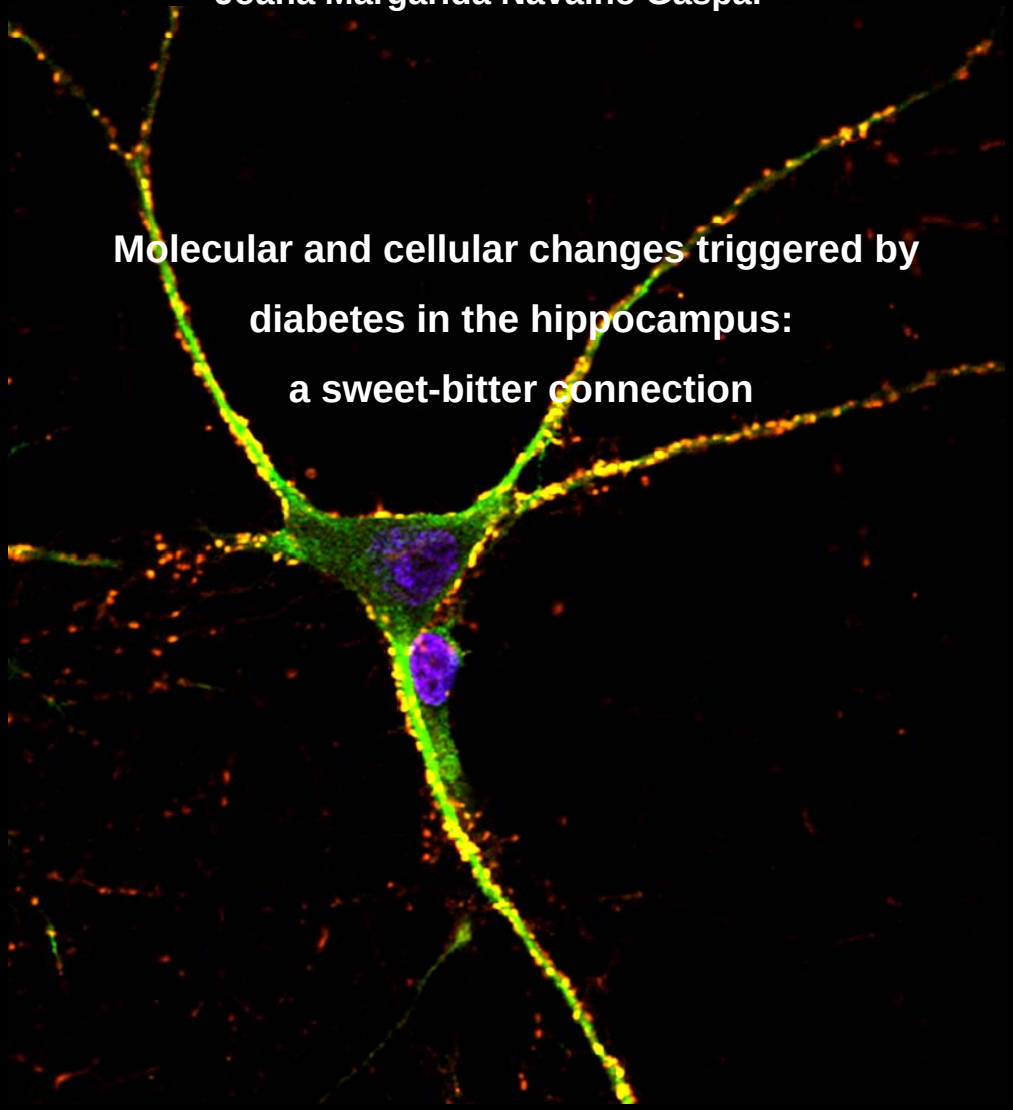
Joana Gaspar

Cellular and molecular changes triggered by diabetes in hippocampus: a sweet-bitter connection

• UC •
2010

Joana Margarida Navalho Gaspar

Molecular and cellular changes triggered by diabetes in the hippocampus: a sweet-bitter connection



Universidade de Coimbra
2010

Alterações moleculares e celulares induzidas pela diabetes no hipocampo

Molecular and cellular changes triggered by diabetes in the hippocampus: a sweet-bitter connection

Joana Margarida Navalho Gaspar

Universidade de Coimbra

2010

Cover:

Image showing a double immunolabeling for synapsin-1 (red) and tuj-1 (green) in cultured rat hippocampal neurons, obtained by confocal microscopy. Cell nuclei are stained with DAPI (blue). 630x magnification.

Alterações moleculares e celulares induzidas pela diabetes no hipocampo

Molecular and cellular changes triggered by diabetes in the hippocampus: a sweet-bitter connection

Joana Margarida Navalho Gaspar

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

Este trabalho foi realizado no Centro de Oftalmologia e Ciências da Visão, no Instituto Biomédico de Investigação da Luz e Imagem (IBILI) da Faculdade de Medicina da Universidade de Coimbra, e no Centro de Neurociências e Biologia Celular (CNC) da Universidade de Coimbra, sob orientação do Doutor António Francisco Ambrósio e co-orientação do Doutor Paulo Fernando Santos, ao abrigo de uma bolsa de doutoramento atribuída pelo Programa Doutoral em Biologia Experimental e Biomedicina do Centro de Neurociências e Biologia Celular da Universidade de Coimbra, financiada pela Fundação para a Ciência e a Tecnologia (SFRH/BD/32949/2006).

Universidade de Coimbra

2010

Agradecimentos

Agradeço ao Doutor Francisco Ambrósio, pela orientação deste trabalho, e pelo apoio, discussões e sugestões para a elaboração deste trabalho. Obrigada pela confiança, pelo estímulo e incentivo. Obrigada pela amizade e convívio ao longo destes últimos anos.

Ao Doutor Paulo Santos, pela co-orientação e discussão deste trabalho, e pela disponibilidade, ajuda e simpatia ao longo destes anos.

Ao Doutor Attila Köfalvi por todas as sugestões, ajuda e disponibilidade para a realização deste trabalho. Obrigada pelo apoio, paciência, amizade e boa disposição mesmo quando tudo parecia perdido.

Ao Doutor Rodrigo Cunha pelas sugestões, ajuda e disponibilidade para a realização deste trabalho.

A todos os meus colegas e amigos de laboratório pela partilha dos bons e maus momentos, pelo incentivo, amizade e boa disposição, que contribuíram para a realização desta tese. Obrigada à Alda, Ana Paula, Áurea, Carla, Célia, Ermelindo, Joana Galvão, Joana Gonçalves, Joana Liberal, Joana Viegas, João, Paula, Raquel, Rosa e Sofia.

À minha amiga e companheira desta caminhada, Filipa, pela partilha deste trabalho, dos problemas e também das vitórias, e pelas sugestões e críticas que contribuíram para a elaboração desta tese. Obrigada pelos cházinhos, pelas conversas, desabafos e por aturares as minhas crises existenciais.

À Áurea, Carla, Célia, Ermelindo, Filipa, Joana Gonçalves, Joana Liberal e Paula pelos momentos extra-laboratório. Pelos nossos jantares, jogos de “pictionary” e conversa fiada. Obrigada pelas palavras de incentivo e por estarem sempre presentes quando era e quando não era preciso.

A todos os meus amigos fora do laboratório, pela amizade e apoio ao longo destes anos, em especial à Amara, Catarina, Fátima, Magda e Raquel, pelas horas de conversa fiada, cafés e pelos fantásticos lanches na Vénus. Obrigada amigas!

Babi e Cátia, obrigada pela cumplicidade e amizade verdadeira. Obrigada também por estarem sempre presentes, por acreditarem em mim, pelos bons e "maus" conselhos, pelas gargalhadas e por todos os "nossos" momentos.

A toda a minha família, que de uma forma ou de outra sempre me apoiou ao longo deste percurso.

Ao Humberto por me teres acompanhado, e acreditado em mim mesmo quando eu já não acreditava, por teres partilhado todos os sonhos, alegrias e tristezas deste trabalho. Obrigada por todo o apoio, carinho e compreensão.

Aos meus pais, por sempre acreditarem que era capaz, pelas palavras de encorajamento, pelo apoio, paciência e carinho. Um obrigado muito especial à minha mãe pela preocupação constante e por compreenderes as longas ausências.

Agradeço ao Centro de Oftalmologia e Ciências da Visão, IBILI, por me ter acolhido e por me ter proporcionado condições para a realização deste trabalho. Agradeço também ao Programa Doutoral em Biologia Experimental e Biomedicina por me ter seleccionado na sua quinta edição. Por último agradeço também à Fundação para a Ciência e a Tecnologia pelo financiamento do meu trabalho (SFRH/BD/32949/2006).

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

PhD Programme
Biologia Experimental e Biomedicina

POPH

QREN QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL
PORTUGAL 2007-2013

UNIÃO EUROPEIA
Fundo Social Europeu

Publications

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

Gaspar JM, Baptista FI, Galvão J, Castilho AF, Cunha RA, Ambrósio AF. Diabetes differentially affects the content of exocytotic proteins in hippocampal and retinal nerve terminals. Neuroscience 2010 September 15;169(4):1589-1600.

Gaspar JM, Castilho AF, Baptista FI, Liberal J, Ambrósio AF. Long-term exposure to high glucose induces changes in the content and distribution of some exocytotic proteins in cultured hippocampal neurons. Neuroscience 2010 October 13 [Epub ahead of print].

Baptista FI, Gaspar JM, Cristóvão A, Santos PF, Köfalvi A, Ambrósio AF. Diabetes induces changes in vesicular transporters and neurotransmitter release in hippocampus and retina. Submitted to Brain Research.

Gaspar JM, Baptista FI, Castilho AF, Liberal J, Gonçalves J, Ambrósio AF. Type 1 diabetes induces neural changes in rat hippocampus. Submitted to *Diabetologia*.

Note: The results presented in this dissertation, included in Chapters 2-5, are formatted according to the style of the journal where the papers were published or submitted for publication, with minor modifications.

Table of Contents

Abbreviations

AD	Alzheimer's disease
ADA	American Diabetes Association
ACTH	Adrenocorticotropic hormone
AGEs	Advanced glycation end products
AIF	Apoptosis inducing factor
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
AR	Aldose reductase
ATP	Adenosine triphosphate
AVP	Vasopressin
BBB	Blood-brain barrier
BB/Wor	BioBreeding/Worcester
BBZDR/Wor	BioBreeding Zucker diabetic rat/ Worcester
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	*Cornu Ammon*
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CASK	CaMK/SH3/guanylate kinase domain protein
CAZ	Cytomatrix assembled at the active zone
CHR	Corticotropin-releasing hormone
CNS	Central nervous system
cPKC	Conventional protein kinase C
DAG	Diacylglycerol
DG	Dentate gyrus
DOC	Deoxycholate
DPM	Disintegrations per minute
DTT	Dithiothreitol
ECDCDM	The expert committee on the diagnosis and classification of diabetes *mellitus*
ECF	Enhanced chemifluorescence
EPSP	Excitatory postsynaptic potential
EU	European Union
FBS	Fetal bovine serum
FR%	Fractional release %
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GFAT	Glutamine:fructose-6 phosphate amidotransferase
GlcNac	N-acetylglucosamine
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
HA1c	Hemoglobin A1c
HPA	Hypothalamic-pituitary-adrenal

IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase isoform
IPSP	Inhibitory postsynaptic potential
KHR	Krebs-Henseleit ringer
KO	Knockout
LDCV	Large dense-core vesicle
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MAP-2	Microtubule associated protein-2
Mint	Munc18/nSec1-interacting protein
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	Nuclear factor-κB
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic ressonance
NO	Nitric oxide
nPKC	Novel protein kinase C
NSF	N-ethylmaleimide-sensitive fusion
O-GlcNAcase	*O*-GlcNAc β-*N*-acetylglucosaminidase
OPC	Oligodendrocyte precursor cell
OT	Oxytocin
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PITP	Phosphatidylinositol transfer protein
PKA	Protein kinase A
PKC	Protein kinase C
PSD	Postsynaptic density
PSD-95	Postsynaptic density-95
PTP	Post-tetanic potentiation
PVDF	Polyvinyllidene difluoride
PVN	Paraventricular nucleus
RAGE	Receptors for advanced glycation end -products
RIM	Rab3-interacting molecule
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SAP97	Synapse-associated protein 97
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNAP-25	Synaptosomal-associated protein with 25 kDa
SNARE	Soluble NSF attachment protein receptor
SSV	Small synaptic vesicle
STZ	Steptozotocin
TBS-T	Tris-buffered saline-Tween 20
TGF-α	Transforming growth factor-α

TNF-α	Tumor necrosis factor-α
UDP	Uridine diphosphate
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
VAMP-2	Vesicle-associated membrane protein 2
VGAT	Vesicular GABA transporter
VGluT-1	Vesicular glutamate transporter-1
VGluT-2	Vesicular glutamate transporter-2

Resumo

A encefalopatia diabética é uma complicação associada à diabetes, caracterizada por alterações neuroquímicas, electrofisiológicas, estruturais e cognitivas. No hipocampo, a diabetes induz depleção de vesículas sinápticas nos terminais nervosos, atrofia de dendrites em neurónios piramidais, alterações na expressão de proteínas pré-sinápticas envolvidas na exocitose e também altera a libertação de neurotransmissores. Apesar destas evidências, as alterações moleculares e celulares que ocorrem no hipocampo causadas pela diabetes não estão ainda totalmente esclarecidas. Assim, o principal objectivo deste trabalho consistiu em clarificar os efeitos da diabetes no hipocampo, e em particular identificar alterações moleculares que ocorrem no terminal pré-sináptico. Para isso avaliámos o efeito da diabetes, ou de uma concentração elevada de glicose, no conteúdo de proteínas sinápticas envolvidas na exocitose e na libertação de neurotransmissores. Estudámos também o efeito da diabetes nas células neurais do hipocampo.

A diabetes foi induzida em ratos Wistar com uma injecção intraperitoneal de estreptozotocina. O conteúdo proteico das SNAREs (sintaxina-1, VAMP-2 e SNAP-25), sinapsina-1, sinaptotagmina-1, sinaptofisina e rabfilina 3a foi avaliado nos terminais nervosos purificados e extractos totais de hipocampo após duas, quatro e oito semanas de diabetes. Nos terminais nervosos, o conteúdo de sintaxina-1 diminuiu em todos os tempos, e o de SNAP-25 e sinapsina-1 diminuiu apenas após quatro e oito semanas de diabetes. O conteúdo de sinaptofisina aumentou em todos os tempos. Não foram detectadas alterações no conteúdo das restantes proteínas. Em extractos totais, o conteúdo de SNAP-25 e de sintaxina-1 diminuiu nos períodos em que foram detectadas alterações mais significativas nos terminais nervosos. Estes resultados indicam que a diabetes induz alterações diferenciadas no conteúdo de várias proteínas exocitóticas no hipocampo.

Uma vez que a hiperglicémia é considerada o principal factor para o desenvolvimento de complicações da diabetes, avaliou-se se uma exposição prolongada a uma concentração elevada de glicose, que mimetiza hiperglicémia prolongada, altera o conteúdo e localização de proteínas sinápticas envolvidas na exocitose em neurónios de hipocampo em cultura. Os neurónios foram expostos durante sete dias a glicose elevada (50 mM) ou manitol (25 mM; mais 25 mM D-glicose), usado como controlo osmótico.

Avaliou-se a viabilidade celular e o conteúdo proteico e localização das proteínas exocitóticas e de VGluT-1 e VGAT. A glicose elevada não alterou a morfologia celular, embora tenha ocorrido um aumento de apoptose. Além disso, diminuiu o conteúdo de SNAP-25, e aumentou o de sinaptotagmina-1 e VGluT-1. O conteúdo das restantes proteínas não se alterou. Também ocorreu uma acumulação de sintaxina-1, sinaptotagmina-1 e VGluT-1 no corpo celular de alguns neurónios, sugerindo que a hiperglicémia pode comprometer o tráfego destas proteínas para a sinapse. Como a glicose elevada não afectou a maioria das proteínas, isto sugere que outros factores podem contribuir para a alteração nas proteínas sinápticas detectadas em animais diabéticos.

Uma vez que alterações na exocitose podem afectar a libertação de neurotransmissores, investigou-se o efeito da diabetes (duas e oito semanas) na libertação basal e induzida de [^{14}C]glutamato e [^{3}H]GABA em terminais nervosos de hipocampo, tendo-se analisado também o conteúdo proteico de VGluT-1, VGluT-2, VGAT e da subunidade α_{1A} do canal de cálcio do tipo P/Q. Às duas semanas de diabetes, os níveis de VGluT-1 e VGluT-2 não se alteraram. O conteúdo de VGAT e da subunidade α_{1A} diminuiu nos terminais nervosos, mas não nos extractos totais. Às oito semanas de diabetes não foram observadas alterações. Também não ocorreram alterações na libertação de GABA nos animais diabéticos, mas a libertação basal de glutamato aumentou às oito semanas. Estes resultados indicam que a diabetes induz alterações pré-sinápticas, que podem afectar a transmissão sináptica.

Uma vez que as alterações cognitivas podem resultar de alterações neuronais e gliais no hipocampo, avaliou-se o efeito da diabetes (duas, quatro e oito semanas) em células neurais do hipocampo de rato. A neurodegeneração foi avaliada por marcação com violeta de cresilo e por detecção de caspase-3. A análise da imunoreactividade de MAP-2 e sinaptofisina em fatias de cérebro, e do conteúdo proteico de Tuj-1, tau e calbindina D28k, permitiu identificar alterações neuronais. A reactividade de astrócitos (GFAP) e de microglia (CD11b e ED1) também foram analisadas. Embora não se tenha detectado sinais de degeneração no hipocampo dos animais diabéticos, ocorreu uma diminuição de todos os marcadores neuronais após oito semanas de diabetes. A imunoreactividade de MAP-2 diminuiu às duas e quatro semanas no giro dentado e às oito semanas em todas as

sub-regiões. A sinaptofisina aumentou nos animais diabéticos na sub-região CA3 em todos os tempos. Embora não tivesse ocorrido reactividade de astrócitos, o número de microglias activas aumentou nas sub-regiões CA3 e giro dentado após oito semanas de diabetes. Estes resultados indicam que embora a diabetes não induza neurodegeneração no hipocampo em fases iniciais da doença, ocorrem alterações neuronais, principalmente às oito semanas de diabetes, sendo a sub-região CA3 a mais afectada. A activação das células da microglia sugere a existência de uma resposta pro-inflamatória no hipocampo induzida pela diabetes.

Concluindo, a diabetes induz alterações nos terminais pré-sinápticos do hipocampo, nomeadamente ao nível do conteúdo de várias proteínas sinápticas envolvidas na exocitose, e também na libertação de neurotransmissores. Além disso, as alterações neurais e a activação de um processo pro-inflamatório no hipocampo podem contribuir para a disfunção sináptica e para os problemas cognitivos detectados em animais e pacientes diabéticos.

Abstract

Diabetic encephalopathy is a complication of diabetes, characterized by brain neurochemical, electrophysiological and structural alterations, and cognitive and memory impairments. In hippocampus, diabetes induces the depletion of synaptic vesicles in nerve terminals and dendritic atrophy of pyramidal neurons, changes the expression of presynaptic proteins involved in exocytosis and affects neurotransmitter release. However, the molecular and cellular alterations caused by diabetes in hippocampus are not fully clarified. Thus, our main goal was to further clarify the effects of diabetes in hippocampus. We gave a particular attention to the molecular changes occurring at the presynaptic level. For that, we evaluated the effect of diabetes, or high glucose, on the content of synaptic proteins involved in exocytosis and on neurotransmitter release. The effect of diabetes on hippocampal neural cells was also evaluated.

