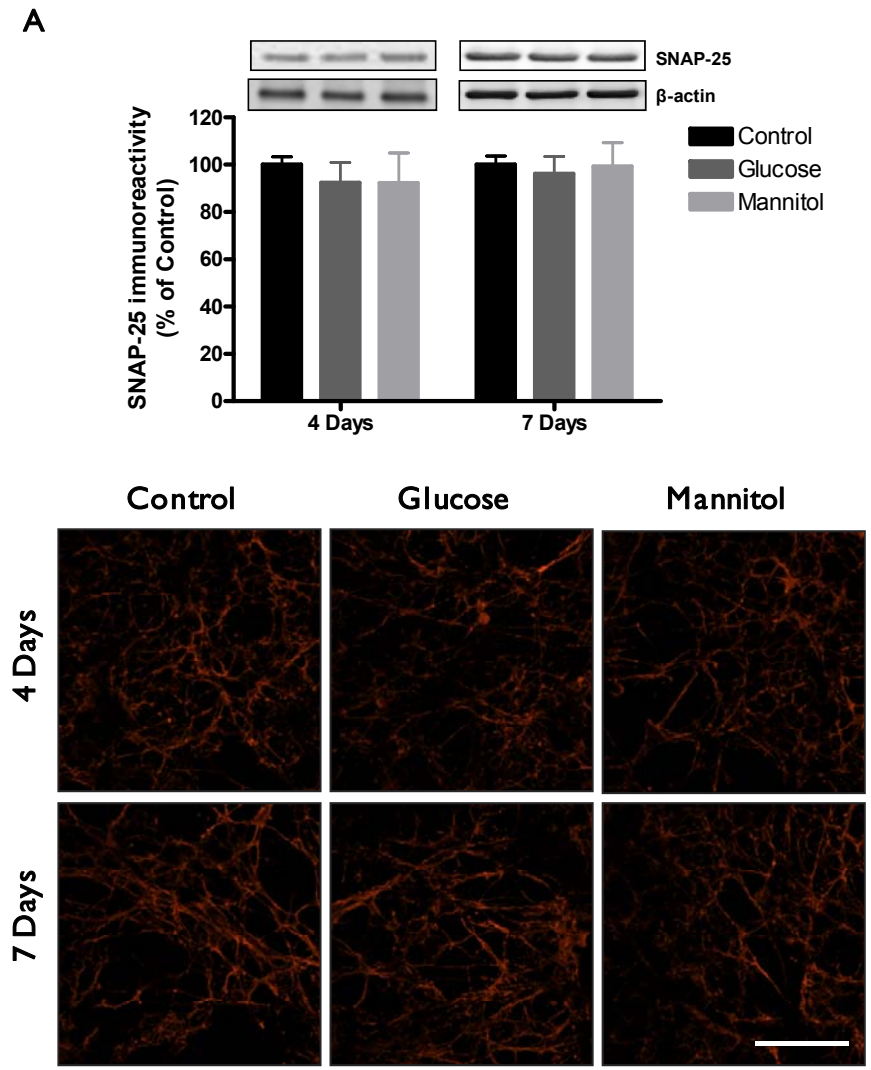


Figure 1. The morphology of retinal neurons is not affected by prolonged exposure to high glucose. Cultured retinal neural cells were exposed to 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, for 4 or 7 days. The concentration of glucose in control conditions was 5 mM. The morphology of retinal neurons was assessed by immunocytochemistry using a Tuj-1 (neuron-specific class III beta-tubulin) antibody. Magnification 630x; Scale bar 50 µm.

Neurotransmission relies on synaptic vesicles fusing with the membrane of nerve cells in order to release their neurotransmitter content into the synaptic cleft. This process requires the assembly of SNAP-25 (synaptosome-associated protein with 25 kDa), syntaxin-1 and VAMP-2 (vesicle-associated membrane protein 2), which constitute the SNARE complex (Rizo and Rosenmund, 2008). Exposure of retinal cells to elevated glucose, or mannitol, did not cause any change in the content of SNAP-25, syntaxin-1 and VAMP-2 at 4 and 7 days of exposure (Fig. 2).

Similarly, no changes were detected in the cellular distribution of these proteins by immunocytochemistry in cells exposed to high glucose or mannitol, when compared to control cells (Fig. 2).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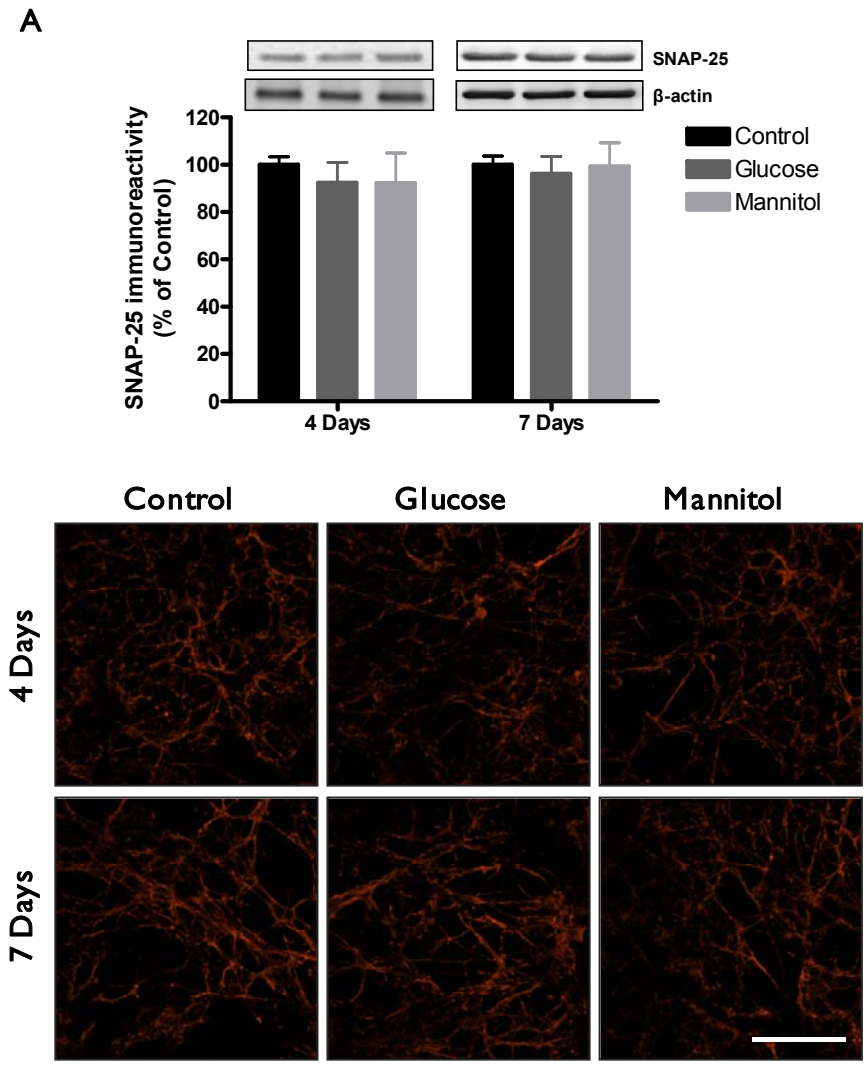
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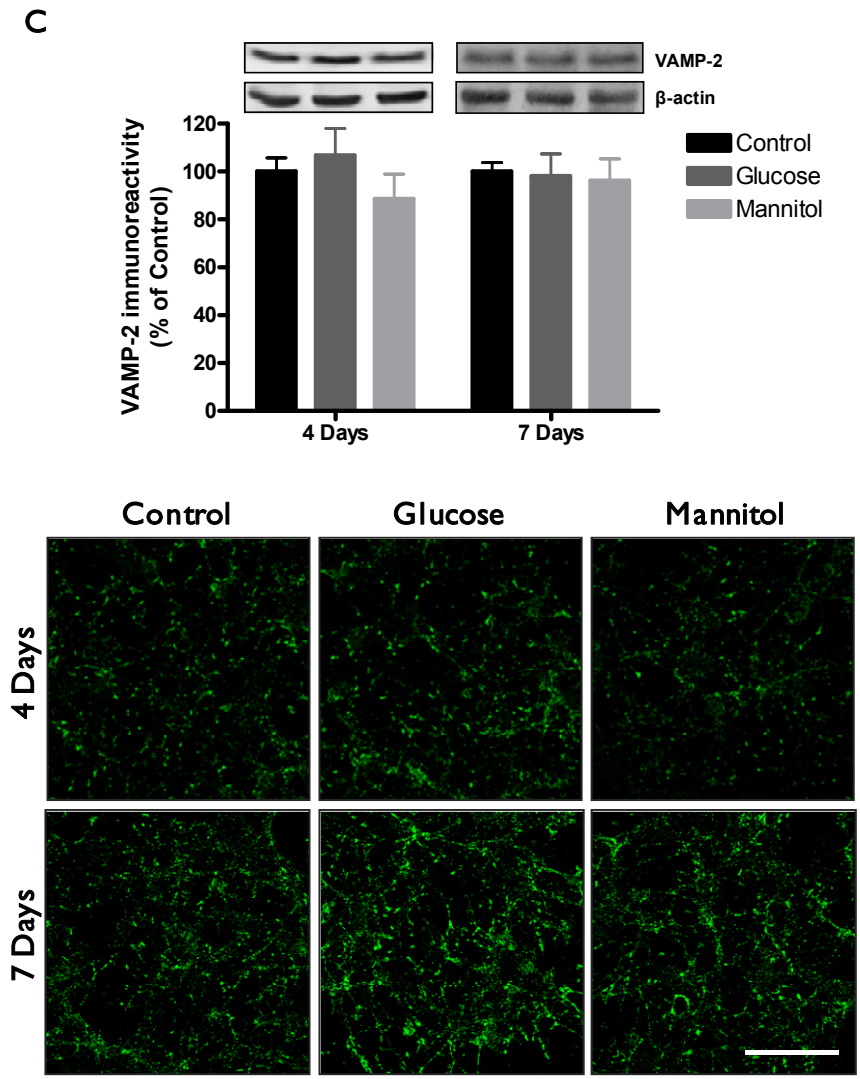
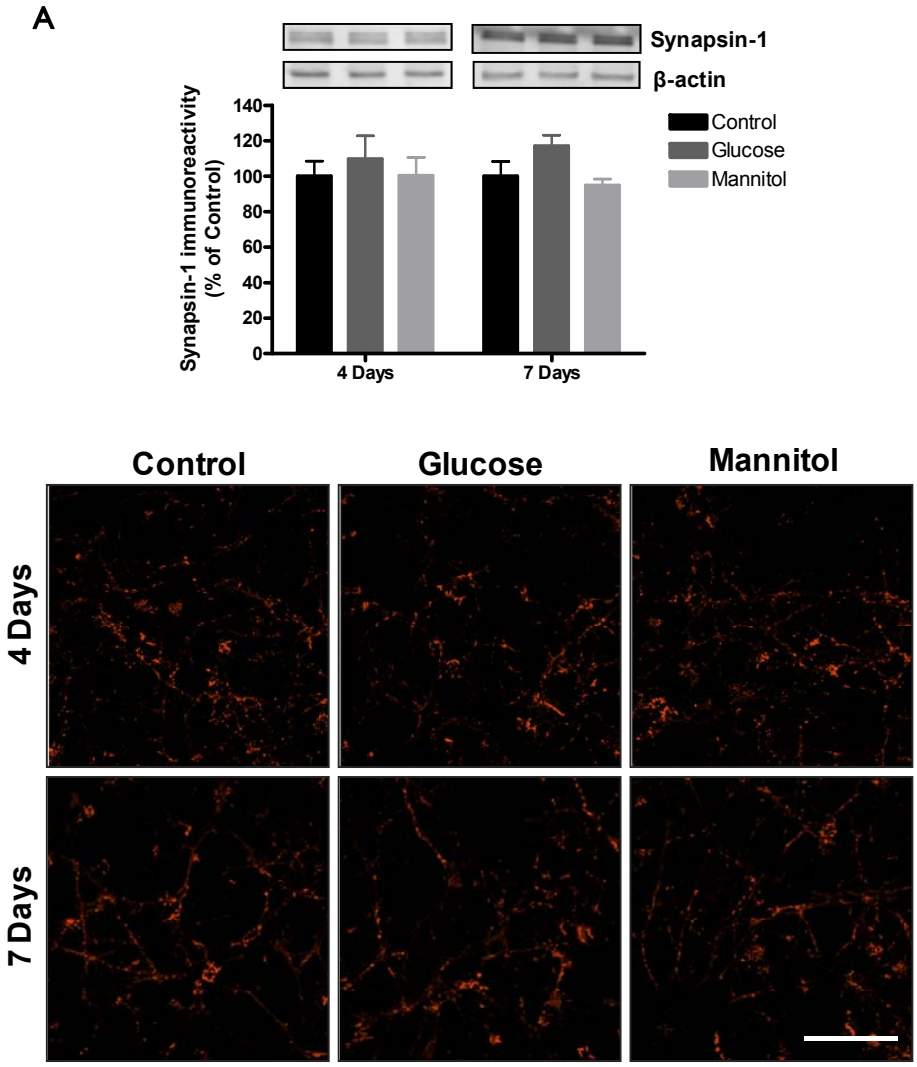


Figure 2. High glucose does not affect the content and localization of SNARE proteins. The protein levels of SNAP-25 (A), syntaxin-1(B) and VAMP-2 (C) were analyzed by Western blotting in total extracts of retinal cell cultures. Representative images of SNAP-25, syntaxin-1 and VAMP-2 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). Results are expressed as percentage of control ± SEM, of at least five independent cell cultures. The immunoreactivity and distribution of SNAP-25, syntaxin-1 and VAMP-2 was analyzed by immunocytochemistry. Representative confocal images are presented below the graphs. Magnification 630x; Scale bar 50 µm.

High glucose does not affect the content and localization of synapsin-1 and synaptophysin

Synapsin-1 binds synaptic vesicles to components of the cytoskeleton, which prevents them from migrating to the presynaptic membrane and release the neurotransmitter (Ceccaldi et al., 1995), while synaptophysin is a membrane glycoprotein of synaptic vesicles with still unknown function and a widely used marker for nerve terminals. The levels of both synaptic vesicle

proteins, synapsin-1 and synaptophysin, were analyzed by Western blotting and no significant changes were found when cells were exposed to high glucose or mannitol for 4 or 7 days. Similarly, by immunocytochemistry, no changes were found in the cellular distribution of these proteins in cells exposed to elevated glucose or mannitol (Figure 3).



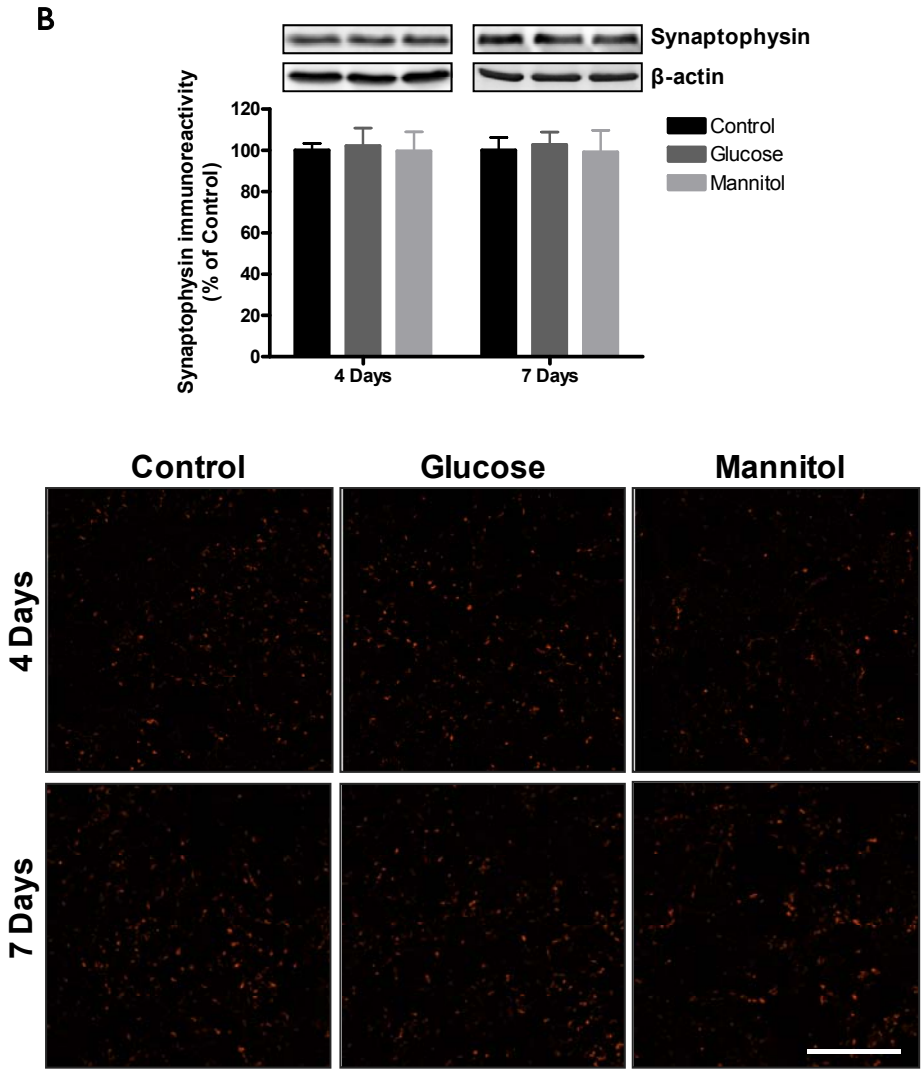
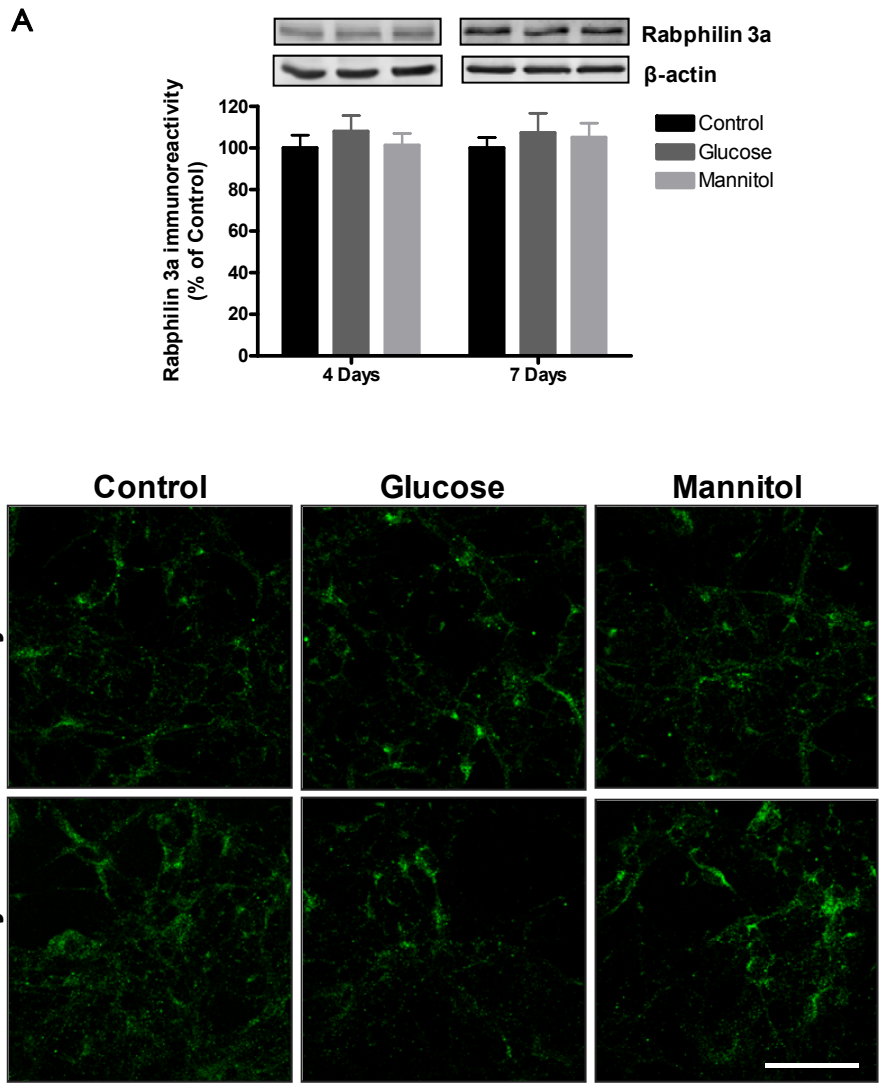


Figure 3. High glucose does not affect the levels and distribution of synapsin-1 and synaptophysin in cultured retinal cells. The protein levels of synapsin-1 (A) and synaptophysin (B) were analyzed by Western blotting in total extracts of retinal cell cultures. Representative images of synapsin-1 and synaptophysin immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). Results are expressed as percentage of control ± SEM, of at least five independent cell cultures. The immunoreactivity and distribution of synapsin-1 and synaptophysin was analyzed by immunocytochemistry. Representative confocal images are presented below the graphs. Magnification 630x; Scale bar 50 μm.

High glucose does not affect the content and distribution of rabphilin 3a and synaptotagmin-1

Rabphilin 3a and synaptotagmin-1 both contain two C2 domains that bind calcium (Chung et al., 1998; Sudhof and Rizo, 1996). These molecules are present in synaptic vesicles, and they have been proposed to act as calcium sensors in synaptic vesicle exocytosis (Geppert and Sudhof, 1998). The exposure of retinal cells to elevated glucose or mannitol for 4 or 7 days did not induce

any changes in the protein levels or in the cellular distribution (Figure 4) of rabphilin 3a and synaptotagmin-1, comparing to control cells.



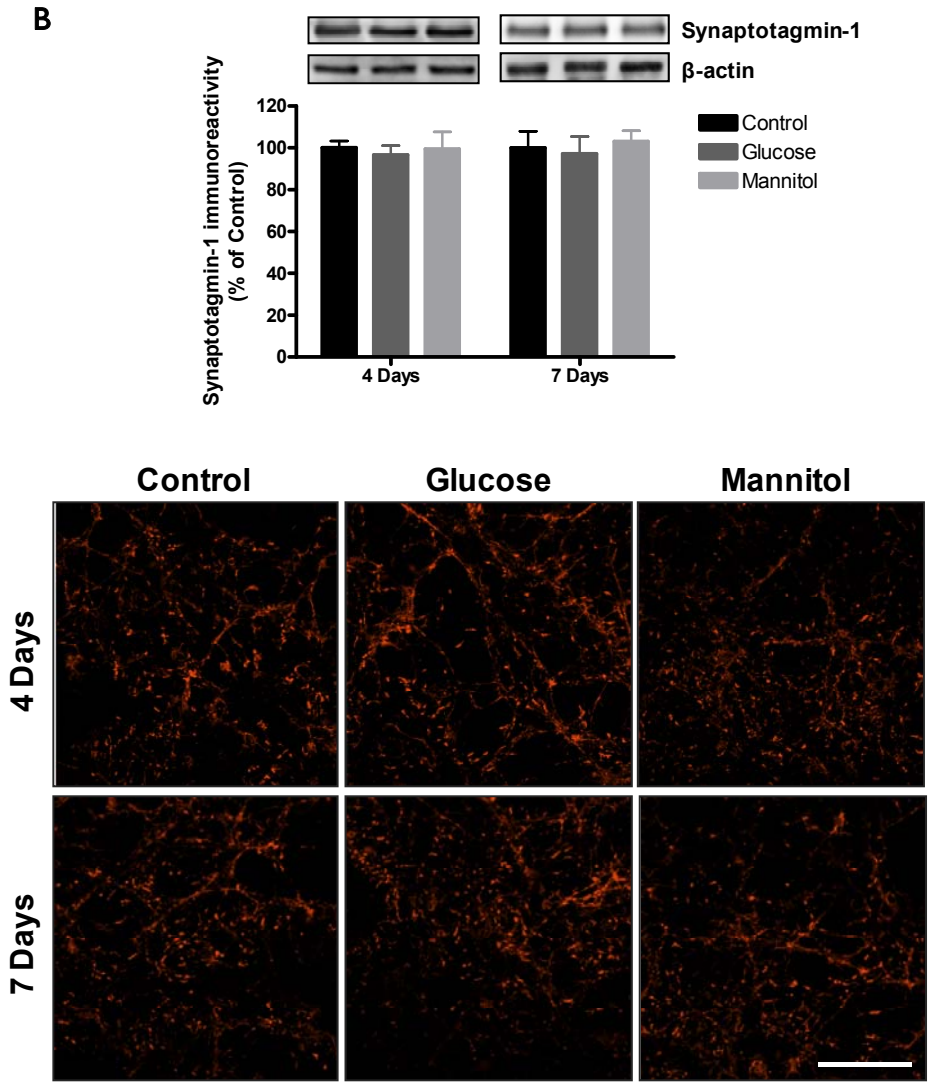
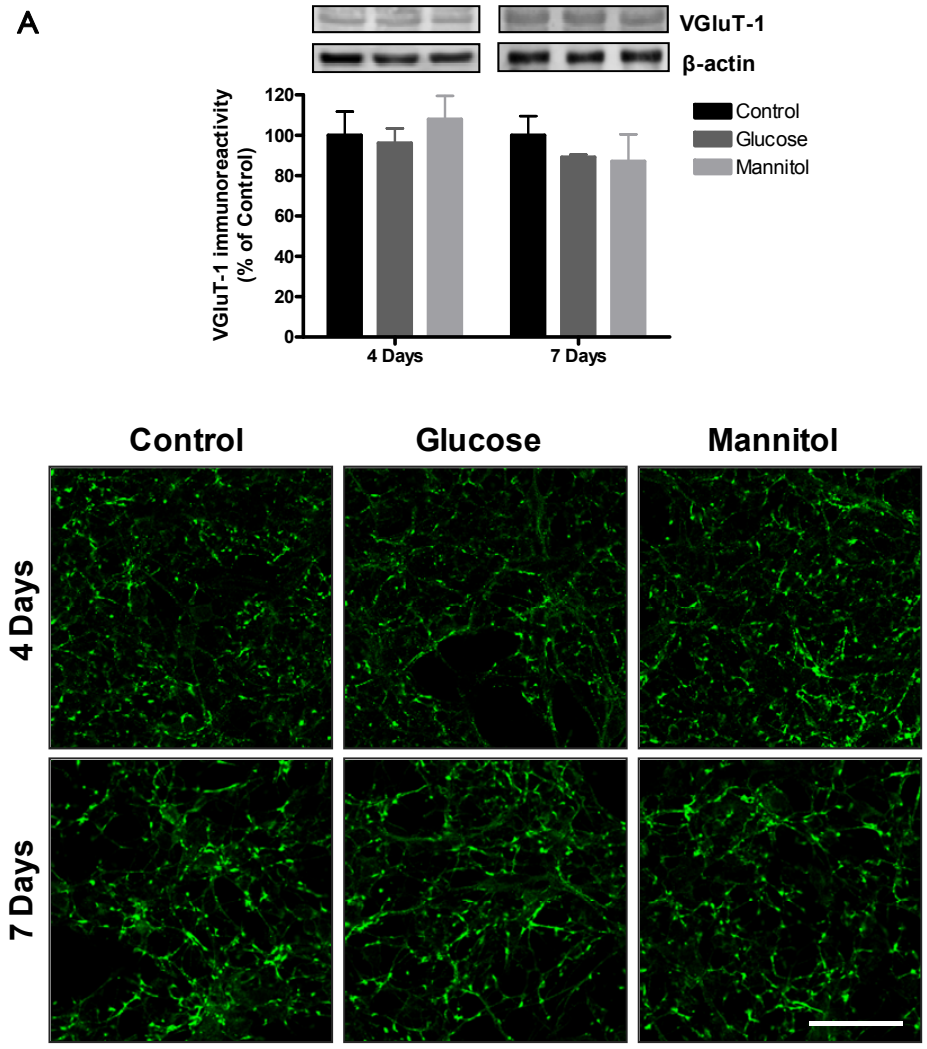


Figure 4. High glucose does not affect rabphilin 3a and synaptotagmin-1 protein levels and cellular distribution in retinal cells. The protein levels of rabphilin 3a (A) and synaptotagmin-1 (B) were analyzed by Western blotting in total extracts of retinal cell cultures. Representative images of rabphilin 3a and synaptotagmin-1 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). Results are expressed as percentage of control ± SEM, of at least five independent cell cultures. The immunoreactivity and distribution of rabphilin and synaptotagmin-1 was analyzed by immunocytochemistry. Representative confocal images are presented below the graphs. Magnification 630x; Scale bar 50 μm.

High glucose does not affect the content and distribution of vesicular glutamate and GABA transporters

The vesicular transporters for glutamate (VGluT-1) and GABA (VGAT) are responsible for the uptake and storage of glutamate and GABA by synaptic vesicles. After 4 or 7 days exposure of cultured retinal neural cells to high glucose, no significant changes were detected in the protein levels or in the cellular distribution (Figure 5) of VGluT-1 and VGAT.



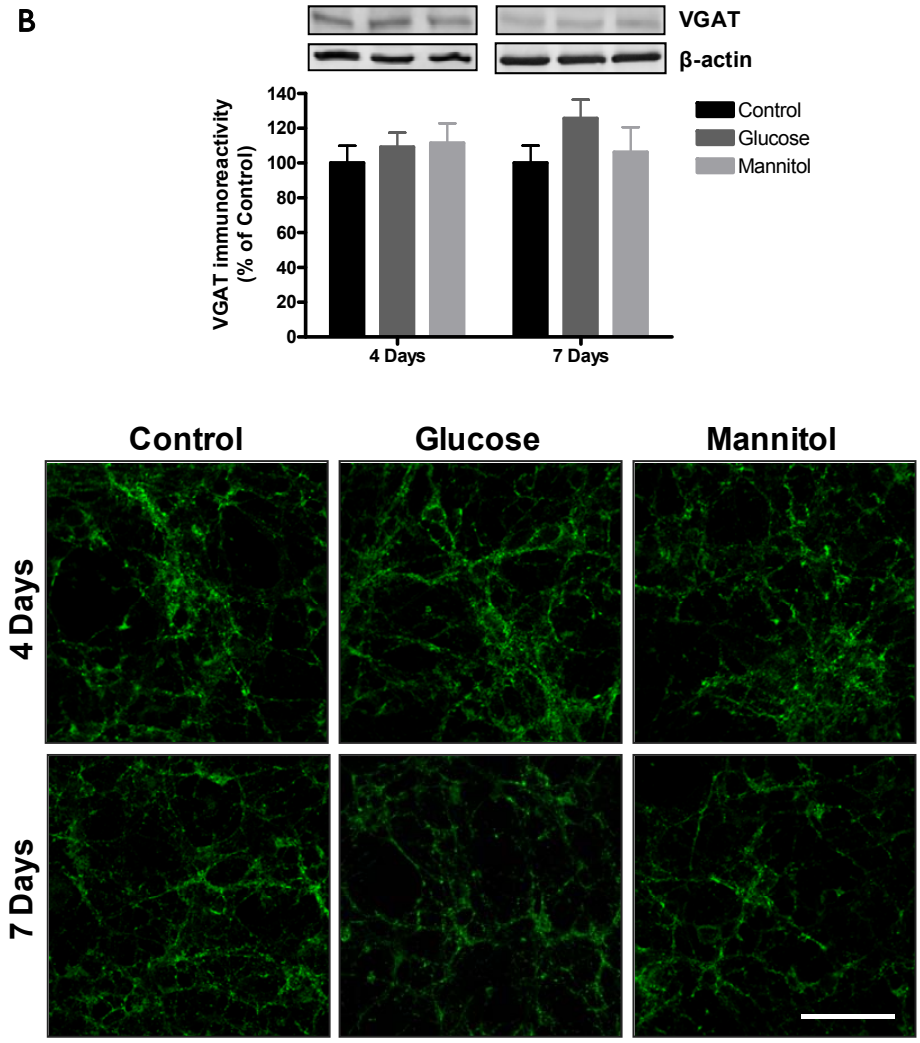


Figure 5. High glucose does not affect the levels of vesicular glutamate and GABA transporters in cultured retinal cells. The protein levels of VGluT-1 (A) and VGAT (B) were analyzed by Western blotting in total extracts of retinal cell cultures. Representative images of VGluT-1 and VGAT immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). Results are expressed as percentage of control ± SEM, of at least five independent cell cultures. The immunoreactivity and distribution of VGluT-1 and VGAT was analyzed by immunocytochemistry. Representative confocal images are presented below the graphs. Magnification 630x; Scale bar 50 μm.

Discussion

In previous works, we investigated the effect of diabetes on the content of several proteins involved in exocytosis in the retina (and hippocampus), analyzing different time points of diabetes duration, and we demonstrated that the content of some of those proteins can be affected, particularly in nerve terminals (Gaspar et al., 2010a). Since hyperglycemia (one factor) is widely

regarded as the main feature contributing for changes found in the retina of diabetic humans and animals, although other factors can contribute to changes detected in several tissues, our main goal was to evaluate whether hyperglycemic conditions could be responsible for the observed effects in synaptic proteins. For that, using cultured retinal neural cells, we checked whether prolonged elevated glucose (4 and 7 days) *per se* (to isolate a single factor) could affect the content of exocytotic/synaptic proteins and/or their cellular distribution in retinal neural cells.