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 hippocampus, during the early stages of diabetes (two, four and eight weeks). In nerve terminals of diabetic animals, the content of syntaxin-1 decreased at all time points, and SNAP-25 and synapsin-1 decreased at four and eight weeks of diabetes, while synaptophysin content increased at all time points. The content of the remaining proteins was not affected. In total extracts, SNAP-25 and syntaxin-1 content also decreased, but only when more drastic changes were detected in nerve terminals. These results indicate that diabetes differentially affects the content of exocytotic proteins in hippocampal nerve terminals.

Since hyperglycemia is considered the main trigger of diabetic complications, we analyzed whether long-term high glucose, which mimics prolonged hyperglycemia, changes the content and localization of synaptic proteins involved in exocytosis in cultured hippocampal neurons. Neurons were exposed for seven days to high glucose (50 mM) or mannitol (25 mM; plus 25 mM D-glucose), which was used as osmotic control. We examined the effect of high glucose in cell death and evaluated the protein levels and cellular localization of the exocytotic proteins mentioned above, and of VGluT-1 and VGAT. Neuronal morphology was not significantly affected by high glucose, although there was a

significant increase in the number of apoptotic nuclei. In high glucose-treated cells, the content of SNAP-25 decreased, whereas the content of synaptotagmin-1 and VGluT-1 increased. The remaining proteins were not affected. Moreover, high glucose induced an accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in the cell body of some neurons, suggesting that hyperglycemia might affect the trafficking of these proteins to the synapse. Because high glucose did not affect the majority of the proteins, it appears that other factors might contribute to the alterations detected in synaptic proteins in diabetic animals.

Since the impairment in exocytosis may contribute to changes in neurotransmitter release, we also evaluated the effect of diabetes (two and eight weeks) on [^{14}C]glutamate and [^{3}H]GABA basal and evoked release in hippocampal nerve terminals. The protein levels of VGluT-1, VGluT-2, VGAT and α_{1A} subunit of P/Q calcium channels were analyzed. At two weeks of diabetes, the content of VGluT-1 and VGluT-2 was not affected, but the content of VGAT and α_{1A} subunit decreased. At eight weeks of diabetes no changes were detected. Regarding neurotransmitter release, diabetes did not affect GABA release at both time points. However, at eight weeks of diabetes the basal release of glutamate increased. These results further indicate that diabetes can affect the pre-synaptic terminal, which may cause impairments in synaptic transmission.

Since cognitive impairments can result from neuronal and glial changes in the hippocampus, we investigated the effect of diabetes (two, four and eight weeks) on rat hippocampal neural cells. Potential neurodegeneration was evaluated by cresyl violet staining and by the detection of caspase-3. To identify neuronal changes, the immunoreactivity of MAP-2 and synaptophysin was examined in brain slices, as well as the protein content of three neuronal markers, Tuj-1, tau and calbindin D28k. Astrocyte (GFAP marker) and microglia (CD11b and ED1 markers) reactivity was also evaluated. No signs of neurodegeneration were detected in the hippocampus of diabetic animals. However, the protein levels of all neuronal markers decreased after eight weeks of diabetes. Moreover, MAP-2 immunoreactivity decreased in DG at two, four and eight weeks of diabetes, and at eight weeks decreased in all subregions. Synaptophysin immunoreactivity increased in CA3 subregion at all time points. Although astrocyte reactivity was not changed, the number of active microglial cells increased in DG and CA3 subregions at eight weeks of

diabetes. The results indicate that diabetes does not induce neuronal degeneration in hippocampus during the early stages of the disease, but neuronal changes occur, mainly after eight weeks of diabetes, being the CA3 subregion the most affected. The activation of microglial cells indicates that diabetes triggers a pro-inflammatory response in the hippocampus.

In conclusion, diabetes induces changes in the presynaptic compartment in the hippocampus, namely in the content of several synaptic proteins involved in exocytosis. Neurotransmitter release can also be affected. Moreover, diabetes induces neuronal changes and triggers a pro-inflammatory response in the hippocampus. Altogether, these alterations might contribute to cognitive impairments detected in diabetic animals and humans.

CHAPTER 1

Introduction

1.1 Hippocampus

The hippocampus is a part of the cerebral cortex and is one of the brain structures within the limbic system, playing a fundamental role in memory and associative learning and is one of the basic building blocks of mammalian higher cognitive functions (Amaral and Witter, 1989).

The hippocampal formation (Figure 1.1) has a peculiar anatomy, with cytoarchitectonically distinct regions, including dentate gyrus (DG), *Cornu Ammon* (CA1 and CA3), *subiculum, presubiculum* and entorhinal cortex (Amaral and Witter, 1989).

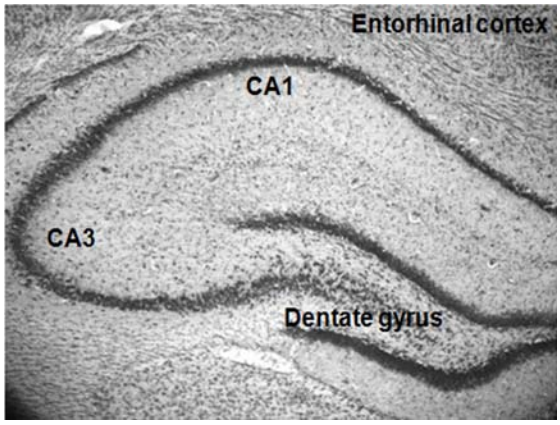


Figure 1.1: Image of hippocampal subregions.¦Histological cross-section of a rat hippocampus showing the different subregions: *Cornu Ammon* (CA1 and CA3) and dentate gyrus; 5x Magnification.

1.1.1 Cell types in hippocampus

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: the principal or granular cell layer, the molecular cell layer and the polymorphic cell layer (Amaral et al., 2007). CA1 and CA3 have only a principal cell layer, called pyramidal cell layer (Figure 1.2). Besides these principal neurons, hippocampus also has interneurons and glial cells (astrocytes, oligodendrocytes and microglial cells).

1.1.1.1 Principal neurons

The principal neurons of DG are granular cells, which have a small cell body and are arranged into the granular cell layer (Figure 1.2). The dendrites of granular cells, that emerge only from the top of the cell body (monopolar neurons), extend perpendicularly to the granular cell layer, into the overlying molecular layer, where they form synapses with several neurons from different regions. 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).

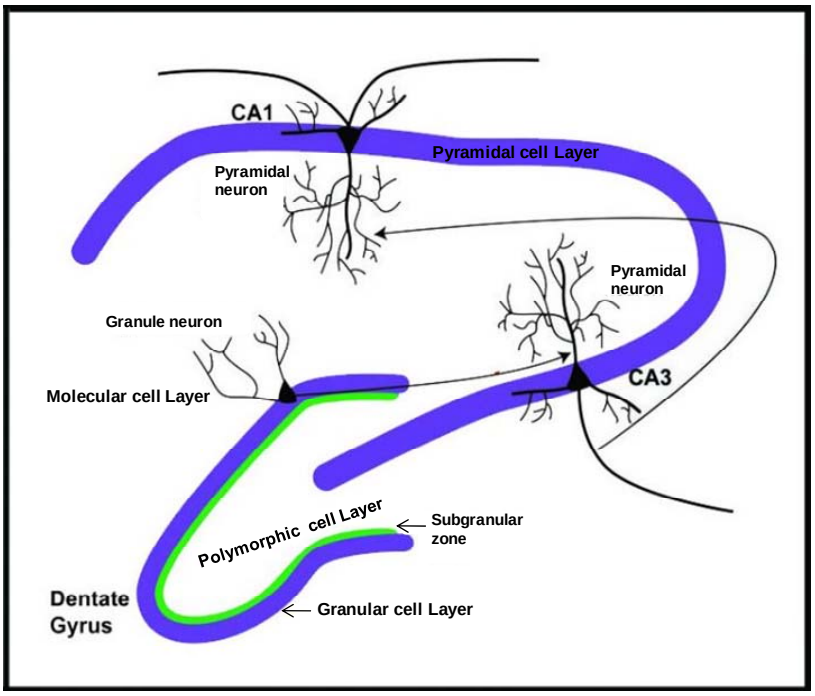


Figure 1.2: Principal neurons in the hippocampal formation. Structural organization of neurons in hippocampal cell layers in each subregion (Adapted and modified from www.sites.lafayette.edu).

In the CA subregions, the principal neurons are pyramidal cells (Figure 1.2), 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 (Johnston and Amaral, 2004; Anderson et al., 2007).

1.1.1.2 Interneurons

The vast majority of interneurons in DG, CA1 and CA3 subregions has no spines and are gabaergic neurons, which are specifically localized in restricted target regions. In DG, the most common interneurons are pyramidal basket cells, whose cell body is located at the border between the granular and the polymorphic cell layers. Axons of pyramidal basket cells enervate cell bodies of granular cells. There are also interneurons in molecular and polymorphic cell layers that can be divided into three different groups, based on their synaptic targets:

- axo-axonic cells: synapse with the initial segments of pyramidal neurons, exerting a strong control over action potential initiation;

- basket cells: make synapses with the soma of pyramidal cells, and each basket cell can make multiple contacts with a pyramidal neuron;

- bistratified cells: make synaptic contacts with apical and basal dendrites of pyramidal neurons (Johnston and Amaral, 2004).

1.1.1.3 Astrocytes

Astrocytes are a subtype of glial cells in the central nervous system (CNS). Astrocytes provide structural and metabolic support to neighboring neurons, and also maintain ion homeostasis in the extrasynaptic space, clearing glutamate from the synaptic cleft (Kimelberg, 2010; Figure 1.3). Astrocytes also play active roles in controlling the number and strength of neuronal synapses contributing to synaptic transmission and plasticity (Newman, 2003; Barker and Ullian, 2010).

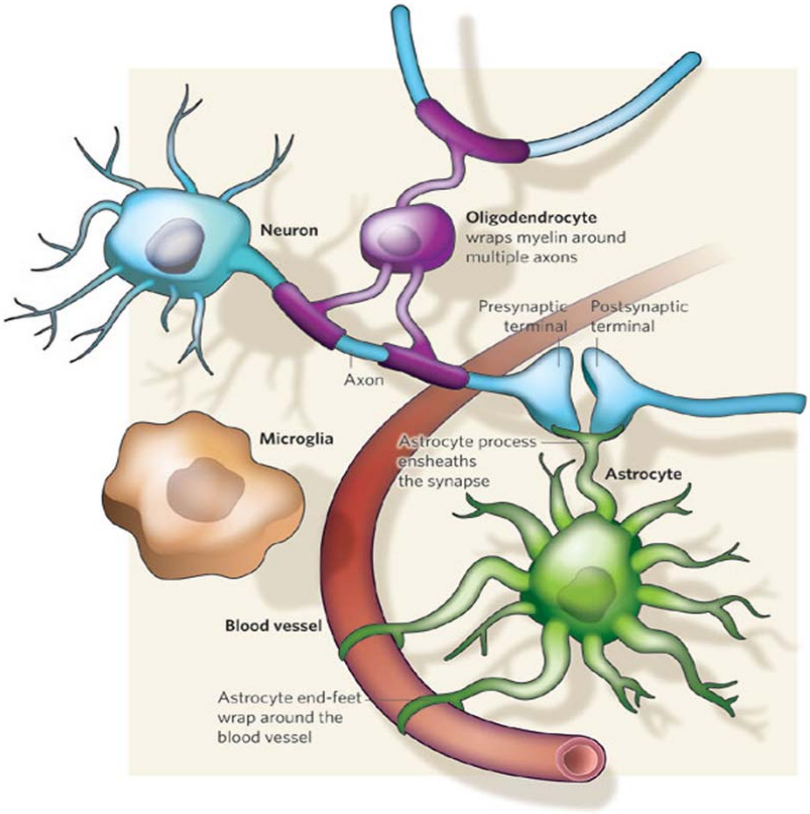


Figure 1.3: Glial cells interacting with neurons and the surrounding blood vessels. Astrocytes extend processes that ensheath blood vessels and synapses, providing structural and metabolic support to neighboring neurons. Oligodendrocytes wrap myelin around axons. Microglia keeps the brain under surveillance for damage or infection (adapted from Allen and Barres, 2009).

1.1.1.4 Oligodendrocytes

Oligodendrocytes are the myelinating cells of the CNS, providing a support role for neurons (Figure 1.3). Oligodendrocytes are derived from neuroectodermal cells within the subventricular zone. At an immature stage these cells are termed oligodendrocyte precursor cells (OPCs) and express a proteoglycan, NG2. Later in development, OPCs mature into oligodendrocytes that form the myelin insulation on rapidly conducting axons. Curiously, NG2-positive cells also persist in the adult CNS, where they compose up to 5% of brain cells and are the main population of cells that proliferate in the adult brain (Fields, 2008; Bradl and Lassmann, 2010). Myelin is a lipid-rich membrane that electrically insulates the axon, allowing for rapid and efficient propagation of impulses. In addition,

myelin also provides trophic support to axon and maintains its long-term integrity (Jackman et al., 2009).

1.1.1.5 Microglial Cells

Microglial cells are the primary immune effector cells in the brain (Figure 1.3). Microglia represents the major population of resident macrophages in the CNS, accounting for 10% of the non-neuronal cells in the brain parenchyma. Microglial cells are derived from myeloid precursor cells, which enter in CNS during embryogenesis and maturate according to the microenvironment (Perry et al., 2010), originating regional differences in microglia distribution and morphology in the brain. The expression of tissue macrophage markers on microglia also varies in different brain regions, although the functional significance of these regional differences is not known (Lawson et al., 1990). In a healthy brain, microglia has an immune function and plays an integral role in maintaining brain homeostasis. Some studies also indicate that these cells might perform a nutritive or supportive function for neurons (Nimmerjahn et al., 2005).

In neurodegenerative disorders occurs an activation of microglial cells that sustain a local inflammatory response (see review Amor et al., 2010). When activated, microglial cells change their morphology and upregulate cell surface receptors. Moreover, these cells transform themselves into phagocytic cells with the ability of presenting antigens to circulating immune cells, participating in the immune responses. In addition, microglia activation can respond to these changes expressing neurotrophic factors and releasing pro- and anti-inflammatory cytokines (Nimmerjahn et al., 2005; Mandrekar-Colucci and Landreth, 2010; Perry et al., 2010).

1.1.2 Basic circuits in hippocampus

The excitatory pathway in hippocampus is organized in a trisynaptic circuit (Figure 1.4).

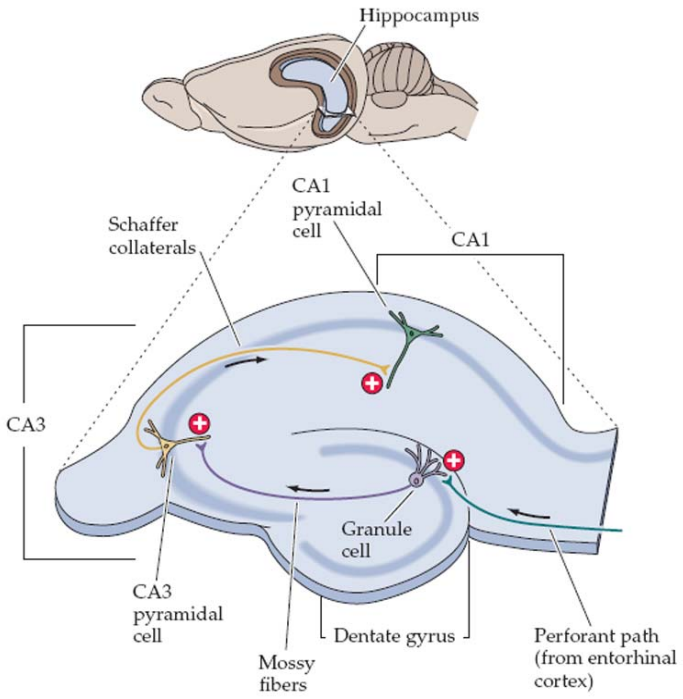


Figure 1.4: Trisynaptic circuit. Illustration of a section of the rodent hippocampus showing the major regions, excitatory pathways and synaptic connections (Adapted from Purves, 2004a).

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. Stimulation of mossy fibers evokes an 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 Schaffer collateral

projections are excitatory but can be followed by inhibitory postsynaptic potentials (IPSPs) (Lopes da Silva and Arnolds, 1978; Johnston and Amaral, 2004).

1.2 Synaptic transmission

Sophisticated and highly efficient mechanisms are needed to allow synaptic communication among the neuronal network. 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). 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. 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 (Augustine, 2004; Margeta and Shen, 2010). This mechanism of synaptic transmission is also the basis of neuronal plasticity and memory occurring in the hippocampus.

1.2.1 Synaptic plasticity in hippocampus

Synapses are also able to modulate the strength of the transmitted signals. This ability to modify the strength of transmission is known as synaptic plasticity, which is essential to information storage and memory formation within the hippocampus. Two components of plasticity can be distinguished: short-term plasticity (which endures for a few hours) and long-term plasticity (which persists for several days or longer). Synaptic strength can be potentiated, known as long-term potentiation (LTP), or depressed, in this case named long-term depression (LTD; Figure 1.5). Different molecular and cellular mechanisms can be involved in LTP or LTD at different synapses (Purves, 2004a).

Repetitive high frequency synaptic stimulation commonly induces LTP. A prolonged depolarization removes Mg^{2+} from *N*-methyl-D-aspartate (NMDA) ionotropic glutamate receptors allowing the entry of Ca^{2+} through these receptors in postsynaptic neurons. The

increase in calcium concentration activates signal transduction cascades in postsynaptic neurons that induce LTP. The activation of protein kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) is a common feature of LTP. Changes in postsynaptic densities, such as the insertion of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) ionotropic glutamate receptors into the postsynaptic membrane, mediated by these kinases contribute to the enhancement of LTP (Figure 1.5A). Changes in the presynaptic component, such as the increase in glutamate release, can also contribute to the enhancement of LTP. This presynaptic potentiation requires a retrograde signal, namely nitric oxide (NO), which spreads from the postsynaptic region to presynaptic terminals (Malenka and Nicoll, 1999; Lynch, 2004).