In the present work, we demonstrated that exposure of rat retinal neural cell cultures to high glucose does not alter the content and the cellular distribution of synaptic proteins involved in exocytosis and vesicular glutamate and GABA transporters. Although in this study we did not observe any changes in the content of synaptic proteins in retinal cultures, in a previous study, using the same cultures exposed to high glucose for 7 days, we observed changes in the content of glutamate receptor subunits. We found a significant decrease in the protein content of GluR1 and GluR6/7 subunits, and an increase in the content of GluR2 and KA2 subunits (Santiago et al., 2006b). These observations guarantee that elevated glucose is able to induce molecular changes in neural cells in general, and particularly in retinal cells. Additionally, in hippocampal neurons, we had previously found that long-term exposure to elevated glucose induces changes in the protein content of SNAP-25, synaptotagmin-1 and VGluT-1, as well as in the cellular localization of syntaxin-1, synaptotagmin-1 and VGluT-1, occurring an accumulation of these proteins in the cell bodies of hippocampal neurons (Gaspar et al., 2010b). The differences observed between retinal and hippocampal neurons exposed to prolonged elevated glucose might be due to several reasons. For instance, it is possible that the exposure time of retinal neurons to high glucose (maximum 7 days) was not sufficient to induce changes in the content or distribution of these proteins. Another possibility is that hippocampal neurons may be more susceptible to elevated glucose than retinal neurons. In fact, we did not detect changes in the content of several synaptic proteins in retinal total extracts of diabetic rats, whereas in hippocampal total extracts we did (Gaspar et al., 2010a). Additionally, it is also possible that changes may be occurring specifically at the presynaptic level in cultured retinal neurons. However, by immunocytochemistry, we did not detect any change in the cellular distribution of the synaptic proteins in cells exposed to high glucose, indicating that other factors beside hyperglycemia may underlie changes detected in exocytotic proteins induced by diabetes.

Previous studies have suggested that hyperglycemia might not be responsible for all clinical aspects of diabetic retinopathy, since there are numerous cases of this pathology without evident presence of hyperglycemia (Chan et al., 1985; Harrower and Clarke, 1976), leading to the question if hyperglycemia *per se* is necessary or sufficient for the establishment of the disease (Antonetti et al., 2006; Barber et al., 2001). Other factors, such as the lack (or decrease) of insulin secretion or action, and inflammation may play a role in changes occurring in nerve terminals detected in diabetic animals. Retinal neurons depend on insulin receptor activity for survival (Barber et al., 2001). Long-term instability in retinal insulin signalling may impair insulin-dependent anabolic activities such as protein synthesis in the retina (Chihara, 1981) and increase cell death (Reiter and Gardner, 2003), suggesting that insulin signalling provides neurotrophic actions in the

retina. Therefore, diabetic retinopathy may result in part from neurotrophin deficiency (Whitmire et al., 2011), similarly to peripheral neuropathy (Brussee et al., 2004; Pierson et al., 2003). Early intervention with systemic insulin-like growth factor-I (IGF-I) administration can prevent retinal cell death despite of ongoing hyperglycemia (Seigel et al., 2006). Moreover, it has been shown that insulin treatment starting at the onset of diabetes prevents the development of cognitive deficits in diabetic rats (Biessels et al., 1998). Insulin also prevents diabetes-induced alterations in astrocyte glutamate uptake and the decrease in GFAP expression detected in the cerebral cortex, hippocampus and cerebellum of untreated diabetic rats (Coleman et al., 2010). Moreover, VanGuilder and colleagues (2008) have demonstrated that basal synapsin-I phosphorylation is decreased in the retina of diabetic rats at 1 month of diabetes, which may impede the recruitment of vesicles to the presynaptic terminal, and systemic insulin treatment re-established synapsin-I phosphorylation levels similar to controls, suggesting the importance of insulin for proper neurotransmission in the retina.

Although impaired insulin action is the primary defect of diabetes, it has also been suggested that diabetic retinopathy may result from combinatorial insults including inflammation and increased levels of glucocorticoids (Antonetti et al., 2006). Cytokine-activated microglia release cytotoxins that are responsible for retinal neuronal death (Krady et al., 2005), and the inhibition of microglial activity may be an important strategy in the treatment of diabetic retinopathy. Moreover, a recent study found that $[Ca^{2+}]_i$ changes triggered by purinergic receptors activation, both in retinal neurons and microglial cells, are potentiated in cultured retinal cells exposed to high glucose. This augmented calcium response might account for the increase in the release of inflammatory mediators and neurotransmitters found in diabetic retinas (Pereira et al., 2010).

So, although we did not detect any changes in the expression or localization of exocytotic proteins and glutamate and GABA transporters under high glucose conditions and though we have to consider that other factors, such as the lack of insulin, increased level of glucocorticoids and inflammatory processes may play an important role in the pathogenesis of diabetic retinopathy, this study adds a new perspective regarding hyperglycemia and neural changes in diabetes. In this way, this work made possible to demonstrate that hyperglycemia, commonly regarded as the primordial factor for the development of diabetic complications, may in fact, not be the main feature leading to changes in synaptic proteins observed in diabetic retinas. Eventually, the role of other factors in neuronal dysfunction in diabetic retinopathy might be even more important than hyperglycemia, but this issue needs to be clarified.

Conclusions

The present study shows that exposure of cultured retinal neural cells to high glucose does not induce changes in the content and distribution of synaptic proteins of the exocytotic machinery and vesicular glutamate and GABA transporters, thus suggesting that hyperglycemia might not be the main factor contributing for neuronal changes occurring in retinal neurons in diabetic retinopathy. Our findings, together with previous studies, put forward that other factors

such as the lack of insulin, increased levels of glucocorticoids or inflammation may contribute to changes that have been previously observed in synaptic proteins in retinal nerve terminals of diabetic animals, as well as for alterations in electroretinograms and loss of colour and contrast sensitivity observed in diabetic patients.

Acknowledgments

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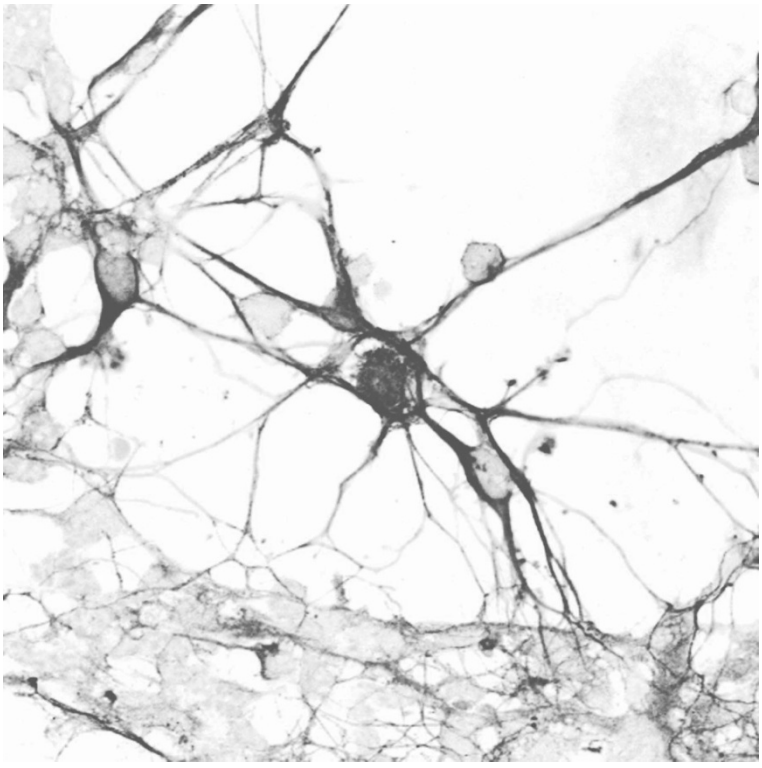
Chapter 5

Cell-specific effects of high glucose and interleukin-1β in retinal neural cell cultures



The results presented in this chapter were submitted for publication by Filipa I. Baptista, Célia A. Aveleira, Áurea F. Castilho, Carolina Fernandes, Núria Simões and António F. Ambrósio.

Abstract

Diabetic retinopathy is considered a microvascular disease, but increasing evidence shows that the neural components of the retina are also affected. Despite hyperglycemia being considered the hallmark of this pathology, it also presents characteristics of a low-grade chronic inflammatory disease, characterized by increased production of cytokines, like interleukin-1beta (IL-1β). However, little is known about the effects of IL-1β on different retinal cell types and how these cells may interact under an inflammatory condition. Using primary rat retinal neural cell cultures, we evaluated if high glucose *per se* was capable of changing the expression of IL-1β in retinal cells, and identified which cell types produce IL-1β and express IL-1RI. Moreover, the effects of high glucose and IL-1β on cell death and proliferation, giving a particular attention to microglial and macroglial cells, and the effect of IL-1β on synaptic proteins, was also evaluated.

In primary retinal cultures, neurons, glial and microglial cells express IL-1β and IL-1RI. Moreover, high glucose *per se* upregulates the levels of IL-1β in these cultures, and high glucose (24h) and IL-1β transiently downregulate IL-1RI protein content. When retinal neural cells were exposed to 10 ng/ml IL-1β for 24 h or 30 mM glucose for 7 days, changes in glial and microglial cells proliferation were detected. High glucose decreased glial and microglial cell proliferation, whereas IL-1β increased their proliferation. Nevertheless, IL-1β did not increase retinal cell death or induce changes in synaptic proteins and neuronal markers content.

IL-1β may play an important role in the pathogenesis of diabetic retinopathy, affecting glial and microglial cells. Consequently, the dysfunction of both cell types may ultimately affect neurons and contribute to neural changes observed in diabetic patients. Particularly, since IL-1β may have an important role in retinal microglial activation and proliferation under diabetes, limiting IL-1β-triggered inflammatory processes could be important to prevent the progression of diabetic retinopathy.

Keywords: Diabetic retinopathy, IL-1β, IL-1RI, High glucose, Retinal neural cells.

Introduction

Diabetic retinopathy is the most common complication of diabetes and a leading cause of blindness in the western countries. Hyperglycemia is considered the primary pathogenic factor for the development of diabetic retinopathy, and the breakdown of blood-retinal barrier (BRB), is one of the first alterations clinically evident and a hallmark of the disease (Cunha-Vaz, 2004). Although this pathology is considered a microvascular disease, increasing evidence has shown that the neural components of the retina are also affected, even before the detection of microvascular changes (Antonetti et al., 2006). Alterations in electroretinograms in diabetic patients and animals, and the loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). Despite the progress in understanding the pathogenesis of diabetic retinopathy, the mechanisms leading to neural dysfunction are far from being completely elucidated.

Several evidences indicate that diabetic retinopathy has characteristics of a low-grade chronic inflammatory disease. Increased production of cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), up-regulation of cyclooxygenase-2 (COX-2), increased expression of adhesion molecules and increased leukocyte adhesion and vascular permeability (Carmo et al., 2000; Kowluru et al., 2003; Miyamoto et al., 1999) have been demonstrated in the retina of diabetic animals. Several genes involved in inflammatory processes are up-regulated early in the diabetic rat retina (Brucklacher et al., 2008; Joussen et al., 2001). In the vitreous fluid of diabetic patients the levels of IL-1β, IL-6 and TNF-α are increased (Abu el Asrar et al., 1992; Patel et al., 2008; Yuuki et al., 2001). Moreover, in the retinas of streptozotocin (STZ)-induced diabetic rats the levels of IL-1β are also increased (Carmo et al., 1999; Gerhardinger et al., 2005; Kowluru and Odenbach, 2004a; Krady et al., 2005), and this increase was correlated with an increase in BRB permeability (Carmo et al., 1999, 2000). It has been shown that Müller glial cells isolated from diabetic rats acquire a complex reactive phenotype in response to diabetes, increasing the expression of inflammation-related genes (Gerhardinger et al., 2005).

A recent study reported that $[Ca^{2+}]_i$ changes triggered by purinergic receptors activation, both in retinal neurons and microglial cells, are potentiated in cultured retinal cells exposed to high glucose (Pereira et al., 2010). This augmented calcium response might account for the increase in the release of inflammatory mediators and neurotransmitters found in diabetic retinas.

Early microglia activation in the retina is a common response to diabetic retinopathy, which is associated with progressive neurodegeneration in the retina. Activated microglia exhibit enhanced proliferation, migration, phagocytosis, and production of different bioactive molecules (Schuetz and Thanos, 2004). Thus, whereas the retina has traditionally been viewed as an immune privileged tissue, evidence is accumulating to support a role for local inflammation in the pathogenesis of diabetic retinopathy (Joussen et al., 2004; Kern, 2007; Krady et al., 2005).

IL-1β is a proinflammatory cytokine that upregulates several inflammatory mediators, including IL-1β itself, TNF-α, COX-2, prostaglandins, inducible nitric oxide synthase (iNOS) and

chemokines (Chai et al., 1996; Chung and Benveniste, 1990; Rothwell and Luheshi, 2000; Sparacio et al., 1992). IL-1β elicits responses in cells only through the activation of IL-1 type I (IL-1RI) receptor, although it can also bind to IL-1 type II receptor (IL-1RII) and to the soluble forms of IL-1RI and IL-1RII. The IL-1R accessory protein (IL-1RAcP) is a key element on the activation of the signal transduction cascade (Boutin et al., 2003; Watkins et al., 1999). Since IL-1β regulates the expression of IL-1RI (Pousset et al., 2001), it will be important to know how diabetes and IL-1β itself affect the expression of IL-1RI in the retina. We previously demonstrated that IL-1RI is highly expressed in retinal microvascular endothelial cells and that high glucose, by osmotic stress, and IL-1β downregulate IL-1RI protein content in retinal endothelial cells (Aveleira et al., 2010a).

In this study, using primary rat retina neural cell cultures, we aimed to evaluate if high glucose *per se* is capable of changing the expression of IL-1β in retinal neural cells. Moreover, we aimed to identify which cell types produce IL-1β and express IL-1RI and study the cell-specific effects of high glucose and IL-1β in retinal cultures trying to clarify which cell types can be mainly affected. We analyzed cell viability/death, cell proliferation, and the expression of several specific cell markers.

Materials and Methods

Primary cultures of rat retinal neural cells

Procedures involving animals were conducted in accordance to the EU guidelines for the use of experimental animals (86/609/EEC) and in accordance to the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals in vision research. Primary rat retinal neural cell cultures were obtained from the retinas of 3–5 days-old Wistar rats, as previously described (Santiago et al., 2006a). Briefly, rat pups were decapitated, and the retinas were dissected under sterile Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4), under a dissecting microscope. The retinas were digested with trypsin (0.1%, 15 min, at 37°C; Gibco Invitrogen Corporation; Carlsbad, CA, USA), in Ca^{2+}- and Mg^{2+}-free Hank's solution. After digestion, the cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (Gibco Invitrogen Corporation; Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were plated at a density of $2.0x10^6$ cells per cm^2 on poly-D-lysine substrate (0.1 mg/ml). The cells were maintained at 37°C in a humidified incubator with 5% CO_2/air. After 2 days in culture, cells were incubated with 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for further 7 days in culture. The concentration of glucose in control conditions was 5 mM. Cells were also exposed to IL-1β (10 ng/ml) or LPS (1μg/ml) for 1 h, 3 h, 6 h or 24 h at DIV (day *in vitro*) 9.

Preparation of total protein extracts of cultured retinal cells

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at 4°C) and then lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4). The lysates were incubated on ice for 30 min and then centrifuged at 16,100 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. Equal amounts of protein were loaded per lane and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 8%-12% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were then incubated with primary antibodies (listed in Table 1) overnight at 4°C. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated with anti-mouse or anti-rabbit alkaline phosphatase-linked IgG secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK) in TBS-T with 1% low-fat milk for 1 h at room temperature. The membranes were processed for protein detection using the enhanced chemifluorescence substrate (ECF; GE Healthcare). Fluorescence was detected on an imaging system (Storm 860 gel and blot image system, Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden) and the digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed for β-actin immunoreactivity (1:5,000) to prove that similar amounts of protein were loaded in the gels.

Immunocytochemistry

Retinal cell cultures were washed three times with PBS and fixed with 4% paraformaldehyde and 4% sucrose for 10 min at room temperature. Cells were then washed three times with PBS and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. Non-specific binding was prevented incubating cells with 10% goat serum/0.2% Tween-20 in PBS for 20 min. Cells were then incubated with the primary antibodies (listed in Table 1) for 2 h at room temperature. After incubation, cells were rinsed three times with PBS and incubated with the secondary antibodies Alexa Fluor 594-conjugated anti-mouse IgG (1:250) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:250) for 1 h at room temperature in the dark. The nuclei were

stained with DAPI (1:5,000). Upon rinsing three times with PBS, the coverslips were mounted on glass slides using glycergel mounting medium (Dako, Denmark). The preparations were visualized in a laser scanning confocal microscope LSM 710 META (Zeiss, Germany).

Table 1. List of primary antibodies

Antibody	Western Blot dilution	Immunocytochemistry dilution	Company
Mouse anti-Tuj-1	1:1,000	1:1,000	Covance
Mouse anti-SNAP-25	1:1,000	1:100	Synaptic Systems
Rabbit anti-VAMP-2	1:2,000	1:100	Chemicon
Mouse anti-Syntaxin-1	1:5,000	1:50	Synaptic Systems
Mouse anti-Synaptotagmin-1	1:5,000	1:100	Synaptic Systems
Mouse anti-Synapsin-1	1:5,000	1:500	Synaptic Systems
Mouse anti-Synaptophysin	1:5,000	1:50	Chemicon
Rabbit anti-Rabphilin 3a	1:1,000	1:100	Synaptic Systems
Rabbit anti-VGAT	1:1,000	1:100	Synaptic Systems
Rabbit anti-VGluT-1	1:5,000	1:100	Synaptic Systems
Mouse anti-NeuN	1:100	_	Chemicon
Rabbit anti-GFAP	1:5,000	1:500	Sigma
Mouse anti-Cd11b	_	1:100	Serotec
Mouse anti-ED-1	1:1,000	1:250	Serotec
Goat anti-IL-1β	_	1:50	RD
Mouse anti-IL-1RI	1:500	1:100	RD
Mouse anti MAP-2	1:1,000	1:500	Sigma
Ki-67	_	1:100	Abcam

Assessment of cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the assessment of cell viability. MTT, when taken up by living cells, is converted from a yellow- to a water-insoluble blue-colored precipitate by cellular dehydrogenases (Mosmann, 1983). Briefly, cells were washed with saline buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.4 $CaCl_2$, 6 glucose, 10 HEPES; pH 7.4), and then saline buffer with MTT (0.5 mg/ml) was added to the cultures and incubated for 1 h at 37°C in a humidified atmosphere with 5% CO_2/air in the incubation chamber. The precipitated dye was dissolved in 0.04 M HCl in isopropanol and the absorbance was measured at 570 nm, with a reference filter at 620 nm. The results are presented as mean ± SEM and represent the percentage of control (no treatment). All experiments were carried out in triplicate.

Terminal transferase dUTP nick end labeling (TUNEL) staining

Cells undergoing apoptosis were identified by TUNEL assay. DeadEnd Fluorimetric TUNEL system, in which fluorescein-12-dUTP is catalytically incorporated at 3´-OH DNA ends using recombinant Terminal Deoxynucleotidyl Transferase, was performed according to the

manufacturer's instructions (Promega Corporation, Madison, WI, USA). Cells were also stained with DAPI to label the nuclei. The images were acquired with an inverted fluorescence microscope (DM IRE2, Leica Microsystems, Cambridge, UK). At least a minimum of 10 random fields in each coverslip were counted.

Isolation of total RNA from retinal cells and cDNA synthesis

Total RNA was isolated using the RNeasy Mini Kit from QIAGEN (GmbH, Hilden, Germany) according to the manufacturer instructions. Briefly, cells were lysed and the extracts kept at −80°C until RNA isolation. Total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 50 µl of RNase-free water by centrifugation. Total amount of RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, Waltham, USA) and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. RNA samples were treated with RNase-free DNAse (QIAGEN) to eliminate any contamination with genomic DNA. Reverse transcription into cDNA was carried out using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 1 µg of total RNA from each sample was reverse transcribed into cDNA in accordance with the manufacturer instructions. Reverse transcription reactions were performed in a thermocycler at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and 4° C for 5 min. cDNA samples were then stored at -20°C until use.

Quantitative real time PCR

Quantitative real-time PCR was performed in an iQ5 thermocycler (Bio-Rad) using 96-well microtitre plates and the QuantiTect SYBR Green PCR Master Mix (QIAGEN)/ iTaq™ SYBR® Green Supermix with ROX (Bio-Rad). The primers for the target rat genes (IL-1β, NM_031512) and the reference gene (rat HPRT, NM_012583) were pre-designed and validated by QIAGEN (QuantiTect Primers, QIAGEN). A master mix was prepared for each primer set containing the appropriate volume of 2x QuantiTect SYBR Green PCR Master Mix and 10× QuantiTect Primer (both from QIAGEN). For each reaction, 18 µl of master mix were added to 2 µl of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 20 µl per well. The reactions were performed according to the manufacturer's recommendations: 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The melting curve protocol started immediately after amplification. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the iQ5 Optical System Software (Bio-Rad). Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency.

ELISA

At DIV 9, the conditioned medium of each well containing primary retinal neural cells exposed to different experimental conditions (control, high glucose, mannitol and LPS) was

removed and stored at -80ºC until performing the ELISA assay. Rat IL-1β ELISA development kit (PeproTech, UK), containing the key components required for the quantitative measurement of natural and/or recombinant rat IL-1β in a sandwich ELISA format (within the range of 47–3000 pg/ml) was used. Each sample was assayed in duplicate using 100 µL of culture medium per well.

Flow Cytometry

The analysis of cells undergoing apoptosis was performed using the Annexin V-FITC Assay Kit (BD Biosciences), following the manufacturer's instructions and using PI staining. Primary cultures were treated as described above. After treatments, cells were detached from plates with trypsin-EDTA (0.05%) and pelleted at 1,000 rpm for 5 min. Harvested cells were washed twice by centrifugation with cold PBS at 1,500 rpm and then diluted in 100 µl of annexin binding buffer per assay. Cells were incubated with annexin V-FITC and PI for 15 min at room temperature in the dark. Later, 200 µl of annexin binding buffer was added to the cells, which were then mixed gently and kept in the dark. The stained cells were analyzed immediately with a FACSCalibur (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser and a 635-nm red diode laser. The collected events per sample were 20,000. Flow cytometry data was analyzed with CellQuest software (Becton Dickinson) and plotted as a function of fluorescence intensity FL-1 (green) vs. FL-3 (red) fluorescence. We used annexin V-FITC (emission 518 nm) vs. PI (emission 617 nm) to identify populations of viable cells (annexin V^-PI^-), early apoptotic cells (annexin V^+PI^-), necrotic cells (annexin V^-PI^+), and late apoptotic cells (annexin V^+PI^+).

Statistical analysis

Results are presented as mean ± SEM. Statistical significance was determined by using ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p<0.05$.

Results

Rat retinal neurons and glial and microglial cells produce IL-1β and express IL-1RI

IL-1β is a pro-inflammatory cytokine that can be synthesized by several cell types such as leukocytes, endothelial cells, neurons and glial cells. Since the retina is composed by different cell types that potentially may produce IL-1β, we first analyzed whether the retinal cells present in culture (neurons, macroglial and microglial cells) were able to synthesize IL-1beta and identified those that can be directly affected by IL-1beta, *i.e.*, cells that express IL-RI. The expression of IL-1beta and the distribution of IL-1RI receptor in primary rat retinal neural cell cultures were investigated by double-labelling immunocytochemistry. Specific cell markers were used to identify the different cell types present in the cell culture: MAP-2/TUJ-I (neurons), GFAP (macroglial cells), CD11b (microglial cells).

IL-1β immunoreactivity appeared with a diffuse cytosolic punctate pattern in MAP-2-positive cells, indicating that retinal neurons are able to produce IL-1β (Figure. 1A). IL-1β-positive cells were

also immunoreactive for GFAP, a marker of astrocytes and Müller cells, and CD11b (Figure. 1A), a marker for microglia, indicating that both glial and microglial cells also express IL-1β.
A punctate immunoreactivity was also observed in retinal cells when the expression of IL-1RI was analyzed. In neurons, IL-1RI was localized in the cell body and distributed along the cell processes (Figure. 1B). In macroglial and microglial cells, IL-1RI was diffusively distributed through the cytosol (Figure. 1B). These observations indicate that retinal neural cells express IL-1β and can be responsive to it, since they also express IL-1RI.

High glucose increases IL-1β production in primary rat retinal neural cell cultures

Since hyperglycemia is considered the hallmark of diabetes (King, 1986; Klein, 1995) and since in the retinas of diabetic rats the levels of IL-1β are increased (Carmo et al., 1999; Gerhardinger et al., 2005; Kowluru and Odenbach, 2004a; Krady et al., 2005), we evaluated the effect of elevated glucose on IL-1β expression in retinal neural cultures in order to evaluate if high glucose *per se* is capable of increasing the expression of IL-1β.
Using an ELISA assay, we measured the content of IL-1β in the conditioned medium of primary retinal neural cell cultures. A significant increase in IL-1β levels was detected in high glucose-treated cells (from 35.4 ± 5.8 pg/ml in control to 79.5 ± 7.9 pg/ml in elevated glucose condition). No changes were detected in cells exposed to mannitol, demonstrating that the effect was not due to the increase in osmolarity. As expected, a significant increase in IL-1β levels was detected in LPS-treated cells (positive control; increase to 798.7 ± 330.2 pg/ml) (Figure. 2A).
To determine if alterations in the protein content were due to alterations in mRNA expression, the mRNA expression of IL-1β was evaluated by qPCR in these retinal cell cultures. A significant increase in IL-1β mRNA content was detected (increase to 248.5 ± 55.5% of the control) in high glucose-treated cells. However, no changes were observed in cells exposed to mannitol, demonstrating again that the effect of glucose was not due to the increase in osmolarity. In addition, as expected, a significant increase in IL-1β mRNA was detected in LPS-treated cells (increase to 39,868.7 ± 4,043.4%) (Figure. 2B).

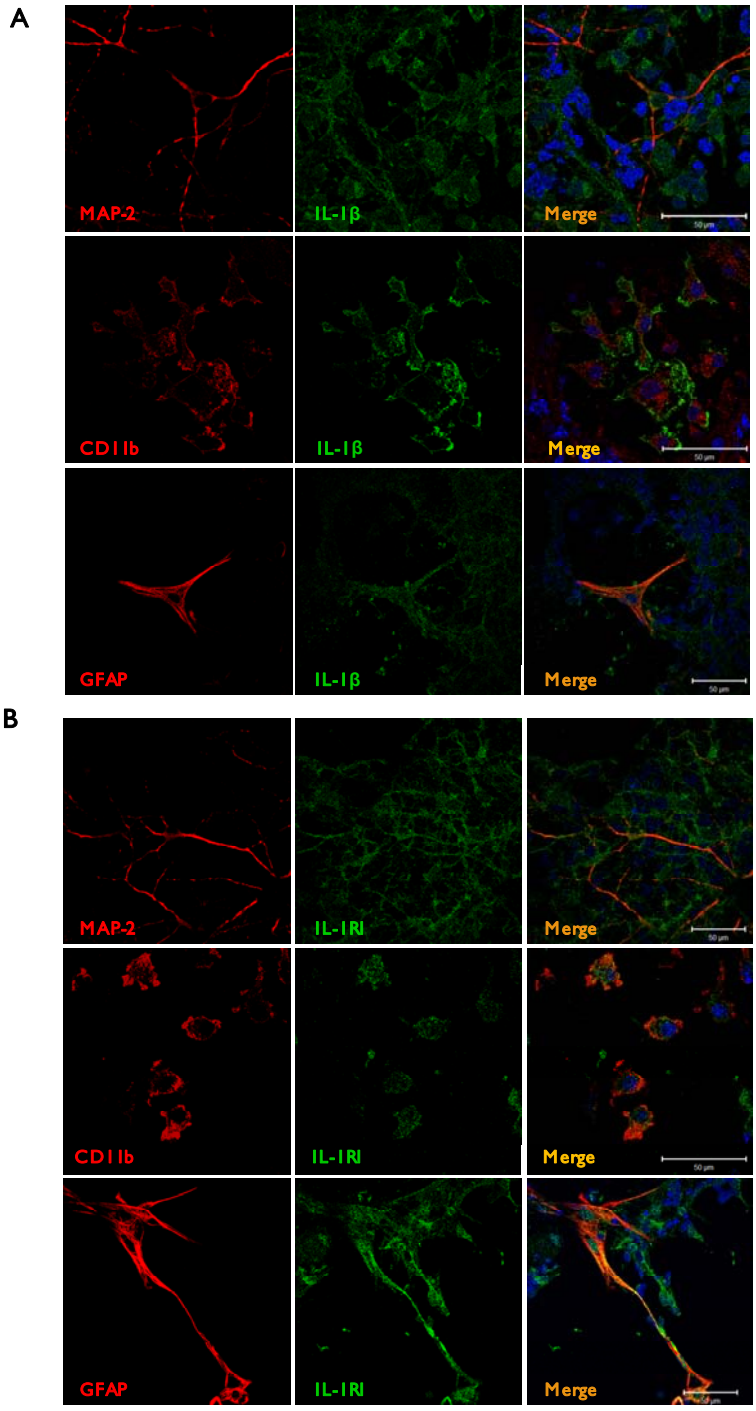


Figure 1. Rat retinal neurons and glial cells produce IL-1β and express IL-1RI. IL-1β and IL-1RI immunostaining (green) can be observed in rat retinal neurons, glial cells (Müller cells and astrocytes) and microglia (red), showing that retina neural cells express IL-1β and can be responsive to it, since they also express IL-1RI. Magnification: 630x (neurons and microglia) or 400x (glial cells).

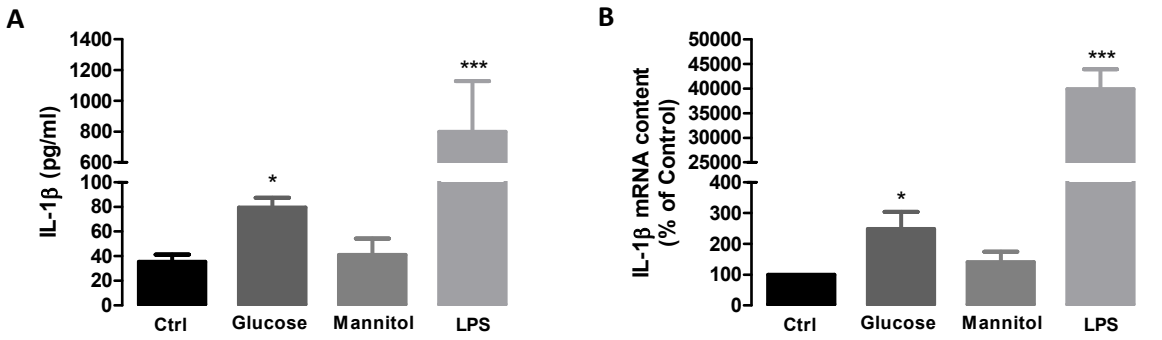


Figure 2. Upregulation of IL-1β in rat retinal cell cultures exposed to elevated glucose. Elevated glucose increases the levels of IL-1β in the culture medium, as measured by ELISA (A); Elevated glucose upregulates the IL-1β mRNA expression, as assessed by RT-qPCR (B). The results represent the mean ± SEM of at least 4 independent experiments and are presented as percentage of control. *p<0.05, ***p<0.001, significantly different from control; one-way ANOVA followed by Dunnett's post-hoc test.

High glucose decreases IL-1RI protein levels in primary retinal neural cell cultures

Retinal cells were also exposed to 30 mM glucose (short and long-term exposures) to investigate the effect of high glucose (hyperglycemic-like condition) on IL-1RI protein levels. Short-term exposure (1–24 h) to high glucose significantly downregulated IL-1RI protein levels at 24 h of exposure (78.2 ± 5.7% of control; Figure. 3A). Short-term exposure to mannitol did not significantly affect IL-1RI protein levels, proving that the effect of glucose on IL-IRI content was not due to an increase in osmolarity (Figure. 3B). Since the levels of IL-1β have been shown to be increased in the retinas of diabetic animals (Carmo et al., 1999; Kowluru and Odenbach, 2004a) and that high glucose increases IL-1β in these cultures, the effect of IL-1β on IL-1RI protein expression in retinal neural cells was also evaluated. Cells were exposed to 10 ng/ml IL-1β for 1, 3, 6 and 24 h. IL-1β induced a transient downregulation of IL-1RI (76.9 ± 3.5% and 78.9 ± 3.0% of control, at 3 and 6 h of exposure, respectively; Figure. 3C), recovering basal levels at 24 h exposure. We also analyzed the effect of a chronic exposure (7 days) to high glucose and mannitol on IL-1RI protein content, and in both experimental conditions no changes in IL-1RI protein levels were detected.

IL-1RAcP is an essential component of the IL-1RI complex, being required for the activation of the signal transduction cascade upon ligand binding to IL-1RI. Since high glucose and IL-1β decreased IL-1RI protein levels in retinal cultures, we also evaluated whether IL-1RAcP protein content could be affected under these conditions. Short- (1–24 h) and long-term (7 days) exposure to high glucose and mannitol did not change IL-1RAcP protein levels in retinal cultures. The protein content of IL-1RAcP was also unchanged by IL-1β treatment (data not shown).

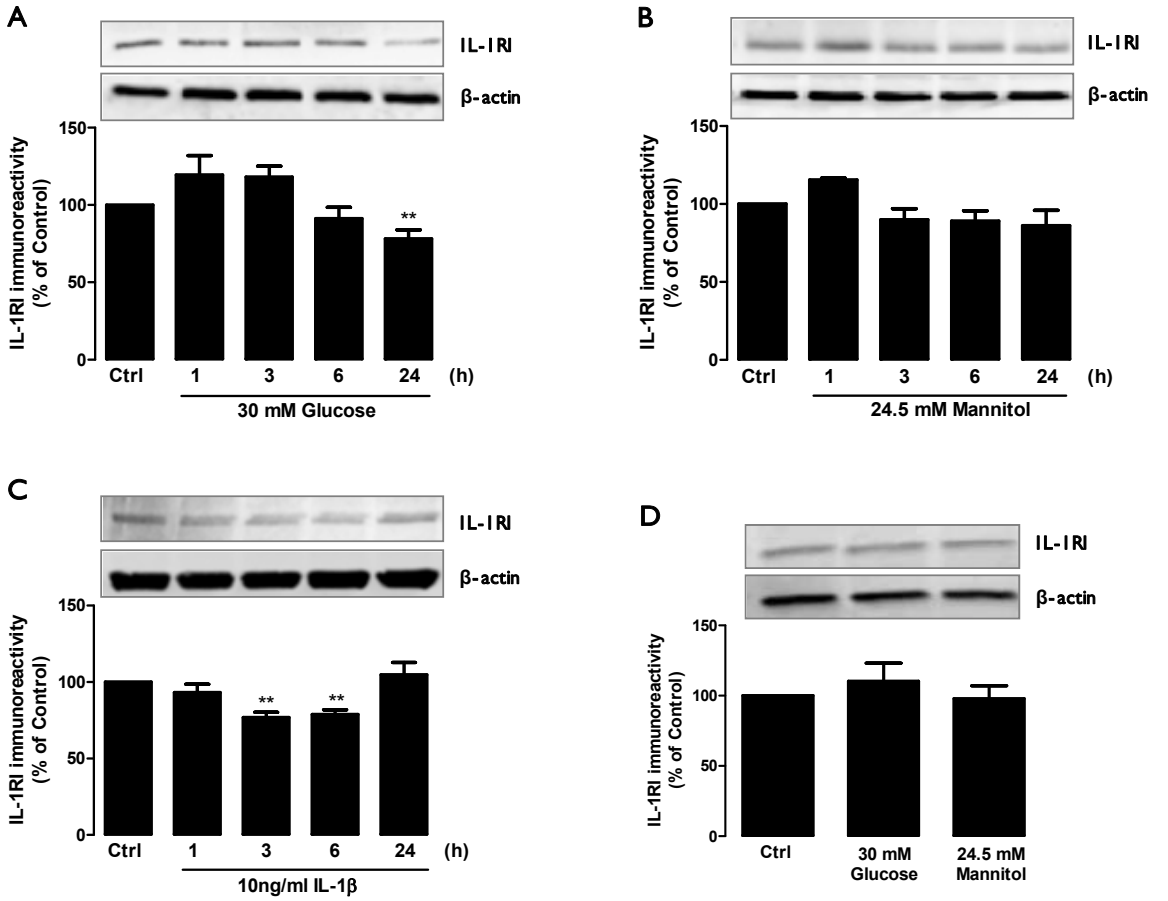
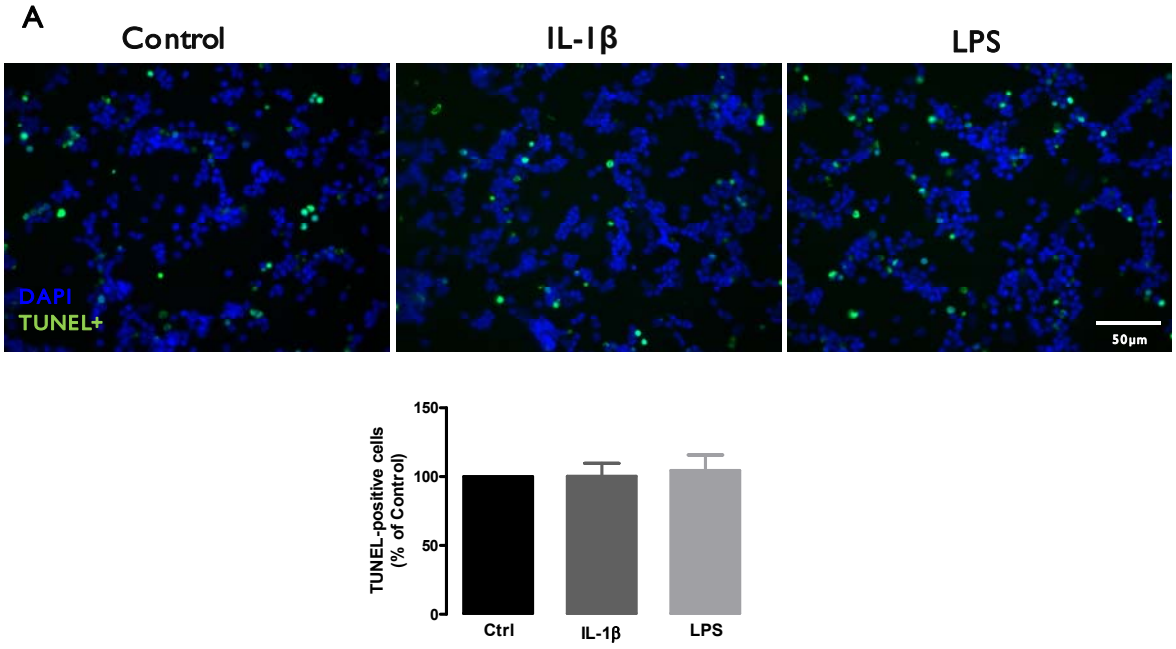


Figure 3. Effect of high glucose and IL-1β on IL-1RI protein levels in retinal neural cells. Cells were exposed to 30 mM glucose (A), 24.5 mM mannitol (plus 5.5 mM glucose) (B), and IL-1β (C), for 1, 3, 6 and 24 h, or high glucose and mannitol for 7 days (D). IL-1RI immunoreactivity was analyzed by Western blotting in total cell extracts. Representative Western blots for IL-1RI and β-actin (loading control) are presented above the graphs. The intensity of the bands was determined by quantitative densitometric analysis. The results represent the mean ± SEM of, at least, 4 independent experiments and are expressed as percentage of control. **p<0.01 significantly different from control, as determined by ANOVA followed by Dunnett's post-hoc test.

Exposure to IL-1β does not increase retinal neural cell death

Previous studies from our laboratory (Costa et al., 2012; Santiago et al., 2007), showed that after 7 days of exposure to high glucose, a decrease in cell viability was concomitant with an increase on the number of apoptotic nuclei detected by TUNEL assay. Since IL-1β is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis (Lin et al., 2012; Simsa-Maziel and Monsonego-Ornan, 2012), we evaluated whether the exposure to IL-1β *per se* could increase apoptosis in

retinal neural cells. Retinal cell cultures were exposed to IL-1β (10 ng/ml, 24 h), and also to LPS (1 μg/ml, 24 h), which was used as a positive control, and retinal cell viability was evaluated by TUNEL assay and flow cytometry. IL-1β and LPS treatment did not change the number of TUNEL-positive cells as compared to control conditions (Figure. 4A). To confirm these results we additionally performed flow cytometry with annexin V and PI (propidium iodide), aiming to distinguish the features of apoptotic vs. viable cells. A typical representative dot plot analysis of retinal cells after exposure to IL-1β or LPS is shown in Figure. 4B. Dot plot analysis of retinal neural cells treated with IL-1β for 24 h (10 ng/ml) showed that 94.8 ± 0.3% of cells are viable, similarly to control conditions (94.5 ± 0.5% of the cells were viable), demonstrating that IL-1β (and LPS) did not increase cell death in these cultures. Interestingly, the exposure of retinal neural cells to IL-1β for 24 h induced an increase in the reduction of MTT (120.5 ± 4.4% of the control; Figure. 4C), which is frequently used as a viability or proliferative assay. Since IL-1β did not increase cell death, this observation suggests that IL-1β was enhancing cell proliferation. In order to verify whether the increase in the MTT reduction induced by IL-1β was mediated by the activation of IL-1RI receptor, retinal cells were exposed to IL-1β together with an anti-IL-1RI antibody, for 24 h. The presence of the anti-IL-1RI antibody prevented the increase in the MTT reduction induced by IL-1β (Figure. 4C).



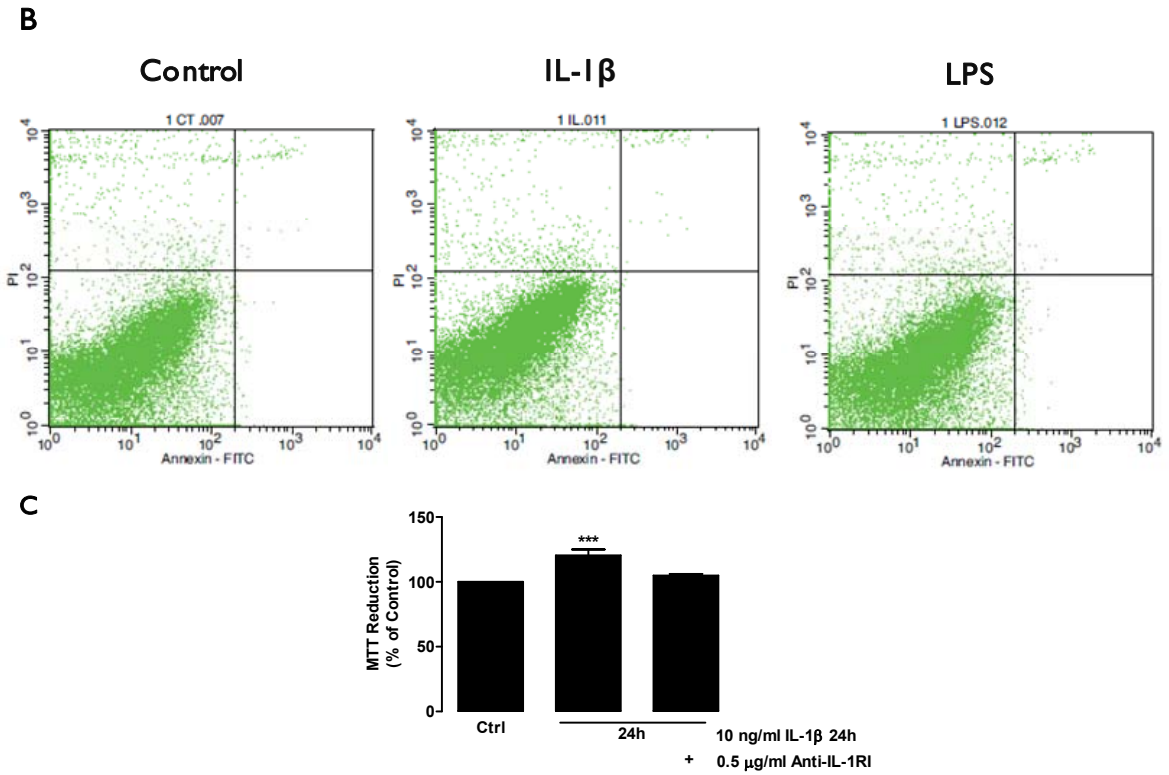
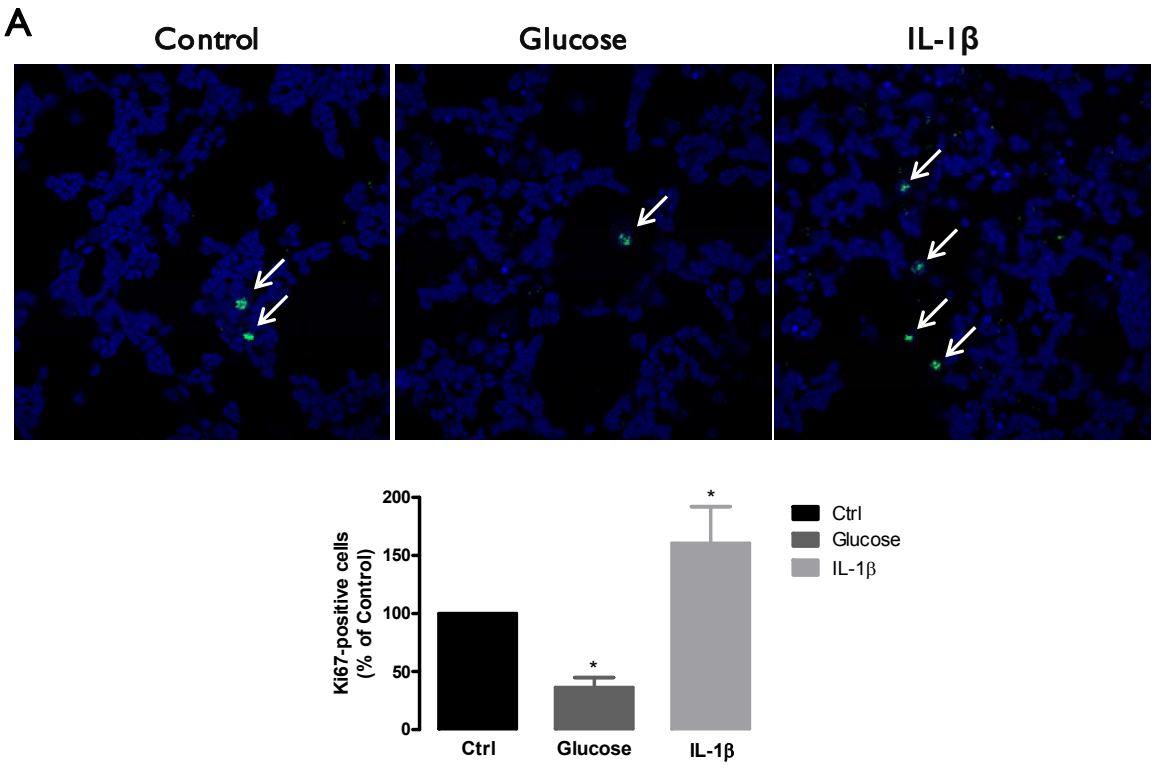


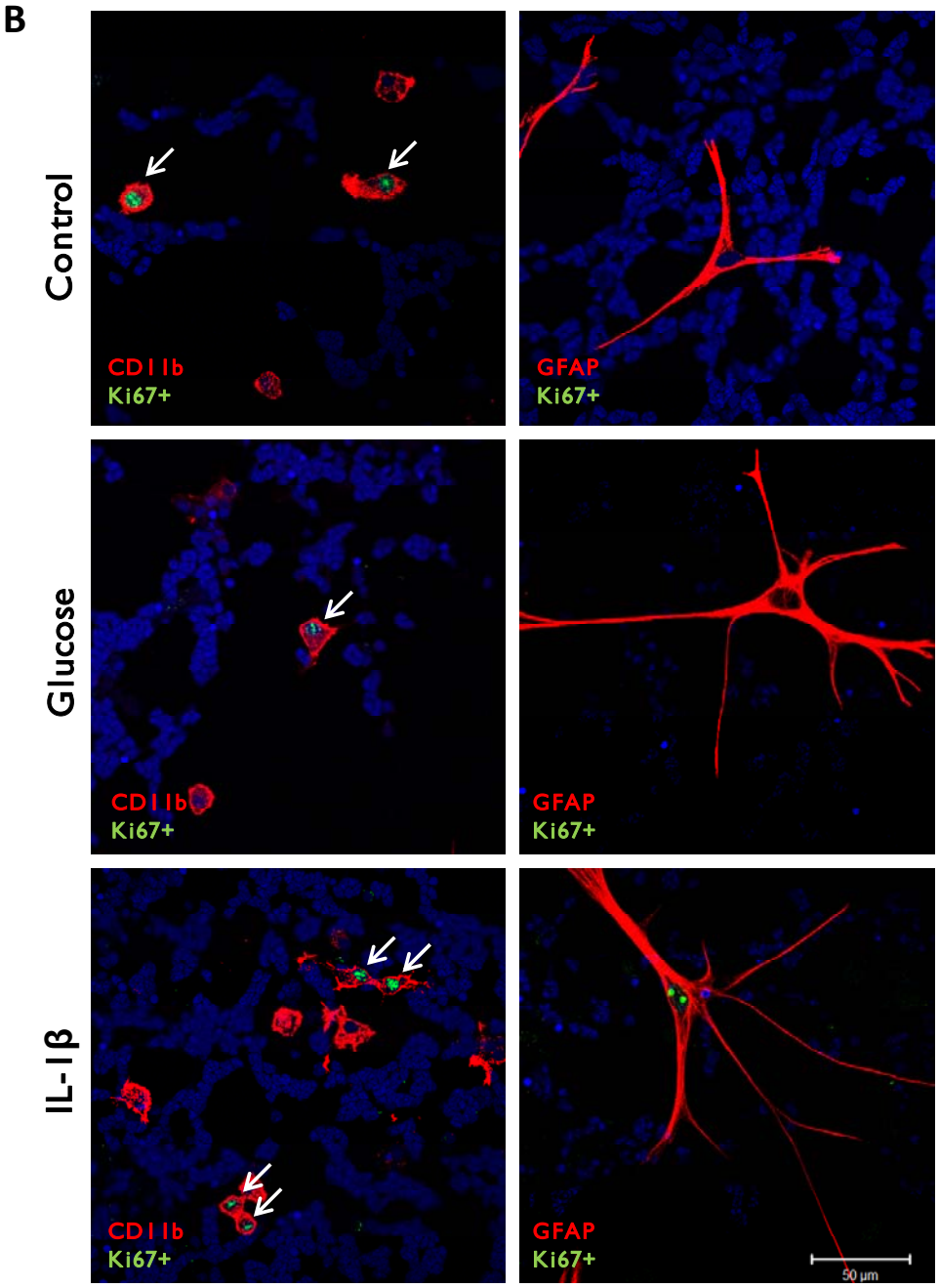
Figure 4. Effect of IL-1β on retinal neural cell death. Rat retinal neural cell cultures were exposed to IL-1β or LPS for 24h and cell death was evaluated by the TUNEL assay. The results represent the mean ± SEM of 6 independent experiments, and are expressed as the number of TUNEL-positive cells per field as percentage of control. Representative images are presented above the graph (A). To further evaluate cell death, retinal cells were labeled with annexin V plus PI and were analyzed by flow cytometry (B). MTT was used as a cell viability assay. The results represent the mean ± SEM of 5 independent experiments (C). ***p<0.001significantly different from control as determined by ANOVA followed by Dunnett's post-hoc test.

IL-1β increases the proliferation of glial and microglial cells

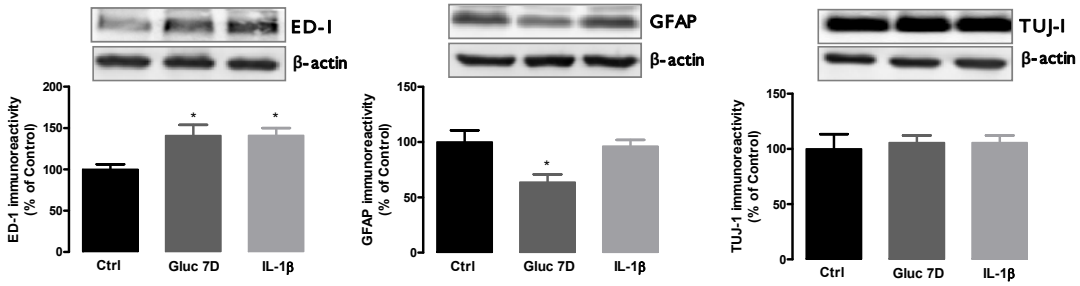
Since we have detected an increase in the MTT reduction and no changes in the number of TUNEL-positive cells in cultures exposed to IL-1β, we hypothesized that IL-1β could be affecting the proliferation of some cell types in these cultures. To evaluate the effect of IL-1β on cell proliferation, retinal neural cells were labeled with an antibody anti-Ki-67. Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0), being strictly associated with cell proliferation. First, we evaluated the number of Ki-67-positive cells in retinal neural cell cultures exposed to high glucose or mannitol, for 7 days, and to IL-1β or LPS for 24 h. A significant decrease in the number of Ki-67-positive cells was observed in cells incubated with elevated glucose for 7 days (decrease to 36.40 ± 8.5% of control) with no changes in cells exposed to mannitol (data not shown). Conversely, a significant increase in the

number of Ki-67-positive cells (increase to 160.6% ± 31.5% of control) was observed in cells incubated with IL-1β (Figure 5A). In these cultures, we noticed that the majority of Ki-67-positive cells appeared to be microglia (CD11b+) and some glial cells (GFAP+; astrocytes and Müller). Therefore, we analyzed the colocalization of CD11b+ and GFAP+ cells with Ki-67. In control conditions, we found an average of 2.3 ± 1.2 Ki-67+CD11+ cells per field. In cells exposed to elevated glucose, there was a decrease to 1.0 ± 0.8 Ki-67+CD11+ cells per field, whereas in cells exposed to IL-1β an increase in the number of Ki-67+CD11+ cells can be observed (3.5 ± 1.1). Representative images are shown in Figure. 5B. Regarding GFAP+Ki-67+ cells, very few or none positive cells were observed in both control (average of 0.1 ± 0.1 Ki-67+ co-localizing with GFAP+ cells per field) and high glucose conditions (0 Ki-67+ cells per field). When cells were exposed to IL-1β, the average number of Ki-67+ cells co-localizing with GFAP+ cells per field was of 0.5 ± 0.2 (Figure 5B). These results suggest that prolonged high glucose and IL-1β are inducing opposite effects in cell proliferation.




B
Control
Glucose
IL-1β
CD11b
Ki67+
GFAP
Ki67+
50 µm

C


ED-1
β-actin
ED-1 immunoreactivity (% of Control)
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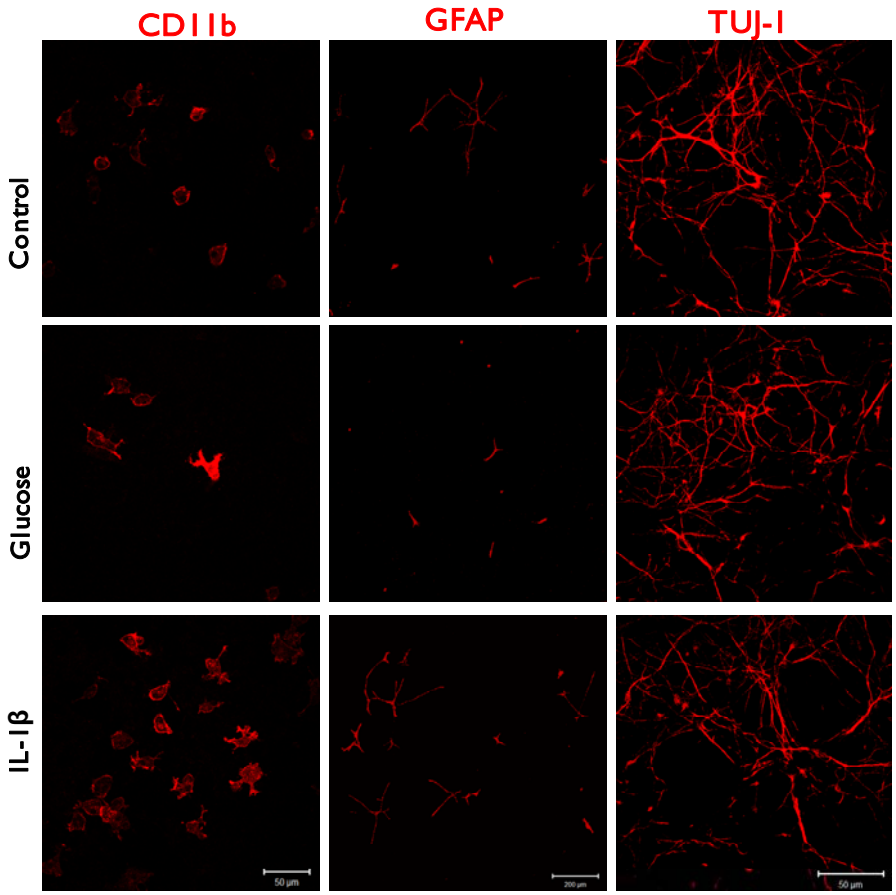
CD11b
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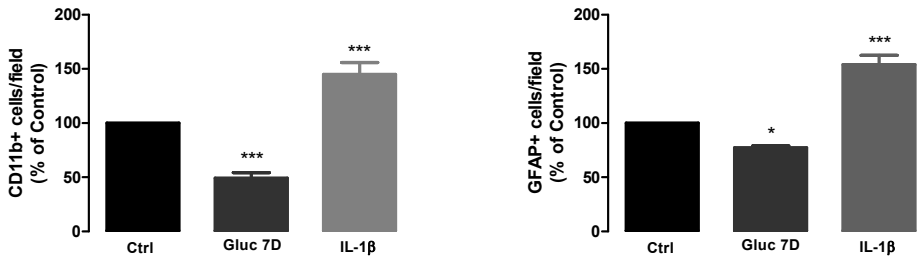
CD11b+ cells/field (% of Control)
Ctrl
Gluc 7D
IL-1β
GFAP+ cells/field (% of Control)
Ctrl
Gluc 7D
IL-1β

Figure 5. IL-1β induces the proliferation of glial and microglial cells in retinal neural cell cultures. Rat retinal neural cell cultures were exposed to high glucose for 7 days or IL-1β for 24 h and stained for Ki-67 (A). Co-localization of CD11b- and GFAP-positive cells with Ki-67 (B). Effect of high glucose for 7 days or IL-1β for 24 h in the levels of ED1, GFAP and TUJ-1 in retinal neural cultures (C). Cells were stained for cell-specific antigens (D) and each cell type was counted (E). Data represent means ± SEM of at least 3 independent experiments. **p<0.01, ***p<0.001, significantly different from control as determined by ANOVA followed by Dunnett's post-hoc test.

Next, we analyzed the effect of high glucose and IL-1β on the levels of ED1 (a marker for activated microglia), GFAP (a marker for macroglial cells), and TUJ-1 (neuron-specific class III beta-tubulin marker). We were not able to quantify CD11b (a microglial cell marker) levels by western blot. A significant increase in ED1 protein levels was detected in cells exposed to high glucose for 7 days and IL-1β for 24 h (increase to 140.0 ± 13.4% and 140.8 ± 8.9% of control, respectively) (Figure. 5C). No changes were observed in mannitol-treated cells, whereas in cells exposed to LPS a significant increase (increase to 153.1 ± 10.76% of control) was detected (data not shown). GFAP levels decreased in cells exposed to high glucose (decrease to 63.8 ± 7.1% of control) (Figure. 5C). No changes were observed in mannitol and LPS conditions (data not shown). No changes were detected in TUJ-1 levels in any condition studied (Figure 5C).

Microglial cells are very sensitive to alterations in its microenvironment, as well as to changes in cell culture conditions. They are considered to be major producers of IL-1β, which in turn can further activate microglial cells. In order to additionally evaluate the effect of high glucose or IL-1β on microglia proliferation, we counted the number of cells immunolabeled for CD11b in retinal neural cell cultures exposed to high glucose or mannitol, for 7 days, or to IL-1β for 24 h. High glucose induced a significant decrease in the number of CD11b-positive cells (49.2 ± 5.1 % of control). Mannitol had no effect on the number of CD11b-positive cells. However, IL-1β induced a significant increase in the number of CD11b-positive cells (144.9 ± 10.9 % of control), as shown in Figure. 5D and E.

We also investigated the effect of high glucose and IL-1β on glial cells and neurons in these cultures. Immunolabelling with anti-GFAP, showed that the number of GFAP-positive cells is significantly decreased by high glucose (77.3 ± 1.6% of control), but is strongly increased by IL-1β (153.9 ± 8.6% of control) (Figure 5C and E). Due to the vast neuronal network, we were not able to accurately quantify the number of TUJ-1-positive cells. Representative images are presented in Figure. 5D.

IL-1β does not induce changes in the content of synaptic proteins and neural markers

Although no changes in TUJ-1 levels were detected, we evaluated whether neurons could be affected by IL-1β at other levels, namely at pre-synaptic level, analyzing several pre-synaptic proteins and other neuronal markers. Previously, we have demonstrated that diabetes affects the content of several synaptic proteins, as well as glutamate and GABA vesicular transporters, in retinal and hippocampal nerve terminals (Baptista et al., 2011; Gaspar et al., 2010a). In a recent

study, we additionally found that prolonged exposure of cultured retinal neural cells to high glucose does not induce changes in the content and distribution of synaptic proteins of the exocytotic machinery and vesicular glutamate and GABA transporters (unpublished results), thus suggesting that hyperglycemia might not be the main factor contributing for molecular changes occurring in retinal neurons triggered by diabetes, particularly in nerve terminals. Our findings, together with previous studies, (Antonetti et al., 2006; Barber et al., 2001) put forward that other factors such as the lack of insulin or increased levels of pro-inflammatory mediators may contribute to changes that have been previously detected in synaptic proteins of diabetic animals. To investigate whether IL-1β by itself could lead to those changes we analyzed, in retinal neural cell cultures, the effect of IL-1β (24 h exposure) on the content of several synaptic proteins: SNARE complex proteins (VAMP-2, SNAP-25 and syntaxin-1), which are critical for exocytosis, synapsin-1 (binds synaptic vesicles to the cytoskeleton), synaptophysin (presynaptic marker), rabphilin 3a (vesicle-associated protein that is a target of rab 3A small GTP-binding protein), synaptotagmin-1 (Ca^{2+} sensor), and vesicular glutamate and GABA transporters, MAP-2 (a Microtubule-associated protein, involved in microtubule assembly) and NeuN (Neuronal Nuclei). Exposure of cultured retinal cells to IL-1β (10 ng/ml) or LPS (1 μg/ml), for 24 h, did not induce any alteration in the content of any of the synaptic proteins and neuronal markers above mentioned (Figure. 6).

Discussion

In this study, we show that high glucose *per se* upregulates the levels of IL-1β in retinal neural cells. Additionally, high glucose and IL-1β differently affect microglial and glial cells, changing their proliferation in retinal neural cell cultures. Hyperglycemia is considered the major risk factor for the development of diabetic retinopathy. Nevertheless, in the last decade, a growing body of evidence has shown that diabetic retinopathy has characteristics of a low-grade chronic inflammatory disease (Kern, 2007). In fact, it has been shown that macroglial and microglial cell activation is an important feature of neuroinflammation present in the diabetic retina (Gardner et al., 2002; Gaucher et al., 2007; Krady et al., 2005; Mizutani et al., 1998; Rungger-Brandle et al., 2000). Moreover, increased production of inflammatory mediators, such as IL-1β (Demircan et al., 2006; Kern, 2007), TNF-α (Demircan et al., 2006) and NO (Carmo et al., 2000; Kern, 2007) has been reported in the retinas of diabetic animals.