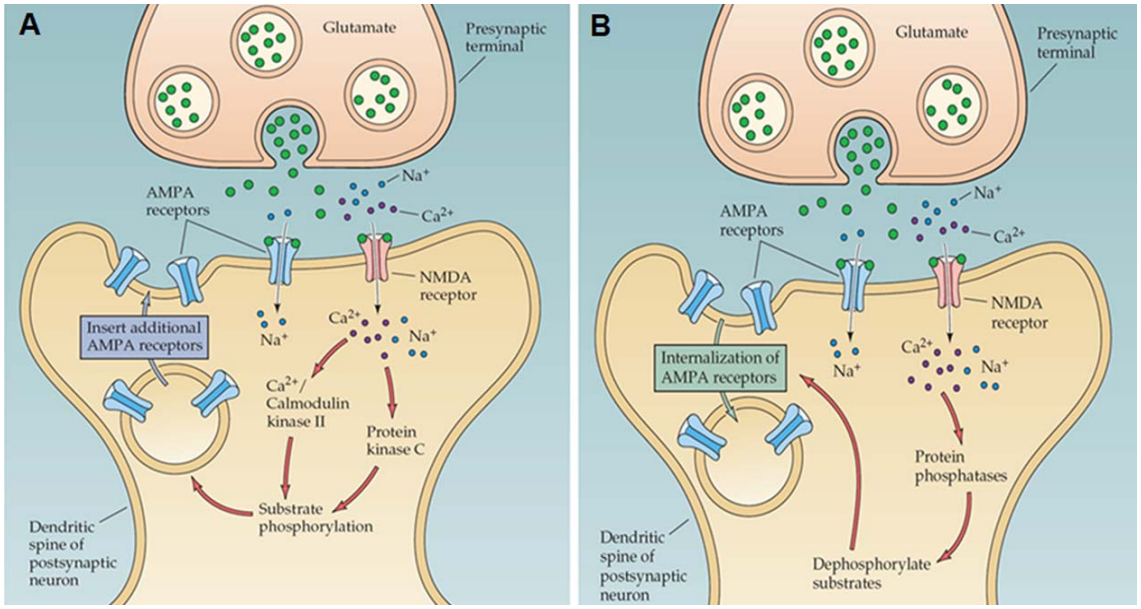


Figure 1.5: Mechanisms underlying LTP and LTD. (A) LTP - Calcium enters through NMDA receptors in the postsynaptic cell and activates postsynaptic protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), leading to the increase of AMPA receptors at postsynaptic membranes; (B) LTD - A low-amplitude increase in Ca^{2+} concentration in the postsynaptic neuron activates postsynaptic protein phosphatases, leading to the internalization of postsynaptic AMPA receptors by endocytosis, thereby decreasing the sensitivity to glutamate released from nerve terminals (adapted from Purves, 2004a).

LTP can be independent of NMDA receptors. In this case, LTP is induced by presynaptic mechanisms, such as the one occurring in synapses between mossy fibers and

CA3 pyramidal neurons. Presynaptic mechanisms are also responsible for short-term forms of plasticity, such as synaptic facilitation and post-tetanic potentiation (PTP) (Byrne and Roberts, 2004; Purves, 2004a). Synaptic facilitation is a transient increase in synaptic strength, occurring when two or more action potentials invade the presynaptic terminal in close succession. Facilitation results of prolonged elevation of presynaptic Ca^{2+} levels following synaptic activity, leading to more neurotransmitter released by each succeeding action potential, causing the postsynaptic end plate potential to increase progressively (Purves, 2004a). PTP results from a high-frequency burst in presynaptic action potentials (known as tetanus) that can yield an even more prolonged elevation of presynaptic Ca^{2+} level. PTP is delayed in its onset and typically enhances transmitter release for up to a few minutes after the sequence of stimuli ends. PTP is thought to arise from calcium-dependent processes, including activation of presynaptic protein kinases, which enhance the ability of incoming Ca^{2+} ions to trigger fusion of synaptic vesicles with the plasma membrane (Purves, 2004a). The difference in duration distinguishes PTP from synaptic facilitation.

LTD occurs when neurons are stimulated at a low frequency for longer periods. This also activates NMDA receptors, promoting a low-amplitude rise in intracellular Ca^{2+} concentration in postsynaptic neurons. The small increase in intracellular Ca^{2+} concentration activates protein phosphatases leading to the internalization of AMPA receptors in the postsynaptic cell (Figure 1.5B), therefore decreasing the sensitivity to glutamate released from presynaptic terminals (Purves, 2004a).

1.2.2 Presynaptic active zone

Within the synapse, the release of neurotransmitters is restricted to highly specialized compartments located at the presynaptic membrane, which are called active zones. These sites are characterized by distinctive electron-dense aggregates of proteins that mediate and regulate neurotransmitter release (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). Within the CAZ, a network of microfilaments and proteins are present (Figure 1.6): (1) synaptic vesicle fusion proteins, including syntaxin-1, synaptosomal-associated protein with 25 kDa (SNAP-25) and Munc18; (2) cytoskeletal

proteins, such as actin, tubulin, myosin and spectrin; (3) scaffolding proteins, such as CaMK/SH3/guanylate kinase domain protein (CASK), Munc18/nSec1-interacting protein (Mint) and synapse-associated protein 97 (SAP97); (4) voltage-gated calcium channels and (5) cell adhesion molecules (Garner et al., 2000; Schoch and Gundelfinger, 2006). All of these CAZ associated proteins are implicated in the functional and spatial organization of the individual steps of the synaptic vesicle cycle.

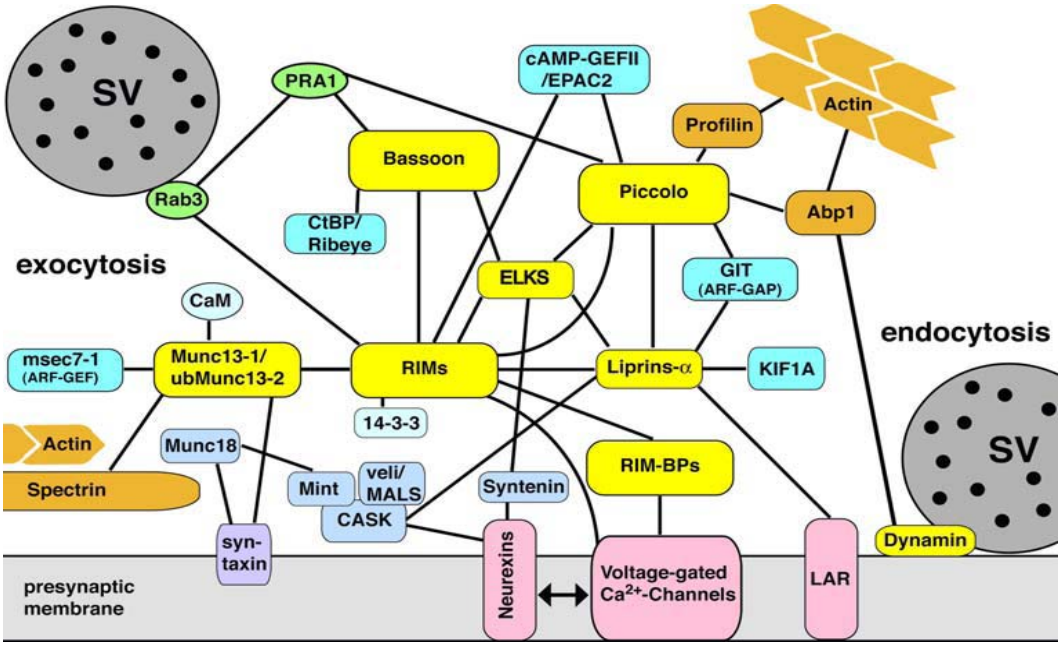


Figure 1.6: Network of interactions of CAZ proteins at the presynaptic active zone (adapted from Schoch and Gundelfinger, 2006).

1.2.3 Neurotransmitter release by exocytosis

Neurotransmitter release is a highly regulated process that occurs mainly by exocytosis of secretory vesicles. In the synapse, classical neurotransmitters are stored in small synaptic vesicles (SSVs; Figure 1.7), which are abundant in the brain. Neuropeptides are stored in large dense-core vesicles (LDCVs; Sudhof, 2008).

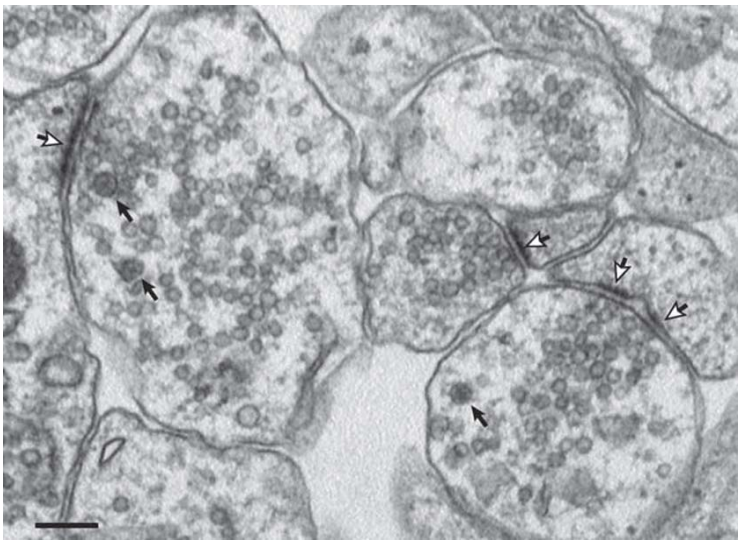


Figure 1.7: Synapses from cultured mouse cortical neurons. Synaptic vesicles are located in nerve terminals adjacent to synaptic junctions that are composed by the active zone of the presynaptic nerve terminal and by postsynaptic densities. Open arrows point to postsynaptic densities of the synaptic junction. Close arrows indicate the presence of large dense core vesicles (LDCVs). Bar 500 nm (Adapted from Sudhof, 2008).

Exocytosis is a highly regulated process divided in five steps (Figure 1.8): 1) incorporation of neurotransmitters into synaptic vesicles; 2) trafficking of the vesicles to nerve terminal; 3) docking; 4) priming and 5) fusion of vesicles with plasma membrane. 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.

Neurotransmitters are actively transported into synaptic vesicles, which undergo a trafficking cycle into the nerve terminal (Figure 1.8). Synaptic vesicles are mobilized and docked at the active zone of the plasma membrane. Docked vesicles are characterized by their close apposition to the plasma membrane. From a molecular point of view, docking ensures that synaptic vesicles are anchored to the active zone of the cell. However, the exact mechanism by which the plasma membrane docking of incoming synaptic vesicles occurs is unknown.

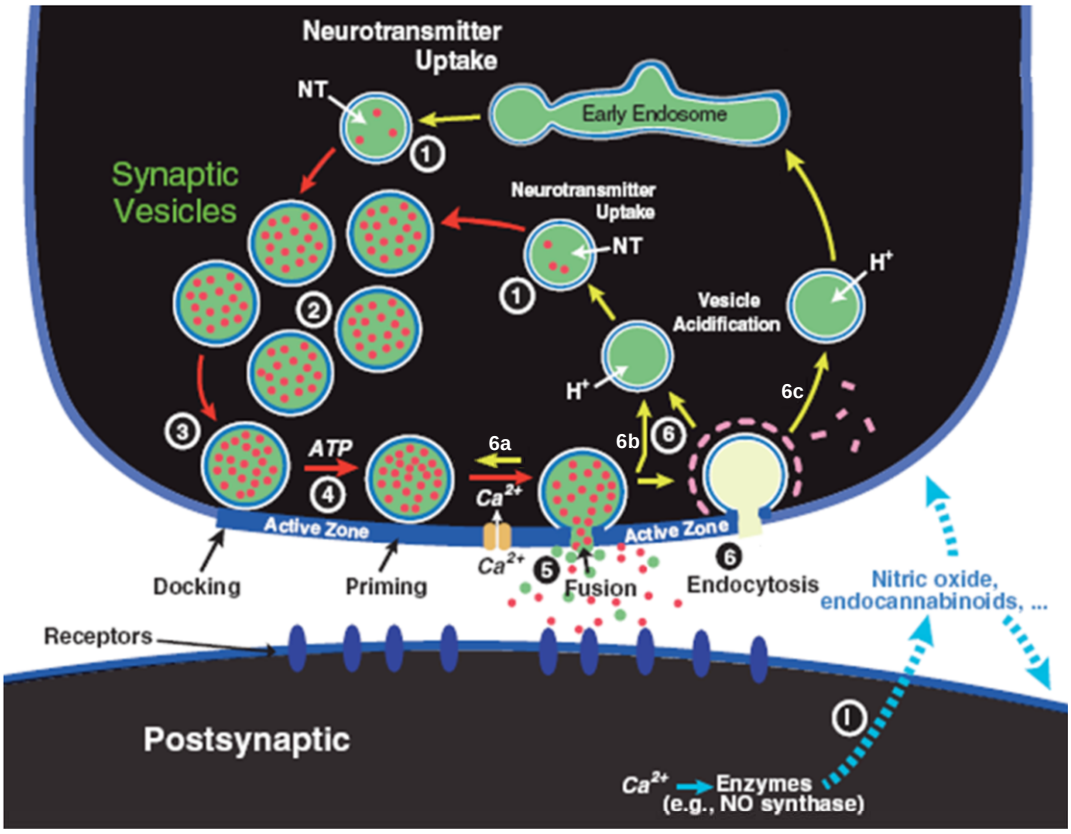


Figure 1.8: Diagram representing the steps of neurotransmitter release by exocytosis. Synaptic vesicles are filled with neurotransmitters by active transport (1), and then a synaptic vesicle cluster is formed (2). Filled vesicles dock at the active zone (3), where they undergo priming (4), which makes vesicles competent for Ca^{2+}-triggered fusion with the plasma membrane (5). After fusion, synaptic vesicles undergo endocytosis and recycling (6). The steps of exocytosis are indicated by red arrows. Endocytosis and recycling steps are indicated by yellow arrows (Adapted and modified from Sudhof, 2008).

Once in the active zone of presynaptic terminals, synaptic vesicles undergo a priming process in which they become fusion competent. This step is adenosine triphosphate (ATP)-dependent and precedes fusion triggered by Ca^{2+} entry through voltage-gated Ca^{2+} channels into the synaptic terminal. After Ca^{2+} entering into the presynaptic terminal, primed vesicles fuse with the plasma membrane releasing neurotransmitters. The assembly of N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor (SNARE) complex, which is composed by the vesicle-associated membrane protein 2 (VAMP-2), SNAP-25 and syntaxin-1, brings the synaptic vesicle membrane close to the plasma membrane promoting the vesicle fusion (Lin and Scheller, 2000). After fusion-pore opening

and the release of neurotransmitter, synaptic vesicles undergo endocytosis and are recycled by alternative pathways: local reuse (Figure 1.8 step 6a); vesicles (called “kiss-and-run”) undock and recycle locally to reacidify and refill with neurotransmitters without endosomal intermediate (Figure 1.8 step 6b); and vesicles endocytose via clathrin-coated pits, reacidify and refill with neurotransmitters (Figure 1.8 step 6c; Sudhof, 2004). Modifications in any of these steps may change the strength of synaptic connections and play a role in synaptic plasticity.

1.2.3.1 Synaptic vesicle pools and vesicle mobilization

A presynaptic vesicle cluster can be subdivided into distinct pools based on morphological or physiological criteria. Morphologically, a distinction is made between vesicles closely apposed to the plasma membrane (docked vesicle pool) and those more distant from the plasma membrane (reserve pool) (Fdez and Hilfiker, 2006).

Physiologically vesicle cluster can be distinguished based on their ability and rate of release. The readily releasable pool includes docked vesicles that are immediately available to be exocytosed under high frequency stimulation (Rosenmund and Stevens, 1996). The recycling pool participates in exo-endocytosis cycle under resting conditions (Rizzoli and Betz, 2005). The reserve pool constitutes the vast majority of vesicles in the nerve terminal. Under experimental conditions, this pool is mobilized to the active zone by intense stimulation, and might only occur once the other two pools are saturated (Rizzoli and Betz, 2005). The size of each pool depends on the transitional dynamics of vesicles and on the type of synapse (Rizzoli and Betz, 2004; Fdez and Hilfiker, 2006).

Synapsins

Synapsins are abundant proteins that are associated with the synaptic vesicles. 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). These proteins are encoded by three distinct genes, synapsin I, II and III, and can undergo alternative splicing, thus generating different variants. Synapsin I is expressed specifically in neurons and binds to actin (Baines and Bennett, 1986), tubulin (Bahler and Greengard, 1987) and neurofilaments (Huttner et al., 1983). This ability to bind several components of

the cytomatrix indicates that this protein may mediate the attachment of synaptic vesicles to cytoskeleton, regulating their trafficking within the presynaptic terminal (Valtorta et al., 1992). The number of synaptic vesicles decreases in synapsin knockout (KO) animals, suggesting that synapsins can also influence the turnover of synaptic vesicles (Takei et al., 1995).

Synapsins are substrates for several protein kinases, and the phosphorylation of synapsins is an important regulatory switch involved in vesicle mobilization. During stimulation, synapsins are phosphorylated and dissociate from vesicles, being dispersed into the cytosol. In the end of stimulation, synapsins are dephosphorylated and reassociate with vesicles. The cycles of phosphorylation-dephosphorylation are involved in the movement of synaptic vesicles between the reserve pool and the releasable pool, regulating the efficiency of neurotransmitter release (Chi et al., 2001).

1.2.3.2 Vesicle docking

Docked vesicles are characterized by their close apposition to the plasma membrane. These vesicles are preferentially located around sites of Ca^{2+} entry (called microdomains). 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.

Three proteins have been identified as potential vesicle docking proteins: piccolo (Cases-Langhoff et al., 1996) and bassoon (tom Dieck et al., 1998), both 420 kDa proteins, and aczonin (Wang et al., 1999), with 550 kDa. These proteins share several characteristics that suggest a role in tethering vesicles at the active zone. Their large size and multiple protein-protein interaction domains make them candidates to form complexes that dock vesicles to the plasma membrane or cytoskeleton. These proteins are localized specifically at the active zones (see Figure 1.6) and are tightly associated with the presynaptic cytoskeletal matrix.

Rab are guanine triphosphate (GTP)-binding proteins that interact with various effectors [Rabphilins and Rab3-interacting molecule (RIM)]. Rab3A, 3B, 3C and 3D belong to the family of Rab proteins that are present at synaptic vesicles, mediating vesicle

docking (Richmond, 2005; Sudhof, 2008). Rab3A KO mice have a decreased pool of docked vesicles and consequently spontaneous neurotransmitter release is diminished (Coleman et al., 2007). RIM is a large multidomain protein that is localized at presynaptic active zones and that interacts simultaneously with Rab3A, Munc-13, SNAP-25 and Ca^{2+} channels (Coppola et al., 2001; Dulubova et al., 2005; Schoch and Gundelfinger, 2006). The interaction of Rab3A with RIM may have a role in the recruitment of vesicles to the active zone, thus mediating the docking process.

In conclusion, the docking process requires the involvement of multiple proteins working in combination to bring the vesicle close enough to the plasma membrane. Further characterization of these proteins and the identification of their binding partners are necessary to elucidate the process of vesicle docking (Lin and Scheller, 2000).

1.2.3.3 Vesicle priming

Membrane contact established by docking mechanisms is not a sufficient condition for membrane fusion. Additional biochemical events termed “priming” are crucial to make vesicles competent for Ca^{2+}-triggered fusion. In neural and neuroendocrine cells, only a small fraction of docked vesicles at the plasma membrane are fusion-competent and undergo rapid fusion in response to Ca^{2+} elevation. Priming occurs after docking of a synaptic vesicle and before fusion. It has been proposed that the formation of the 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 involvement of ATP-dependent proteins (Figure 1.9; Klenchin and Martin, 2000). Also important in priming are NSF and phosphatidylinositol transfer protein (PITP), as well as the interactions between synaptic vesicle proteins, such as Rab3A and rabphilin, with constituents of the active zone, such as RIM and Munc-13 (Kavalali, 2002).