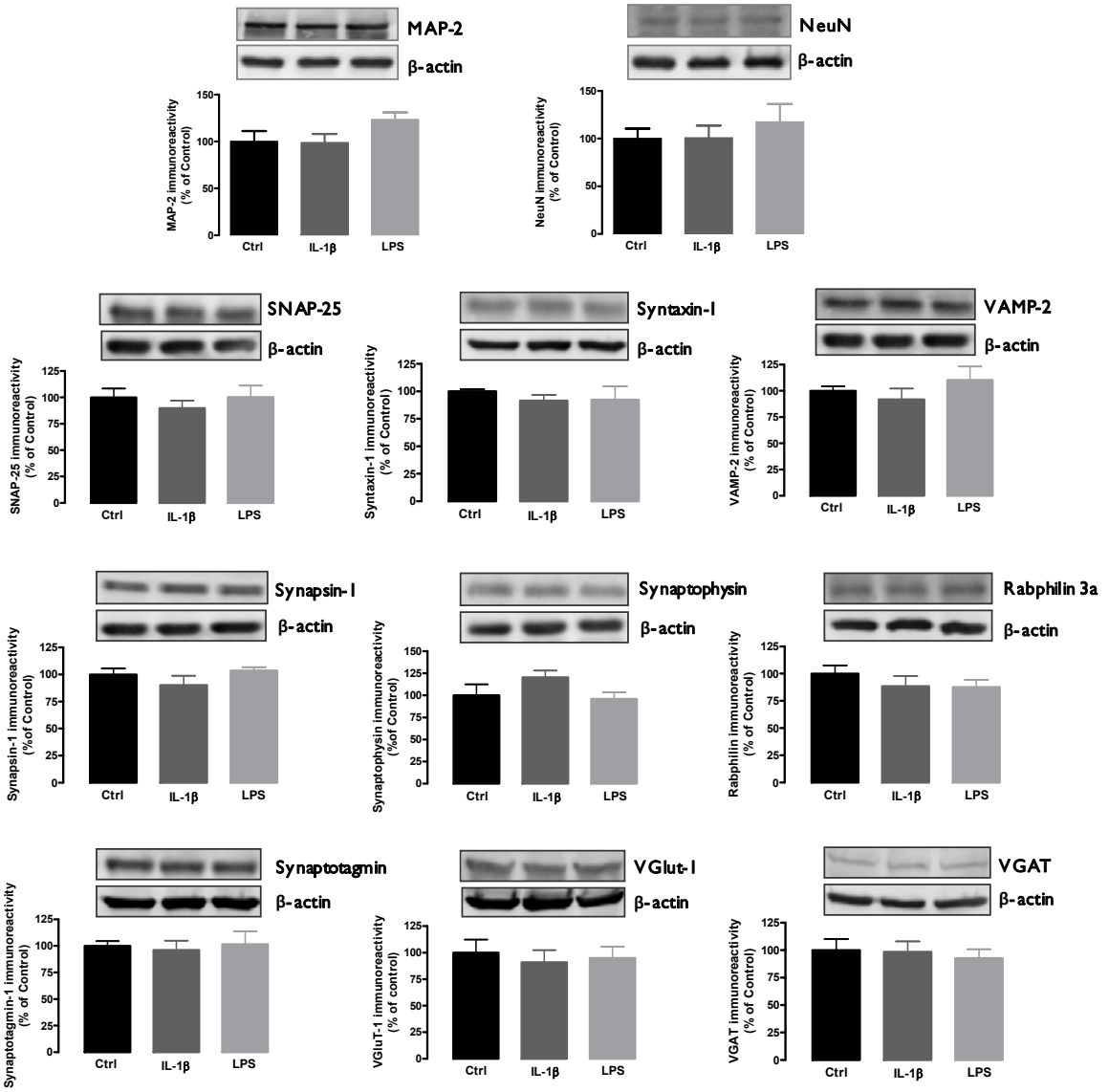

Figure 6. Effect of IL-1β on the content of synaptic proteins and neural cell markers. The protein levels of synaptic proteins and neural markers were analyzed by western blotting in total extracts of retinal cell cultures. Representative images of immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). Results are expressed as percentage of control ± SEM, of at least 3 independent cell cultures.

The results presented in this work show that high glucose is sufficient to induce IL-1β expression in retinal neural cell cultures. In the CNS, it has been shown that the main cellular source of IL-1β in response to stressors is the activated microglia, with a consequent upregulation

of the cytokine in macroglia as well (Rothwell and Luheshi, 2000). However, a previous study demonstrated that exposure to high glucose, for 4 days, is not sufficient to induce IL-1β overexpression in microglial cells (Liu et al., 2012). Nevertheless, this study was performed using brain-derived microglial cells and less time of exposure to high glucose (4 days) than in our study (7 days exposure) in retinal cell cultures. Moreover, when exposed to high glucose *in vitro*, monocytes upregulate and/or secrete increased amount of IL-1β. In this case, the upregulation of IL-1β by high glucose is mediated by a PKCα-dependent activation of p38MAPK, ERK, and NF-kB (Dasu et al., 2007). In retinal endothelial cells, it was found that high glucose induced a 3-fold increase on IL-1β expression, which was prevented by calphostin C, a protein kinase C inhibitor (Liu et al., 2012).

Regarding IL-1RI, we had previously found a time-dependent downregulation of IL-1RI protein levels in retinal endothelial cells exposed (1–24 h) to high glucose, mannitol or IL-1β. Long-term exposure (7 days) to high glucose or mannitol also decreased IL-1RI protein content. In endothelial cells, IL-1RI downregulation was due to its activation by IL-1β and was prevented by lysosome inhibitors. After high glucose or IL-1β treatment, IL-1RI translocates to the nucleus which could also be accounting for the observed downregulation (Aveleira et al., 2010a). We also observed a downregulation of IL-1RI in rat retinal neural cell cultures under short-term exposure to glucose but the downregulation was transient. Contrarily to IL-1RI, the protein levels of IL-1RAcP, which is an essential component of the IL-1RI complex, remained unchanged (data not shown), suggesting that this protein is more resistant to stress conditions and that adaptive changes to IL-1β-triggered responses are regulated essentially at the IL-1RI level.

Previous studies from our group have already shown that a long-term exposure (7 days) to high glucose decreases cell viability in retinal neural cell cultures (Santiago et al., 2007). Exposure to high glucose also alters the $[Ca^{2+}]_i$ responses evoked by purine receptor activation in neurons and microglial cells in retinal neural cell cultures (Pereira et al., 2010). This augmented calcium response might account for the increase in the release of neurotransmitters and inflammatory mediators contributing for retinal cell death. More recently, it was demonstrated that as early as 3 days of exposure to high glucose also induces a small, but significant, decrease in cell viability, and a constant decline in cell viability was found for longer incubation periods, reaching a minimum at the last time point studied, 7 days (Costa et al., 2012). The increase in the number of apoptotic nuclei detected by TUNEL assay after 7 days of incubation with high glucose was concomitant with a decrease in cell viability assessed with the MTT assay (Costa et al., 2012; Santiago et al., 2007). Using several markers to identify the type of cells undergoing apoptosis in cultures exposed to high glucose, it was shown that very few TUNEL-positive cells were immunoreactive for neuronal (NeuN, TUJ-1), microglial (CD11b) or macroglial (GFAP, vimentin) cell markers. A high percentage of apoptotic cells in high glucose condition were TUNEL+Rhodopsin+ or TUNEL+PKCα+ and were ascribed to be rod photoreceptors and rod bipolar cells (Costa et al., 2012). In the present study, exposure to IL-1β did not induce changes in the number of apoptotic cells in retinal cell cultures. Interestingly, IL-1β increased the reduction of MTT. Despite being

frequently used as a cell viability assay, it is also used as a proliferative assay, and these results led us to suspect that exposure to IL-1β was triggering cell proliferation. Therefore, we further evaluated the effect of IL-1β and high glucose on cell proliferation by Ki-67 immunostaining. By cell counting, we detected a significant decrease in the number of CD11b+Ki-67+ cells and in the number of CD11b+ cells in cultures exposed to high glucose. Given that it was not detected an increase in TUNEL+CD11b+ cells in these cultures exposed to high glucose (Costa et al., 2012), this observation suggests that the decrease was not due to cell death.

Since IL-1β increased the number of CD11b+ cells, and since high glucose increased IL-1β production in retinal neural cell cultures, we would also expect an increase in CD11b+ cells in cells exposed to high glucose. However, as mentioned above, high glucose led to a decrease in the number of CD11b+ cells. One explanation for these apparent contraditory results is that IL-1β concentration present in high glucose condition is probably not sufficient to induce microglia proliferation. The IL-1 β levels detected in high glucose condition were about 79.5±7.9 pg/ml, a much smaller concentration than the one used when cells were exposed to IL-1β in this study (10 ng/ml). Another explanation is that high glucose might decrease the proliferation of CD11b+ cells due to cell cycle arrestment (Rao et al., 1999). Nevertheless, there was an increase in ED1 levels in cells exposed to high glucose, indicating that high glucose activates microglia. In fact, this possibility is supported by the increase in IL-1β levels when cells were exposed to high glucose, since activated microglia are prone to release increased levels of cytokines.

Microglial cells in the retina are sensors for disturbances in their neuronal environment. These cells can provide trophic support to neurons and are able to assist in synaptic plasticity, surround damaged neurons and participate, in an anti-inflammatory manner, in synaptic stripping, a process of removing branches from damaged neurons to promote repair and regrowth (Lull and Block, 2010). Moreover, they are also able to influence Müller cells directly, and initiate a program of bidirectional microglia-Müller cell signaling that can mediate adaptive responses within the retina following injury (Wang et al., 2011).

Similarly to what was observed for microglial cells, IL-1β increased the number of GFAP+ cells, as well as the the number of GFAP+ cells stained with Ki-67, indicating that glial cells are also proliferating. After injury, Müller cells become activated and undergo reactive gliosis, which is characterized by proliferation and changes in gene expression (Dyer and Cepko, 2000). However, when retinal cells were exposed to high glucose there was a decrease in GFAP levels and a decrease in the number of GFAP+ cells, indicating that elevated glucose per se has a negative effect of glial cell proliferation, despite enhancing the levels of IL-1β in these cultures. The reduction in glial cell proliferation is not due to glial cell death (Costa et al., 2009). These observations suggest that the effect of IL-1β on glial cell proliferation is clearly dependent on its concentration in the cell culture media. For relatively low levels of IL-1β, this cytokine is not able to induce glial cell proliferation.

Continuous stimulation with stress signals can lead to chronic overactivation of microglia and loss of autoregulatory mechanisms, amplifying inflammation (Toda et al., 2002), which may

consequently be harmful for neurons. When retinal cultures were exposed to IL-1β, we did not detect any changes in the levels of several neuronal markers. Nevertheless, we cannot exclude the possibility that for longer periods of exposure to IL-1β, microglia may have detrimental effects in these cultures changing from a protective to a pro-inflammatory modus (Karlstetter et al., 2010; Lull and Block, 2010).

In this study, we found no changes in the levels of several synaptic proteins in retinal cultures exposed to IL-1β. A similar result was obtained when cells were exposed to high glucose (Chapter 4). Previously, we demonstrated that diabetes affects the content of several synaptic proteins, as well as glutamate and GABA vesicular transporters, in retinal nerve terminals (Baptista et al., 2011; Gaspar et al., 2010a). Since diabetes is a multifactorial disease, other factors, besides inflammation or hyperglycemia per se, such as the lack (or decrease) of insulin levels or action may play a role in changes occurring in nerve terminals detected in diabetic animals. Retinal neurons depend on insulin receptor activity for survival (Barber et al., 2001). Long-term instability in retinal insulin signalling may impair insulin-dependent anabolic activities such as protein synthesis in the retina (Chihara, 1981) and increase cell death (Reiter and Gardner, 2003), suggesting that insulin signalling provides neurotrophic actions in the retina. Therefore, changes in neural retina under diabetes may result in part from neurotrophin deficiency. Additionally, the combinatory effect of two or more factors may induce changes in neurons, synergistically being responsible for the previous alterations detected in the retina under diabetic conditions.

It was previously demonstrated that exposure to high glucose increases TNF-α expression (Costa et al., 2012). The blockade of TNF receptor 1, which is expressed in retinal neurons, is capable of preventing the increase in cell death induced by high glucose. Additionally, it was demonstrated that the secretion of TNF-α and monocyte chemotactic protein-1 (MCP-1) by rat cortical microglia, triggered by exposure to high glucose, is mediated by ROS and NF-κB pathway activation, which may be a mechanism underlying neuronal injury and the pathogenesis of diabetic encephalopathy (Quan et al., 2011). Moreover, the inhibition of the IL-1β signalling pathway by using IL-1RI-deficient mice protected the animals from diabetes-induced retinal pathology (Vincent and Mohr, 2007), suggesting that IL-1RI may play a key role in the development of diabetic retinopathy. Since IL-1RI provides a crucial locus of control of IL-1β activity, blocking the activation of IL-1RI should be taken into account as a possible therapeutic strategy for the treatment of diabetic retinopathy. Likewise, strategies aiming to inhibit the activity of TNFR1 will also be important.

Responses of activated Müller cells and microglia in the retina are described not to be independent but involve bidirectional feedback signals that help initiate and propagate a coordinated adaptive response (Wang et al., 2011). Therefore, in this study, changes in cell proliferation and expression of cell-specific markers suggest that there are adaptive responses that may help to limit cell death of neurons by directing and amplifying inflammatory processes in order to restore and maintain homeostasis in neuronal cell cultures.

In summary, our findings show that high glucose increases IL-1β production in retinal cell cultures. Moreover, we found that high glucose and IL-1β differently affect microglial and glial

cells. High glucose decreased glial and microglial proliferation, whereas IL-1β increased their proliferation. These apparently opposing effects might be related with the levels of IL-1β. When cells are exposed to high glucose, the levels of IL-1β reached are significantly lower comparing to the condition when cells are exposed to IL-1β. Since overactivation of microglial cells may have deleterious effects in the retina, limiting IL-1β-triggered inflammatory processes could be a mechanism to prevent the progression of diabetic retinopathy.

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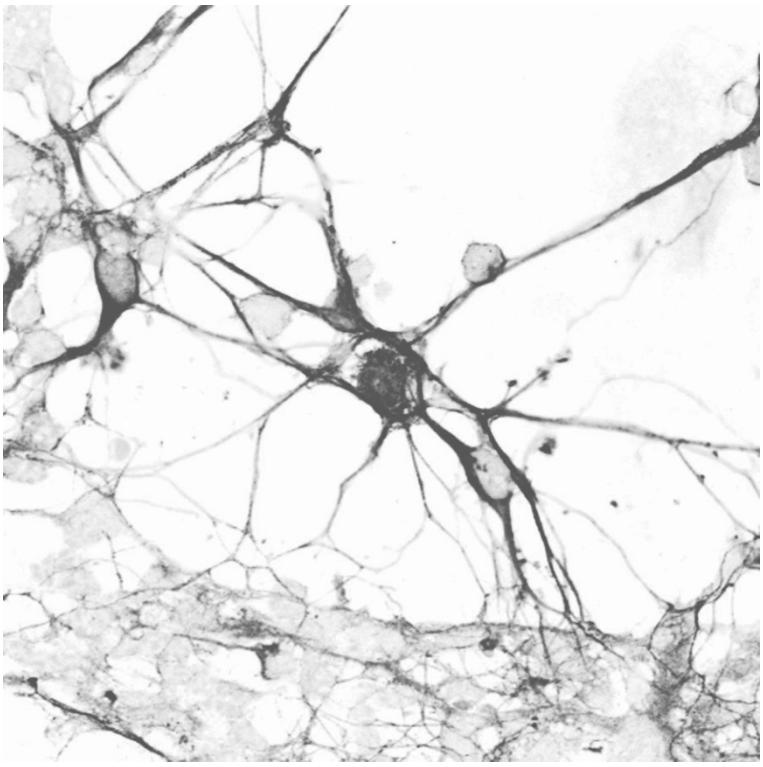
Chapter 6

Diabetes induces alterations in the content and distribution of kinesin motor proteins in the rat retina



The results presented in this chapter were submitted for publication by Filipa I. Baptista, Maria J. Pinto, Filipe Elvas, Ramiro D. Almeida and António F. Ambrósio.

Abstract

Diabetic retinopathy is a leading cause of vision loss and blindness. The disruption of axonal transport underlies many neurodegenerative diseases and might also play a role in diabetes-associated disorders affecting nervous system. The effect of diabetes in motor proteins (kinesins and dynein) in the retina has not been addressed. Therefore, we investigated the impact of diabetes (2 and 8 weeks duration) on KIF1A, KIF5B and dynein motor proteins in the retina. The immunoreactivity of those proteins was evaluated by immunohistochemistry in retinal sections and by immunoblotting in total retinal extracts from streptozotocin-induced diabetic and age-matched control animals. Diabetes decreased the content of KIF1A at 8 weeks of diabetes and KIF1A immunoreactivity in all retinal layers analyzed, with the exception of the photoreceptor layer. Changes in KIF5B immunoreactivity were also detected by immunohistochemistry in the retina at 8 weeks of diabetes, being increased at the photoreceptor layer and outer nuclear layer, and decreased in the inner plexiform layer and ganglion cell layer. No significant changes were detected in dynein immunoreactivity in the retina.

Since hyperglycemia is considered the trigger of diabetic complications, we investigated whether prolonged exposure to elevated glucose per se could affect the content and distribution of motor proteins in retinal cultures. Primary retinal cultures were exposed to high glucose (30 mM) or mannitol (osmotic control; 24.5 mM plus 5.5 mM glucose), for seven days. The exposure to elevated glucose did not induce any changes in the content or distribution of motor proteins, neither in the distribution of mitochondria. It is likely that other factors, such as the lack of insulin, may contribute for the alterations in motor proteins detected in diabetic animals. These alterations might in turn contribute to early signs of neural dysfunction in the retina of diabetic patients and animal models.

Keywords: Diabetes; retina; axonal transport; kinesin; dynein.

Introduction

Diabetic retinopathy is the most common microvascular complication of diabetes *mellitus* and is a leading cause of vision loss and blindness among working-age adults in Western countries. However, increasing evidence has shown that the neural components of the retina are also affected (Antonetti et al., 2006). Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995), demonstrating that the neural retina can be also affected by this disease.

Neurons are highly polarized cells, with long axons, which constitute a major challenge to the movement of proteins, vesicles, and organelles between cell bodies and presynaptic sites. To overcome this, neurons possess specialized transport machinery consisting of cytoskeletal motor proteins (kinesins and dynein) generating directed movements along cytoskeletal tracks. Axonal transport motor proteins require ATP demands, this implying the localization of functional mitochondria along the axons. Mobile mitochondria can become stationary or pause in regions that have a high metabolic demand and can move again rapidly in response to physiological changes. Defects in mitochondrial transport are implicated in the pathogenesis of several major neurological disorders (Sheng and Cai, 2012). Axonal transport is therefore crucial to maintain neuronal viability, and any impairment in this transport may play a role in the development or progression of several diseases (De Vos et al., 2008).

A decrease in the levels of mRNAs encoding for neurofilament proteins was found in the dorsal root ganglia of streptozocin-induced diabetic (Mohiuddin et al., 1995). Additionally, slow axonal transport of neurofilament and microtubule components was also found to be reduced, leading to a decrease in axonal caliber (Medori et al., 1988). These evidences suggest that deficits in axonal transport may contribute to neuronal changes observed in diabetes in neural tissues.

A few studies have evaluated the effect of diabetes on axonal transport in the retina and most of them have focused in studying fluoro-gold labelling in retinal ganglion cells (RGCs) (Ino-Ue et al., 2000; Zhang et al., 2000; Zhang et al., 1998). Despite these evidences, the impact of diabetes in motor proteins (kinesins and dynein) has not been addressed. Yet, potential changes in their content and distribution might underlie some changes already observed in axonal transport in the retina and visual pathway under diabetic conditions (Fernandez et al., 2012; Zhang et al., 2000). Previously, we found that diabetes changes the levels of several synaptic proteins in retinal nerve terminals, with no changes in total retinal extracts, further suggesting that axonal transport of those proteins may be impaired in diabetes (Gaspar et al., 2010a). Hyperglycemia has been considered the main pathogenic factor for the development of diabetic complications. In hippocampal cell cultures, we found that high glucose leads to an accumulation of vesicular glutamate transporter-1, syntaxin-1 and synaptotagmin-1 at the cell body, further suggesting that axonal transport of these proteins to nerve terminals might be affected under hyperglycaemic conditions (Gaspar et al., 2010b). In this work, we aimed to study the effect of diabetes and also high glucose *per se* (prolonged exposure for 7 days), mimicking hyperglycemic conditions, on the content and distribution of the motor proteins KIF1A (kinesin that transports synaptic vesicle

precursors), KIF5B (kinesin involved in mitochondrial transport and in the transport of synaptic vesicle precursors and membrane organelles) and dynein (motor protein for retrograde axonal transport) in diabetic animals and primary rat retinal cell cultures. Since motor proteins need ATP to carry cargoes along the axons, the distribution of mitochondria was also analyzed in retinal neural cultures exposed to high glucose.

Experimental Procedures

Animals

Male Wistar rats (Charles River Laboratories), eight weeks-old, were randomly assigned to control or diabetic groups. All animals were handled according with the EU guidelines for the use of experimental animals (86/609/EEC). Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, freshly dissolved in 10 mM sodium citrate buffer, pH 4.5) (Sigma, St. Louis, MO, USA). Hyperglycemic status (blood glucose levels exceeding 250 mg/dl) was confirmed two days after STZ injection with a glucometer (Elite, Bayer, Portugal). Before sacrifice, rats were weighted and blood samples were collected for measurement of glucose. Diabetic rats and age-matched controls were anesthetized with halothane and then sacrificed, two and eight weeks after the onset of diabetes.

Preparation of total retinal extracts

The eyes of diabetic and age-matched control animals were enucleated and placed in cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at 4°C). The retinas were dissected and lysed in RIPA buffer supplemented with complete mini protease inhibitor cocktail tablets and 1 mM DTT, pH 7.2, at 4°C. Then, the lysates were sonicated and centrifuged at 16,000 x *g* for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Immunohistochemistry in retinal sections

Preparation of cryosections

Rats from each experimental group were deeply anesthetized with ketamine/xylazine and intracardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The eyes were enucleated, washed in ice-cold PBS and fixed in 4% PFA in PBS for 1 h. The cornea was removed and the posterior segments were fixed in 4% PFA in PBS for an additional period of 5 h. The tissue samples were transferred to 20% sucrose buffer overnight at 4°C for cryoprotection and then were embedded in OCT (optimal cutting temperature gel, Shandon Cryomatrix, Shandon, Pittsburg, USA). The blocks were stored in a deep freezer (-80°C) until use. Transverse sections with 12 µm were obtained on a cryostat (Leica CM3050S, Nussloch, Germany) at -20°C. The cryosections were then collected on gelatin-coated glass slides and allowed to air dry for 1 h. Retina sections were then stored at -20°C for later use.

Immunohistochemistry

For immunostaining, frozen sections were placed 45 min at RT. After thawing, the sections were fixed in cold acetone (-20°C) during 10 min and subsequently hydrated 3 times in PBS, during 10 min each time, to remove OCT. Sections were permeabilized with 0.25% Triton X-100 in PBS, for 30 min, and blocked with 5% fetal bovine serum (FBS) in PBS, for 30 min. Then the sections were incubated with primary antibodies against KIF1A (1:50 in PBS with 1% FBS), KIF5B (1:100 in PBS with 1% FBS), or dynein (1:100 in PBS with 1% FBS) at 4°C overnight in a humid atmosphere, to avoid tissue dehydratation. After washing in PBS, 3 times for 10 min, a conjugated secondary antibody plus DAPI (1:5,000), to stain cell nuclei, were added for 1 h in the dark, at RT. After washing the sections, 3 times for 10 min in PBS, coverslips were mounted over the retinal sections using glycerogel (Dako mounting medium). Stained sections were observed with a laser scanning confocal microscope LSM 710 META (Zeiss, Germany).

Quantitative analysis of immunofluorescence data was performed using ImageJ.

Primary cultures of rat retinal neural cells

Retinal cell cultures were obtained from the retinas of 3–5 days-old Wistar rats as previously described (Santiago et al., 2006a). Briefly, rat pups were decapitated, and the retinas were dissected under sterile Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4), with a dissecting microscope. The retinas were digested with trypsin (0.1%, 15 min, at 37°C; Gibco Invitrogen Corporation; Carlsbad, CA, USA), in Ca^{2+}- and Mg^{2+}-free Hank's solution. After digestion, the cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated FBS (Gibco Invitrogen Corporation; Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were plated at a density of $2.0x10^6$ cells per cm^2 on poly-D-lysine substrate (0.1 mg/ml) and were maintained at 37°C in a humidified incubator with 5% CO_2/air. The concentration of glucose in control conditions was 5 mM. After 2 days in culture, cells were incubated with 25 mM D-glucose (30 mM final concentration, with 5 mM from culture medium) or 25 mM D-mannitol (plus 5 mM glucose from culture medium), which was used as an osmotic control, and maintained for additional 7 days in culture (nine days in culture).

Preparation of extracts of cultured retinal cells

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at 4°C) and then lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4). The lysates were incubated on ice for 30 min and then centrifuged at 16,100 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Immunocytochemistry

Retinal cell cultures were washed three times with PBS and fixed with 4% PFA and 4% sucrose for 10 min at RT. Cells were then washed three times with PBS and permeabilized with 1% Triton X-100 in PBS for 10 min at RT. Non-specific binding was prevented incubating cells with with 5% fetal bovine serum (FBS)/0.2% Tween-20 in PBS for 20 min. Cells were then incubated with the primary antibodies (listed in Table 1) for 2 h at RT. After incubation, cells were rinsed three times with PBS and incubated with the secondary antibodies for 1 h at RT in the dark. The nuclei were stained with DAPI (1:5,000). Upon rinsing three times with PBS, the coverslips were mounted on glass slides using Dako Fluorescence mounting medium (Dako, Denmark). The preparations were visualized in a laser scanning confocal microscope LSM 710 META (Zeiss, Germany).

Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, using 6%-8% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature (RT). The membranes were then incubated with primary antibodies (listed in Table 1) overnight at 4°C. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated with anti-mouse or anti-goat alkaline phosphatase-linked IgG secondary antibody (1:10,000; GE Healthcare, Buckinghamshire, UK) in TBS-T with 1% low-fat milk for 1 h at RT. The membranes were processed for protein detection using the enhanced chemifluorescence substrate (ECF; GE Healthcare). Fluorescence was detected on an imaging system (Thyphoon FLA 9000, GE Healthcare) and the digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed and tested for β-actin immunoreactivity (1:5,000) or β-III tubulin (TUJ-1) (1:5,000) to prove that similar amounts of protein were applied in the gels.

Table 1. List of primary antibodies

Primary Antibody	Sample	Antibody Dilution	Protein (µg)	Source
Mouse anti-KIF1A	Total Extracts Retina	1:1,000	40	BD Biosciences
	Total Extracts Primary cultures	1:1,000	40	
Goat anti-KIF1A	Immunocytochemistry	1:50	_	Santa Cruz
	Immunohistochemistry	1:50	_	
Goat anti-KIF5B	Total Extracts Retina	1:2,000	10	Abcam
	Immunohistochemistry	1:100	_	
	Total Extracts Primary cultures	1:2,000	20	
	Immunocytochemistry	1:100	_	
Mouse anti-Dynein	Total Extracts Retina	1:2,000	20	Abcam
	Immunohistochemistry	1:100	_	
	Total Extracts Primary cultures	1:2,000	40	
	Immunocytochemistry	1:100	_	
Rabbit anti-TUJ-1	Total Extracts Primary cultures	1:1,000	20	Covance

Statistical analysis

Statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Student's *t*-test. Variance analysis was not undertaken since the effect of age on the content of motor proteins was not the aim of this study. Thus, gels were always loaded with samples from age-matched animals and not from animals with different ages. Statistical significance for the analysis of retinal cell cultures protein content was determined by using one-way ANOVA, followed by Dunnett's post hoc test. Quantitative analysis of immunofluorescence data was performed using ImageJ and statistical analysis between control and diabetic animals were analyzed using the unpaired Student's *t*-test. Differences were considered significant for $p<0.05$.

Results

Animal body weight and glucose blood levels

The animal body weight assessed prior the induction of diabetes was similar between the two groups (255.7±3.5 g for control animals and 253.4±3.4 g for diabetic group) as well as blood glucose levels (89.1±1.4 mg/dl for controls and 86.7±5.7 mg/dl for diabetic group). The average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table 2. A marked impairment in weight gain occurred in diabetic rats comparing to age-matched controls in all time points analyzed. Diabetic animals also presented significantly higher blood glucose levels when compared to age-matched controls.

Table 2. Average weight and blood glucose levels of diabetic and aged-matched control rats.

		Weight (g)	Blood Glucose (mg/dL)
2 Weeks	Control	319.5±5.46	102.33±3.54
	Diabetic	233.22±8.54***	377.44±21.18***
8 Weeks	Control	394.62±16.74	89.85±2.88
	Diabetic	245.75±13.67***	488.85±38.54***

Measurements were made immediately before the sacrifice of the animals. ***p<0.001.

Diabetes decreases the content of KIF1A in total retinal extracts

Since the impact of diabetes in motor proteins involved in axonal transport in the retina is unknown, the content of KIF1A, KIF5B, and dynein in total retinal extracts from diabetic animals and age-matched controls was analyzed by immunoblotting. At 2 weeks of diabetes no significant changes were detected in the content of both kinesins. However, a significant decrease was found at 8 weeks of diabetes in KIF1A levels in total retinal extracts (reduction to 69.6±6.0% of control), whereas KIF5B levels remained unchanged. Moreover, no significant differences were detected between diabetic and age-matched control animals in dynein content at 2 and 8 weeks of diabetes (Figure 1).

Diabetes decreases the KIF1A immunoreactivity along the retinal layers

The immunoreactivity of KIF1A along the retinal layers was also analyzed in diabetic and age-matched control rats. No significant changes were observed in KIF1A immunoreactivity in the retina at 2 weeks of diabetes, comparing to control. However, at 8 weeks of diabetes there was a significant decrease in KIF1A immunoreactivity in the majority of retinal layers (Figure 2A and B). There was a reduction to 58.8±16.8% in the outer nuclear layer (ONL), a reduction to 49.3±20.1% in the outer plexiform layer (OPL), a reduction to 48.6±18.7% in the inner nuclear layer (INL), a reduction to 55.2±10.1% in the inner plexiform layer (IPL), and a reduction to 40.5±8.2% in the ganglion cell layer (GCL).

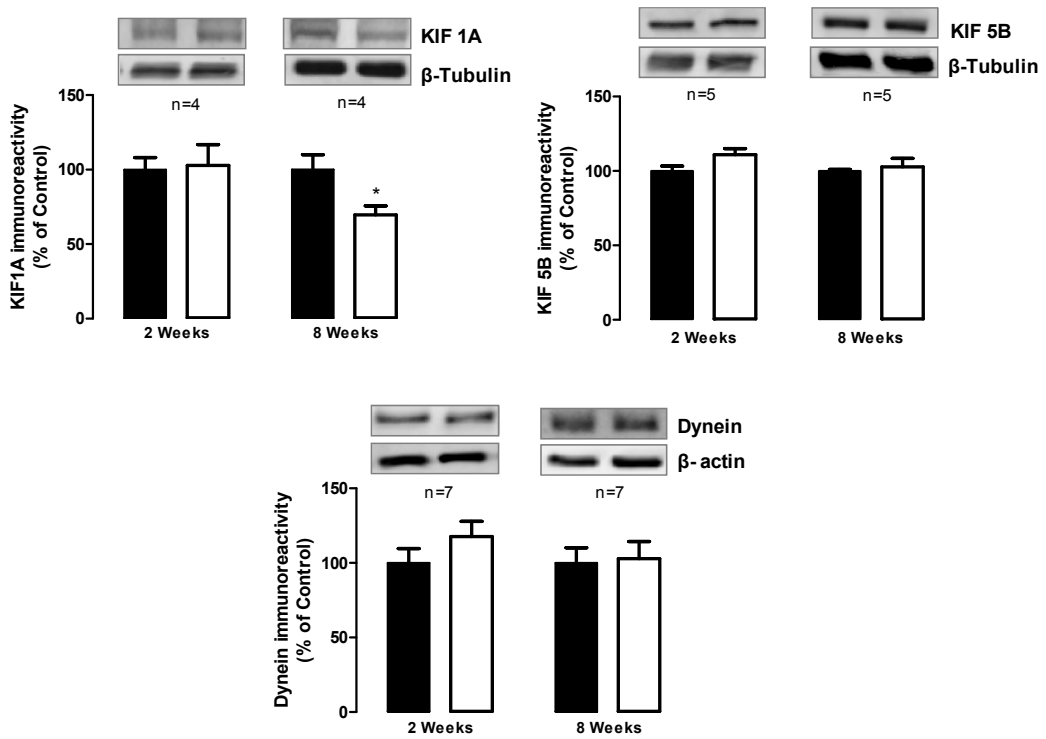
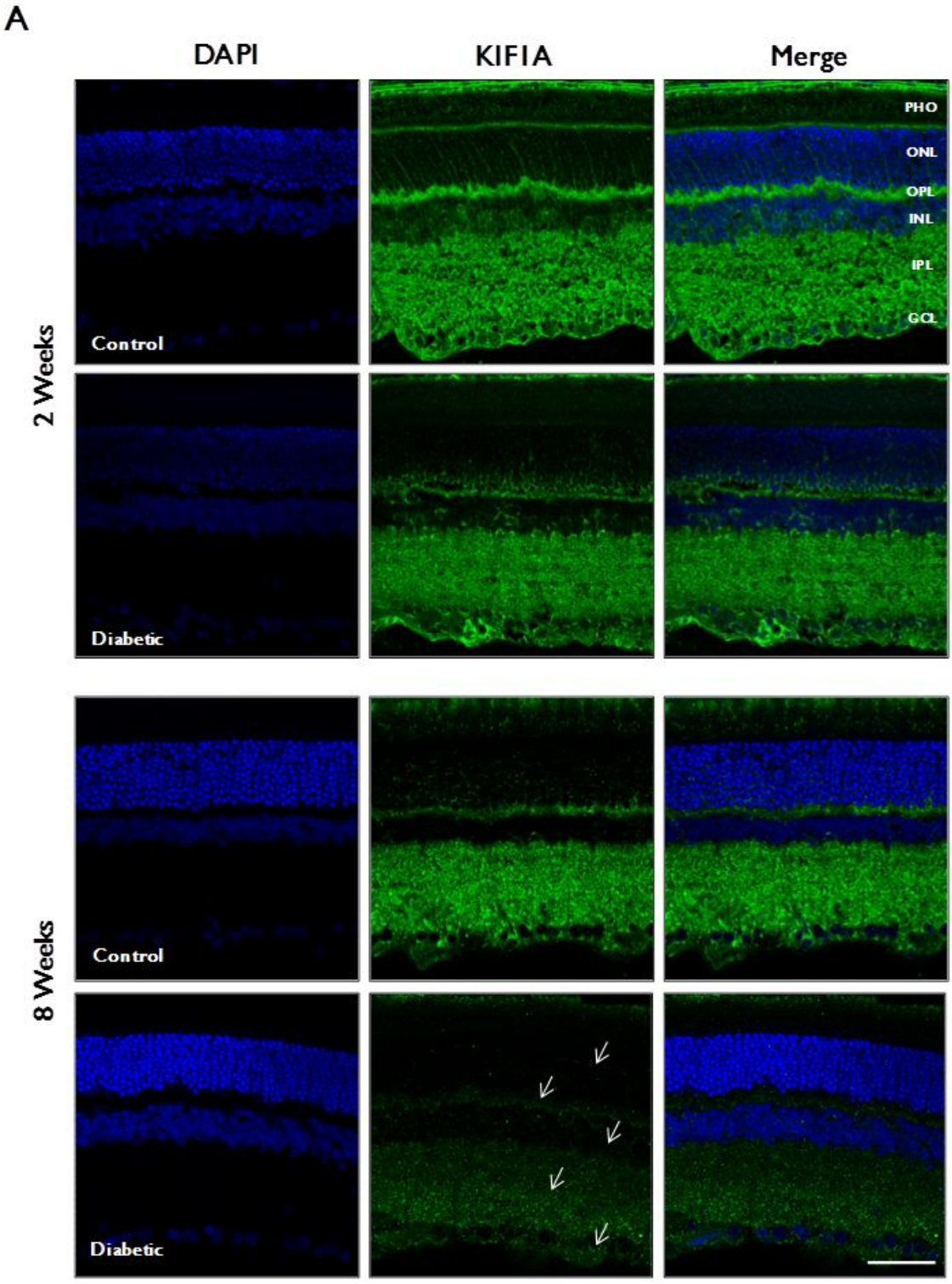


Figure 1. Diabetes decreases KIF1A protein content in the retina. The protein levels of KIF1A, KIF5B and dynein were analyzed by immunoblotting in total retinal extracts obtained from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes). Representative Western blots are presented above the graphs, with the respective loading controls (β-actin or β-III tubulin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-7 animals. *p<0.05, significantly different from control as determined by the unpaired Student's *t*-test.


A
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8 Weeks
Control
Diabetic

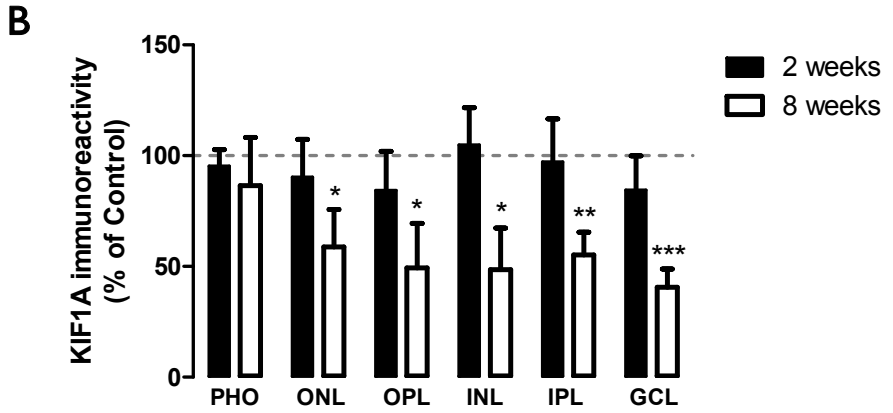
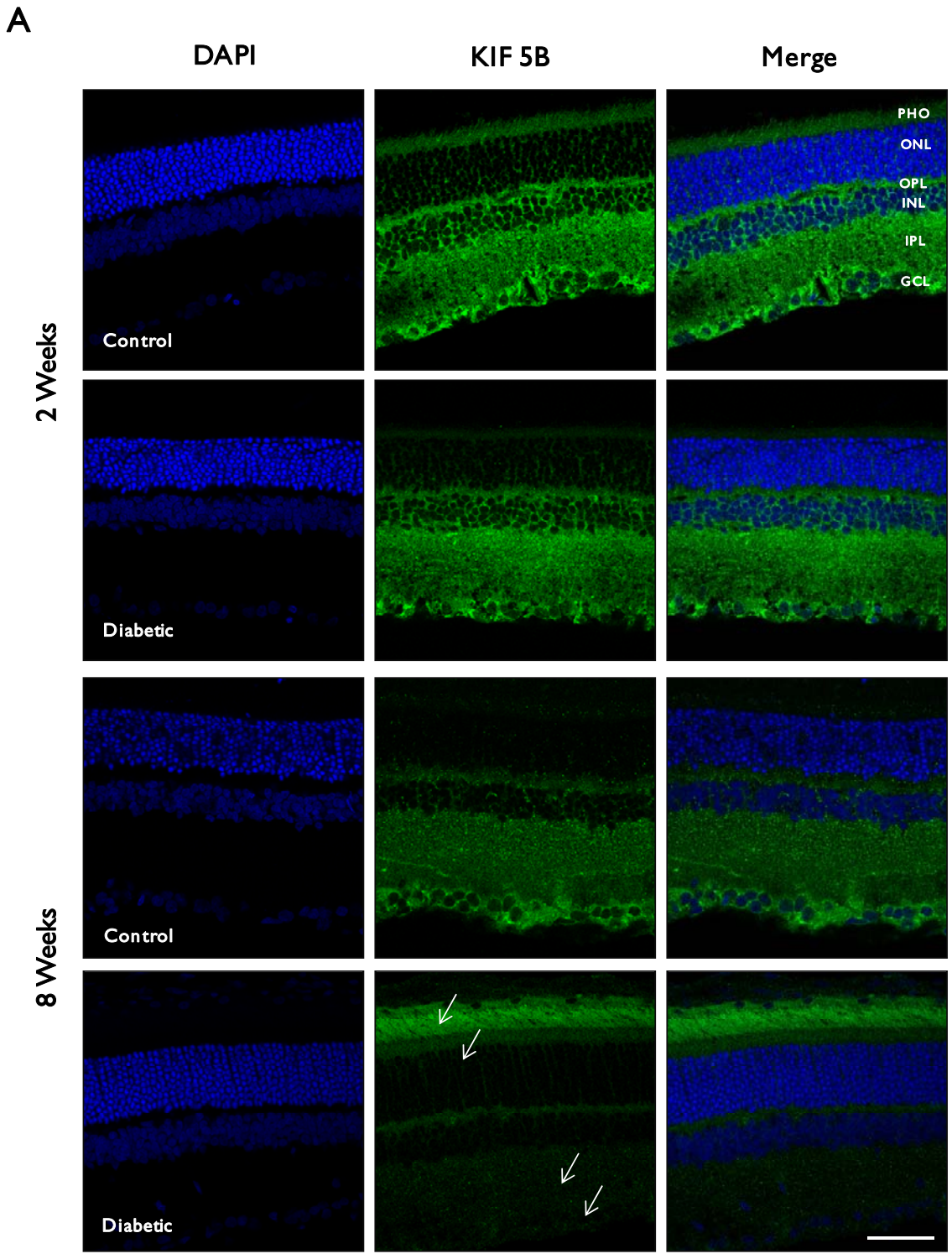


Figure 2. Diabetes decreases KIF1A immunoreactivity along retinal layers. (A) The distribution of KIF1A was evaluated in retinas isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes) by immunohistochemistry. Magnification 400x; Scale bar: 50 µm. *White arrows*: significantly different from control. (B) The immunoreactivity of KIF1A was quantified in each retinal layer by ImageJ. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of at least 5 animals per condition. *p<0.05, **p<0.01, ***p<0.001, significantly different from control as determined by the unpaired Student's *t*-test.

Diabetes changes KIF5B immunoreactivity in the retina

Although no differences were found in the content of KIF5B in total retinal extracts, the distribution of KIF5B along the different retinal layers appeared to be altered, either increased or decreased, but only at 8 weeks of diabetes. By immunohistochemistry, it was detected a significant increase in KIF5B immunoreactivity in the outermost retinal layers (Figure 3A and B), namely at the outer and inner segments of photoreceptor layer (PHO) and at the outer nuclear layer (ONL), respectively to 201.3±19.6% and 154.4±14.8%, comparing to age-matched controls. Conversely, a significant decrease in IPL and GCL was detected at 8 weeks of diabetes (decrease to 74.4±11.5% and to 72.5±12.0% comparing to age-matched controls, respectively) (Figure 3A and B).


A
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Diabetic
8 Weeks
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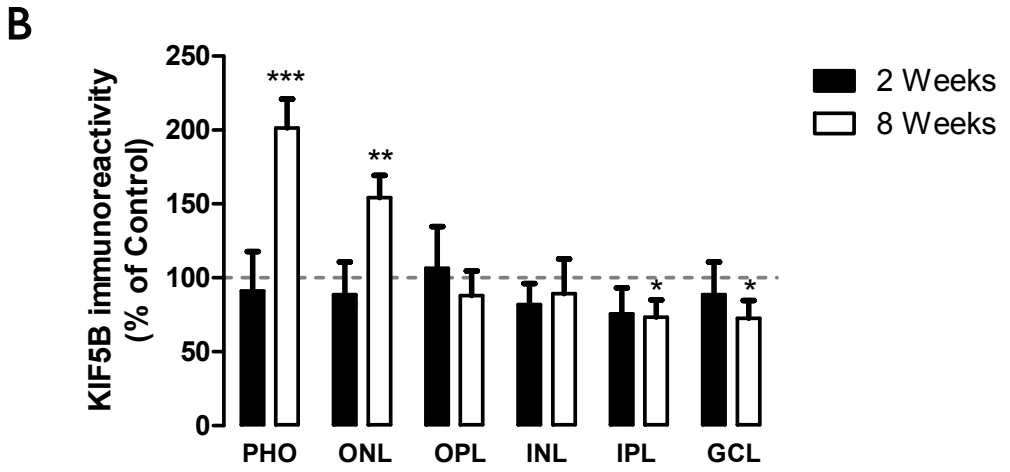
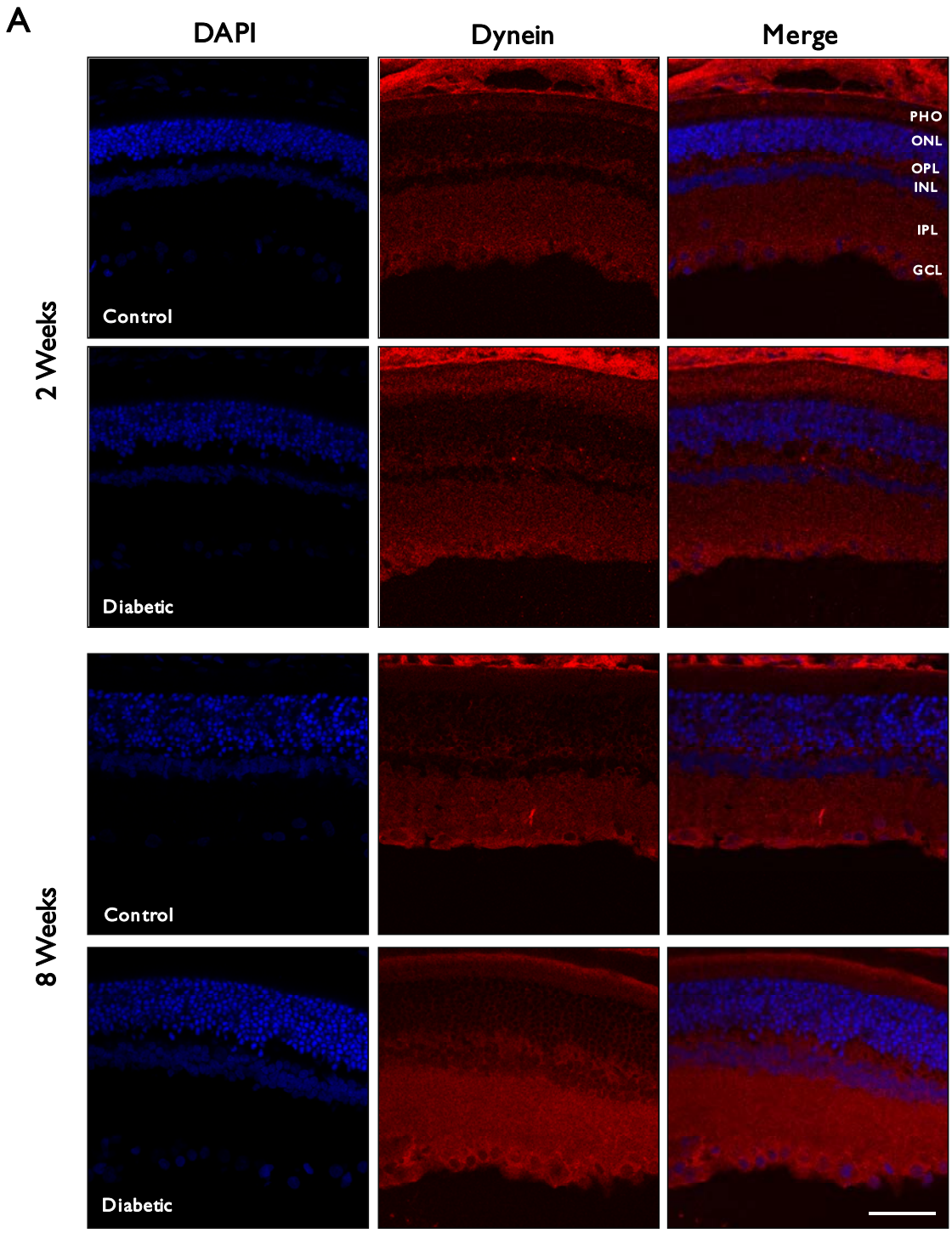


Figure 3. Diabetes alters the distribution of KIF5B in the retina. (A) The distribution of KIF5B was evaluated in retinas isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes) by immunohistochemistry. Magnification 400x; Scale bar: 50 µm. *White arrows*: significantly different from control. (B) The immunoreactivity of KIF5B was quantified in each retinal layer by ImageJ. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of at least 5 animals per condition. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from control as determined by the unpaired Student's *t*-test.

Diabetes does not alter the content and distribution of dynein in the retina

In total retinal extracts, dynein levels remained similar to those found in control animals at 2 and 8 weeks of diabetes. We also analyzed the immunoreactivity of dynein in the retina, and no significant changes were observed along the different retinal layers between diabetic and control animals (Figure 4A and B)


A
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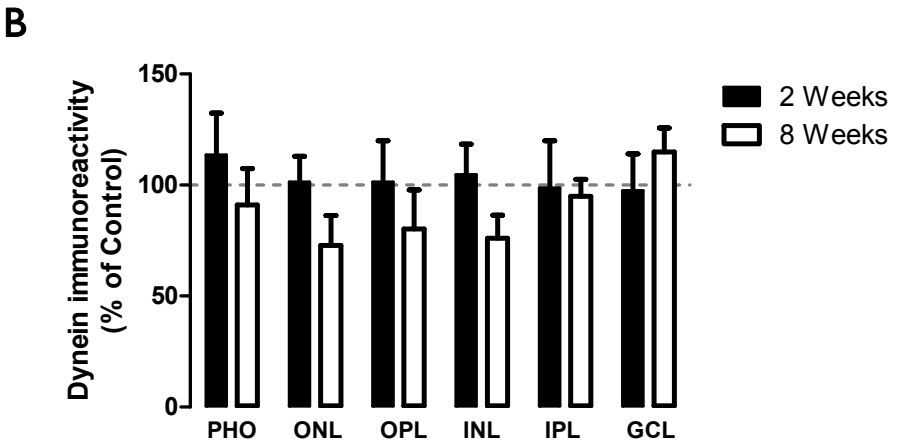
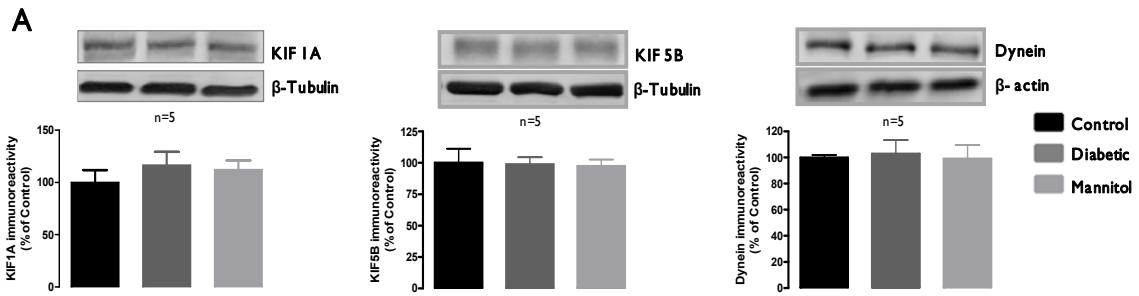


Figure 4. Diabetes does not induce significant alterations in dynein in the retina. (A) The distribution of dynein was evaluated in retinas isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes) by immunohistochemistry. Magnification 400x; Scale bar: 50 μm. (B) The immunoreactivity of dynein was quantified in each retinal layer by ImageJ. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of at least 5 animals.

High glucose does not affect the content and localization of KIF1A, KIF5B, and dynein in retinal cultures

Hyperglycemia is considered the main cause of diabetes complications, triggering various processes that may induce cell dysfunction. KIF1A and KIF5B are motor proteins that transport cargoes from the cell body to the synapse, whereas dynein is responsible for retrograde axonal transport. Exposure of cultured retinal cells to elevated concentrations of D-glucose (30 mM) or D-mannitol (24.5 mM + 5.5 mM glucose), for 7 days, did not induced changes in total protein content of KIFA, KIF5B and dynein (Figure 5A). Additionally, the morphology of retinal neurons was analyzed by immunocytochemistry using a TUJ-1 (neuron-specific class III β-tubulin) antibody. High glucose and mannitol did not induce any alteration in the neuronal morphology (Figure 5B). The immunoreactivity of KIF1A, KIF5B and dynein, as well as the fluorescence of mitotracker (fluorescent dye that stains mitochondria in live cells) also showed that high glucose and mannitol did not induce any change in protein distribution when compared to control (Figure 5B).



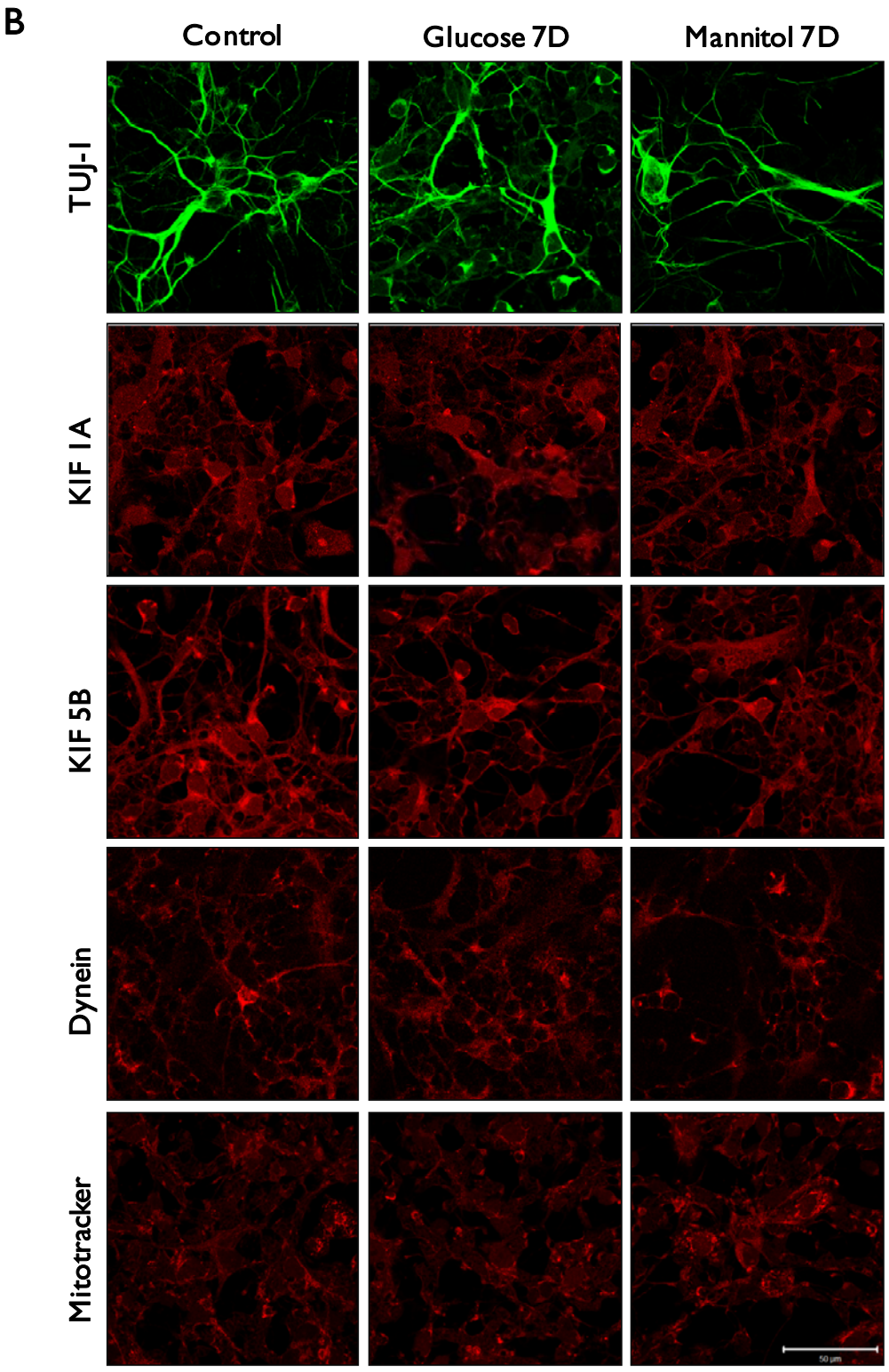

B
Control
Glucose 7D
Mannitol 7D
TUJ-I
KIF 1A
KIF 5B
Dynein
Mitotracker

Figure 5. High glucose does not affect the content and distribution of KIF1A, KIF5B and dynein in retinal neural cell cultures. Cultured retinal cells were incubated with 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for additional 7 days in culture. The concentration of glucose in control conditions was 5 mM. (A) The protein levels of KIF1A, KIF5B and dynein were analyzed by western blotting. Representative images of protein immunoreactive bands are presented above the graphs, with the respective loading control (β-actin or β-III tubulin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of five independent experiments. Regarding total protein content, statistical significance was determined by using ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p<0.05$. (B) The immunoreactivity and distribution of TUJ-1, KIF1A, KIF5B and dynein in the culture was analyzed by immunocytochemistry, as well as the intensity of fluorescence of mitotracker. Magnification 630x; Scale bar: 50 μm.

Discussion

In the current study, we evaluated the impact of diabetes and elevated glucose on key proteins involved in axonal transport in retinal cells. We show that diabetes alters the content of KIF1A and the distribution of KIF1A and KIF5B along retinal layers at 8 weeks of diabetes, suggesting that anterograde transport mediated by these kinesins may be impaired.

Previously, we have found a decrease in the content of several synaptic proteins important for neurotransmission in retinal nerve terminals at 2 and/or 8 weeks of diabetes (Baptista et al., 2011; Gaspar et al., 2010a). Since anterograde axonal transport is responsible for carrying proteins to nerve terminals, which are involved in synaptic transmission, the decrease in the content of synaptic proteins in nerve terminals may be a consequence of deficits in their transport. In cones lacking KIF3A (kinesin present in photoreceptors), changes in photoreceptor properties occur, showing the importance of kinesin in the visual pathway (Avasthi et al., 2009). Trafficking of membrane proteins involved in phototransduction to the outer segments is impaired, resulting in progressive cone degeneration and absence of a photopic electroretinogram. Additionally, rod photoreceptors lacking KIF3A degenerate rapidly between 2 and 4 weeks (Avasthi et al., 2009). Moreover, another study also in the retina, showed that after 24 and 72 h of intravitreal injection of NMDA, occurs an early elevation of KIF5B protein levels in the retina, whereas a significant decrease occurs in the optic nerve, suggesting that a depletion of KIF5B precedes axonal degeneration of the optic nerve in NMDA-induced neurotoxicity (Kuribayashi et al., 2010). Recently, it was found a deficit in the anterograde transport from the retina to the superior colliculus, 6 weeks after the induction of diabetes with STZ (Fernandez et al., 2012). Possibly, similar changes as those we detected in our diabetic model, namely in the content of KIF1A and distribution of KIF1A and KIF5B in the retinal layers, may also be occurring at 6 weeks of diabetes contributing therefore to the deficits observed in the anterograde transport from the retina to the superior colliculus.

In the opposite direction, there is the retrograde axonal transport system, which transports, among other molecules, neurotrophic factors that influence steady-state activities in the cell body. It was previously reported a progressive deficit in the retrograde axonal transport,

mainly in large axons, that is evident 1 month after diabetes induction, that is not associated with RGC loss (Ino-Ue et al., 2000). Here, we studied the 74 kDa dynein intermediate chain subunit that forms a bridge between the heavy chain and the dynactin subunits, which bind microtubules and the cargo to be transported. No changes were found in the total content of dynein in whole retinal extracts from diabetic rats (2 and 8 weeks duration). In an experimental model of glaucoma, the expression of dynein light chain in RGC is downregulated, which could contribute to neuron dysfunction and apoptosis (van Oterendorp et al., 2011). On the other hand, it was demonstrated that dynein heavy chain (chain that contains the site of ATP hydrolysis and is the force-generating part of the protein) accumulates at the optic nerve head with experimental intraocular pressure elevation in the rat, supporting the hypothesis that disrupted axonal transport in RGC may be involved in the pathogenesis of glaucoma (Martin et al., 2006). Although we did not found significant changes in dynein immunoreactivity, we cannot exclude the possibility that for longer periods of diabetes these changes may become significant.

Since hyperglycemia is considered the main factor underlying the development of diabetic complications, we evaluated whether prolonged exposure to high glucose *per se*, which simulates hyperglycemic conditions, could change the content of proteins involved in axonal transport in primary retinal cultures. Moreover, since KIF5 motors are responsible for the axonal transport of mitochondria, and decreased number of mitochondria in axons will likely decrease ATP supply to molecular motors, leading to decreased anterograde and retrograde movement of mitochondria and vesicles, the fluorescence of mitotracker, a mitochondrial probe, was also evaluated. High glucose did not induce any significant changes in the content and distribution of motor proteins and mitochondria, suggesting that hyperglycemia per se might be not the main trigger of molecular alterations in the retinal tissue. Other factors, such as the lack of insulin or the increase in the levels of pro-inflammatory mediators, may contribute for the changes in motor proteins detected in the retina of diabetic animals. In sensory neurons, the loss of insulin-dependent neurotrophic support may contribute to mitochondrial membrane depolarization, establishing a link between insulin and mitochondrial dysfunction in diabetic neuropathy (Fernyhough et al., 2003). Retinal neurons depend on insulin receptor activity for survival (Barber et al., 2001). Long-term instability in retinal insulin signalling may impair insulin-dependent anabolic activities such as protein synthesis in the retina (Chihara, 1981) and increase cell death (Reiter and Gardner, 2003), suggesting that insulin signalling provides neurotrophic actions in the retina. Therefore, diabetic retinopathy may result in part from neurotrophin deficiency (Whitmire et al., 2011), similarly to peripheral neuropathy.

Several evidences indicate that diabetic retinopathy has characteristics of a low-grade chronic inflammatory disease. Increased production of cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), up-regulation of cyclooxygenase-2, increased expression of adhesion molecules and increased leukocyte adhesion and vascular permeability (Carmo et al., 2000; Kowluru et al., 2003; Miyamoto et al., 1999) have been demonstrated in the retina of diabetic animals. Additionally, in the retinas of STZ-induced diabetic rats the levels of IL-1β are also increased (Carmo et al., 1999; Gerhardinger et al., 2005; Kowluru and Odenbach,

2004a; Krady et al., 2005). Therefore, inflammation may be also a factor contributing to changes in axonal transport in diabetes. Previously, it was shown that TNF-α induces perinuclear clustering of mitochondria in L929 cells. This clustering of mitochondria was microtubule-dependent and mimicked by immunoinhibition of conventional kinesin, therefore suggesting that TNF-α-induced mitochondrial clustering is caused by impaired kinesin-mediated transport of mitochondria (De Vos et al., 1998). Afterwards, it was shown that TNF receptor-1 induces activation of kinase pathways, resulting in hyperphosphorylation of kinesin light chain and inhibition of kinesin activity. These data provided evidence for direct regulation of kinesin-mediated organelle transport by extracellular stimuli via cytokine receptor signaling pathways (De Vos et al., 2000). Moreover, it was previously demonstrated that nitric oxide (NO) released from activated microglia inhibits directed axonal movement of synaptic vesicle precursors containing synaptophysin and synaptotagmin in hippocampal neurons, suggesting that disturbance of axonal transport by NO from microglia origin may therefore be responsible for axonal injury and synaptic dysfunction in inflammatory brain diseases (Stagi et al., 2005). Later, it was demonstrated that exposure of hippocampal neuronal cultures to TNF-α enhanced the phosphorylation of c-Jun N-terminal kinase (JNK) in neurites. TNF-α treatment induced the dissociation of KIF5B from tubulin in axons and inhibited axonal transport of mitochondria and synaptophysin by reducing the mobile fraction via JNK (Stagi et al., 2006). Therefore, inflammatory cytokines, can affect axonal transport motors and consequently contribute to the previous detected changes in synaptic proteins in the retina (Baptista et al., 2011; Gaspar et al., 2010a).

In summary, our data demonstrate that the content of KIF1A and KIF5B motor proteins is affected by diabetes in the retina, which may contribute to the impairment of the anterograde axonal transport and consequently to neuronal dysfunction in the retina. The changes observed may be probably due to insulin deficiency or inflammation rather than hyperglycemia, or to a synergistic combination of these factors.

Acknowledgments

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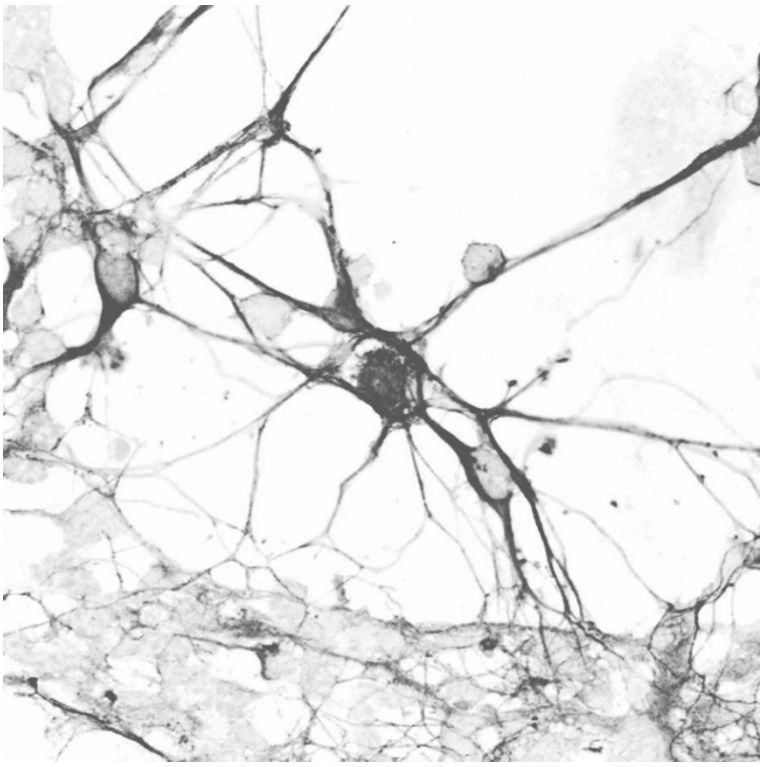
Chapter 7

Diabetes affects anterograde axonal transport motor proteins in the hippocampus



The results presented in this chapter were submitted for publication by Filipa I. Baptista, Maria J. Pinto, Ramiro D. Almeida and António F. Ambrósio.