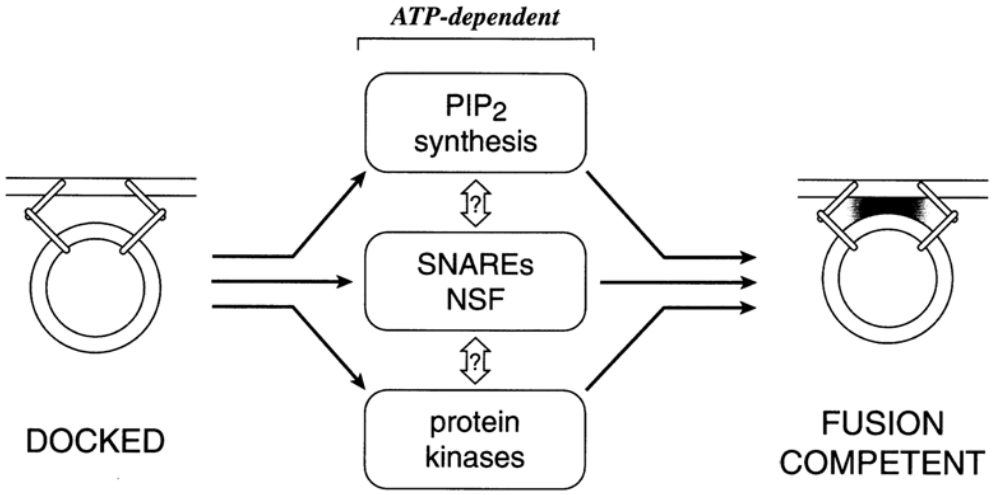


Figure 1.9: Scheme illustrating known biochemical reactions occurring at the "priming" step of exocytosis. ATP-dependent reactions include NSF/SNAP rearrangement of SNARE complexes, phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis and protein phosphorylation by protein kinases, such as protein kinase A (PKA) and PKC (Adapted and modified from Klenchin and Martin, 2000).

SNARE complex

The formation of the SNARE complex is essential for synaptic vesicle fusion during exocytosis. The SNARE complex is formed through the interaction of VAMP-2 with the plasma membrane proteins syntaxin-1 and SNAP-25, originating a very stable complex that brings vesicles and plasma membrane into a close proximity that can be sufficient to overcome the energy barrier to fusion (Kavalali, 2002).

All SNARE proteins share a characteristic motif (SNARE motif) of approximately 60 amino acids that is responsible for protein-protein interactions (Jahn and Scheller, 2006). They also have hydrophobic post-translational modifications that allow membrane anchorage. The complex is composed of four parallel helices (Figure 1.10) with syntaxin-1 and VAMP-2 contributing with one helix each and SNAP-25 contributing with two helices (Sutton et al., 1998).

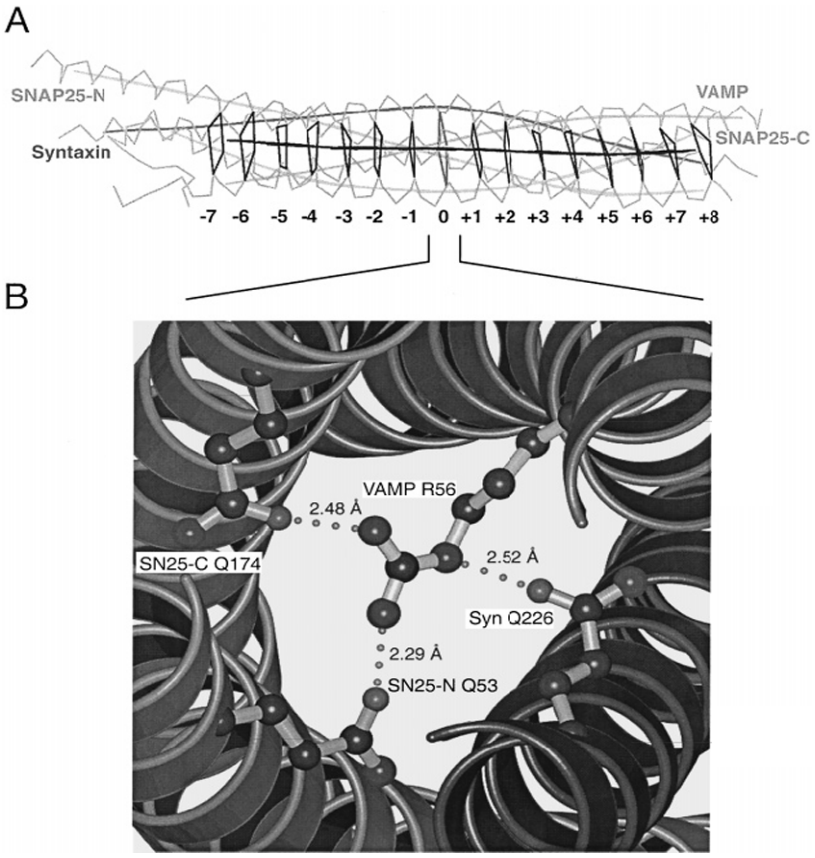


Figure 1.10: Structure of the SNARE complex. (A) The complex is composed of four parallel helices: syntaxin-1 H3 domain, VAMP-2 coiled-coil domain, and SNAP-25 amino- and carboxi-terminal helices. (*B*) At layer 0, syntaxin-1 and SNAP-25 possess glutamine residues that interact ionically with the arginine residue from VAMP-2. This position (layer 0) has the greatest diameter of the layers and may be important in helix alignment or dissociation of the complex (Adapted from Sutton et al., 1998).

When syntaxin-1 is in its closed conformation, it does not bind to VAMP-2 and SNAP-25 (Dulubova et al., 1999). This conformation is maintained by the tight binding of Munc-18 (Figure 1.11A) to full-length syntaxin-1, preventing the binding of VAMP-2 and SNAP-25 (Calakos et al., 1994; Shen et al., 2010). 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 (Fujita et al., 1996; 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 (Richmond et al., 2001) to form the syntaxin-1/SNAP-25 heterodimer and promote the priming of vesicles.

Rab3A is also strongly implicated in SNARE formation (Fischer von Mollard et al., 1991; 1994). The interaction of Rab and Rab effectors, such as RIM and rabphilin, causes a conformational change in Munc-18 that destabilizes the link between syntaxin-1 and Munc-18 (Figure 1.11B; Stahl et al., 1996), leading to the formation of the SNARE complex (Figure 1.11C and D; Lin and Scheller, 2000).

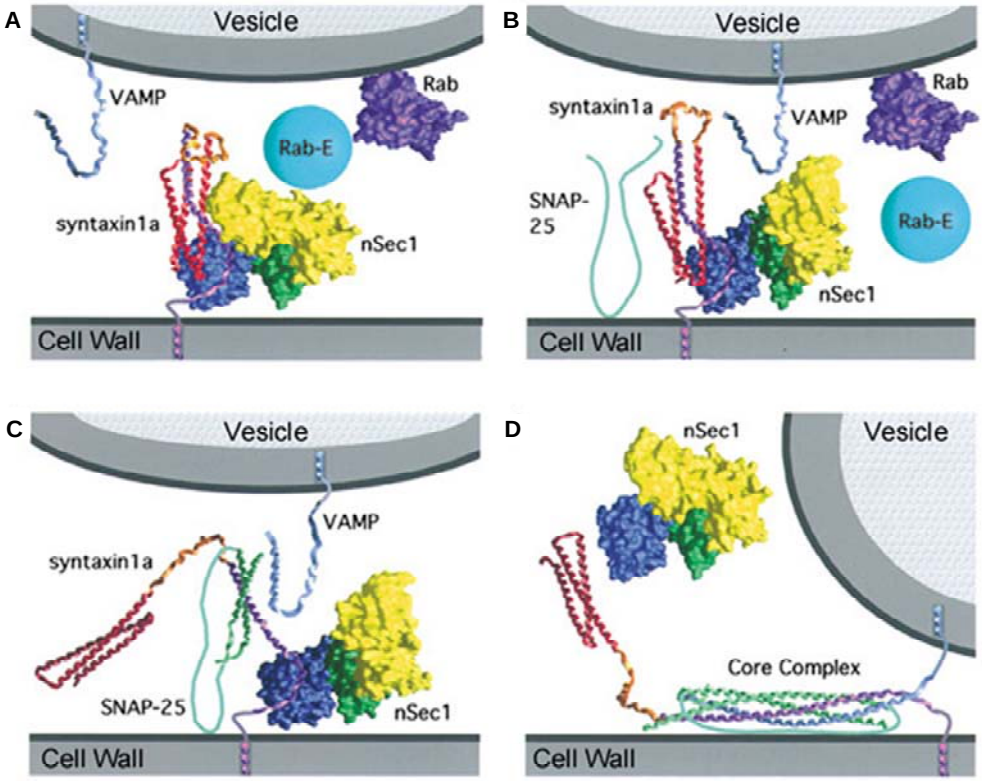


Figure 1.11: Model of SNARE complex formation. (A) Syntaxin-1 is in its closed conformation bound to Munc-18 (nSec1). (B) The action of Rab/Rab effector causes a conformational change in Munc-18, leading to the destabilization of syntaxin-1 linker region. (C) The conformational change in syntaxin-1 allows the formation of the ternary complex. (D) The complex completes the assembly of the helical bundle and promotes membrane fusion (Adapted from Misura et al., 2000).

1.2.3.4 Vesicle fusion

The last step in exocytosis is vesicle fusion that results in the merging of vesicles with plasma membrane to deliver neurotransmitter into the extracellular space. The fusion of synaptic vesicles requires relatively high intracellular Ca^{2+} concentrations (50 - 100 μM). When an action potential arrives to the nerve terminal leads to the opening of voltage-sensitive Ca^{2+} channels (Figure 1.12B), the intracellular Ca^{2+} concentration rises and vesicles fuse releasing neurotransmitters.

Calcium channels and synaptotagmin-1

Synaptic transmission is mediated by N-type and P/Q-type Ca^{2+} channels (Seagar et al., 1999; Zamponi, 2003) that are localized at low density in dendrites and at high density in presynaptic terminals. Besides mediating Ca^{2+} entry to initiate neurotransmitter release, they also interact directly with proteins of the synaptic vesicle docking/fusion machinery (Catterall, 1999). Voltage-sensitive Ca^{2+} channels are heterometric proteins composed by an α_1 subunit that forms the ion-conducting pore. This subunit is associated with two auxiliary subunits, $\alpha_2\delta$ and β, that regulate the properties of the channel pore. The α_1 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+} (Sheng et al., 1994; Wiser et al., 1996).

Synaptotagmin-1 is a 65 kDa protein localized at synaptic vesicles. This protein has a large cytoplasmic domain, consisting of tandem C2 domains (conserved motifs of ~140 amino acid residues, which often bind Ca^{2+} and membranes), connected by a linker. These two C2 domains serve as Ca^{2+} sensors and interact with syntaxin-1 in a Ca^{2+}-dependent manner. These characteristics suggest that synaptotagmin-1 regulates exocytosis (Chapman, 2008). 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).

In conclusion, calcium binding to synaptotagmin-1 induces molecular rearrangements in the SNARE complex (Figure 1.12C) and in lipid bilayers allowing vesicle fusion and neurotransmitter release (Chapman, 2008).

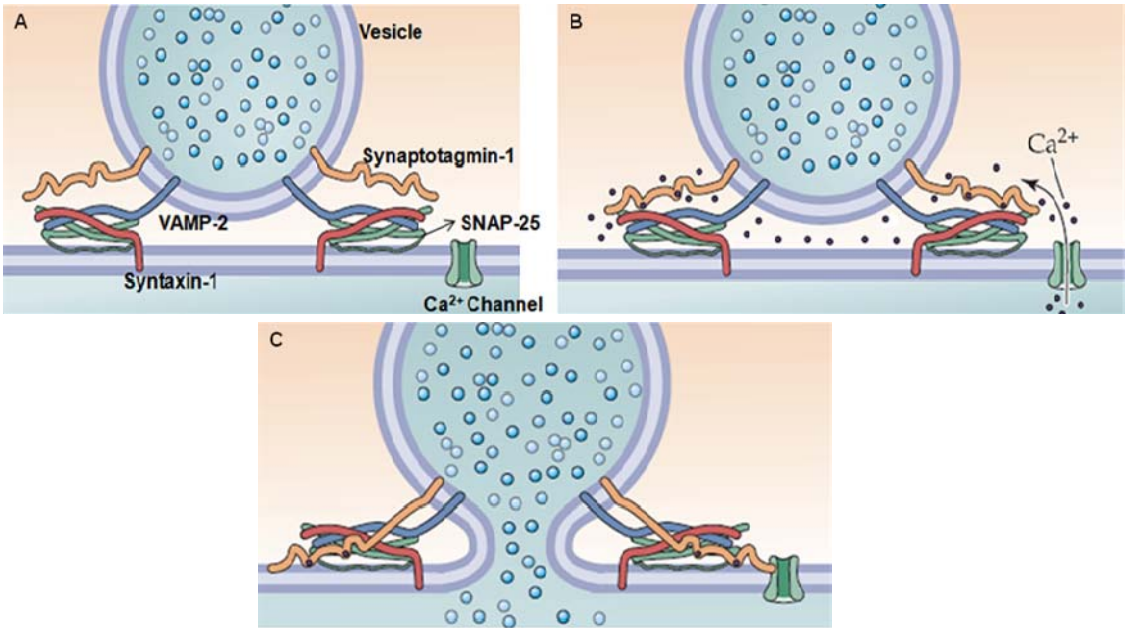


Figure 1.12: Diagram for Ca^{2+}-triggered vesicle fusion. (A) In priming, SNARE proteins at the synaptic vesicle and plasma membrane form a complex that brings together the two membranes. (B) Ca^{2+} enters in presynaptic terminal through voltage-sensitive Ca^{2+} channels and then binds to synaptotagmin-1. (C) Ca^{2+} changes the conformation of synaptotagmin-1, the cytoplasmic region of this protein is inserted into the plasma membrane, synaptotagmin-1 binds to SNAREs and catalyzes membrane fusion (Adapted and modified from Purves, 2004b).

1.3 Calcium-independent release of neurotransmitters

To terminate synaptic transmission, neurotransmitters are inactivated by either enzymatic degradation or by membrane neurotransmitter transporters into neuronal and/or glial cells by neurotransmitter transporters. Once inside the neuronal cell, neurotransmitters can be further transported into synaptic vesicles by vesicular transporters. These processes are responsible for the homeostasis of neurotransmitter pools within nerve terminals (Masson et al., 1999). Membrane transporters are members of a large superfamily including: Na^+- and Cl^--dependent symporters [e.g., for γ-aminobutyric acid (GABA), monoamines and glycine], Na^+- and K^+-dependent antiporters (e.g., for glutamate), among others. These transporters utilize 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+} (Bernath, 1992; Richerson and Wu, 2003).

1.4 Diabetes *mellitus*

Diabetes *mellitus* is the most common metabolic disorder worldwide. It is characterized by hyperglycemia resulting from defects in either insulin secretion or insulin action, or both. Diabetes incidence is increasing dramatically, and it is estimated that in 2030 around 366 million people will have diabetes (Wild et al., 2004).

1.4.1 Classification of diabetes

Four types of diabetes *mellitus* have been recognized: type 1, type 2, other specific types and gestational diabetes. However, type 1 (insulin-dependent or juvenile onset diabetes) and type 2 (non-insulin-dependent) diabetes are the two major types of diabetes.

Type 1 diabetes

Type 1 diabetes is an autoimmune disease that leads to the destruction of pancreatic β-cells, resulting in the lack of insulin. The rate of β-cells destruction is variable, being fast in some individuals, and slow in others. Genetic and environmental factors have been suggested to contribute to the etiology of type 1 diabetes, along with other factors, such as viral infections. Type 1 diabetes generally occurs in children and young people, usually under 30 years of age, affecting 5 to 10% of diabetic population (ADA, 2010).

Type 2 diabetes

Type 2 diabetes or adult-onset diabetes accounts for 90% of all cases of diabetes and occurs mostly in adult individuals. It is primarily characterized by diminished insulin secretion from β-cells and/or insulin resistance, which results from impaired ability of insulin to regulate the glucose levels. Its incidence increases with age, especially after 40 years of age, and it is mostly associated with obesity and a sedentary lifestyle (Zimmet et al., 2001).

Hyperglycemia develops gradually and at the earlier stages is often not severe enough for the patient to notice any symptoms of diabetes. However, these patients are at increased risk of developing macro and microvascular complications (ADA, 2010).

Other specific types of diabetes

This group of other specific types of diabetes has various known reasons to occur. Diabetes may develop in persons with genetic defects that affect β-cell function, defects in insulin action, pancreatic dysfunction, such as pancreatitis or hemochromatosis, dysfunction associated with other endocrinopathies, and pancreatic dysfunction caused by drugs, chemicals or infections (ADA, 2010).

Gestational diabetes

This form of diabetes is defined as any degree of glucose intolerance with onset 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. The hyperglycemia resolves in most women after delivery, but places them at increased risk of developing type 2 diabetes *mellitus* later in life.

Diabetes can cause a wide range of health complications, affecting almost every tissue of the body. These complications result from a complex interplay between direct and indirect metabolic consequences of long-term hyperglycemia, insulin deficiency and additional genetic and environmental factors. Diabetic complications can be delayed by proper glycemic control by the patients. However, many of these complications can develop and become quite severe (ECDCDM, 2003). Long-term complications of diabetes include retinopathy, with potential loss of vision, nephropathy, leading to renal failure, peripheral neuropathy, with risk of foot ulcers and amputation, and encephalopathy, with alteration in several cognitive domains, among others complications.

1.5 Diabetic encephalopathy

Long-term diabetes is associated with complications in the brain, commonly referred as diabetic encephalopathy, which manifests by poorer cognitive ability. Diabetic encephalopathy has been associated with structural alterations in the brain, brain atrophy, and with electrophysiological changes, that culminate in deficits in memory and cognitive performance. The cognitive decline occurs in type 1 and type 2 diabetes, but the degree of cognitive dysfunction and the way that abnormalities are manifested is different in both types (Gispen and Biessels, 2000; Wrighten et al., 2009). Type 1 diabetic patients have impairments in learning and memory, problem solving, and mental and motor speed (Ryan, 1988). 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 are relatively unaffected (Ryan et al., 2006). Cortical and subcortical atrophy has been detected in type 2 diabetic patients by brain magnetic resonance imaging (Manschot et al., 2006).

In diabetic animal models, impaired performance in complex tasks, such as in Morris water maze or in spatial-object learning task, have been detected (Biessels and Gispen, 1996; Biessels et al., 1998; Popovic et al., 2001; Stranahan et al., 2008a). 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; b).

It is accepted that several factors underlie the development of complications in CNS in diabetic subjects. 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.

1.5.1 Contributors to diabetic encephalopathy

Several factors can contribute to diabetes-induced cognitive impairments. The complex pathophysiological characteristics of diabetes (Figure 1.13) may include decreased insulin secretion or activity, dysregulation of glucose homeostasis, and

impairment in the hypothalamic-pituitary-adrenal (HPA) axis, among others (Wrighten et al., 2009).