Abstract

Diabetes *mellitus* is the most common metabolic disorder in humans. Diabetic encephalopathy is characterized by cognitive and memory impairments, which have been associated with changes in the hippocampus, but the mechanisms underlying those impairments triggered by diabetes, are far from being elucidated. The disruption of axonal transport is associated with several neurodegenerative diseases and might also play a role in diabetes-associated disorders affecting nervous system. We investigated the effect of diabetes (2 and 8 weeks duration) on KIF1A, KIF5B and dynein motor proteins, which are important for axonal transport, in the hippocampus. The levels of those proteins were evaluated by immunohistochemistry in hippocampal slices and immunoblotting in total extracts of hippocampus from streptozotocin-induced diabetic and age-matched control animals. Diabetes increased the immunoreactivity of KIF1A and KIF5B in the hippocampus, but no alterations in dynein were detected. Since hyperglycemia is considered a major player in diabetic complications, the effect of a prolonged exposure to high glucose on motor proteins, mitochondria and synaptic proteins in hippocampal neurons was also studied, giving particular attention to changes in axons. Hippocampal cell cultures were exposed to high glucose (50 mM) or mannitol (osmotic control; 25 mM plus 25 mM glucose) for 7 days. In hippocampal cultures incubated with high glucose no changes were detected in the fluorescence intensity or number of accumulations related with mitochondria in the axons of hippocampal neurons. Nevertheless, high glucose increased the number of fluorescent accumulations of KIF1A and synaptotagmin-1 and decreased KIF5B, SNAP-25 and synaptophysin immunoreactivity specifically in axons of hippocampal neurons. These changes suggest that anterograde axonal transport mediated by these kinesins may be impaired in hippocampal neurons, which may lead to changes in synaptic proteins, thus contributing to changes in hippocampal neurotransmission and to cognitive and memory impairments.

Keywords: Diabetic encephalopathy; axonal transport; kinesin; dynein; synaptic proteins; mitochondria

Introduction

Diabetes has been associated with cognitive and memory impairments, indicating that the hippocampus can be affected by this disease. Several studies have demonstrated that diabetes impairs synaptic structure and function in the hippocampus both presynaptically (Gaspar et al., 2010a; Grillo et al., 2005) and postsynaptically (Biessels et al., 1996; Kamal et al., 1999). Previously, we found that diabetes changes the levels of several synaptic proteins involved in exocytosis in hippocampal and retinal nerve terminals, suggesting that axonal transport of those proteins to distal synaptic sites may be impaired under diabetes (Baptista et al., 2011; Gaspar et al., 2010a). Moreover, in hippocampal cell cultures, we also found that prolonged exposure to high glucose leads to an accumulation of syntaxin-1, VGluT-1 and synaptotagmin-1 at the cell body of hippocampal neurons, further suggesting that axonal transport may be affected (Gaspar et al., 2010b). Potential alterations in axonal transport can somehow contribute to the development of cognitive impairment and memory loss under diabetes.

The impairment of axonal transport is an early and perhaps causative event in many neurodegenerative diseases, and might be due to alterations and/or loss of motor proteins (kinesin and dynein), microtubules, cargoes (by inhibiting their attachment to motor proteins) and ATP fuel supply (mitochondria) which enables molecular motors to undertake the axonal transport (De Vos et al., 2008). The inhibition of axonal transport leads to a rapid loss of function in the distal axon and to a "dying back" axonal degeneration. The axonal transport is known to be affected in experimental models of diabetes. Most studies regarding nerve dysfunction in diabetes focus on the peripheral nervous system, however increasing evidence also shows that the central nervous system can be affected by diabetes. At peripheral nervous system level, a reduction in retrograde transport has been reported, namely the transport of nerve growth factor in the sciatic nerve of diabetic rats, and endogenous neurotrophins on the cervical and vagus nerve of diabetic rats (Jakobsen et al., 1981; Lee et al., 2002; Lee et al., 2001). Moreover, alterations in the axonal caliber in nerves of diabetic animals are likely to be secondary to the impairment of slow anterograde axonal transport, which is correlated with reduced local levels of neurofilament (Medori et al., 1985; Yagihashi et al., 1990). Studies using fluoro-gold labelling showed that diabetes affects the retrograde axonal transport in retinal ganglion cells (Ino-Ue et al., 2000; Zhang et al., 2000), and recently, a deficit in anterograde transport from the retina to the superior colliculus was detected at 6 weeks of diabetes (Fernandez et al., 2012). Furthermore, it was also shown that hyperglycemia impairs axonal transport in olfactory receptor neurons in mice (Sharma et al., 2010). Nevertheless, to our knowledge, no studies have been performed to analyze the effect of diabetes on axonal transport in the hippocampus, or to investigate local changes in motor proteins in hippocampal neurons. Therefore, the goal of this work, was to evaluate the impact of early diabetes in the hippocampus, namely in the content and distribution of KIF1A (kinesin that transports synaptic vesicle precursors containing synaptophysin and synaptotagmin), KIF5B (kinesin that transports mitochondria and membrane organelles that contain presynaptic membrane proteins such as syntaxin-1 and SNAP-25) and

dynein (motor protein responsible for the retrograde axonal transport of organelles, such as mitochondria). Moreover, since hyperglycemia has been considered the main pathogenic factor underlying the development of diabetic complications, we aimed to evaluate whether high glucose *per se*, giving particular attention to changes occurring in the axons, could affect the levels and distribution of motor and synaptic proteins, and the distribution of mitochondria in the axons of hippocampal neurons.

Experimental Procedures

Animals

All animals were handled according to the EU guidelines for the use of experimental animals (86/609/EEC), and the experiments were approved by our Institutional Ethics Committee (Comissão de Ética da Faculdade de Medicina da Universidade de Coimbra). Approval ID: FMUC/08/11. Male Wistar rats (Charles River Laboratories), eight weeks-old, were randomly assigned to control or diabetic groups. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, freshly dissolved in 10 mM sodium citrate buffer, pH 4.5) (Sigma, St. Louis, MO, USA). Hyperglycemic status (blood glucose levels exceeding 250 mg/dl) was confirmed two days later with a glucometer (Elite, Bayer, Portugal). Before sacrifice, rats were weighted and blood samples were collected to measure glucose levels. Diabetic rats and age-matched controls were anesthetized with halothane and then sacrificed, two and eight weeks after the onset of diabetes.

Immunohistochemistry in brain slices

Brain slices preparation

Rats from each experimental group were deeply anesthetized with ketamine/xylazine and intracardially perfused with 0.1 M phosphate-buffered saline solution (PBS, in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.47 KH_2PO_4; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and post-fixed for 24 h in 4% PFA and then dehydrated in 20% sucrose in 0.1 M PBS for 24 h. Brain slices (30 µm thickness) were cut in a cryostat (Leica CM3050S, Nussloch, Germany) and collected in 0.1 M PBS with 0.01% sodium azide. Brain slices were used for free-floating immunohistochemistry.

Free-floating immunohistochemistry

Slices were washed twice with 0.1 M PBS, blocked with 0.25% Triton X-100 and 5% normal fetal bovine serum (FBS) in 0.1 M PBS for 1 h at room temperature, and then incubated with the appropriate primary antibodies (listed in Table 1) for 24 h at 4°C. Incubation with primary antibodies was followed by incubation with conjugated secondary antibody Alexa Fluor-488 (donkey anti-goat IgG, 1:250), for sections stained for KIF1A and KIF5B, or Alexa Fluor-568 (goat anti-mouse IgG, 1:250), for sections stained for dynein, plus DAPI (1:5,000), to stain cell nuclei, for 2 h 30 min at room temperature. From this point forward, the slices were protected from

light. Sections were then washed three times with 0.1 M PBS in the dark and then mounted on slides with glycergel (Dako mounting medium). Sections were examined with a LSM 710 Meta Confocal laser scanning microscope (Zeiss, Germany).

Immunofluorescence quantification in hippocampal subregions

Quantitative analysis of immunofluorescence in brain slices was performed using ImageJ. Typically, images of the hippocampal subregions from each animal group (control and diabetic animals), were captured using the same settings and an equivalent area of each subregion was considered. The total fluorescence intensity of each hippocampal subregion was quantified and measurements were expressed in arbitrary units (AU).

Preparation of total hippocampal extracts

After dissection, the hippocampi from each rat were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, supplemented with complete miniprotease inhibitor cocktail tablets and 1 mM DTT). The resulting homogenate was sonicated (4 pulses, 2 seconds each) and then centrifuged at 16,100 x *g* for 10 min. All procedure was performed at 4°C. The supernatant was stored at -80°C until use.

Primary cultures of rat hippocampal neurons

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E17–E19 Wistar rat embryos. The hippocampi were dissected under sterile conditions, using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hank's solution (in mM: 5.36 KCl, 0.44 KH_2PO_4, 137 NaCl, 4.16 $NaHCO_3$, 0.34 $Na_2HPO_4.2H_2O$, 5 glucose, 1 sodium piruvate, 10 HEPES and 0.001% phenol red, pH 7.4). The hippocampi were digested with trypsin (0.06%, 15 min, at 37°C; Gibco Invitrogen, Life Technologies, Scotland, UK), in Ca^{2+}- and Mg^{2+}-free Hank's solution. The hippocampi were then washed with Hank's solution containing 10% fetal bovine serum (Biochrom, Cambridge, UK) to stop digestion. The cells were dissociated in Neurobasal medium (Gibco Invitrogen) supplemented with B27 (1:50 dilution; Gibco Invitrogen), 0.5 mM glutamine, 25 µM glutamate and 50 µg/ml gentamycin. The cells were plated in six-well plates ($8.75x10^4$ cells/cm^2) or in coverslips ($2.25x10^4$ cells/cm^2) coated with poly-D-lysine (0.1 mg/ml). For experiments in which axon segments were analyzed, neurons were plated in the center of the coverslip (approximately 10,000 cells). Under these conditions, axons grow outward the center (were soma and dendrites are located) and away from the dense neuronal network, where they can be imaged and analyzed independently. The cultures were maintained in a humidified incubator with 5% CO_2/ 95% air at 37°C for 14 days. The concentration of glucose in control conditions was 25 mM. After seven days in culture, half of the medium was replaced by fresh medium, and cells were incubated with 25 mM of glucose (yielding a total of 50 mM glucose) or with 25 mM mannitol (plus 25 mM glucose in normal medium), which was used as an osmotic control, and maintained for further seven days.

Preparation of extracts of cultured hippocampal neurons

Cells were rinsed twice with ice-cold PBS and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4). The lysates were incubated on ice for 30 min and then centrifuged at 16,100 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C until use.

Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 6-8% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were then incubated with primary antibodies (listed in Table 1) overnight at 4°C. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated with an anti-mouse or anti-goat alkaline phosphatase-linked IgG secondary antibody (1:10,000; GE Healthcare, Buckinghamshire, UK) in TBS-T with 1% low-fat milk for 1 h at room temperature. The membranes were processed for protein detection using the enhanced chemifluorescence substrate (ECF; GE Healthcare). Fluorescence was detected on an imaging system (Thyphoon FLA 9000, GE Healthcare) and the digital quantification of bands immunoreactivity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed and tested for β-actin immunoreactivity (1:5,000) or β-III tubulin (1:5,000) to prove that similar amounts of protein were applied to the gels.

Immunocytochemistry

Hippocampal cell cultures were washed three times with PBS and fixed with 4% paraformaldehyde and 4% sucrose for 10 min at room temperature. Cells were then washed three times with PBS and permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. Non-specific binding was prevented incubating cells with 3% BSA/0.2% Tween-20 in PBS for 30 min. Cells were then incubated with the primary antibodies (listed in Table 1) for 2 h at room temperature. After incubation, cells were rinsed three times with PBS and incubated with the secondary antibodies for 1 h at room temperature in the dark. The nuclei were stained with DAPI (1:5,000). Upon rinsing three times with PBS, the coverslips were mounted on glass slides using Dako Fluorescent mounting medium (Dako, Denmark). The preparations were visualized under a confocal laser scanning microscope LSM 710 META (Zeiss, Germany) or under

an inverted microscope Zeiss Axiovert 200 (Zeiss, Germany), as indicated in the corresponding figure legends. Quantitative analysis of immunofluorescent data was performed using ImageJ.

Table1. List of Primary antibodies

Primary Antibody	Sample	Antibody Dilution	Protein (µg)	Source
Mouse anti-KIF1A	Total Extracts Hippocampus	1:1,000	20	BD Biosciences
	Total Extracts Primary cultures	1:1,000	80	
Goat anti-KIF1A	Immunocytochemistry	1:50	_	Santa Cruz
	Immunohistochemistry	1:50	_	
Goat anti-KIF5B	Total Extracts Hippocampus	1:2,000	10	Abcam
	Total Extracts Primary cultures	1:2,000	20	
	Immunocytochemistry	1:100	_	
	Immunohistochemistry	1:100	_	
Mouse anti-Dynein	Total Extracts Hippocampus	1:2,000	20	Abcam
	Total Extracts Primary cultures	1:2,000	40	
	Immunocytochemistry	1:100	_	
	Immunohistochemistry	1:100	_	
Mouse anti-Tau	Total Extracts Primary cultures	1:1,000	30	Cell Signaling
	Immunocytochemistry	1:500	_	
Rabbit anti-TUJ-1	Immunocytochemistry	1:1,000	_	Covance
Mouse anti-SNAP-25	Immunocytochemistry	1:100	_	SYSY
Mouse anti-Synaptophysin	Immunocytochemistry	1:50	_	Chemicon
Mouse anti-Syntaxin-1	Immunocytochemistry	1:100	_	SYSY
Mouse anti-Synaptotagmin-1	Immunocytochemistry	1:200	_	SYSY

Statistical analysis

For Western blotting analysis, statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Student's *t*-test (variance analysis was not undertaken since the effect of age on the content of motor proteins was not the aim of this study). Thus, gels were always loaded with samples from age-matched animals and not from animals with different ages. Statistical comparisons for the analysis of Western blotting data from hippocampal cell cultures was determined by using one-way ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p<0.05$.

In order to quantitatively analyze immunofluorescence in brain slices, all values were compiled for statistical analysis and significant difference between control and diabetic animals was performed using the unpaired Student's *t*-test. Additionally, for quantitative analysis of

immunofluorescence in hippocampal cultures, significant difference between control and high glucose condition was also analyzed using the unpaired Student's *t*-test. Differences were considered significant for $p<0.05$.

Results

Animals

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (255.7±3.5 g for control animals and 253.4±3.4 g for diabetic animals). The glucose levels were also similar in both groups (89.1±1.4 mg/dl for controls and 86.7±5.7 mg/dl for diabetic animals). Average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table 2. A marked impairment in weight gain occurred in diabetic rats comparing to age-matched controls in all time points analyzed. Diabetic animals also presented significantly higher blood glucose levels when compared to age-matched controls.

Table 2. Average weight and blood glucose levels of diabetic and age-matched control rats

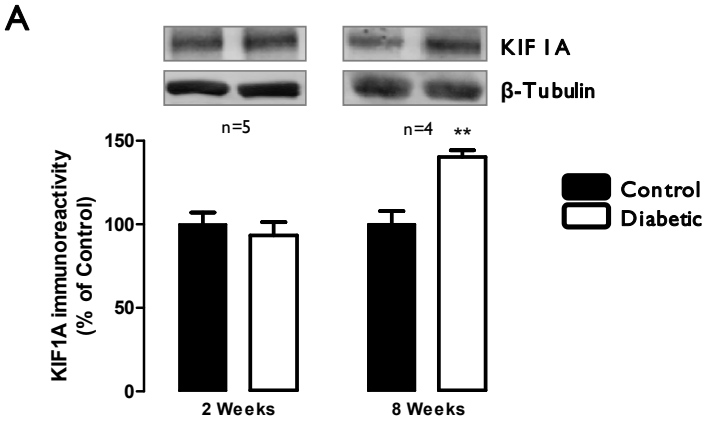
Diabetes duration	Weigth (g)	Blood Glucose (mg/dL)
2 Weeks		
Control	323.7±6.1	104.9±4.1
Diabetic	231.1±10.5***	372.3±24.1***
8 Weeks		
Control	394.6±16.7	89.9±2.9
Diabetic	245.8±13.7***	488.9±38.5***

Measurements were made immediately before the sacrifice of animals. *** $p<0.001$.

Diabetes increases KIF1A and KIF5B content and immunoreactivity in the hippocampus at 8 weeks of diabetes

KIF1A is an anterograde motor protein that transports membranous organelles along axonal microtubules (Hirokawa et al., 2009). It is thought that this protein may play a critical role in the development of axonal neuropathies, which may result from impaired axonal transport. Our previous results suggest that hyperglycemic conditions might impair axonal transport in hippocampal neurons (Gaspar et al., 2010a; Gaspar et al., 2010b). To our knowledge, the effect of diabetes on the content of motor proteins in the hippocampus has never been addressed, and so we analyzed the content of KIF1A and KIF5B in hippocampal total extracts by western blotting and also the immunoreactivity of both proteins in hippocampal *Cornu Ammonis* (CA1 and CA3) and dentate gyrus (DG) subregions. No changes were detected neither in the levels of KIF1A in

total extracts of hippocampus, nor in its immunoreactivity in hippocampal subregions at 2 weeks of diabetes (Figure 1A and B). However, at 8 weeks of diabetes, KIF1A levels significantly increased in hippocampal total extracts (increase to 140.3±4.0%) compared to age-matched control animals (Figure 1A). A significant increase in the immunoreactivity of this protein was also observed by immunohistochemistry at 8 weeks of diabetes in CA1, CA3 and DG hippocampal subregions (increase to 156.6±14.9%, 210.1±31.6% and 213.7±16.6% of the control, respectively; Figure 1B).



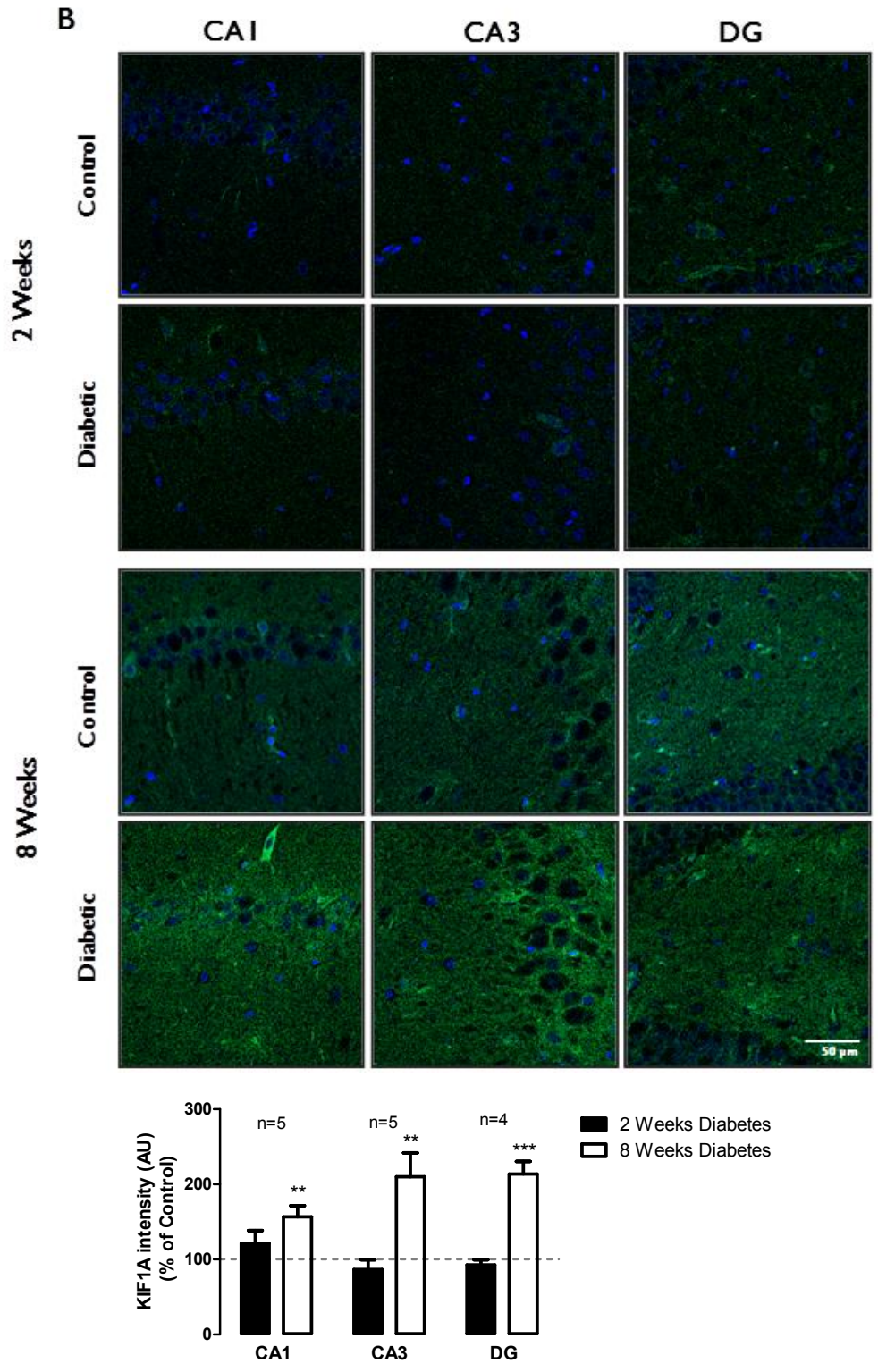
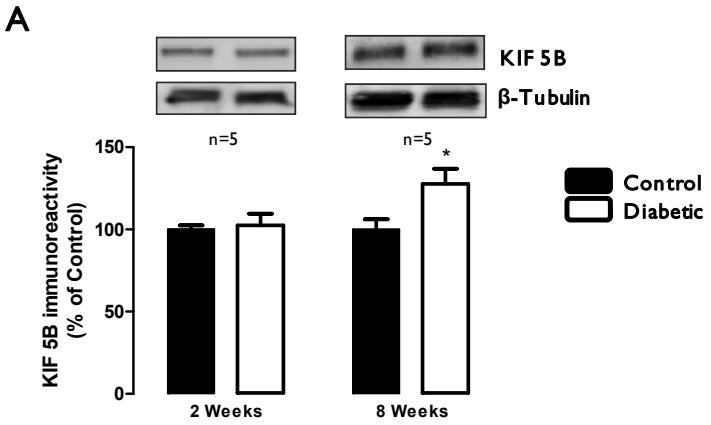

B
CA1
CA3
DG
2 Weeks
Control
Diabetic
8 Weeks
Control
Diabetic
50 µm
KIF1A intensity (AU)
(% of Control)
300
200
100
0
n=5
n=5
n=4
**
**
***
2 Weeks Diabetes
8 Weeks Diabetes
CA1
CA3
DG

Figure 1. Diabetes induces changes in the protein content of KIF1A in the hippocampus. (A) KIF1A protein levels were analyzed by immunoblotting in total extracts of hippocampus isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes). Representative Western blots are presented above the graphs, with the respective loading controls (β-III tubulin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 4-5 animals. **p<0.01 compared to age-matched control using Student's t test. (B) The distribution of KIF1A protein in hippocampal subregions of control and STZ-induced diabetic animals was also analyzed by immunohistochemistry. KIF1A immunoreactivity increased at 8 weeks of diabetes. The preparations were visualized under a laser scanning confocal microscope LSM 710 META (Zeiss, Germany). Scale bar: 50 μm. The quantification of immunoreactivity (fluorescence intensity arbitrary units) was performed and presented below the images. **p<0.01, ***p<0.01, significantly different from control as determined by the unpaired Student's *t*-test.

KIF5B is a microtubule-dependent motor protein required for normal distribution of presynaptic cargoes and mitochondria. At 2 weeks of diabetes, no significant changes were observed in KIF5B immunoreactivity in the hippocampus by Western blotting (Figure 2A and B). However, at 8 weeks of diabetes, it was observed a significant increase in the content of KIF5B by Western blotting (increase to 127.7±9.3% of the control; Figure 2A). Moreover, by immunohistochemistry, it was also detected an increase in the immunoreactivity of this protein in CA1, CA3 and DG hippocampal subregions (increase to 131.3±11.0%, 170.9±23.7% and 146.2±21.4% of the control, respectively; Figure 2B).



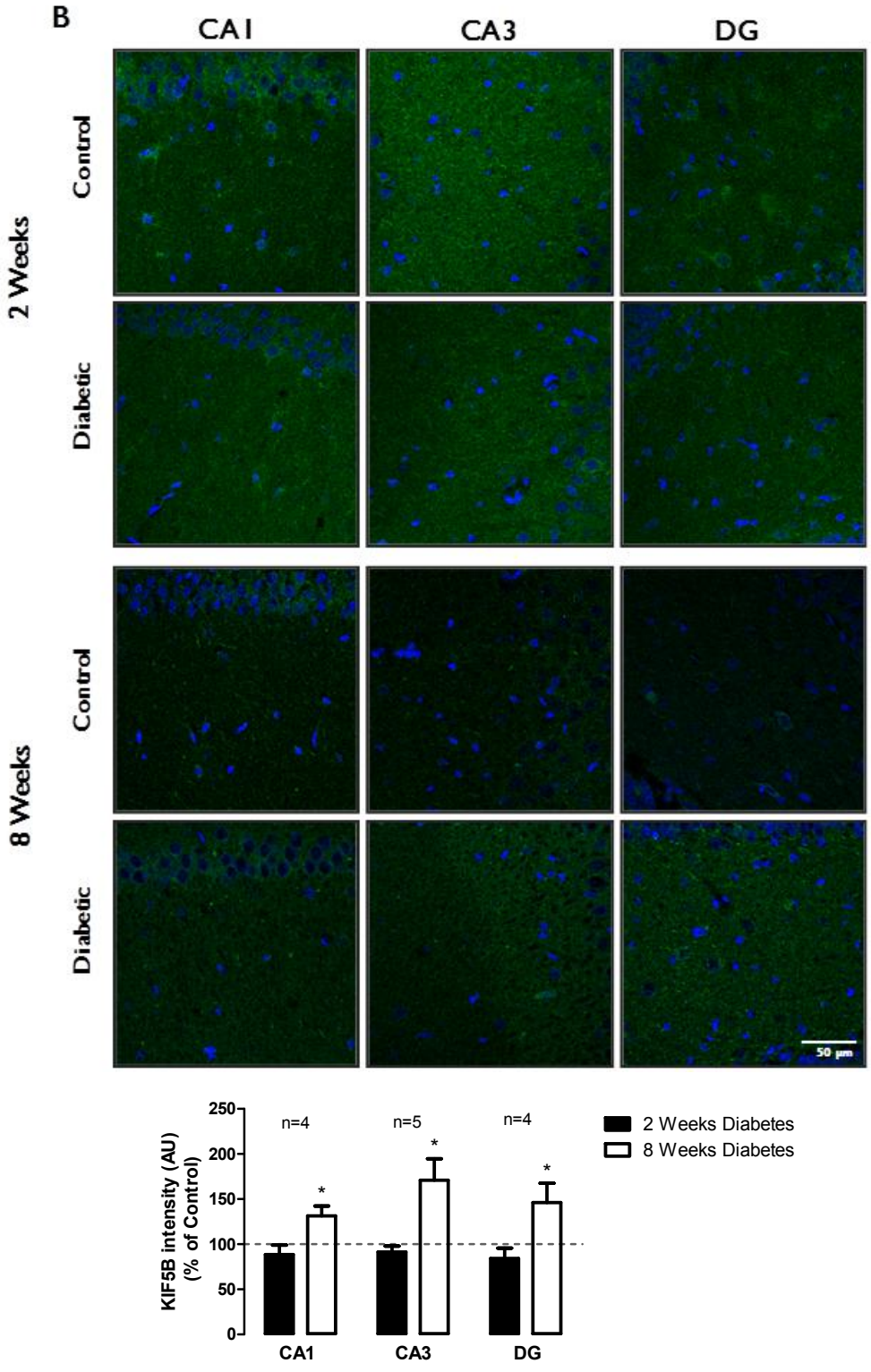
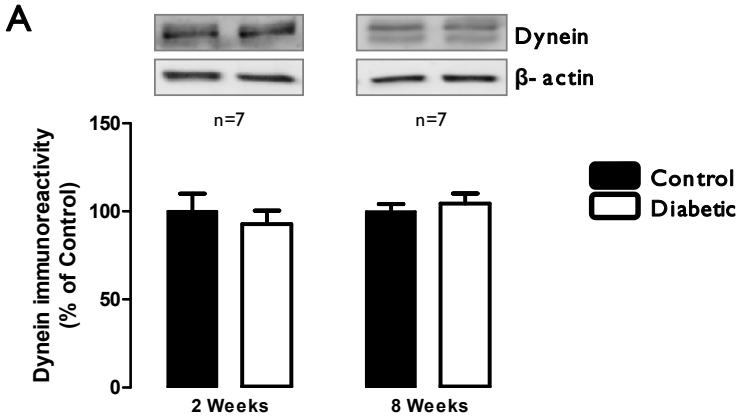

B
CA1
CA3
DG
2 Weeks
Control
Diabetic
8 Weeks
Control
Diabetic
50 µm
KIF5B intensity (AU)
(% of Control)
250
200
150
100
50
0
n=4
n=5
n=4
*
*
*
2 Weeks Diabetes
8 Weeks Diabetes
CA1
CA3
DG

Figure 2. Diabetes induces changes in the protein content of KIF5B in the hippocampus. (A) KIF5B protein levels were analyzed by immunoblotting in total extracts of hippocampus isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes). Representative Western blots are presented above the graphs, with the respective loading controls (β-III tubulin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 5 animals. *p<0.05 compared to age-matched control using Student's t test. (B) The distribution of KIF5B protein in hippocampal subregions of control and STZ-induced diabetic animals was also analyzed by immunohistochemistry. KIF5B immunoreactivity increased at 8 weeks of diabetes. The preparations were visualized under a laser scanning confocal microscope LSM 710 META (Zeiss, Germany). Scale bar: 50 μm. The quantification of immunoreactivity (fluorescence intensity arbitrary units) was performed and presented below the images.*p<0.05, significantly different from control as determined by the unpaired Student's *t*-test.

Diabetes does not affect the content of dynein in the hippocampus

Dynein is the major molecular motor protein that moves cargoes such as mitochondria, organelles and proteins towards the minus end of microtubules, thus being responsible for retrograde transport in neurons. In hippocampal total extracts, dynein levels remained similar to those found in control samples at 2 and 8 weeks of diabetes (Figure 3A). Similarly, no changes were detected in CA1, CA3 and DG hippocampal subregions by immunohistochemistry in both time-points of diabetes, 2 and 8 weeks (Figure 3B).