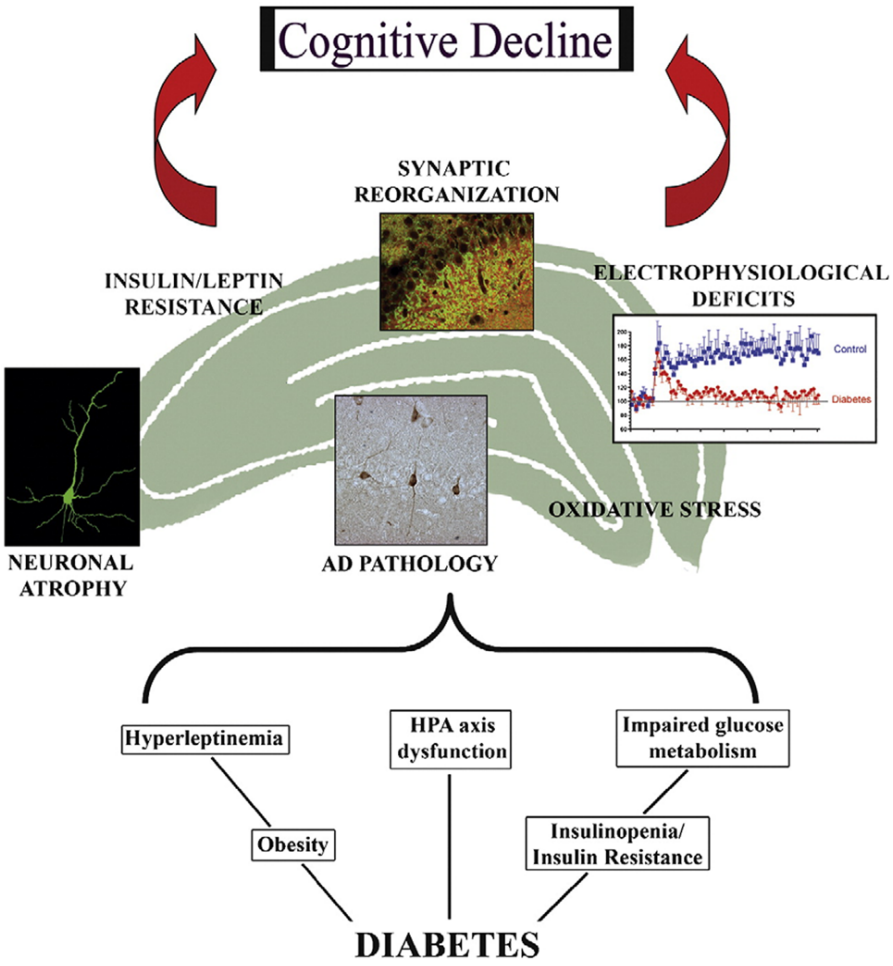


Figure 1.13: Potential mechanistic mediators of diabetes-induced brain dysfunction. The complex pathophysiological features of diabetes include decrease in insulin activity, impaired glucose homeostasis and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis function, among others. Changes observed in hippocampus under diabetic conditions include neuronal/dendritic atrophy, changes in synapse formation, oxidative stress, the appearance of Alzheimer's disease (AD)-like histopathology, and electrophysiological deficits (adapted from Wrighten et al., 2009).

1.5.1.1 Hyperglycemia

Brain preferentially uses glucose as the main metabolic fuel source. Glucose is transported across the blood-brain barrier (BBB) through the glucose transporter (GLUT)-1, which is localized in microvessels, and then glucose is transported into neurons through GLUT-3. To guarantee an appropriate transport of glucose to the tissues, it is essential a homeostasis of glucose in the blood. Under poor controlled diabetes, glucose transport is disturbed leading to up to fourfold increase in neuronal glucose levels, which can lead to a dysregulation of brain metabolism involving inadequate glucose utilization (Tomlinson and Gardiner, 2008; Duarte et al., 2009).

Hyperglycemia is considered the main pathogenic factor for the development of diabetic complications. In diabetic animals, there are increased levels of glucose in hippocampus (Duarte et al., 2009). It was demonstrated that a better control of blood glucose levels leads to a better cognitive performance in type 2 diabetic patients, suggesting that hyperglycemia can contribute to cognitive impairments and dementia in diabetic patients (Ryan et al., 2006). Brain structures, including hippocampus, are sensitive to changes in glucose homeostasis. Elevated glucose levels trigger various processes and activate several biochemical pathways that ultimately induce cell dysfunction and eventually cell death, conducting to slowly progressive functional and structural abnormalities in the brain (Ryan, 1988; Roriz-Filho et al., 2009; Wrighten et al., 2009).

Biochemical alterations in hippocampus

The normal fate of intracellular glucose is to be phosphorylated by hexokinase into the glycolytic pathway. However, under high levels of glucose, hexokinase saturates and glucose is derived into other metabolic pathways. Multiple biochemical pathways (Figure 1.14) have been proposed to link hyperglycemia and cognitive deficits associated with diabetes. The activation of polyol and hexosamine pathways, the formation of advanced glycation end-products (AGEs), the activation of PKC and the increase in oxidative stress contribute to neuronal cell dysfunction induced by diabetes (Klein and Waxman, 2003; Tomlinson and Gardiner, 2008).

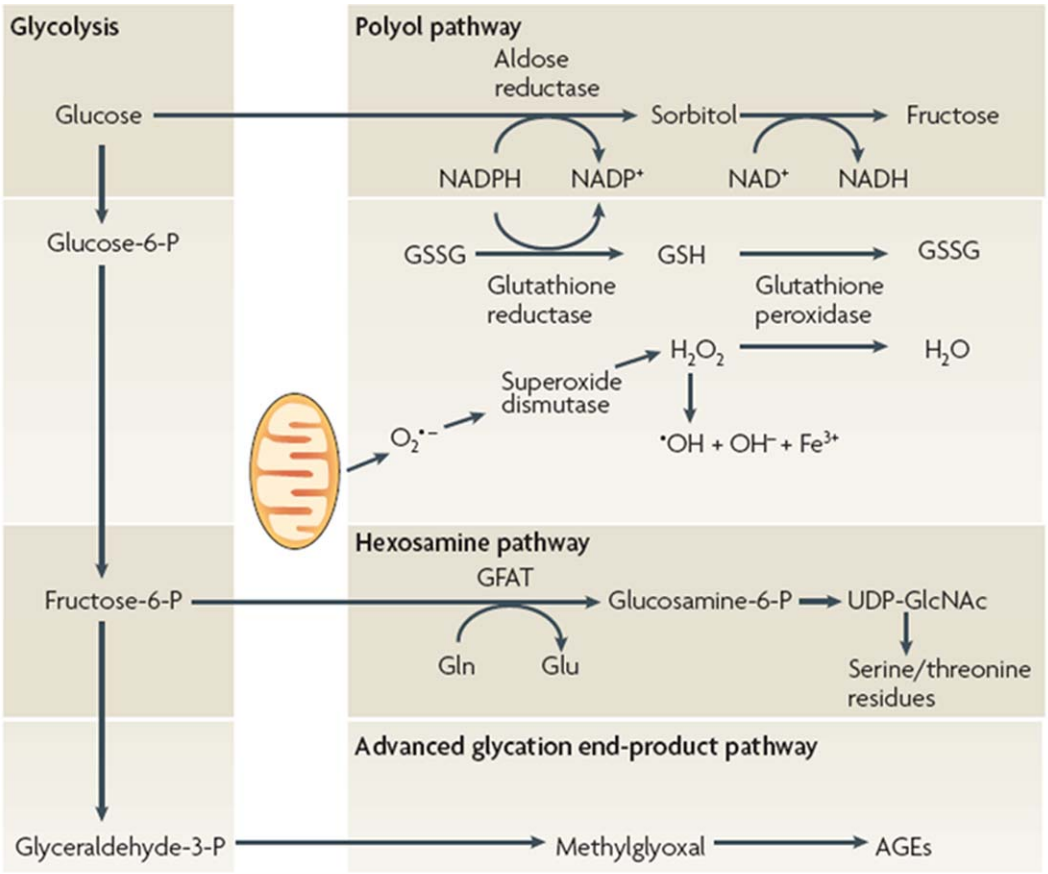


Figure 1.14: Scheme of the biochemical pathways activated by hyperglycemia. Under hyperglycemic conditions, the enzyme hexokinase saturates and glucose is derived into the polyol pathway. Hyperglycemia also induces the activation of the hexosamine pathway, the formation of advanced glycation end-products and increases the production of reactive oxygen species (ROS) (Adapted from Tomlinson and Gardiner, 2008).

Polyol pathway

In the polyol pathway, glucose is reduced to sorbitol and fructose by the enzyme aldose reductase (AR), using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (Greene et al., 1987). Under hyperglycemia, substantial amounts of sorbitol are formed and accumulate in the cell, being toxic 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). In the hippocampus of diabetic animals, the activity of AR is increased (Aragno et al., 2005), leading to higher sorbitol levels (Malone et al., 2008) and evidencing that the BBB does not protect the brain from

elevations in blood glucose levels. The increase in the activity of the polyol pathway in peripheral nerves has been associated with a reduction of intracellular inositol, a major component of phospholipids, and is required for the proper function of several neurotransmitters (Gillon et al., 1983). The inhibition of AR protects against brain and retinal damage in diabetic animals (Yeung et al., 2010).

Advanced glycation end-products

Non-enzymatic glycation results from the interaction of aldoses, such as glucose, with free amino groups of polypeptides or lipids. The formation of early glycation products, such as Schiff bases and Amadori products, as is the case of the well-known hemoglobin A1c (HA1c), is reversible. Along the time, these intermediates are transformed into irreversibly bound and chemically reactive adducts, the AGEs. The formation of AGEs occurs especially in the presence of high glucose concentrations. AGEs have the ability to establish a covalent crosslink between proteins altering their structure and function, which may lead to cellular damage (Figure 1.15; Tomlinson and Gardiner, 2008).

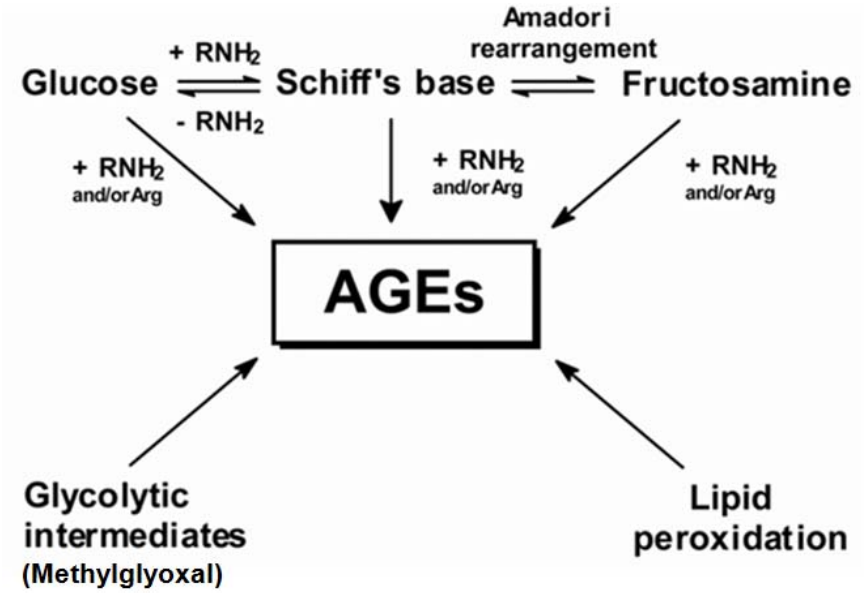


Figure 1.15: Diagram showing the processes and intermediates involved in the formation of advanced glycation end-products (Adapted and modified from Rabbani and Thornalley, 2008).

AGEs induce cellular responses through the activation of the AGEs receptor (RAGE), which in turn triggers intracellular signals and destabilizes biological processes. AGEs generation also increases inflammation and enhances oxidative stress in vessels. Since this pathway can destabilize several biological processes, it has been suggested to have an important role in the pathogenesis of diabetic complications and in neurodegenerative disorders (Yamagishi and Imaizumi, 2005; Wang et al., 2009). In fact, high levels of AGEs have been detected in brain and spinal cord of diabetic animals, being responsible for the glycosylation of some cytoskeletal proteins, such as tubulin and actin (Williams et al., 1982; Pekiner et al., 1993). These proteins have a central role in the maintenance of axonal structure and function, and their modification by glycation may alter the structural and functional properties of the axon, slowing the axonal transport, and contributing to axonal atrophy and degeneration (Mayer et al., 1984).

Protein kinase C activation

The PKC family is a large group of structurally related serine/threonine kinases that includes at least twelve isoforms, classified into three subgroups: classical PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC). The classification depends on the structure of their catalytic and regulatory domains and on co-factor requirements for its activation (Table 1.1) (Sun and Alkon, 2009; Jaken, 1996).

Table 1.1: PKC isoforms.

Conventional (cPKC)	Novel (nPKC)	Atypical (aPKC)
α	δ	ξ
β1	ε	ζ
β2	η	λ
γ	θ	
	μ	
Calcium-dependent; Phospholipid-dependent	Calcium-independent Phospholipid-dependent	Calcium-independent; Phospholipid-independent

PKCs are ubiquitously expressed in CNS and are involved in synaptic plasticity. The activation of PKCs enhances action potentials, increasing neurotransmitter release, and modulates glutamatergic and GABAergic systems. Phorbol esters, which are activators of cPKCs, increase the size of the readily releasable transmitter pool and the rate at which the pool refills at glutamatergic hippocampal synapses (Stevens and Sullivan, 1998).

Hyperglycemia can lead to an increase in glycolysis, resulting in increased *de novo* synthesis of diacylglycerol (DAG; Figure 1.13), that is the main activator of PKC. The protein levels and activity of cPKCs increase in diabetic animals and these findings correlate with increased levels of serotonin in hypothalamus, hippocampus, striatum and mid brain (Ramakrishnan et al., 2004; 2009). Moreover, the activation of PKC results in an increase in synaptic transmission in diabetic rats although to a much lesser extent than in control rats, and these diabetic animals present a reduction in LTP (Kamal et al., 2003). These evidences indicate that PKC underlies at least some of the neuronal alterations induced by diabetes.

Hexosamine pathway activation

Under normal conditions, glucose enters in the glycolytic pathway and is converted to glucose-6-phosphate and then to fructose-6-phosphate. Only a small amount of the intracellular glucose is metabolized in the hexosamine pathway (around 2% - 5% of the total glucose). In this pathway, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT) (Dias and Hart, 2007). N-acetylglucosamine-6-phosphate is metabolized to uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc) and other hexosamines that are important substrates for the O-linked glycosylation involved in the synthesis of glycoproteins, proteoglycans and glycolipids.

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 (Du et al., 2000; Dias and Hart, 2007).

Oxidative stress

Increased oxidative stress might result, not only from the increased generation of free radicals, but also from an impairment of the antioxidant defense system, responsible for scavenging free radicals and maintaining redox homeostasis. In hyperglycemic conditions, overproduction of reactive oxygen species (ROS) may occur through the increase in AGEs formation, the increase flux in the polyol pathway and the increase in O^{2-} production due to increased oxidative metabolism of glucose in mitochondria (Giugliano et al., 1996). On the other way, ROS can increase the formation of AGEs, the activation of AR, as well as DAG production with a consequent enhancement of PKC activity (Nishikawa et al., 2000).

In the brain of diabetic animals there is an increase in oxidative and nitrosative stress (Kuhad et al., 2009; Wang et al., 2010), which can contribute to neuronal damage and cognitive and behavioral deficits (Fukui et al., 2001). Increased concentrations of lipid peroxidation products, which are indicative of oxidative damage, were found in the cerebral microvasculature (Mooradian, 1995) and brain tissue (Kumar and Menon, 1993) of diabetic animals. Together with the increase in ROS production, there is a decrease in the activity of antioxidant enzymes, namely superoxide dismutase and catalase, in the brain of diabetic rats (Kumar and Menon, 1993). Moreover, treatment with antioxidants prevents or ameliorates neuronal changes in the brain of diabetic animals (Tuzcu and Baydas, 2006; Comin et al., 2010; Wang et al., 2010).

1.5.1.2 Insulin

Insulin is a small protein synthesized by pancreatic β-cells that regulates the uptake of glucose in cells. Insulin is actively transported across the BBB, but it can also be produced locally in the brain (Clarke et al., 1986). The expression of insulin receptors throughout the brain (Marks et al., 1990) suggests that insulin has an important role in the physiology of the CNS. In particular, the expression of insulin receptors in hippocampus (Dore et al., 1997) has driven the hypothesis that insulin is an important contributor or regulator of certain behaviours, and learning and memory (Zhao and Alkon, 2001). This hypothesis was also supported by clinical evidence. Chronic intranasal insulin administration improves cognitive performance in both AD (Reger et al., 2008) and non-demented individuals (Benedict et al., 2007), and acute insulin administration improves

declarative memory in AD patients (Craft et al., 1996). Cognitive performance is also improved in animal models of insulin resistance after insulin treatment (Francis et al., 2008).

In diabetic animals, the expression of insulin receptor is decreased in hippocampus (Dou et al., 2005), which suggests that insulin signaling can be impaired. Insulin treatment prevents morphological changes in hippocampal CA3 subregion induced by diabetes (Magarinos et al., 2001), preventing also the decrease in glial fibrillary acidic protein (GFAP) levels and the increase in glutamate uptake (Coleman et al., 2010). Insulin or pro-insulin C-peptide can also prevent apoptotic neuronal cell death (Sima and Li, 2005; Jafari Anarkooli et al., 2009), the upregulation of RAGE and nuclear factor-κB (NF-κB) and the release of pro-inflammatory factors in the hippocampus of diabetic animals (Sima et al., 2009). Moreover, replacement of insulin in type 1 diabetic rats prevents cognitive deficits and protects against hippocampal damage (Biessels et al., 1998). Altogether, these facts suggest that the impairment in insulin signaling may be an important factor for the development of cognitive impairments under diabetes.

1.5.1.3 Hypothalamic-pituitary-adrenal axis dysfunction

HPA axis is a complex set of homeostatic interactions between hypothalamus, pituitary gland and adrenal glands (Figure 1.16). The HPA axis regulates the responses to stress and modulates various body processes such as metabolism, growth, immune response and mood. In the paraventricular nucleus (PVN) of the hypothalamus, neuroendocrine neurons (called parvocellular neurons) synthesize and secrete vasopressin (AVP) and corticotropin-releasing hormone (CRH). These two peptides can act in synergy on the pituitary gland to stimulate the secretion of the adrenocorticotropic hormone (ACTH) from corticotrope cells. In turn, ACTH enters peripheral circulation where it reaches the adrenal cortex and induces the production of glucocorticoid hormones (cortisol in humans, corticosterone in rats and mice). Glucocorticoids exert a negative feedback on the PVN and pituitary to suppress CRH and ACTH production, respectively (Mormede et al., 2007; Bose et al., 2009).

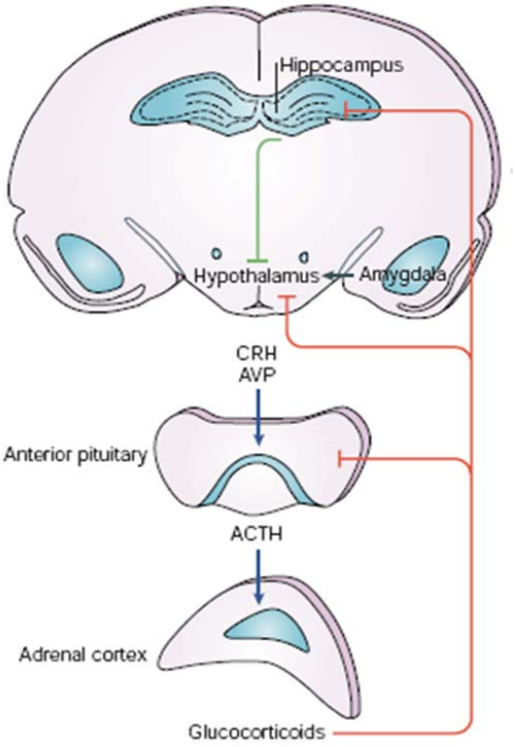


Figure 1.16: Diagram of hypothalamic-pituitary-adrenocortical (HPA) axis. The activation of the HPA axis results in the production of corticotropin-releasing hormone (CRH) and vasopressin (AVP) in the paraventricular nucleus of the hypothalamus. These peptides are released into the bloodstream and then they stimulate the production and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH stimulates the synthesis and release of glucocorticoids from the adrenal cortex (Adapted and modified from Sandi, 2004).

Corticosterone is a major stress hormone that has deleterious effects on several organs in the body, including the brain, where it acts via two types of receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Both receptors are widely expressed throughout the brain by different cells types.

The dysregulation of the HPA axis is associated with many pathological conditions, including the complications associated with diabetes (Reagan et al., 2008). A high expression of hypothalamic hormones, AVP and oxytocin (OT), was detected in the paraventricular nucleus of diabetic animals (Dheen et al., 1994; Saravia et al., 2001). Diabetic patients with poor controlled glycemic status had elevated plasma cortisol levels (Couch, 1992), and adrenal glucocorticoids are elevated in diabetic rodents (Magarinos and McEwen, 2000). The exposure to corticosteroids induces an increase in the expression and

a redistribution of synaptophysin in the hippocampus (Grillo et al., 2005). Corticosteroids also induce the depletion and clustering of synaptic vesicles in hippocampal mossy fiber terminals, and similar observations were found in diabetic animals (Magarinos and McEwen, 2000). Moreover, high levels of glucocorticoids contribute to the impairment in synaptic plasticity and neurogenesis in the hippocampus, being associated with learning and memory deficits in rodent models of diabetes (Stranahan et al., 2008a). Lowering the corticosterone levels attenuates the impairment in hippocampus-dependent learning (Stranahan et al., 2008b). Therefore, under diabetic conditions, the increase in HPA hormones might reflect a dysregulation of this system, influencing hippocampal structure and function, and consequently leading to cognitive impairments (Beauquis et al., 2008).

1.5.2 Molecular, biochemical and cellular alterations in the hippocampus in diabetes

Diabetic encephalopathy is a late complication of diabetes that is characterized by cognitive deficits and memory impairment. Although several brain areas contribute to the cognitive deficits associated with diabetes, hippocampal-dependent tasks seem to be particularly sensitive to this disease. The multifactorial pathogenesis of diabetic encephalopathy is not completely understood, but clearly shares features with brain ageing and with Alzheimer's disease, such as alterations at neurochemical, electrophysiological, structural and neurobehavioural level (Biessels et al., 1994).

Structural changes

The cognitive impairments in diabetic encephalopathy have been associated with structural alterations (Hernandez-Fonseca et al., 2009) and brain atrophy (Ferguson et al., 2005; Manschot et al., 2006). Swelling of neurons, glia, oligodendrocytes, synaptic boutons and mitochondria occur in several brain regions in diabetic animals (Hernandez-Fonseca et al., 2009). Diabetes also reduces the density and length of neuronal dendrites (Martínez-Tellez et al., 2005; Malone et al., 2008) and induces remodeling of dendrites, which could be associated with the shrinkage of hippocampus (McEwen et al., 2002). Presynaptic changes have also been reported in the hippocampus of diabetic animals, such as depletion and dispersion of synaptic vesicles (Magarinos and McEwen, 2000) and changes

in the content of presynaptic proteins (Grillo et al., 2005; Duarte et al., 2009). Alterations in axonal neurofilaments, including fragmentation of neurofilaments, were also observed in diabetic animals (Hernandez-Fonseca et al., 2009). Moreover, neurofilaments can undergo an addition of N-acetylglucosamine (GLcNAcylation) or phosphorylation (Dias and Hart, 2007; Sugimoto et al., 2008), which can impair axonal transportation and neuronal function. Tau is a microtubule-associated protein that regulates neurotransmission, and excessive tau phosphorylation disrupts microtubule binding and stability leading to changes in trafficking (Billingsley and Kincaid, 1997). In diabetic animals, tau phosphorylation increases (Kim et al., 2009; Zhang et al., 2010), which can lead to synaptic dysfunction and cognitive impairments.

Changes in synaptic transmission

Changes in synaptic strength are thought to provide a cellular basis for information storage in the brain. Impairments in hippocampal synaptic plasticity and cognition have been demonstrated in a number of rodent models of diabetes. Impairment in LTP and enhancement of LTD, which are correlated with impairments in spatial memory tasks, have been shown in diabetic animals (Biessels et al., 1996; 1998; Kamal et al., 1999; Stranahan et al., 2008a). Despite high frequency stimulation, LTP does not occur in more than 50% of diabetic animals. Moreover, when LTP is induced in diabetic animals, the magnitude of the EPSP slope is slower and is not sustained comparing to control animals (Biessels et al., 1998).

The impairments in synaptic plasticity in diabetic animals have also been associated with increased levels of adrenal glucocorticoids (Stranahan et al., 2008a) and with the lack of insulin (Biessels et al., 1998). Maintaining physiological corticosterone levels and replacing insulin in diabetic rats prevents plasticity changes (Biessels et al., 1998; Stranahan et al., 2008a).

Changes in synaptic transmission can occur due to alterations at both pre and/ or postsynaptic sites (Purves, 2004). Diabetes affects the synthesis and release of several neurotransmitters (Guyot et al., 2001; Morris and Pavia, 2004; Miyata et al., 2007; Satoh and Takahashi, 2008). In hippocampus, diabetes decreases the basal levels of serotonin and dopamine (Yamato et al., 2004) and the basal levels of glutamate in DG. The basal

levels of GABA are not affected (Reisi et al., 2009). Furthermore, diabetes decreases the content of proteins involved in exocytosis (Duarte et al., 2009) and decreases the number of synaptic vesicles in nerve terminals (Magarinos and McEwen, 2000).

NMDA receptors are located postsynaptically and regulate LTP induction. In the hippocampus of diabetic animals, there is a decrease in NMDA receptors that contain the NR2B subunit, causing a decrease in EPSPs (Di Luca et al., 1999; Gardoni et al., 2002). Besides the reduction of the NR2B subunit, its phosphorylation by CaMKII also decreases in postsynaptic densities (Di Luca et al., 1999). The affinity of glutamate for AMPA receptors, but not for NMDA receptors, is decreased in the hippocampus of diabetic rats (Chabot et al., 1997; Gagne et al., 1997), which can be due to a decrease in GluR1 subunits. AMPA receptors devoid of GluR1 subunit are not competent to induce LTP in CA1 subregion (Vanderklish et al., 1992), which might contribute to deficits in synaptic plasticity. Also in the postsynaptic side, the increase in postsynaptic density-95 (PSD-95) protein expression and changes in its distribution may contribute to changes in the content and functional properties of glutamate receptors in the hippocampus of diabetic rats (Grillo et al., 2005).

ATP signaling is also compromised in the hippocampus of diabetic rats. There is a decrease in ATP release and a down-regulation of synaptic P2 receptors (Duarte et al., 2007). Extracellular ATP plays a role in synaptic efficiency processes through the activation of P2 receptors (Cunha, 2008), through combined effects of different P2 receptors acting both in the presynaptic cell to control the release of glutamate (Rodrigues et al., 2005) and in the postsynaptic cell facilitating the activation of ionotropic glutamate receptors (Ortinau et al., 2003; Kloda et al., 2004).

Altogether, these findings indicate that diabetes elicits alterations in synaptic neurotransmission, at pre- and postsynaptic levels, and in different neurotransmitters, which may lead to changes in synaptic plasticity.

Calcium dysregulation

Mechanisms that regulate intracellular Ca^{2+} concentration and signaling pathways activated by Ca^{2+} play a critical role in brain aging. Increasing evidence reveals that

diabetes impairs Ca^{2+} homeostasis, increasing its intracellular concentration in several cell types, including neurons (Levy et al., 1994; 1999; Biessels et al., 2002).

Alterations in Ca^{2+}-dependent forms of synaptic plasticity in the hippocampus of diabetic animals have been associated with learning impairments. Slow afterhyperpolarization, which is a Ca^{2+}-dependent feature of synaptic transmission occurring following a sequence of action potentials, is increased in the hippocampus of diabetic rats (Biessels et al., 2002).

It has also been shown that the Ca^{2+} currents (Hall et al., 2001; Voitenko et al., 2000) and the expression of voltage-sensitive Ca^{2+} channels (Yusaf et al., 2001) are increased in dorsal root ganglion neurons from diabetic rats. In retinal neurons exposed to high glucose the increase in the intracellular Ca^{2+} concentration evoked by cell depolarization in enhanced, and the recovery to basal Ca^{2+} levels takes more time than in control retinal neurons (Santiago et al., 2006). The dysregulation of Ca^{2+} homeostasis certainly contribute to alterations in neurotransmitter release in diabetic animals.

Neuronal apoptosis

Apoptosis is implicated in neurodegenerative disorders like AD and Parkinson's disease, in brain aging (Stefanis et al., 1997), and in diabetes complications such as diabetic retinopathy (Barber et al., 1998). It is therefore also possible that apoptosis might contribute to changes induced by diabetes in the hippocampus. Indeed, in the last decade some reports have demonstrated that apoptosis occurs in the hippocampus of diabetic animals (Li et al., 2002; Li et al., 2005; Sima and Li, 2005; Jafari Anarkooli et al., 2008). In type 1 diabetic BioBreeding/Worcester (BB/Wor) rats occurs apoptosis in CA1 and CA2 hippocampal neurons, which is accompanied with impairments in Morris water maze performances (Li et al., 2002; Li et al., 2005; Sima and Li, 2005). Apoptosis was also detected in a type 2 diabetes animal model, BioBreeding Zucker diabetic rat/Worcester (BBZDR/Wor), in a lesser extent than in the type 1 model, and being more severe in CA1 pyramidal cells (Li et al., 2005). Treatments with the insulin mimetic C-peptide protects against hippocampal neuronal apoptosis, suggesting that cell death is in part caused by impaired insulin/C-peptide action (Li et al., 2005; Sima and Li, 2005). An increase in caspase-3 activity and Bax expression, and a decrease in the expression of Bcl-2 and Bcl-

xL in the hippocampus of diabetic animals were also reported. Bax/Bcl-2 and Bax/Bcl-xL ratios, used as index of apoptotic cell death, were significantly increased (Jafari Anarkooli et al., 2008).

In vitro studies have shown that high glucose induces caspase-dependent apoptosis in PC12 cells (Sharifi et al., 2009). However, in retinal cell cultures, cell death induced by exposure to elevated glucose occurs by a caspase-independent apoptotic pathway, involving the translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus (Santiago et al., 2007).

Inflammation

The levels of pro-inflammatory cytokines are increased in the brain of patients and in animal models of neuropathological disorders associated with impairments in cognitive function, such as in AD and depression (Minghetti et al., 2005; Leonard, 2007). Pro-inflammatory cytokines induce the expression of cytokines, chemokines and adhesion molecules, the activation and recruitment of immune cells to the brain parenchyma, and the activation of glial cells (Rothwell and Luheshi, 2000). Chronic inflammation (Somfai et al., 2006) plays important roles in neuronal damage in diabetic patients and animals (Arvanitakis et al., 2004; Kuhad and Chopra, 2008). In the hippocampus of diabetic animals, the expression of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-2 and IL-6 are increased (Sima et al., 2009). The increase in the expression of pro-inflammatory mediators in diabetic animals is associated with the increase in NO levels and the upregulation of inducible nitric oxide synthase (iNOS), RAGE and NF-kB (Kuhad et al., 2009).

The increase in the expression of pro-inflammatory cytokines under diabetic conditions indicates that the innate immune system is activated (Kuhad et al., 2009; Sima et al., 2009). Human *post-mortem* studies demonstrated that the activation of microglia in the hippocampus is similar to what is observed in Alzheimer's brains (Valente et al., 2010). Moreover, inflammation, and memory and learning deficits are prevented when animals are treated with anti-inflammatory (Kuhad and Chopra, 2008) and antioxidant (Kuhad et al., 2009) compounds, and also when animals receive the insulin mimetic C-peptide (Sima et

al., 2009). Altogether, these evidences indicate that pro-inflammatory cytokines can have an important role in the development of cognitive deficits associated with diabetes.

1.5 Objectives

Growing 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. Several studies clearly indicate that synaptic transmission is impaired in the hippocampus under diabetic conditions, leading to memory and learning deficits, and it appears that these impairments can occur at both pre- and postsynaptic level. Recent findings 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 hippocampus induced by diabetes, giving a particular attention to changes occurring in nerve terminals.

Since the impairment of exocytosis might affect neurotransmission, contributing to alterations in hippocampal physiology detected under diabetes, and recent evidences point to alterations in some exocytotic proteins, in Chapter 2 we investigated the effect of diabetes (type 1 animal model, streptozotocin (STZ)-induced diabetes) on the content of several exocytotic proteins, at different time points, during the early stages of the disease. Given that exocytosis depends on specialized key 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.

Hyperglycemia is considered the main trigger of diabetes complications. Hyperglycemia triggers various processes that induce cell dysfunction and eventually cell death, leading to slowly progressive functional and structural abnormalities in the brain. In Chapter 3, we evaluated whether long-term elevated glucose *per se* (in order to mimic hyperglycemia) changes the content and localization of synaptic proteins involved in exocytosis, and of the vesicular transporters for glutamate and GABA, in hippocampal neurons.

The release of neurotransmitters is a highly regulated process. Changes detected in the exocytotic machinery may affect, at least partially, synaptic transmission, thus contributing to the development of cognitive impairments. A growing body of evidence indicates that diabetes affects neurotransmitter release in different brain regions. Despite all studies showing that neurotransmitter release is impaired in diabetes, few studies were performed in hippocampus. In Chapter 4, we evaluated the effect of diabetes on basal and evoked glutamate and GABA release in hippocampal nerve terminals. The protein content of vesicular glutamate and GABA transporters was also analyzed.

Diabetic encephalopathy shares many features with brain aging and functional disorders of the CNS. However, the pathogenesis of diabetic encephalopathy is not completely clarified yet. In Chapter 5, we aimed to elucidate the effect of diabetes, at different time points, on rat hippocampal neuronal and glial cells. Neurodegeneration and the protein levels and immunoreactivity of several neuronal markers, microtubule associated protein-2 (MAP-2), synaptophysin, Tuj-1, tau and calbindin D28k, were evaluate. Furthermore, we also analyzed the potential reactivity of astrocytes and microglial cells.

CHAPTER 2

Diabetes affects the content of exocytotic proteins in hippocampal nerve terminals

Joana M. Gaspar; Filipa I. Baptista; Joana Galvão; Áurea F. Castilho; Rodrigo A. Cunha; António F. Ambrósio

This chapter is part of an original research manuscript, published in Neuroscience, 2010 Sep 15; 169(4): 1589-1600. The manuscript is a comparative study related with the effect of diabetes on exocytotic proteins in hippocampus and retina. The results obtained in retina are part of the PhD thesis of Filipa I. Baptista.

Abstract

Diabetes has been associated with cognitive and memory impairments, suggesting that hippocampus is affected by this disease. A few studies have shown that diabetes differentially affects neurotransmitter release in different brain regions and induces molecular and structural changes in nerve terminals in hippocampus. We now detailed the impact over time of diabetes (two, four and eight weeks of diabetes) on a large array of exocytotic proteins in hippocampus. The exocytotic proteins density was evaluated by immunoblotting in purified synaptosomes and in total extracts of hippocampus from streptozotocin-induced diabetic and age-matched control animals.

Diabetes affected differentially the content of several synaptic proteins (VAMP-2, SNAP-25, syntaxin-1, synapsin-1 and synaptophysin) in hippocampal nerve terminals. Changes were pronounced and persistent. The content of synaptotagmin-1 and rabphilin 3a in hippocampal nerve terminals was not affected. In total extracts, SNAP-25 and syntaxin-1 content decreased, particularly when more drastic changes were also detected in nerve terminals.

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

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

2.1. Introduction

Diabetes *mellitus* is the most common metabolic disorder in humans, and it has been associated with several complications, such as diabetic encephalopathy (ECDCDM, 2003). Diabetes-induced cognitive impairments have been associated with changes in the integrity and function of hippocampus (Trudeau et al., 2004; Stranahan et al., 2008). Diabetes impairs long-term potentiation (LTP) and facilitates the induction of long-term depression (LTD) (Kamal et al., 1999; Artola et al., 2005), 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; Morris and Pavia, 2004; Yamato et al., 2004; Miyata et al., 2007; Misumi et al., 2008; Satoh and Takahashi, 2008).

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 (Vanguilder et al., 2008; Duarte et al., 2009). Elevated glucose and diabetes also downregulate the expression of several genes coding for exocytotic proteins in pancreatic β-cells (Zhang et al., 2002; Abderrahmani et al., 2006b;Ostenson et al., 2006), whereas in pancreatic α-cells high glucose increases the expression of exocytotic proteins (McGirr et al., 2005).

Since the impairment of exocytosis might affect neurotransmission, contributing to alterations in hippocampal 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 in hippocampal nerve terminals and in total extracts, focusing on different time points during the early stages of the disease, analyzing a broader array of proteins associated with the vesicular release machinery.

2.2 Methods

Materials

Reagents were purchased 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 European Union (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 was confirmed two days later with a glucometer (Elite, Bayer, Portugal). Animals with blood glucose levels exceeding 250 mg/dL were considered diabetic. 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 hippocampal synaptosomal extracts

Percoll purified synaptosomes were isolated as previously described (Duarte et al., 2006), with minor changes. From each animal, one hippocampus (the other hippocampus was used for the preparation of total extracts) was dissected and homogenized in a sucrose-HEPES solution [0.32 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 3,000 x *g* for 10 min at 4ºC. The supernatant was collected and centrifuged at 14,000 x *g* for 12 min at 4ºC. The

resulting pellet was resuspended in 45% (v/v) Percoll solution prepared in Krebs–Henseleit Ringer (KHR) solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose, pH 7.4). After centrifugation at 16,100 x *g* for 2 min at 4ºC the top layer was removed (synaptosomal fraction), and then washed in 1 mL KHR solution and resuspended in lysis buffer [RIPA: 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland) and 1 mM dithiothreitol (DTT)]. The samples were stored at -80ºC until use.

Preparation of total hippocampal extracts

After dissection, one hippocampus from each rat was 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 done at 4ºC. The supernatant was stored at -80ºC 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 95ºC. 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 polyvinylidene difluoride (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 directed against the respective protein (listed in Table 2.1) overnight at 4ºC. 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 band 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 (1:20,000) to prove that similar amounts of protein were applied in the gels.

Table 2.1. List of primary antibodies.

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

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 $p<0.05$.

2.3. Results

2.3.1 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 2.2. 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.

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. **$p<0.01$, ***$p<0.001$.

2.3.2 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 (synaptosomal-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 hippocampal nerve terminals by Western blotting. In hippocampal nerve terminals obtained from diabetic animals, the protein levels of VAMP-2 were similar to those found in control animals at two, four and eight weeks after diabetes induction (Figure 2.1A). 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 hippocampal total extracts was also evaluated. In this case, no changes were found in the protein levels of VAMP-2 in hippocampal extracts from diabetic and age-matched control animals (Figure 2.1B).