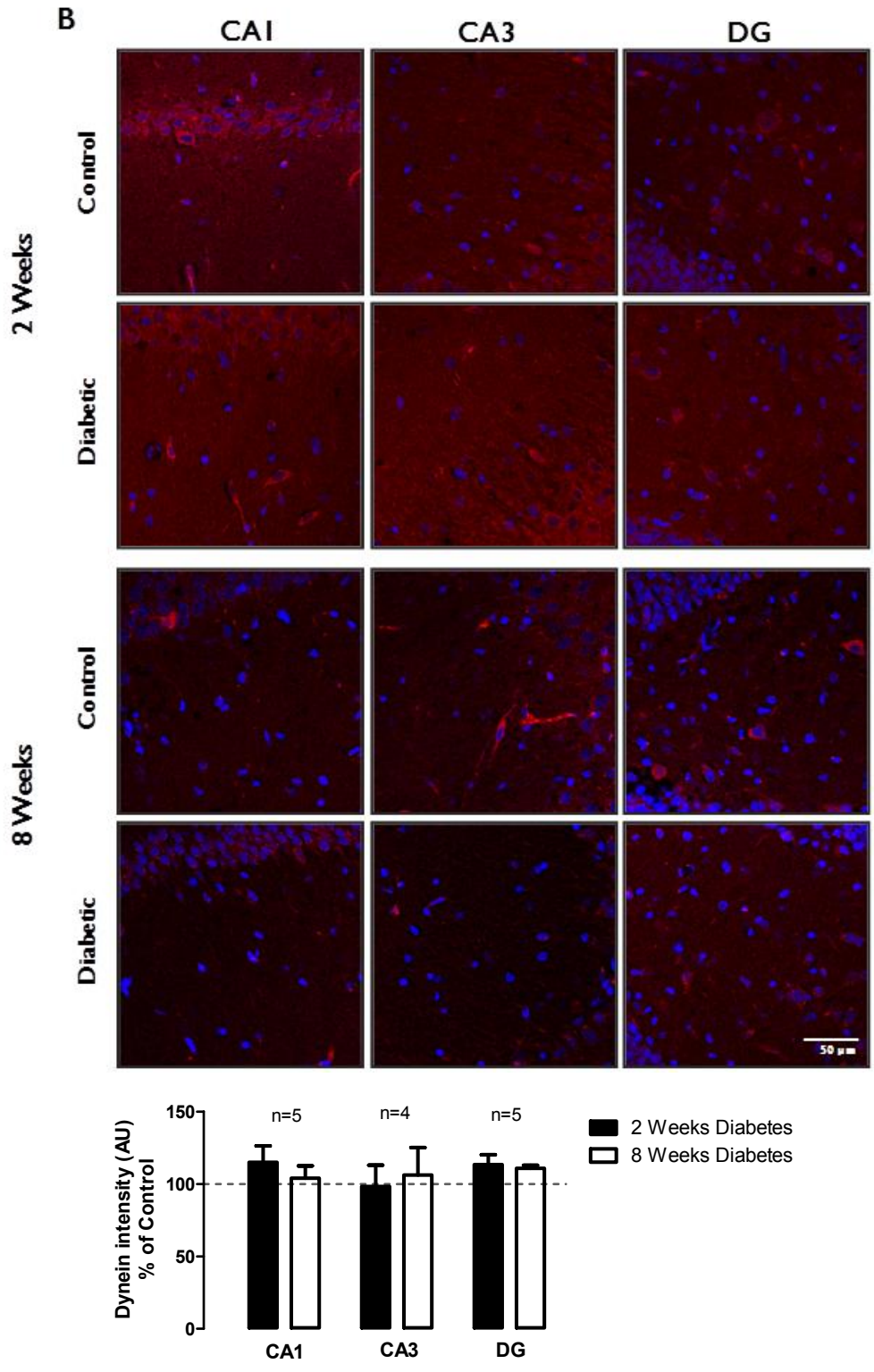
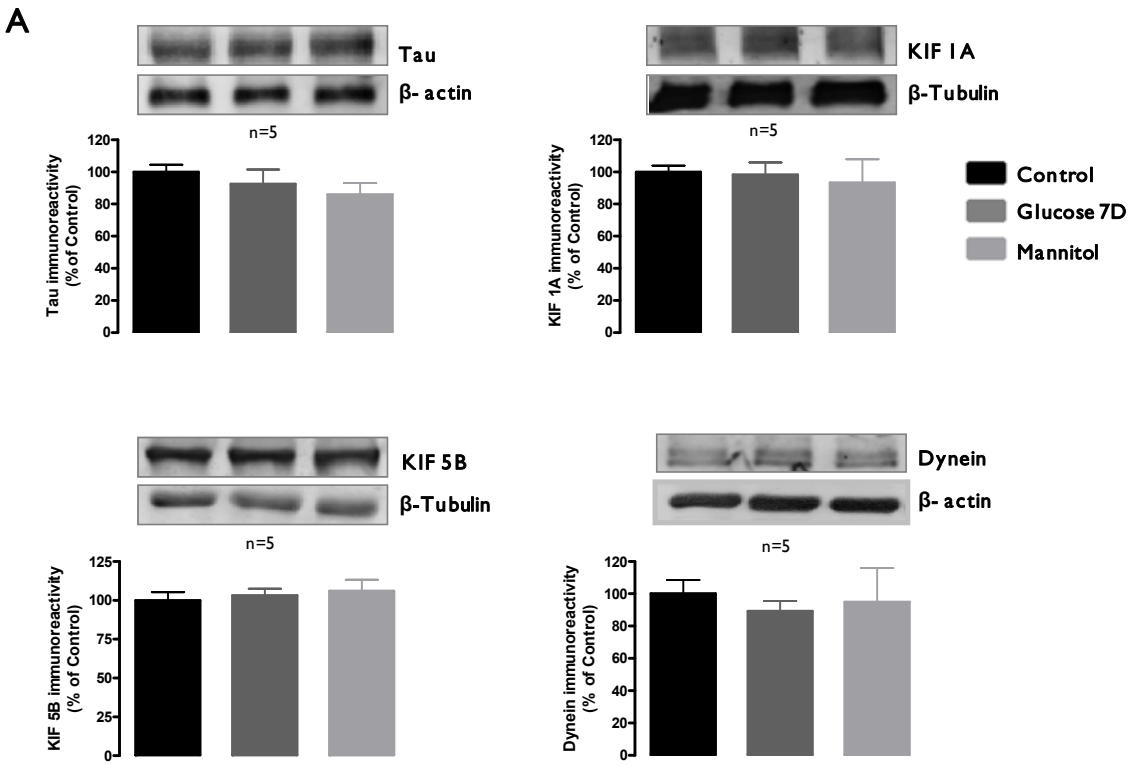

B
CA1
CA3
DG
2 Weeks
Control
Diabetic
8 Weeks
Control
Diabetic
50 µm
Dynein intensity (AU)
% of Control
150
100
50
0
n=5
n=4
n=5
CA1
CA3
DG
2 Weeks Diabetes
8 Weeks Diabetes

Figure 3. Diabetes does not induce changes in the protein content of dynein in the hippocampus. (A) Dynein protein levels were analyzed by immunoblotting in total extracts of hippocampus isolated from control and STZ-induced diabetic animals (2 and 8 weeks of diabetes). Representative Western blots are presented above the graphs, with the respective loading controls (β-III tubulin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM of 7 animals. (B) The distribution of dynein in hippocampal subregions of control and STZ-induced diabetic animals was also analyzed by immunohistochemistry. The preparations were visualized in a laser scanning confocal microscope LSM 710 META (Zeiss, Germany). Scale bar: 50 μm. The quantification of immunoreactivity (fluorescence intensity arbitrary units) was performed and presented below the images.

High glucose does not affect the overall content and localization of tau, KIF1A, KIF5B and dynein in hippocampal cell cultures

Hyperglycemia has been considered the main pathogenic factor underlying the development of diabetic complications, triggering various metabolic and signaling processes that may induce cell dysfunction. Here, we evaluated whether high glucose *per se*, mimicking hyperglycemic conditions, was able to change the content of proteins involved in axonal transport in cultured hippocampal neurons. Exposure of hippocampal neurons to high glucose, or mannitol (osmotic control), did not affect the total protein content of tau, KIF1A, KIF5B and dynein, as it can be observed in Figure 4A. These results were confirmed by immunocytochemistry, since high glucose and mannitol did not induce any change in the immunoreactivity of these proteins in the overall hippocampal culture, when compared to control cells (Figure 4B).


A
Tau
β- actin
n=5
Tau immunoreactivity (% of Control)
KIF 1A
β-Tubulin
n=5
KIF 1A immunoreactivity (% of Control)
Control
Glucose 7D
Mannitol
KIF 5B
β-Tubulin
n=5
KIF 5B immunoreactivity (% of Control)
Dynein
β- actin
n=5
Dynein immunoreactivity (% of Control)

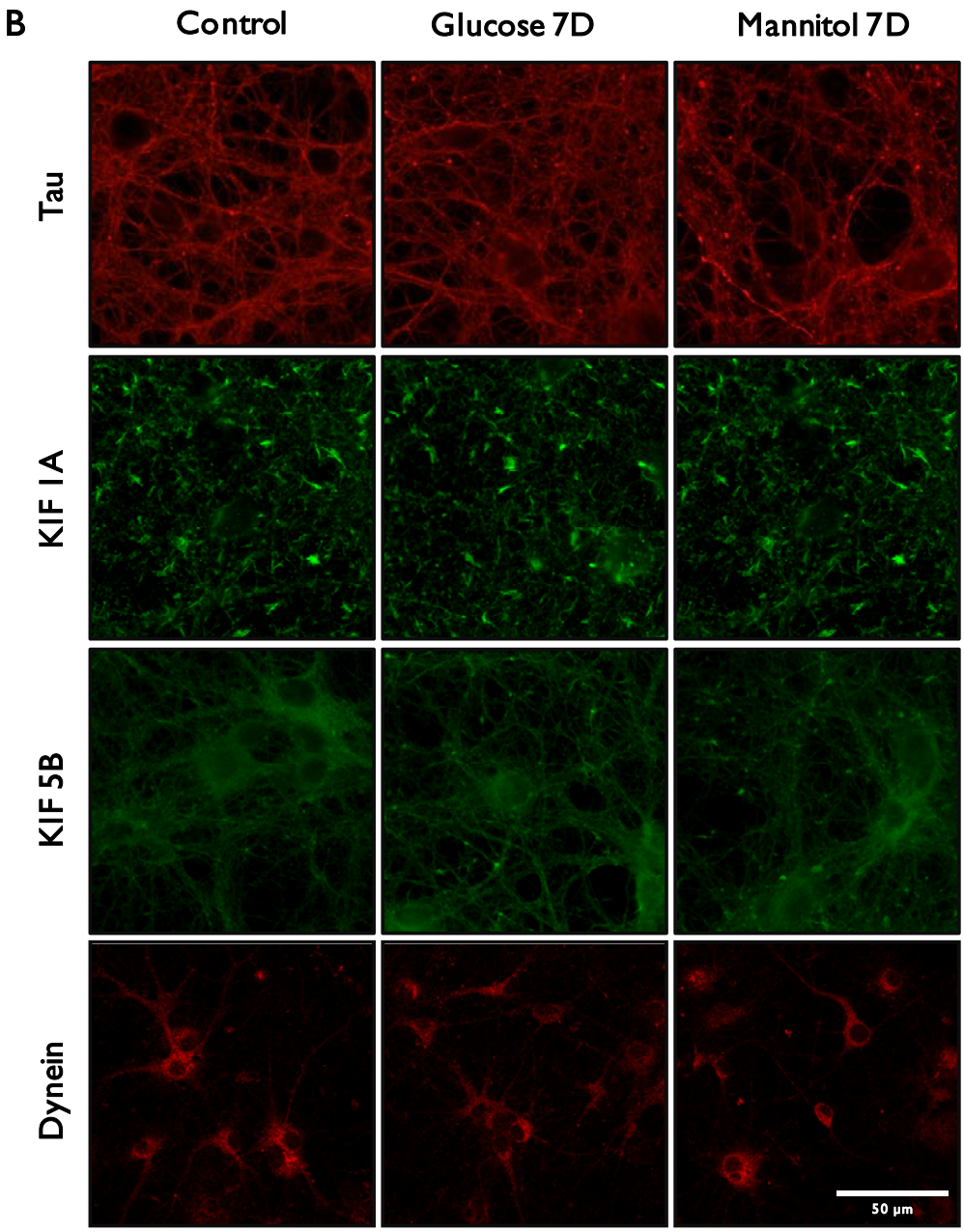


Figure 4. High glucose does not affect the global content and distribution of tau, and motor proteins. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of tau, KIF1A, KIF5B and dynein were analyzed by western blotting. Representative images of protein immunoreactive bands are presented above the graphs, with the respective loading control (β-actin or β-III tubulin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM of 5 independent experiments. Statistical significance for the analysis of the protein content of hippocampal cell cultures was determined by using ANOVA, followed by Dunnett's post hoc test. (B) The immunoreactivity and distribution of tau,

KIF1A, KIF5B and dynein in cultures was analyzed by immunocytochemistry. The preparations were visualized under an inverted microscope Zeiss Axiovert 200 (Zeiss, Germany). Scale bar: 50 μm.

High glucose alters the content and distribution of anterograde motor proteins in axons

Although no changes were detected in the total content of motor proteins in hippocampal cultures, changes can occur specifically in axons, being those changes masked in the overall context. In fact, we previously have shown that diabetic conditions specifically affect the content of several synaptic proteins only in nerve terminals (Baptista et al., 2011; Gaspar et al., 2010a), thus suggesting that their transport to distal sites might be impaired. Taken this into account, we wonder whether exposure to high glucose could induce changes in the content and distribution of motor proteins specifically along axons of hippocampal neurons. Exposure of hippocampal neurons to elevated glucose for 7 days increased the immunoreactivity of KIF1A (KIF1A immunoreactivity/axonal area) in axons (increase to 144.3±5.5% compared to control conditions; Figure 5A). As it can be observed in the axon segments shown in Figure 5A, KIF1A has a characteristic punctate pattern along axons of hippocampal neurons, as it was already described (Lee et al., 2003). To investigate whether high glucose levels could affect this distribution pattern, we measured the number of fluorescent KIF1A accumulations along axon. An increase to 149.4±7.4% in the number of KIF1A accumulations per axonal area compared to control conditions was observed (Figure 5A). Conversely, regarding KIF5B, we did not detect fluorescent KIF5B accumulations in the axons of hippocampal neurons, but there was a decrease (64.1±4.3% of the control, Figure 5B) in its immunoreactivity in the axons of hippocampal neurons exposed to high glucose for 7 days compared to control conditions. Due to the weak signal for dynein in axons, it was not possible to quantify this protein in hippocampal axons. These results indicate that hyperglycemia can have a prominent effect on the axonal content and distribution of motor proteins involved in the anterograde transport.

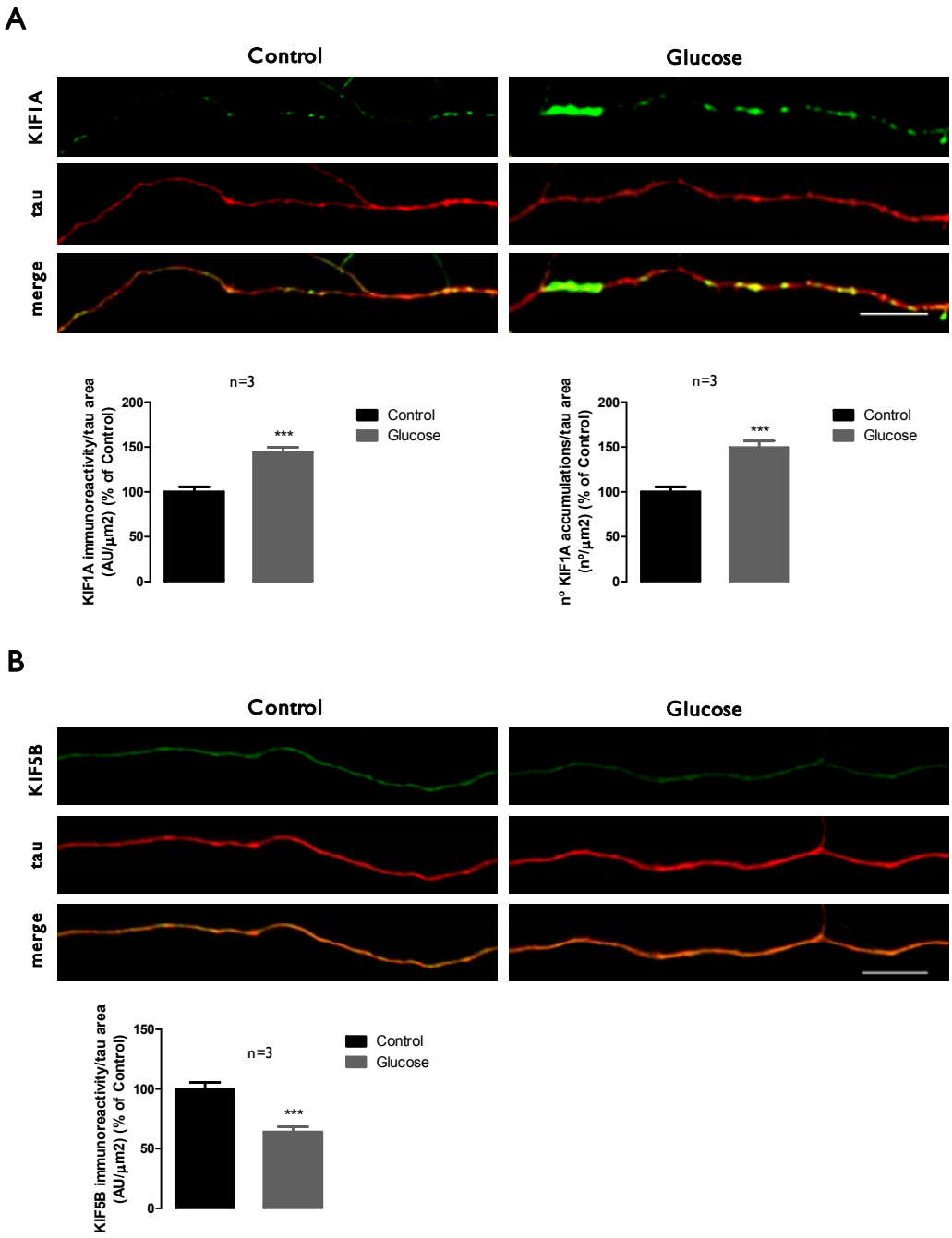
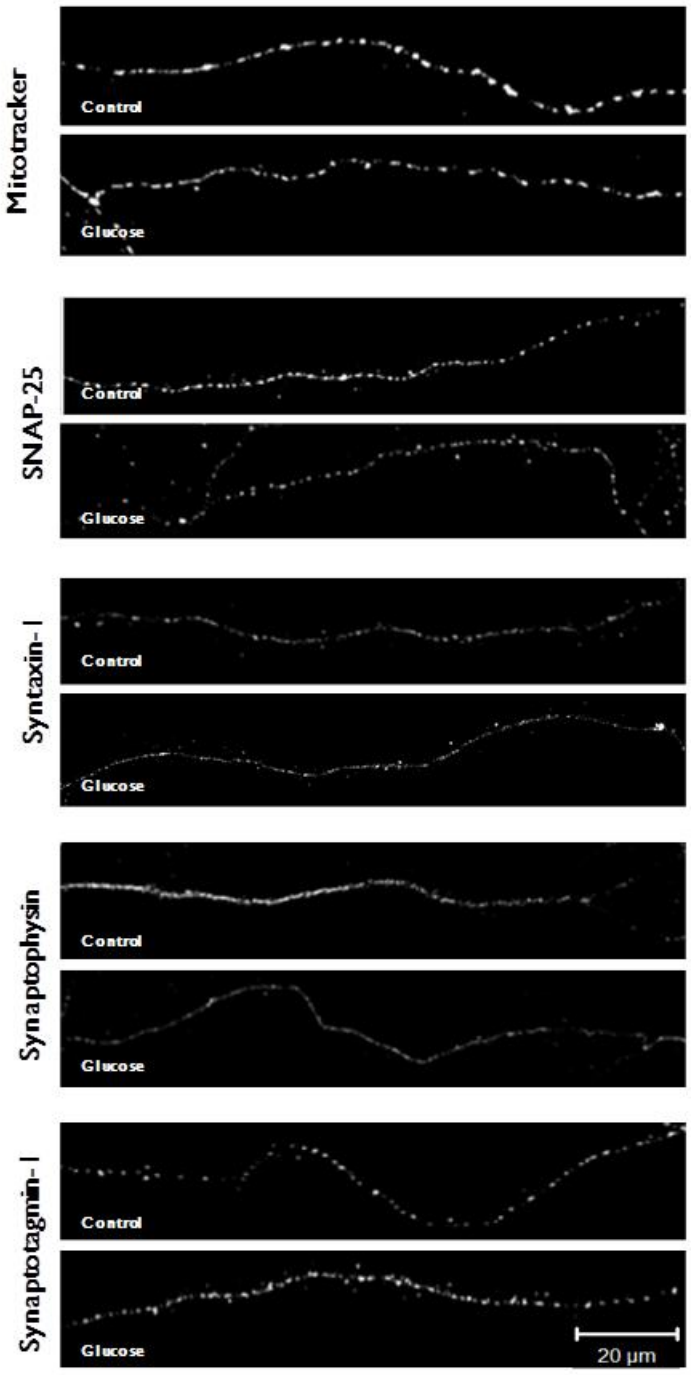


Figure 5. High glucose changes the number of fluorescent accumulations of KIF1A and KIF5B in hippocampal neurons axons. Cultured hippocampal neurons were exposed to 25 mM glucose (Control) and 50 mM glucose (Glucose) for 7 days. The immunoreactivity and number of fluorescent accumulations related to KIF1A (A) and KIF5B (B) immunoreactivity were analyzed. The preparations were visualized under an inverted microscope Zeiss Axiovert 200 (Zeiss, Germany). ***p<0.001, significantly different from control as determined by the unpaired Student's *t*-test. Scale bar: 10 µm.

High glucose decreases SNAP-25 and synaptophysin immunoreactivity and increases the number of accumulations of synaptotagmin-1 in hippocampal axons

KIF1A is a neuron-specific kinesin motor protein that transports synaptic vesicle precursors containing synaptophysin and synaptotagmin, whereas KIF5B is known to transport mitochondria and synaptic vesicle precursors containing syntaxin-1 and SNAP-25 (Hirokawa et al., 2009). Since we detected changes in the number of KIF1A accumulations and changes in KIF1A and KIF5B immunoreactivity in hippocampal axons, we evaluated the effect of elevated glucose on the immunoreactivity/intensity of fluorescence and on the number of accumulations of synaptic proteins and mitochondria (stained with mitotracker, a fluorescent dye that stains mitochondria in live cells; Invitrogen, Life Technologies, Scotland, UK). No significant changes were observed in the intensity of fluorescence or in the number of fluorescent accumulations related to mitochondria in the axons of cultured hippocampal neurons exposed to high glucose compared to control (Figure 6A and B). Nevertheless, regarding synaptic proteins, we found that SNAP-25 and synaptophysin immunoreactivity was significantly decreased (70.5±5.8% and 61.1±6.8% of the control, respectively) in the axons of hippocampal neurons exposed to elevated glucose (Fig. 6A and B). Concerning the number of fluorescent accumulations of synaptic proteins in the axons of hippocampal neurons exposed to elevated glucose, we found that the number of accumulations of SNAP-25, syntaxin-1 and synaptophysin were similar to the control condition, with the exception of synaptotagmin-1, since the number of accumulations of this protein was significantly increased to 143.2±18.1% of the control.

A


Mitotracker
Control
Glucose
SNAP-25
Control
Glucose
Syntaxin-1
Control
Glucose
Synaptophysin
Control
Glucose
Synaptotagmin-1
Control
Glucose
20 µm

B

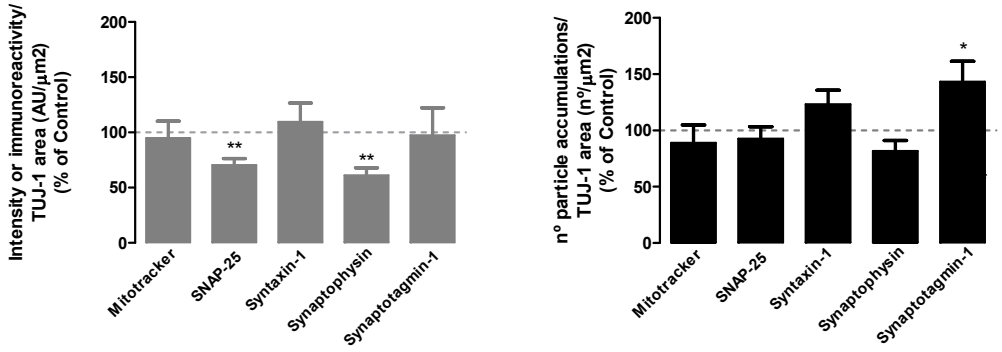


Figure 6. High glucose decreases SNAP-25 and synaptophysin immunoreactivity and increases the number of synaptotagmin-1 fluorescent accumulations. Cultured hippocampal neurons were exposed to 25 mM glucose (Control) and 50 mM glucose (Glucose) for 7 days. (A) The fluorescence intensity and the number of fluorescent accumulations related to mitochondria and synaptic proteins were analyzed in the axons of hippocampal neurons. The preparations were visualized under a laser scanning confocal microscope LSM 710 META (Zeiss, Germany). Scale bar: 20 µm. (B) The quantification of the number of fluorescent accumulations and intensity of fluorescence of mitochondria or immunoreactivity of synaptic proteins was performed and was expressed as percentage of the control. .*p<0.05, **p<0.01, significantly different from control as determined by the unpaired Student's *t*-test.

Discussion

In the present study, we addressed whether early diabetes can affect axonal motor proteins that are important for adequate transport of synaptic proteins and mitochondria in the hippocampus. Specifically, we showed that diabetes alters the content and immunoreactivity of KIF1A and KIF5B motor proteins in the hippocampus of diabetic rats. Moreover, in hippocampal neuronal cultures, we demonstrated that elevated glucose is able to change the immunoreactivity and number of fluorescent accumulations of motor and synaptic proteins in axons.

Due to their high polarity, neurons are particularly dependent on active intracellular transport. Deficits in this transport have been considered to contribute to the pathogenesis of multiple neurodegenerative diseases (Chevalier-Larsen and Holzbaur, 2006). Direct evidence from genetic studies demonstrates that mutations in major components of the cytoskeleton and axonal transport result in axonal defects in Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis and Alzheimer disease (De Vos et al., 2008). Post-translational modifications of cytoskeleton proteins also result in axonal defects in diabetic neuropathy (McLean, 1997). In previous studies, we found that diabetes changes the levels of several synaptic proteins involved in exocytosis in hippocampal nerve terminals, with no changes in total extracts, suggesting that axonal transport of those proteins to distal synaptic sites may be impaired in diabetes (Baptista et al., 2011; Gaspar et al., 2010a). In this study, we found that there is an increase in KIF1A and KIF5B levels in the

hippocampus at 8 weeks after the onset of diabetes, with no changes in dynein levels, suggesting that the anterograde transport may be impaired in the hippocampus. An impairment of axonal transport of certain cargoes may lead to their accumulation in the cell body. In a rat model of α-synucleinopathy, elevated levels of KIF1A were observed in substantia nigra (Chung et al., 2009), and the authors suggested the possibility that accumulation of these motor proteins may be due to the imbalance in protein degradation and synthesis or to axonal transport deficit. Moreover, we found that long-term exposure to elevated glucose induces an accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in the cell bodies of cultured hippocampal neurons (Gaspar et al., 2010b), further suggesting that axonal transport may be impaired. These observations directed us to further analyze the effect of hyperglycemia, which is considered the main factor underlying the development of diabetic complications, on motor proteins, namely KIF1A, KIF5B and dynein. KIF1A transports synaptic vesicle precursors of synaptophysin and synaptotagmin-1, but does not transport organelles that contain plasma membrane proteins, such as syntaxin-1 or SNAP-25. These are transported by KIF5 motors. The number of fluorescent accumulations of KIF1A increased in the axons of hippocampal neurons exposed to elevated glucose for 7 days. Likewise, increased number of accumulations of synaptotagmin-1 was also detected. The accumulation of these particles may be due to impairments at the microtubule network and/or impairment in KIF1A motor function, leading to the accumulation of KIF1A.

KIF5B protein immunoreactivity in the axons of hippocampal cells incubated with high glucose for 7 days decreased and similarly SNAP-25 immunoreactivity was also decreased. Likewise, in our previous studies, a significant decrease in the content of SNAP-25 was detected in hippocampal cultures (Gaspar et al., 2010b), as we had also demonstrated in hippocampal nerve terminals from diabetic rats (Gaspar et al., 2010a). These observations suggest that SNAP-25 appears to be particularly affected by hyperglycemic conditions, at least in hippocampal neurons, but the mechanisms underlying these effects are unknown. The reduction in SNAP-25 levels might significantly impair neurotransmission. In SNAP-25 KO neuronal cultures neurotransmitter release is almost abolished (Bronk et al., 2007). Moreover, synaptophysin immunoreactivity decreased in axons of hippocampal neurons exposed to high glucose. When analyzing the whole distribution of synaptophysin in hippocampal cultures (Gaspar et al., 2010b), we did not detect any change in the immunoreactivity of this protein, but when we analyzed potential changes at hippocampal axons, a significant decrease in the immunoreactivity of this protein was detected. Since synaptophysin is an integral protein of the synaptic vesicle membranes that has been correlated with synaptic density and neurotransmitter release, this decrease may contribute to impair neurotransmitter release. The changes reported here in motor proteins, specifically those occurring in axons, namely the increase in the number of fluorescent accumulations of KIF1A and decreased immunoreactivity of KIF5B strongly suggest that axonal transport may be compromised. As a consequence, a decrease in the number of synaptic vesicles and synaptic density may ultimately account for changes in synaptic transmission in the hippocampus. Nevertheless, we must also keep in mind that other KIFs might partially compensate for the

function of the kinesins here studied, and synaptic vesicle precursors might be transported by KIFs other than KIF1A and KIF5B (Yonekawa et al., 1998).

Retrograde transport is powered mainly by cytoplasmic dynein, but some kinesins can also be involved in this transport (Hirokawa et al., 2009). KIF1A is responsible for the transport of dense-core vesicles in the axons of hippocampal neurons, remaining associated with dense-core vesicles during retrograde axonal transport, demonstrating that these vesicles retain the molecular machinery necessary for transport in both directions (Lo et al., 2011). Defects in axonal transport of synaptophysin-containing vesicle precursors have been observed in KIF1A mutant mice (Yonekawa et al., 1998). Recent findings also demonstrate that KIF1A is necessary for BDNF-mediated hippocampal synaptogenesis and learning enhancement induced by environmental enrichment (Kondo et al., 2012), which reinforces the importance of this kinesin in the hippocampus.

Tau is a microtubule associated protein, which main function is to modulate the stability of axonal microtubules. Excessive tau phosphorylation is known to disrupt its binding to microtubules altering molecular trafficking, which ultimately may lead to synaptic dysfunction (Ebneth et al., 1998; Obulesu et al., 2011). Diabetes induces abnormal hyperphosphorylation of tau in the brain, including the hippocampus (Qu et al., 2011), and proteolytic tau cleavage (Kim et al., 2009), both of which are associated with Alzheimer's disease (Iqbal et al., 2009). In fact, tau modification can be induced by insulin dysfunction and hyperglycemia, which may contribute to the increased incidence of Alzheimer's disease in diabetic patients (Liu et al., 2011b). We did not detect any change in tau immmunoreactivity in hippocampal cultures exposed to high glucose. However, we cannot exclude the possibility of changes in tau phosphorylation state. Evidence obtained in kinesin-1 deficient mice suggested that defects in axonal transport can initiate biochemical changes that induce the activation of axonal stress kinase pathways leading to abnormal tau hyperphosphorylation. This further impairs axonal transport by disrupting the microtubule network and blocking axonal highways that ultimately will give rise to compromised synapse function and neurodegeneration (Falzone et al., 2010; Falzone et al., 2009).

KIF5 motors are also responsible for axonal transport of mitochondria. In KIF5A$^{-/-}$ neurons, the velocity of mitochondrial transport is reduced both in anterograde and retrograde direction (Karle et al., 2012). Decreased number of mitochondria in axons will likely decrease ATP supply to molecular motors leading to decreased anterograde and retrograde movement of both mitochondria and vesicles (De Vos et al., 2008). Growing evidence suggests that mitochondrial dysfunction play a significant role in neurodegenerative diseases like Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Brownlees et al., 2002; Reddy, 2011; Stamer et al., 2002). Mitochondrial dysfunction has also been proposed as a mediator of neurodegeneration in diabetes (Fernyhough et al., 2010), but as far as we are concerned there are no studies addressing the effect of diabetes in mitochondria transport in the central nervous system. In our work, we did not detect changes in the intensity of fluorescence, neither in distribution or number of accumulations related with mitochondria in the axons of hippocampal neurons exposed to elevated glucose when compared to control. Nevertheless, we cannot

exclude the possibility that hippocampal axons are being affected by diabetes since probably other factors, besides hyperglycemia, may also have an effect in mitochondria transport.

Hyperglycemia appears to be an important determinant for the changes observed in this study. However, under diabetic conditions, the lack or reduced levels of insulin, a potent trophic factor, might also play an important role in axonal transport impairment and synaptic changes observed in diabetic animals (Li et al., 2005; Sima and Li, 2005), thus contributing to changes in hippocampal physiology. For instance, short-term replacement of insulin in type I diabetic rats has shown to prevent cognitive deficits (Biessels et al., 1998).

Inflammation may be also a factor contributing to changes in axonal transport in diabetes. Previously, it was reported that pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β, are upregulated in the hippocampus of diabetic BB/Wor rats (Sima et al., 2009) and STZ-induced diabetic animals (Kuhad et al., 2009). TNF induces perinuclear clustering of mitochondria caused by impaired kinesin-mediated transport (De Vos et al., 1998) and the activation of TNF receptor-1 induces the activation of kinase pathways, resulting in hyperphosphorylation of kinesin light chain (KLC) and inhibition of kinesin activity, evidencing direct regulation of kinesin-mediated organelle transport by extracellular stimuli via cytokine receptor signaling pathways in L929 cells (De Vos et al., 2000). Moreover, it was previously demonstrated that nitric oxide released from activated microglia inhibits axonal movement of synaptic vesicle precursors containing synaptophysin and synaptotagmin in hippocampal neurons, suggesting that disturbance of axonal transport by microglial nitric oxide may therefore be responsible for axonal injury and synaptic dysfunction in brain diseases characterized by neuroinflammation (Stagi et al., 2005). TNF produced by activated glial cells in inflammatory or degenerative neurological diseases affects neurites by acting on the kinesin-tubulin complex and inhibiting axonal mitochondria and synaptophysin transport via JNK in hippocampal cultures (Stagi et al., 2006). Very recently, it was demonstrated that hydrogen peroxide, a common reactive oxygen species elevated during inflammation, also inhibits axonal transport in hippocampal cultures (Fang et al., 2012). Further studies will be needed to determine if similar pathways may be active under diabetic conditions, therefore contributing for the detected changes in the levels of synaptic and motor proteins in the hippocampus.

In summary, our data demonstrate that the content of KIF1A and KIF5B motor proteins are altered in the hippocampus of diabetic rats. Furthermore, we showed that high glucose leads to an increase in the number of fluorescent accumulations of KIF1A and synaptotagmin-1 and decreased immunoreactivity of KIF5B, SNAP-25 and synaptophysin specifically in the axons of hippocampal neurons. Altogether, these changes suggest that the anterograde axonal transport may be impaired in the hippocampus, which may lead to changes in the content of synaptic proteins in nerve terminals, since their transport is mediated by these kinesins, and ultimately contribute to neural changes underlying diabetic encephalopathy.

Acknowledgments

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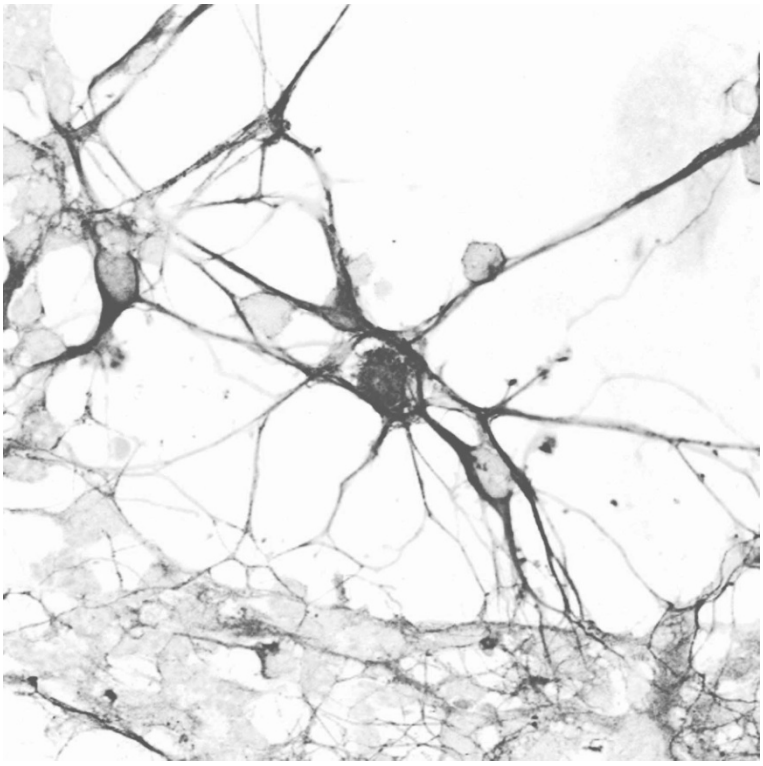
Chapter 8

General Discussion



Discussion

Diabetic retinopathy is a leading cause of vision loss and blindness worldwide. Emerging evidence has indicated that retinal neurons may also be affected by diabetes, even before the detection of microvascular dysfunction (Antonetti et al., 2006). Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neural dysfunction in the retina (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). Diabetes increases apoptosis in neural cells in human and rat retina early in the course of the disease (Barber et al., 1998; Gastinger et al., 2006) and elevated glucose increases neural cell death in primary retinal neural cell cultures, (Santiago et al., 2007). Moreover, diabetes increases the evoked release of [^{3}H]D-aspartate from the retina increasing also in retinal neural cultures exposed to high glucose (Santiago et al., 2006a). Similarly, the release of ATP in retinal cell cultures exposed to high glucose is also increased (Costa et al., 2009). Despite these evidences, the molecular and cellular alterations caused by diabetes in the neural retina are not fully understood, and little is known about the presynaptic changes that might underlie the detected impairments in retinal neurotransmission.

Abnormalities in the expression of exocytotic proteins might account for alterations in transmitter release. In pancreatic islets of diabetic humans and rats, the expression of exocytotic proteins is impaired (Ostenson et al., 2006; Zhang et al., 2002) and it was also reported that elevated glucose downregulates the expression of several exocytotic proteins in pancreatic beta cells (Abderrahmani et al., 2006a). Moreover, it was demonstrated that diabetes induces a decrease in the content or expression of some exocytotic proteins in hippocampal and retinal nerve terminals (Duarte et al., 2009; Vanguilder et al., 2008). Together, these evidences strongly suggest that diabetes induces alterations at synaptic level that might contribute to the impairment of synaptic transmission, but there are no evidences clearly demonstrating this hypothesis. In this work, we aimed to further clarify the effects of diabetes in the neural retina, and we gave a particular attention to changes occurring at presynaptic level.

In chapter 2, we found that diabetes affects several synaptic proteins important for neurotransmitter release in the retina. Concerning, SNARE proteins, the decrease in VAMP-2 levels observed at two weeks of diabetes may affect vesicle fusion and consequently, neurons may transiently overexpress VAMP-2 at four weeks of diabetes (Shirasu et al., 2000), as a compensatory mechanism, recovering to values similar to controls at eight weeks of diabetes. We did not find significant changes in SNAP-25 levels. Nevertheless, in a previous study SNAP-25 levels were decreased in nerve terminals of diabetic animals after one and three months of diabetes. However, these apparently contradictory results may be related with the use of different rat strains, Wistar versus Sprague-Dawley. Syntaxin-1 levels decreased at 2 weeks of diabetes and return to levels similar to control suggesting that the retina is able to recover from or react to the initial insult caused by diabetes.

Synaptophysin levels also decreased in retinal nerve terminals at two weeks of diabetes, but there was a recovered to values similar to control. VanGuilder and colleagues (2008) also showed

a reduction of synaptophysin levels in retinal synaptosomes and suggested that this reduction may change neurotransmitter vesicle pool by decreasing the vesicle recycling (Daly and Ziff, 2002). The reduction of synaptophysin in retinal nerve terminals might be explained by the degradation of this protein by the ubiquitin-proteosome pathway, which is enhanced by activation of angiotensin II type 1 receptors under diabetic conditions (Kurihara et al., 2008). Synapsin-1 content significantly decreased in retinal nerve terminals. Previously, it was demonstrated that diabetes decreases synapsin-1 levels in retinal nerve terminal as well as synapsin-1 phosphorylation in retinal synapses (Vanguilder et al., 2008), which may lead to the immobilization of vesicles within the presynaptic terminal and to the prevention of vesicle fusion. Synapsin-1 KO mice exhibit both decreased vesicle clustering and impaired vesicle recycling (Takei et al.,1995; Ryan et al., 1996). In general, changes were detected early in the retina, and with the exception of synapsin-1, the protein content in retinal nerve terminals was re-established, presenting a tendency to recover. Possibly, for longer periods of diabetes, the impairments may become more permanent, having a deleterious effect in the retina physiology.

Our parallel study, about the effect of diabetes on the content of exocytotic proteins in the hippocampus led us to conclude that retina and hippocampus are differently affected by diabetes (Gaspar et al., 2010a). Most of the changes occurred in the retina at two weeks of diabetes and then recovered, whereas in hippocampus changes were more prominent at 8 weeks of diabetes. Therefore, hippocampal nerve terminals appear to be more affected by diabetes than retinal ones, since changes were more pronounced and persistent in hippocampal nerve terminals, and since for some proteins, changes were even detected in hippocampal total extracts, whereas no changes were observed in the levels of exocytotic proteins in total retinal extracts (Gaspar et al., 2010).

All the alterations detected in the retina occurred specifically at presynaptic level, suggesting that diabetes may increase local degradation of presynaptic proteins and/or their mRNA at retinal synapses. Alternatively, the observed reductions may be due to decreased protein synthesis within synapses. Another possibility is a failure in their transport from the neuronal cell body to synapse. In order to give insight to this last hypothesis, in Chapter 5 we analyzed the effect of diabetes on the content and distribution of KIF1A, KIF5B and dynein motor proteins. Nevertheless, although the changes detected in synaptic proteins in retinal synaptosomes occurred at 2 weeks of diabetes, changes in kinesin motors were detected at later periods (8 weeks of diabetes). However, we cannot exclude the possibility that other kinesins, different from those studied here, may play a role in transporting vesicles, protein complexes or mRNA for the synaptic proteins studied, contributing for decreased levels of synaptic proteins in the retina. Also, although we did not found significant changes in kinesins immunoreactivity at 2 weeks of diabetes, slight changes detected in KIF1A and KIF5B immunoreactivity in some retinal layers may somehow contribute to the impairment in axonal transport in the retina. A progressive deficit in retrograde axonal transport was found in RGCs after 4 weeks of diabetes (Ino-Ue et al., 2000), and more recently, it was demonstrated a deficit in the anterograde transport from the retina to the superior colliculus, 6 weeks after the induction of diabetes (Fernandez et al., 2012). These

evidences clearly show that diabetes impairs axonal transport in the retina, which can account for the decrease in the content of synaptic proteins in retinal nerve terminals (Baptista et al., 2011; Gaspar et al., 2010a).

Since the impairment in exocytosis may contribute to changes in neurotransmitter release, in Chapter 3 we evaluated the effect of diabetes on basal and evoked [^{3}H]GABA release in retinal nerve terminals, as well as on the protein levels of vesicular glutamate and GABA transporters and α1A subunit of P/Q calcium channels. The content of P/Q calcium channels, VGAT, and VGluT-1 and VGluT-2 decreased in synaptosomes at two weeks of diabetes, which might contribute to changes in synaptic transmission. However, the decrease in the levels of the vesicular transporters was transient, similarly to what was observed in Chapter 1, for VAMP-2, syntaxin-1 and synaptophysin, at least in the early stages of diabetes, suggesting that after the initial stress conditions induced by diabetes, the retina is somehow able to react against diabetes induced stress.

Regarding GABA release, at eight weeks of diabetes we found a small, but significant, impairment in the evoked release. Similarly to previous studies (Kamisaki et al., 1991), we found that only 30% of GABA release is exocytotic in our synaptosomal preparation. Since under these conditions, the release is mainly non-exocytotic, it is likely that alterations in exocytotic release might be somehow masked. It was demonstrated that the evoked release of [^{3}H]D-aspartate from the retina increases at four weeks of diabetes, increasing also in retinal neural cultures exposed to high glucose (Santiago et al., 2006a). These observations, namely the decrease in the evoked [^{3}H]GABA release found in this study, and the increase in the evoked release of [^{3}H]D-aspartate in the diabetic rat retinas suggest that diabetes can lead to an unbalanced neurotransmitter release and to the impairment of neurotransmission. If these alterations in transmitter release persist for longer periods, an imbalance between excitation and inhibition might take place and can contribute to neuronal apoptosis detected in the diabetic retina (Barber et al., 1998; Park et al., 2003), which may be correlated to the hypothesis of glutamate induces retinal neurodegeneration in diabetic retinas. This group of results further indicates that diabetes can affect presynaptic terminals, impair synaptic transmission and ultimately contribute to the visual impairments detected in diabetic animals and humans (Daley et al., 1987; Roy et al., 1986; Sakai et al., 1995). As mentioned before, this work was a comparative study related with the effect of diabetes on the protein content of vesicular glutamate and GABA transporters and on neurotransmitter release in the retina and hippocampus. Regarding, vesicular transporters content, changes in these proteins were more pronounced in the retina than in hippocampus, since VGluT-1 and VGluT-2 content was not affected in hippocampus. Moreover, changes occurred early in the retina, at two weeks of diabetes, but at eight weeks no changes were detected in both tissues, with the exception of VGAT in the retina. Regarding neurotransmitter release, slight changes were detected. After eight weeks of diabetes, the basal release of glutamate slightly increased in hippocampus and the evoked GABA release decreased in retina.

Given that hyperglycemia is considered the main trigger of diabetic complications, in Chapter 4 we analyzed whether long-term high glucose *per se* (to isolate a single factor), could

change the content and localization of synaptic proteins involved in exocytosis in primary retinal neural cell cultures. In fact, we aimed to correlate the changes previously detected in synaptic proteins in nerve terminals of diabetic animals with the hyperglycemic status. Prolonged elevated glucose did not alter the total content and cellular distribution of proteins involved in exocytosis, suggesting that hyperglycemia may not be the primordial factor contributing for neuronal changes caused by diabetes, but rather a combination of hyperglycemia with other factors, such as the lack of insulin and inflammation. Although in this study we did not observe any changes in the content of synaptic proteins in retinal cultures, in a previous study, using the same cultures exposed to high glucose for 7 days, we did observe changes in the content of glutamate receptor subunits. We found a significant decrease in the protein content of GluR1 and GluR6/7 subunits, and an increase in the content of GluR2 and KA2 subunits (Santiago et al., 2006b). These observations guarantee that elevated glucose is able to induce molecular changes in neural cells, and particularly in retinal cells. Additionally, in hippocampal neurons, we had previously found that long-term exposure to elevated glucose induces changes in the protein content of SNAP-25, synaptotagmin-1 and VGluT-1, as well as in the cellular localization of syntaxin-1, synaptotagmin-1 and VGluT-1, occurring an accumulation of these proteins in the cell bodies of hippocampal neurons (Gaspar et al., 2010b). The differences observed between retinal and hippocampal neurons exposed to prolonged elevated glucose might be due to several reasons. For instance, it is possible that the period of exposure of retinal neurons to high glucose (maximum 7 days) was not sufficient to induce changes in the content or distribution of these proteins. Another possibility is that hippocampal neurons may be more susceptible to elevated glucose than retinal neurons. However, by immunocytochemistry, we did not detect any change in the cellular distribution of the synaptic proteins in cells exposed to high glucose, further indicating that other factors beside hyperglycemia may underlie changes detected in exocytotic proteins induced by diabetes. Nevertheless, since retinal cultures are composed by several cell types (neurons, microglia, astrocytes and Müller cells) forming a dense neural culture, we cannot totally exclude the possibility that particular changes might be occurring in the axons similarly to what was observed in hippocampal neurons (Chapter 7).

Previous studies have suggested that hyperglycemia might be not responsible for all clinical aspects of diabetic retinopathy, since there are numerous cases of this pathology without evident presence of hyperglycemia (Chan et al., 1985; Harrower and Clarke, 1976). These observations lead to the question if hyperglycemia *per se* is necessary or sufficient for the establishment of the disease (Antonetti et al., 2006; Barber et al., 2001). Other factors, such as the lack (or decrease) of insulin secretion or action, and inflammation, may play a role in changes occurring in nerve terminals detected in diabetic animals. Additionally, since we detected changes in motor proteins involved in axonal transport in diabetic rats (Chapter 6), we also evaluated whether high glucose *per se* could change the content of proteins involved in axonal transport in primary retinal neural cell cultures, trying to correlate those changes with hyperglycemic conditions. Similarly to the results obtained in Chapter 3, high glucose did not induce any changes in the content and distribution of motor proteins and mitochondria, further suggesting that other factors may play a

role in the changes previously detected in motor proteins in diabetic animals. It was reported that retinal neurons depend on insulin receptor activity for survival (Barber et al., 2001). Long-term instability in retinal insulin signalling may impair insulin-dependent anabolic activities, such as protein synthesis (Chihara, 1981), and increase cell death in the retina (Reiter and Gardner, 2003), suggesting that insulin signalling provides neurotrophic actions in the retina. Therefore, diabetic retinopathy may result in part from neurotrophin deficiency (Whitmire et al., 2011), similarly to peripheral neuropathy (Brussee et al., 2004; Pierson et al., 2003). Early intervention with systemic insulin-like growth factor-I (IGF-I) administration can prevent retinal cell death despite of ongoing hyperglycemia (Seigel et al., 2006). Moreover, VanGuilder and colleagues (2008) have demonstrated that basal synapsin-I phosphorylation is decreased in the retina of diabetic rats at 1 month of diabetes, which may impede the recruitment of vesicles to the presynaptic terminal, and systemic insulin treatment re-established synapsin-I phosphorylation levels similar to controls, suggesting the importance of insulin for proper neurotransmission in the retina.

Although impaired insulin action is the primary defect of diabetes, it has also been suggested that diabetic retinopathy may result from combinatorial insults including inflammation (Antonetti et al., 2006). Cytokine-activated microglia release cytotoxins that are responsible for retinal neuronal death (Krady et al., 2005), and the inhibition of microglial activity may be an important strategy in the treatment of diabetic retinopathy. Moreover, a recent study found that $[Ca^{2+}]_i$ changes triggered by purinergic receptors activation, both in retinal neurons and microglial cells, are potentiated in cultured retinal cells exposed to high glucose. This augmented calcium response might account for the increase in the release of inflammatory mediators and neurotransmitters found in diabetic retinas (Pereira et al., 2010). Taking this into account, we further assessed if the changes we have detected in exocitotic proteins in diabetic animals were due to inflammation. Since we did not detect any changes in the expression of synaptic proteins in cultured retinal cells exposed to high glucose or IL-1β, we have to consider that the lack of insulin or the combinatory effects of two or more factors may play an important role in the pathogenesis of diabetic retinopathy.

Given that inflammation has been considered an important player in the pathogenesis of diabetic retinopathy, particularly in the blood-retinal barrier breakdown, and little attention has been given to the potential harmful effects of inflammatory processes in the neural retina, in Chapter 5 we also evaluated the effect of high glucose on IL-1β expression and how these factors could affect the different cell types in the retina. Previous studies have already shown that long-term exposure to high glucose (7 days) decreases retinal cell viability, increasing apoptotic cell death in retinal neural cell cultures (Costa et al., 2012; Santiago et al., 2007). Moreover, exposure to high glucose resulted in an increase in TNF-α expression (Costa et al., 2012). The blockade of TNF receptor 1, which is expressed in retinal neurons, is capable of preventing the increase in cell death induced by high glucose. Moreover, elevated glucose up-regulates IL-1β expression, but decreases microglial cell proliferation which appears to be due to cell cycle arrestment due to elevated glucose (Rao et al., 1999). On the other hand, we found that IL-1β induced microglial

activation and proliferation in retinal neural cultures. Microglia is able to influence Müller cells directly, and initiate a program of bidirectional microglia-Müller cell signaling that can mediate adaptive responses within the retina following injury (Wang et al., 2011). In our work, when retinal neural cultures were exposed to IL-1β, we did not detect any changes in the protein levels of several neuronal markers, suggesting that activated microglia observed in this condition is not having a deleterious effect in neuronal cells, at least for this time of exposure to IL-1β. Nevertheless, we cannot exclude the possibility that for longer periods of exposure to IL-1β, activated microglia may have detrimental effects in these cultures.

The inhibition of the IL-1β signalling pathway by using IL-1RI-deficient mice protected the animals from diabetes-induced retinal pathology (Vincent and Mohr, 2007), suggesting that IL-1β and IL-1RI play a key role in the development of diabetic retinopathy. Since IL-1RI provides a crucial locus of control of IL-1β activity, blocking the activation of IL-1RI should be taken into account as a possible therapeutic strategy for the treatment of diabetic retinopathy. Likewise, strategies aiming to inhibit the activity of TNFR1 will also be important in order to reduce inflammation in the retina and to limit the progression of diabetic retinopathy.

Cognitive impairments induced by diabetes have been linked to changes in the integrity and function of the hippocampus (Trudeau et al.,2004; Stranahan et al., 2008; Wrighten et al., 2009). Diabetes impairs synaptic structure and function in the hippocampus at presynaptic (Grillo et al., 2005) and postsynaptic levels (Biessels et al., 1996; Kamal et al., 1999). We detected changes in the levels of exocytotic proteins in hippocampal synaptosomes induced by diabetes, namely increased content of synaptophysin, and reduction in the content of SNAP-25, syntaxin-1 and synapsin-1 (Gaspar, 2010a). The decrease in the content of SNAP-25 and syntaxin-1 was also detected in hippocampal total extracts, suggesting that changes in hippocampus are also occurring at the level of protein synthesis or degradation. Our results corroborate previous findings (Duarte et al., 2009), where a decrease in the content of these two proteins in hippocampal nerve terminals at 4 weeks of diabetes was reported. Additionally, the decreased levels of synaptic proteins observed in nerve terminals may also be due to impairments in axonal transport of these proteins to the synapse. In Chapter 7, we investigated this hypothesis and found that in the hippocampus of diabetic rats there is an increase in KIF1A and KIF5B levels, 8 weeks after the onset of diabetes, with no changes in dynein levels, suggesting that anterograde transport may be impaired in the hippocampus.

An impairment of axonal transport of certain cargoes may lead to their accumulation in the cell body region. In a rat model of α-synucleinopathy, elevated levels of KIF1A were observed in substantia nigra (Chung et al., 2009), and the authors suggested the possibility that accumulation of these motor proteins may be due to the imbalance in protein degradation and synthesis or to axonal transport deficit. We found that the number of accumulations of KIF1A increased in the axons of hippocampal neurons exposed to elevated glucose for 7 days. Likewise, increased number of accumulations of synaptotagmin-1 was also detected. The accumulation of these particles may be due to impairments at the microtubule network and/or impairment in KIF1A motor function, leading to the accumulation of KIF1A. KIF5B protein immunoreactivity in the

axons of hippocampal cells incubated with high glucose for 7 days decreased, as well as SNAP-25 immunoreactivity, which seems to be well correlated with our previous observations (Gaspar et al., 2010b), where a significant decrease in the content of SNAP-25 was detected. Furthermore, the content of SNAP-25 was also significantly reduced in hippocampal nerve terminals and in total hippocampal extracts from diabetic rats (Gaspar et al., 2010a). The reduction in SNAP-25 levels might significantly impair neurotransmission. In SNAP-25 KO neuronal cultures neurotransmitter release is almost abolished (Bronk et al., 2007). Moreover, synaptophysin immunoreactivity in axons decreased in hippocampal neurons exposed to high glucose. When analyzing the whole distribution of synaptophysin in hippocampal cultures (Gaspar et al., 2010b), we did not detect any change in the immunoreactivity of this proteins, but when we analyze their axons a significant decrease in the immunoreactivity of this protein is observed. Since synaptophysin is an integral protein of the synaptic vesicle membrane that correlates with synaptic density and neurotransmitter release, this decrease in its content may contribute to impair neurotransmitter release. The changes reported on motor proteins, the increased number of accumulations of KIF1A and the decreased immunoreactivity of KIF5B, specifically in axons, strongly suggests that axonal transport may be compromised. As a consequence, a decrease in the number of synaptic vesicles and synaptic density may ultimately account for changes in synaptic transmission in the hippocampus. Nevertheless, other KIFs might also partially compensate for the function of the kinesins studied here, and synaptic vesicle precursors might be transported by KIFs other than KIF1A and KIF5B (Yonekawa et al., 1998). Defects in axonal transport of synaptophysin-containing vesicle precursors have been observed in KIF1A mutant mice (Yonekawa et al., 1998). Recent findings also demonstrate that KIF1A is necessary for BDNF-mediated hippocampal synaptogenesis and learning enhancement induced by environmental enrichment (Kondo et al., 2012), which reinforces the importance of this kinesin in the hippocampus.

Excessive tau phosphorylation is known to disrupt its binding to microtubules, altering molecular trafficking, which ultimately may lead to synaptic dysfunction (Ebneth et al., 1998; Obulesu et al., 2011). Diabetes induces abnormal hyperphosphorylation of tau in the brain, including the hippocampus (Qu et al., 2011), and proteolytic tau cleavage (Kim et al., 2009). In fact, tau modification can be induced by insulin dysfunction and hyperglycemia, which may contribute to the increased incidence of Alzheimer´s disease in diabetic patients (Liu et al., 2011b). We did not detect any change in tau immmunoreactivity in hippocampal cultures exposed to high glucose. However, we cannot exclude the possibility of changes in tau phosphorylation state. Evidence obtained in kinesin-1 deficient mice suggests that defects in axonal transport can initiate biochemical changes that induce the activation of axonal stress kinase pathways leading to abnormal tau hyperphosphorylation. This further impairs axonal transport by disrupting the microtubule network and blocking axonal highways that ultimately will give rise to compromised synapse function and neurodegeneration (Falzone et al., 2010; Falzone et al., 2009).

KIF5 motors are responsible for axonal transport of mitochondria. In KIF5A$^{-/-}$ motor neurons, the velocity of mitochondrial transport is reduced both in anterograde and retrograde direction (Karle et al., 2012). Decreased number of mitochondria in axons will likely decrease

ATP supply to molecular motors leading to decreased anterograde and retrograde movement of both mitochondria and vesicles (De Vos et al., 2008). In our work, we did not detect changes in the number of fluorescent accumulations of mitochondria and their distribution in the axons of hippocampal exposed to elevated glucose when compared to control. However, we did not evaluate the movement and velocity of mithocondria transport in neurons exposed to high glucose, so we cannot exclude the possibility of impaired mitochondria transport in hyperglycemic conditions.

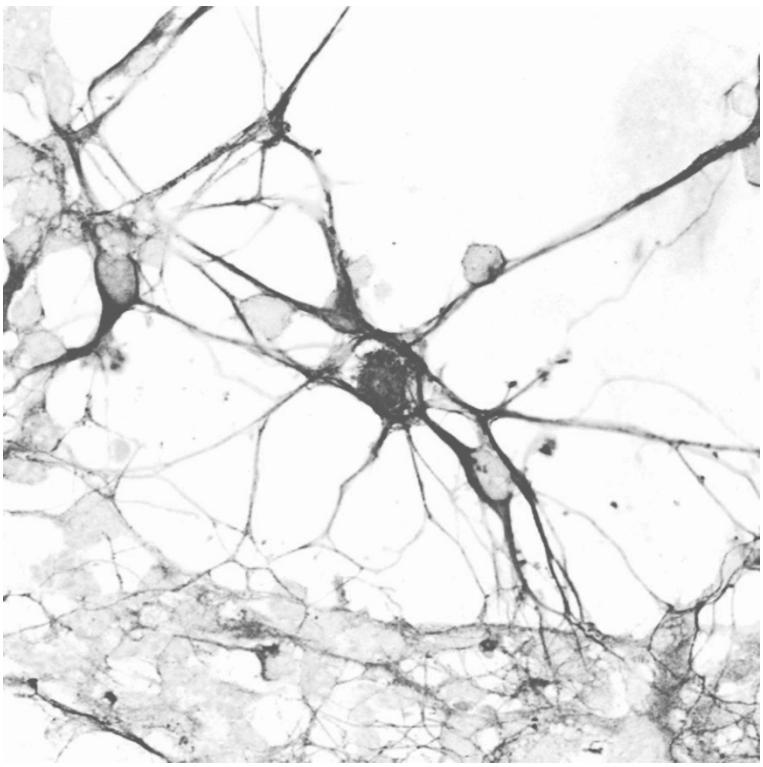
Although hyperglycemia appears to be an important determinant for the changes observed in the hippocampus, the lack or reduced levels of insulin, a potent trophic factor, might also play an important role in axonal transport and synaptic changes observed in diabetic animals (Li et al., 2005; Sima and Li, 2005), thus contributing to changes in hippocampal function. Previously, it was shown that short-term replacement of insulin in type I diabetic rats prevents cognitive deficits (Biessels et al., 1998).

Additionally, inflammation may also be a factor contributing to changes in axonal transport induced by diabetes, which may help to explain previous changes detected in anterograde motor proteins in the retina (Chapter 6) and hippocampus (Chapter 7) of diabetic rats. Previously, it was reported that pro-inflammatory cytokines, such as TNF-α and IL-1β, are upregulated in the hippocampus of diabetic BB/Wor rats (Sima et al., 2009) and STZ animals (Kuhad et al., 2009). Moreover, it has been reported that NO released from activated microglia inhibits axonal movement of synaptic vesicles containing synaptophysin and synaptotagmin in hippocampal neurons (Stagi et al., 2005). On the other hand, TNF-α induces activation of kinase pathways, resulting in hyperphosphorylation of kinesin light chain and inhibition of kinesin activity (De Vos et al., 2000). TNF-α also stimulates the phosphorylation of JNK in hippocampal neurons resulting in the inhibition of axonal transport of mitochondria and synaptophysin (Stagi et al., 2006). Therefore the synergistic effect of hyperglycemia and neuroinflammation might underlie axonal transport deficits in diabetes and similar signaling pathways may be active in a diabetes condition and therefore contributing for the detected changes in the levels of synaptic and motor proteins in the retina and hippocampus.

In summary, diabetes is a multifactorial disease where one or a combinatory range of factors may act sinergistically, resulting in neuronal dysfunction. The results presented in this thesis provide a better insight into the molecular and cellular changes triggered by diabetes in the retina and hippocampus. The alterations induced by diabetes in neural cells, synaptic and motor proteins, vesicular transporters and neurotransmitter release may contribute to synaptic dysfunction and consequently to visual and cognitive impairments detected in diabetic animals and humans.

Chapter 9

Main Conclusions



Main Conclusions

The results presented in this thesis allowed us to elaborate the following main conclusions:

- Diabetes differentially affects the content of several synaptic proteins involved in exocytosis, as well as the content of vesicular transporters and P/Q calcium channels, in retinal nerve terminals. Some changes were transitory suggesting that the retina is able to recover from or react to, the initial insult caused by diabetes, at least temporarily. No changes were observed in total extracts, indicating that the changes detected were specifically occurring at presynaptic level. Moreover, diabetes also decreased the evoked release GABA in retinal nerve terminals. Together, this group of results suggests that diabetes can affect the synapse at the level of pre-synaptic terminals, which may cause impairments in synaptic transmission. An imbalance between excitation and inhibition may lead to neuronal cell dysfunction in the retina.

- High glucose upregulates IL-1β in retinal neural cell cultures and leads to a decrease in glial and microglial cells proliferation. In turn, IL-1β induces microglia activation and increases their proliferation, while it does not lead to an increase cell death and to changes in the levels of synaptic proteins and neuronal markers in retinal cultures. Since overactivation of microglial cells may have deleterious effects in the retina, limiting IL-1β-triggered inflammatory processes could be importante to prevent the progression of diabetic retinopathy.

- Diabetes induces changes in the immunoreactivity of KIF1A and KIF5B motor proteins in the retina, which may contribute to impaired anterograde axonal transport and consequently to neuronal dysfunction in the retina. Moreover, KIF1A and KIF5B motor proteins are also altered in the hippocampus of diabetic rats and high glucose leads to changes in the content and distribution of KIF1A and KIF5B, as well as to changes in some synaptic proteins important for exocytosis, in the axons of hippocampal neurons. These changes might contribute to the alterations in neurotransmission previously detected in the retina and hippocampus of diabetic animal models.

- Long-term exposure to high glucose (maximum 7 days) and to IL-1β (24h) does not change the content and localization of several synaptic proteins involved in exocytosis in retinal neural cultures. Also, high glucose does not induce changes in motor proteins involved in axonal transport in retinal cultures. These observations suggest that other factors such as the lack of insulin or/and inflammation *per se* may contribute to the changes detected in synaptic proteins in the retina of diabetic animals.

In summary, diabetes induces changes in the presynaptic compartment in the retina, namely in the content of several synaptic proteins involved in exocytosis, also affecting neurotransmitter release. The anterograde axonal transport proteins are also affected by diabetes in the retina and hippocampus. However, a prolonged exposure of retinal cell cultures to elevated glucose did not induce significant changes in both synaptic and motor proteins, suggesting that in addition to hyperglycemia, other factors, such as the lack of insulin and inflammation, likely acting synergistically, might contribute to neural changes in the retina, and hippocampus. Probably, each factor alone may not be sufficient to trigger the changes detected in the diabetic retina or hippocampus. Altogether, these alterations might contribute to visual and cognitive impairments detected in diabetic animals and humans.

Chapter 10

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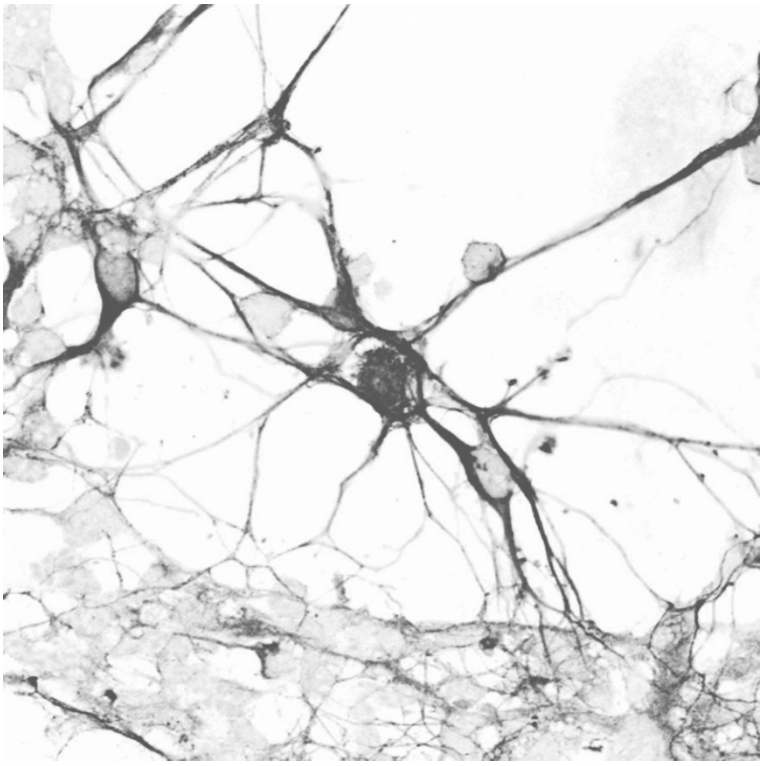

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