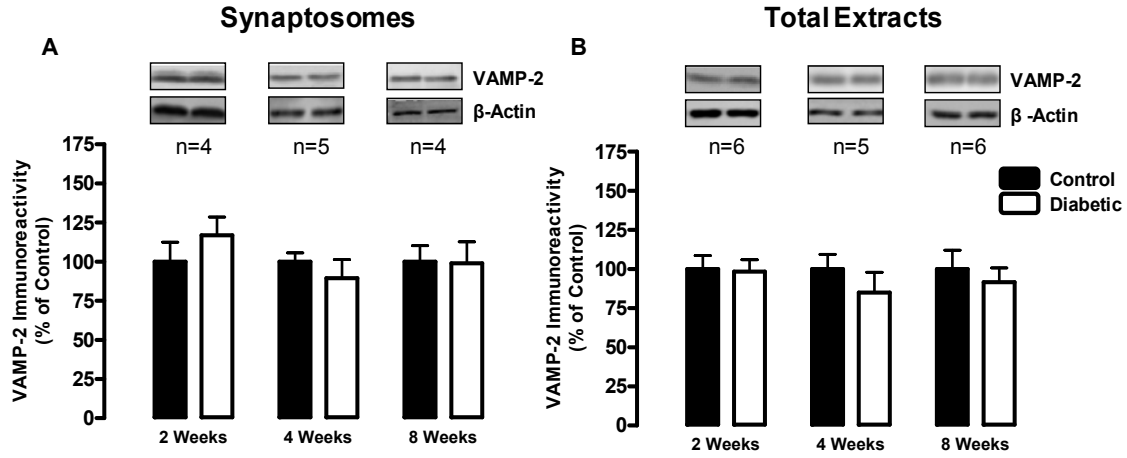


Figure 2.1: Diabetes does not induce changes in the protein content of VAMP-2. The protein levels of VAMP-2 were analyzed by immunoblotting in extracts of hippocampal nerve terminals (A), and also in total extracts (B), isolated from control and STZ-induced diabetic animals. Representative Western blots 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.

Regarding SNAP-25, in hippocampal synaptosomes, after two weeks of diabetes, its protein content was not significantly different from that found in synaptosomes from age-

matched control animals. Nevertheless, after four and eight weeks of diabetes the SNAP-25 protein levels significantly decreased to 50.5 ± 15.2% and 75.1 ± 5.9% of the control, respectively (Figure 2.2A). In total hippocampal extracts, no changes were detected after two and eight weeks of diabetes compared to age-matched controls, but four weeks after the onset of diabetes a significant decrease in the content of SNAP-25 was detected (reduction to 71.2 ± 5.5% of the control; Figure 2.2B).

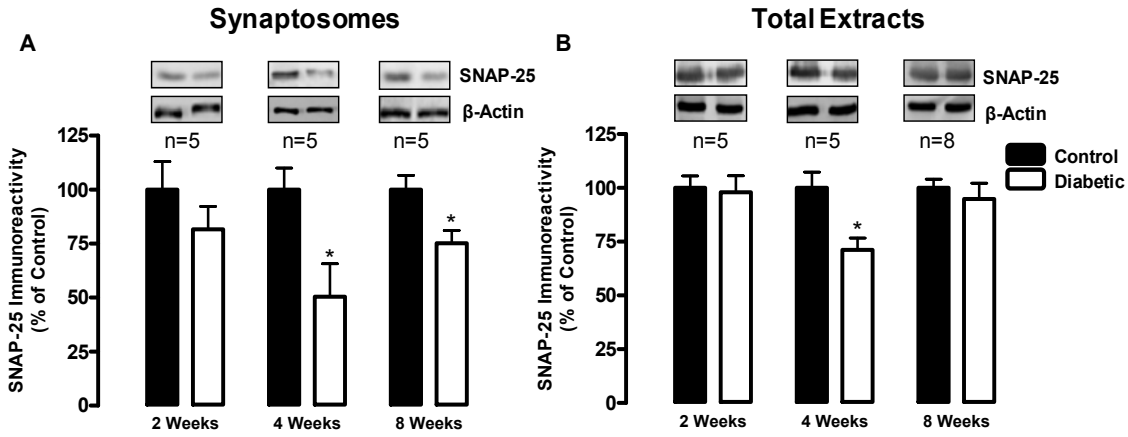


Figure 2.2: Diabetes induces changes in the protein content of SNAP-25 in the hippocampus. The protein levels of SNAP-25 were analyzed by Western blotting in extracts of synaptosomes (A) and also in total extracts (B) isolated from the hippocampus of diabetic and age-matched control animals. Representative 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. *$p < 0.05$ compared to age-matched control using Student's *t* test.

The content of syntaxin-1 significantly decreased in hippocampal nerve terminals isolated from diabetic animals at all time points studied (reduction to 52.7 ± 10.5%, 30.6 ± 12.9%, and 60.0 ± 11.7% of the control after two, four and eight weeks, respectively; Figure 2.3A). In total hippocampal extracts, the protein levels of syntaxin-1 were similar to control after two weeks of diabetes, but decreased significantly after four and eight weeks of diabetes comparing to age-matched controls (reduction to 48.5 ± 11.1% and 86.5 ± 3.8% of the control, respectively; Figure 2.3B).

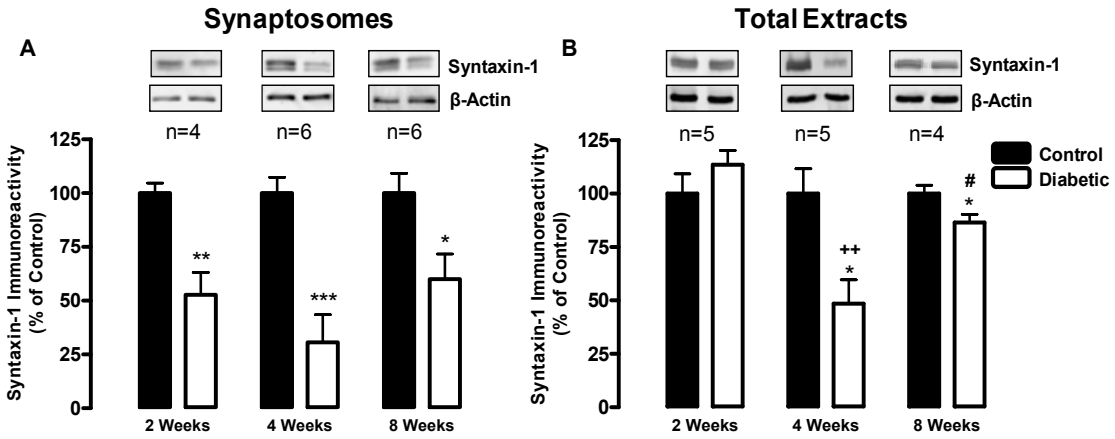


Figure 2.3: Diabetes induces changes in the protein content of syntaxin-1 in the hippocampus. The protein levels of syntaxin-1 were analyzed by immunoblotting in extracts of purified nerve terminals (A), and also in total extract (B), obtained from hippocampus of diabetic and age-matched control animals. 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. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to age-matched control using Student's *t* test; $^{++}p < 0.01$, compared to two weeks of diabetes; $^{\#}p < 0.05$, compared to four weeks of diabetes using a Kruskal-Wallis analysis of variance.

2.3.3 Diabetes decreases the content of synapsin-1 in hippocampal nerve terminals

Synapsins are abundant synaptic vesicle proteins that anchor synaptic vesicles to the cytoskeleton (Ceccaldi et al., 1995). In hippocampal nerve terminals, after two weeks of diabetes, no differences were found in synapsin-1 content between diabetic and control animals, but after four and eight weeks of diabetes there was a significant decrease in synapsin-1 levels (reduction to 65.8 ± 9.2% and 61.8 ± 12.2%, comparing to age-matched controls, respectively; Figure 2.4A). Moreover, significant differences were found between both four and eight weeks of diabetes and the two weeks time point (Figure 2.4A). In total extracts from hippocampus, no significant differences were detected in synapsin-1 content between diabetic and age-matched control animals (Figure 2.4B).

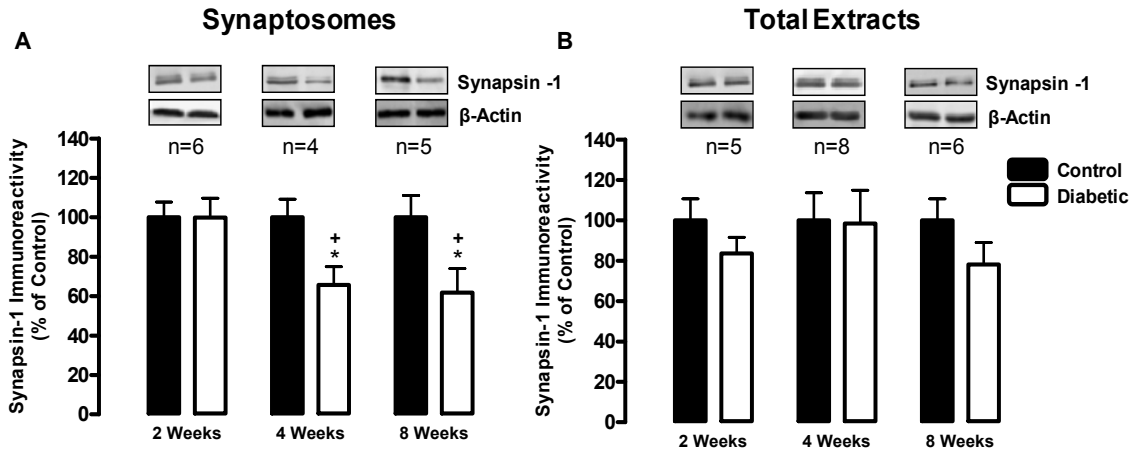


Figure 2.4: Diabetes induces changes in the protein content of synapsin-1 in hippocampal nerve terminals. The protein levels of synapsin-1 were analyzed by immunoblotting in purified synaptosomes and total extracts obtained from the hippocampus (A and B, respectively) from either diabetic or age-matched control animals. Representative Western blots are presented above the 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. **p* < 0.05, compared to age-matched control using Student's *t* test. $^{+}$p < 0.05, compared to two weeks of diabetes using a Kruskal-Wallis analysis of variance**.**

2.3.4 The content of synaptophysin is altered by diabetes in hippocampal nerve terminals

Synaptophysin is a synaptic vesicle protein that is ubiquitously expressed in neurons, being a widely used marker for nerve terminals. In hippocampal nerve terminals, the protein levels of synaptophysin from diabetic animals significantly increased at all time points when compared to the respective controls (Figure 2.5A). In hippocampal extracts, no differences were detected between diabetic and age-matched control animals (Figure 2.5B).

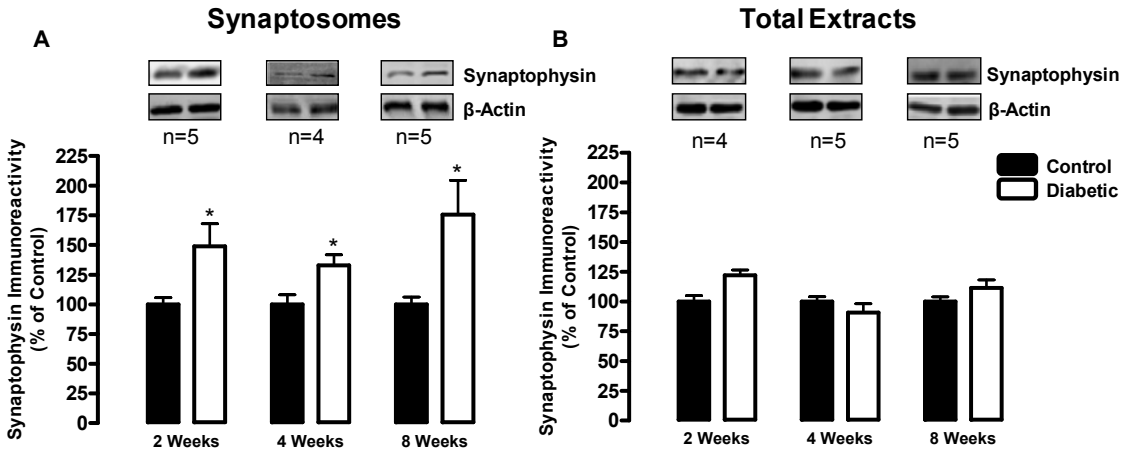


Figure 2.5: Diabetes induces changes in the protein content of synaptophysin in hippocampal nerve terminals. The protein levels of synaptophysin were analyzed by Western blotting in purified hippocampal synaptosomes (A) and total extracts from the hippocampus (B), from control or STZ-induced diabetic 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. *$p < 0.05$, compared to age-matched control animals using Student's *t* test.

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

Synaptotagmin-1 is localized in synaptic vesicles and acts as a Ca^{2+} sensor that regulates exocytosis (Chapman, 2008). The protein content of synaptotagmin-1 was not significantly altered by diabetes in hippocampal nerve terminals (Figure 2.6A). Similar results were obtained for total extracts (Figure 2.6B).

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 either hippocampal nerve terminals (Figure 2.7A) or in total extracts of hippocampus (Figure 2.7B).

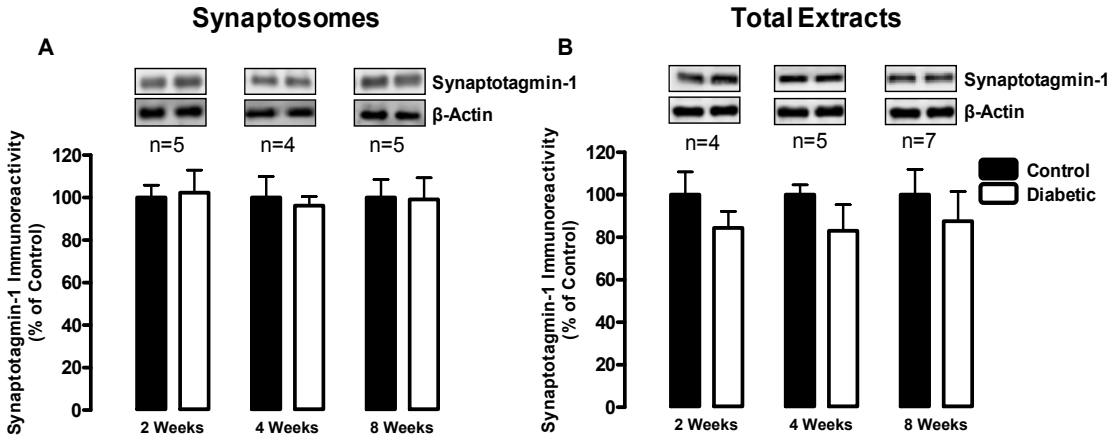


Figure 2.6: Diabetes does not induce changes in synaptotagmin-1 protein levels. The protein levels of synaptotagmin-1 were analyzed by immunoblotting in purified synaptosomes and total extracts from hippocampus (A and B, respectively). Representative Western blots are presented above the graphs, with 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.

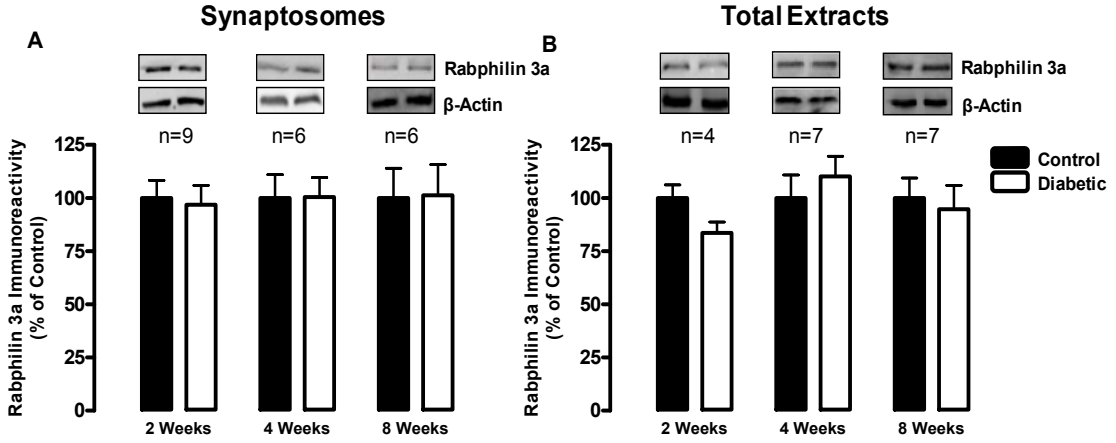


Figure 2.7: Rabphilin 3a protein levels are not affected by diabetes in hippocampus. The protein levels of Rabphilin 3a were analyzed by immunoblotting in purified hippocampal synaptosomes (A), and also in total extracts from the hippocampus (B), from either diabetic animals or aged-matched controls. 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.

2.4. Discussion

In this study, we demonstrate that diabetes alters the content of several synaptic proteins involved in exocytosis in hippocampal nerve terminals. Changes were pronounced and persistent along the time points of the study.

Memory and cognitive deficits (Biessels et al., 1996) induced by diabetes might be due, at least in part, to changes in neurotransmission, at both pre- and postsynaptic level. Diabetes affects neurotransmitter release in various brain regions. Basal or stimulated neurotransmitter release were found to be increased or decreased (Guyot et al., 2001; Morris and Pavia, 2004; Yamato et al., 2004; Misumi et al., 2008; Satoh and Takahashi, 2008). Moreover, diabetes and high glucose increase the release of [^{3}H]-D-aspartate in retina and retinal cell cultures (Santiago et al., 2006), and elevated glucose also increases ATP release in retinal cell cultures (Costa et al., 2009). These evidences strongly suggest that diabetes induces alterations at presynaptic level. A reduction in the density of synaptic proteins was found recently in hippocampal and retinal nerve terminals after four weeks of diabetes (Vanguilder et al., 2008; Duarte et al., 2009). 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 hippocampal nerve terminals. For that, we analyzed more proteins than previous studies and evaluated the profile of changes in hippocampus according to the duration of diabetes. In this work we also evaluated the effect of diabetes on exocytotic proteins on retinal nerve terminals, because one of the goals of this work was to compare the effects of diabetes on exocytotic proteins in hippocampus and retina. 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, SNAP-25 and syntaxin-1, was strongly affected, but VAMP-2 protein levels were not affected. Regarding SNAP-25 and syntaxin-1, a significant decrease was detected in hippocampal nerve terminals and the changes persisted along the time. Furthermore, a decrease in the content of SNAP-25 and syntaxin-1 was also detected in hippocampal total extracts, suggesting that changes 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 after four weeks of diabetes was reported.

Since SNAP-25 and syntaxin-1 constitute around 1% of the total brain protein (Jahn and Scheller, 2006), the substantial decrease in the content of these two proteins in hippocampus certainly impairs neurotransmitter release and might contribute to the development of diabetic encephalopathy. 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).

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 hippocampal nerve terminals of diabetic animals. Diabetes also 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).

In hippocampal nerve terminals, the content of synaptophysin increased at all time points. Grillo and colleagues (2005) also reported that diabetes increases synaptophysin content in CA1, CA3 and dentate gyrus subregions, as determined by autoradiography. However, by Western blot analysis, they did not detect changes in the whole hippocampus. These authors also suggested that changes in the density and distribution of synaptophysin caused by diabetes may take place together with depletion and clustering of synaptic vesicles in hippocampal mossy fiber terminals (Magarinos and McEwen, 2000), and also that the increase in the content of presynaptic synaptophysin might result from a compensatory mechanism of increased synaptic density, as a consequence of dendrite shortening caused by diabetes (Grillo et al., 2005; Martínez-Tellez et al., 2005). Contrary to our findings, it was recently reported a decrease in the content of synaptophysin in

hippocampal nerve terminals (Duarte et al., 2009). However, this might be related with the use of different rat strains, Wistar *versus* Sprague-Dawley.

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 Ca^{2+} sensors in nerve terminals. The content of synaptotagmin-1 and Rabphilin3a was not affected by diabetes in hippocampus. Synaptotagmin-1 has two domains that bind Ca^{2+} (Geppert et al., 1994). Synaptic vesicle fusion is also regulated by Rab3A, GTP-binding protein associated with synaptic vesicles that binds Rabphilin 3a, which has Ca^{2+} binding domains and the ability to bind the cytoskeleton, participating in synaptic vesicle trafficking (Sudhof, 1997).

The effects observed were not aggravated with increased duration of diabetes, at least for the time points studied. Moreover, no alterations were detected in the total protein content for the majority of the proteins analyzed, with the exception of SNAP-25 and syntaxin-1, 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.

As mentioned before, in this work, we also investigated the effect of diabetes on the content of exocytotic protein in the retina. In this case, the alterations occurred only in nerve terminals and mostly at two weeks of diabetes, with the exception of synapsin-1 that decreased at all time points of the study. Moreover, changes in retinal nerve terminals were transient, suggesting that retina is in some way able to recover from the initial stress condition induced by diabetes. Since the retina is known to be early affected by diabetes (Barber, 2003), and memory and cognitive deficits appear later in diabetic patients (Gispen and Biessels, 2000), we would expect more pronounced changes in the retina. Nevertheless, these results demonstrate that hippocampus and retina (Gaspar et al., 2010) are differentially affected by diabetes, being alterations more pronounced and persistent in the hippocampus.

In conclusion, the changes detected in hippocampal nerve terminals might contribute to alterations in neurotransmitter release and synaptic dysfunction and consequently to memory 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

The authors acknowledge to Bárbara Oleiros Paiva for the help in statistical analysis. This work was supported by, Faculty of Medicine, University of Coimbra, Portugal (project GAPI 17/08). Joana M. Gaspar acknowledges a fellowship from Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/32949/2006).

CHAPTER 3

Long-term exposure to high glucose induces changes in the content and distribution of some exocytotic proteins in cultured hippocampal neurons

Joana M. Gaspar; Áurea F. Castilho; Filipa I. Baptista; Joana Liberal;

António F. Ambrósio

Neuroscience. 2010 October 13. [Epub ahead of print]

Abstract

A few studies have reported the existence of depletion of synaptic vesicles, and changes in neurotransmitter release and in the content of exocytotic proteins in the hippocampus of diabetic rats. Recently, we found that diabetes alters the levels of synaptic proteins in hippocampal nerve terminals. Hyperglycemia is considered the main trigger of diabetic complications, although other factors, such as low insulin levels, also contribute to diabetes-induced changes. Thus, the aim of this work was to evaluate whether long-term elevated glucose *per se*, which mimics prolonged hyperglycemia, induces significant changes in the content and localization of synaptic proteins involved in exocytosis in hippocampal neurons. Hippocampal cell cultures were cultured for 14 days and were exposed to high glucose (50 mM) or mannitol (osmotic control; 25 mM plus 25 mM glucose), for seven days. Cell viability and nuclear morphology were evaluated by MTT and Hoechst assays, respectively. The protein levels of VAMP-2, SNAP-25, syntaxin-1, synapsin-1, synaptophysin, synaptotagmin-1, rabphilin 3a, and also of vesicular glutamate and GABA transporters (VGluT-1 and VGAT), were evaluated by immunoblotting, and its localization was analyzed by immunocytochemistry.

The majority of the proteins was not affected. However, elevated glucose decreased the content of SNAP-25 and increased the content of synaptotagmin-1 and VGluT-1. Moreover, there was an accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in the cell body of some hippocampal neurons exposed to high glucose. No changes were detected in mannitol-treated cells.

In conclusion, elevated glucose *per se* did not induce significant changes in the content of the majority of the synaptic proteins studied in hippocampal cultures, with the exception of SNAP-25, synaptotagmin-1 and VGluT-1. However, there was an accumulation of some proteins in cell bodies of hippocampal neurons exposed to elevated glucose, suggesting that the trafficking of these proteins to the synapse may be compromised. Moreover, these results also suggest that other factors, in addition to hyperglycemia, certainly contribute to alterations detected in synaptic proteins in diabetic animals.

Keywords: Diabetic Encephalopathy; Hippocampus; Hyperglycemia; Nerve Terminal; Exocytosis; Synaptic Proteins.

3.1 Introduction

Diabetes *mellitus* is the most common serious metabolic disorder in humans. It is generally accepted that diabetes often results in micro and macrovascular disease, but it can also cause complications in central nervous system (CNS), being diabetic encephalopathy one of the most common complications (Wrighten et al., 2009).

In hippocampus, diabetes causes synaptic vesicle depletion in mossy fiber nerve terminals (Magarinos and McEwen, 2000) and increases the content of the presynaptic marker synaptophysin (Grillo et al., 2005). Electrophysiological changes and impairments in synaptic plasticity (Biessels et al., 1996) and neurogenesis have also been reported in the hippocampus of diabetic animals (Stranahan et al., 2008). It has been suggested that these changes might result in cognitive deficits and increase the risk of depression and dementia (Biessels et al., 1996; Stranahan et al., 2008).

The release of neurotransmitters is a highly regulated process. If this process is affected by diabetes, synaptic transmission will therefore be affected, which might contribute to the development of cognitive impairments. In fact, several reports have demonstrated that diabetes affects neurotransmitter release in different brain regions, including the hippocampus (Guyot et al., 2001; Morris and Pavia, 2004; Yamato et al., 2004; Miyata et al., 2007; Misumi et al., 2008; Satoh and Takahashi, 2008). These alterations might result, at least partially, from changes in the exocytotic machinery. We and others recently found that diabetes changes the protein levels of several synaptic proteins involved in exocytosis in hippocampal and retinal nerve terminals (Vanguilder et al., 2008; Duarte et al., 2009; Gaspar et al., 2010). However, we cannot conclude that those changes were mainly caused by hyperglycemia, since other factors such as the low levels of insulin and the increased levels of corticosteroids could somehow contribute to those changes (Grillo et al., 2005; Li et al., 2005; Wrighten et al., 2009). *In vitro* studies have shown that high glucose downregulates the expression of several genes coding for exocytotic proteins in pancreatic β-cells (Abderrahmani et al., 2006a). Conversely, in pancreatic α-cells the content of SNARE proteins increases in response to high glucose (McGirr et al., 2005), suggesting that the effect of high glucose depends on the cell type affected.

Hyperglycemia has been considered the main pathogenic factor underlying the development of diabetic complications, although other factors (insulin and corticosteroids) have also been implicated. Hyperglycemia triggers various processes that ultimately induce cell dysfunction and eventually cell death, leading to slowly progressive functional and structural abnormalities in the brain (Ryan, 1988; Roriz-Filho et al., 2009). In diabetes, hyperglycemia increases (up to fourfold) neuronal glucose levels (Tomlinson and Gardiner, 2008). Particularly, in the hippocampus of diabetic animals, it was recently reported a significant increase in glucose concentration, as measured by nuclear magnetic resonance (NMR) spectroscopy (Duarte et al., 2009). Taking this into account, the aim of this work was to evaluate whether high glucose *per se*, which simulates hyperglycemic conditions, changes the content and localization of several exocytotic proteins in hippocampal neuronal cultures. Since exocytosis depends on specialized key proteins that control and regulate neurotransmitter release, we analyzed the protein levels of 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), rabphilin3a (vesicle-associated protein that is a target of rab3A small GTP-binding protein), and synaptotagmin-1 (Ca^{2+} sensor). Additionally, we also analyzed the vesicular transporters for glutamate and GABA (VGluT-1 and VGAT, respectively).

3.2 Methods

Primary cultures of rat hippocampal neurons

Pregnant female Wistar rats were handled according with the EU guidelines for the use of experimental animals (86/609/EEC). Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18–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 pyruvate, 10 HEPES and 0.001% phenol red, pH 7.4). The hippocampi were digested with trypsin (0.06%, 15 min, at 37ºC; 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 (FBS; 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). The cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37ºC for 14 days. When supplemented with B27, Neurobasal medium is intended to give optimal growth and long-term survival to rat embryonic hippocampal neurons. Optimal survival rate and neurite growth of hippocampal neurons require 25 mM basal glucose (Brewer et al., 1993), reflecting the fact that neurons have high metabolic rates. Neurobasal medium containing 25 mM glucose (control condition) meets these metabolic requirements. 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 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.

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. Cells were washed with Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.4 $CaCl_2$, 6 glucose, 10 HEPES; pH 7.4) and then MTT (0.5 mg/mL), solubilized in Krebs buffer, was added to the cultures and incubated for 1 h at 37ºC in the incubation chamber. After the incubation, the medium was removed and then the precipitated dye was dissolved in 0.04 M HCl in isopropanol and quantified colorimetrically (absorbance at 570 nm). All experiments were carried out in quadruplicate.

Assessment of apoptosis: nuclear morphology assay

Nuclear morphology was used to assess cells undergoing apoptosis (cells with condensed or fragmented nuclei). Cells were gently washed with warm phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4; pH 7.4) and fixed in 4% paraformaldehyde with 4% sucrose for 10 min at room temperature. Cells were then incubated with the fluorescent dye Hoechst 33342 (2 µg/mL) in PBS for 5 min. Cells were rinsed and mounted using fluorescent mounting medium (Dako, Glostrup, Denmark).

Fluorescence of stained chromatin was examined using an inverted fluorescence microscope (DM IRE2, Leica Microsystems, Cambridge, UK) and cells with condensed/fragmented chromatin were scored. For each preparation nine random fields were counted.

Cellular extracts from hippocampal cultures

Cells were washed twice in fresh cold PBS, and lysed in RIPA buffer [150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5 % sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS)], supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland), 1 mM dithiothreitol (DTT), 10 mM NaF, and 1 mM sodium orthovanadate. The resulting homogenate was centrifuged at 16,100 x g for 10 min to remove cell debris. This procedure was performed at 4ºC. The supernatant was stored at -80ºC 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), and equal amounts of protein were loaded into the gel. 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 95ºC. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 4%-8% or 4%-12% gels, and then 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 (listed in Table 3.1) overnight at 4ºC. 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 (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). 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.

Table 1: List of primary antibodies

Primary Antibody	Western blotting dilution	Immunocytochemistry dilution	Source
Rabbit anti-Tuj-1	----------	1:1,000	Covance
Rabbit anti-VAMP-2	1:2,000	1:100	Chemicon
Mouse anti-SNAP-25	1:1,000	1:100	Synaptic System
Mouse anti-Syntaxin-1	1:10,000	1:100	Synaptic Systems
Mouse anti-Synapsin-1	1:5,000	1:500	Synaptic Systems
Mouse anti-Synaptophysin	1:10,000	1:50	Chemicon
Rabbit anti-Rabphilin 3a	1:1,000	1:100	Synaptic Systems
Mouse anti-Synaptotagmin-1	1:6,000	1:200	Synaptic Systems
Rabbit anti-VGluT-1	1:5,000	1:500	Synaptic Systems
Rabbit anti-VGAT	1:1,000	1:100	Synaptic Systems

Immunocytochemistry

Cells were washed twice with warm PBS and fixed with 4% paraformaldehyde with 4% sucrose for 10 min at room temperature. Cells were rinsed twice in PBS and then permeabilized with 1% Triton X-100 in PBS* (PBS supplemented with 0.03% BSA plus 0.02% sodium azide) for 10 min. After blocking for 30 min with 10% goat serum in PBS*, cells were incubated with the primary antibody (Listed in Table 3.1) for 2 h at room temperature. Cells were rinsed three times with PBS* and incubated for 1 h at room temperature in the dark with Alexa fluor 594-conjugated secondary antibody (goat anti-mouse IgG, 1:250) or Alexa flour 488-conjugated secondary antibody (goat anti-rabbit IgG,

1:250). The preparations were visualized with a Zeiss confocal microscope (LSM 510, Germany).

Stastistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by analysis of variance (ANOVA), followed by Dunnett's post-hoc test. Differences were considered significant for $p<0.05$.

3.3 Results

3.3.1 Effect of long-term exposure to high glucose on morphology and viability of hippocampal neurons

Before analyzing the effects of high glucose on the content and distribution of synaptic proteins, we evaluated if high glucose (50 mM vs 25 mM in control cultures; seven days exposure to mimic chronic hyperglycemia) and mannitol (25 mM; plus 25 mM glucose), which was used as osmotic control, could affect the morphology and viability of cultured hippocampal neurons (14 days in culture). The morphology of hippocampal neurons was evaluated by immunocytochemistry using an antibody anti-Tuj-1 (neuron-specific class III beta-tubulin). No morphological changes were observed in cells exposed to high glucose or mannitol (Figure 3.1A).

The exposure of hippocampal neurons to 50 mM glucose (25 mM added glucose) for seven days did not affect cell viability (96.5 ± 9.5% of control), as assessed by the MTT assay (Figure 3.1B). A similar result was obtained for cells exposed to mannitol (Figure 3.1B). We also analyzed the nuclear morphology of hippocampal neurons using the Hoechst 33342 dye, which allows the assessment of apoptotic nuclei, with condensed/fragmented chromatin (Figure 3.1C). In high glucose-treated cells there was a significant increase of apoptotic nuclei (124.4 ± 5.4% of control). This effect was not due to the increase in osmolarity, since the number of apoptotic cells in mannitol-treated cells and control cells was similar.

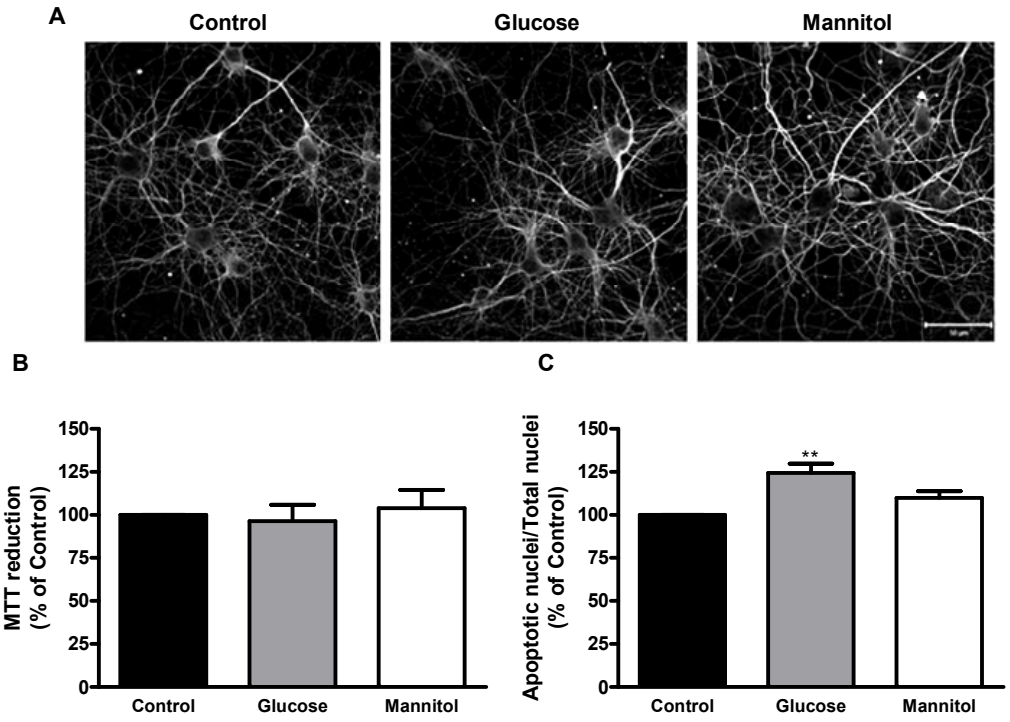


Figure 3.1: Effect of high glucose on the viability of hippocampal neurons. Cultured rat hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The morphology of hippocampal neurons was analyzed by immunocytochemistry using an antibody against neuron-specific class III beta-tubulin (Tuj-1 clone). Magnification 400x; Scale bar 50 µm. (B) Cell viability was assessed by the MTT reduction assay. The results represent the mean ± SEM of at least 4 independent experiments performed in quadruplicate, and are presented as percentage of control. (C) Total cells and cells with condensed/fragmented nuclei (cells undergoing apoptosis) were counted in nine random fields in each coverslip using Hoechst staining. The results represent the mean ± SEM of at least three independent experiments, and are expressed as percentage of control; $^{**}p<0.01$, significantly different from control.

3.3.2 High glucose alters the content and localization of SNARE complex proteins

The exposure of hippocampal neurons to elevated glucose or mannitol did not affect the protein content of VAMP-2 (Figure 3.2A), neither its localization (Figure 3.2B). Similarly, the protein levels of syntaxin-1 were not affected by high glucose or mannitol (Figure 3.3A). However, by immunocytochemistry, we detected an accumulation of syntaxin-1 in cell bodies of hippocampal neurons in cultures exposed to high glucose (Figure 3.3B).

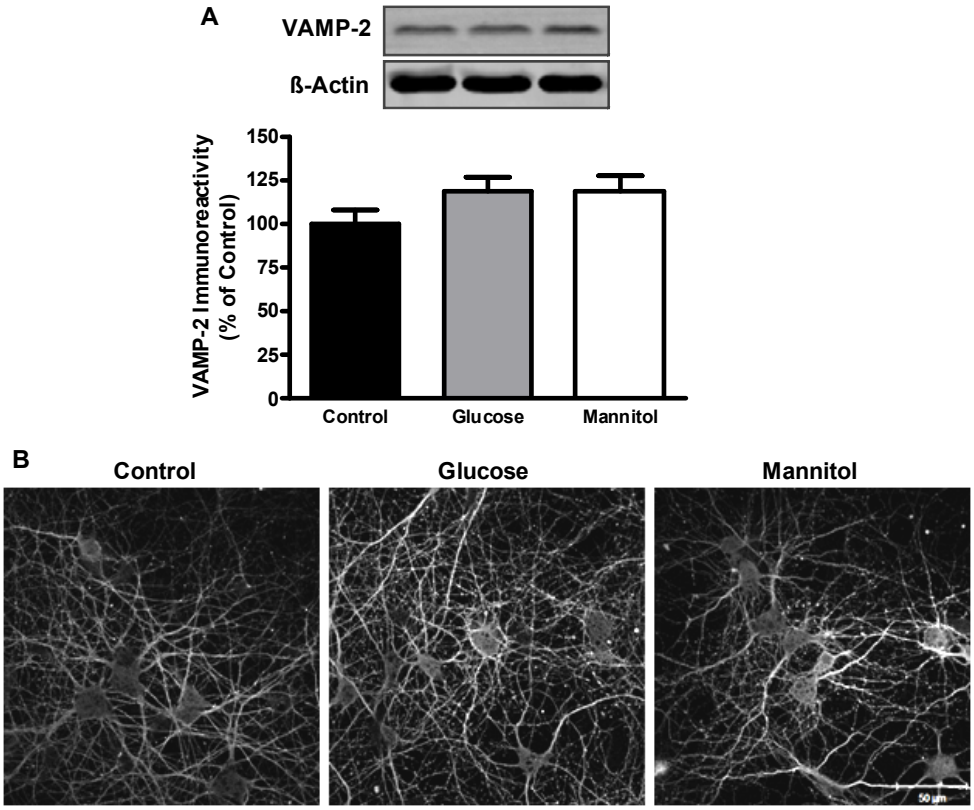


Figure 3.2: High glucose does not affect the content and distribution of VAMP-2. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of VAMP-2 were analyzed by Western blotting. Representative images of VAMP-2 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least five independent experiments. (B) The immunoreactivity and distribution of VAMP-2 was analyzed by immunocytochemistry using an antibody against VAMP-2. Magnification 400x; Scale bar 50 µm.

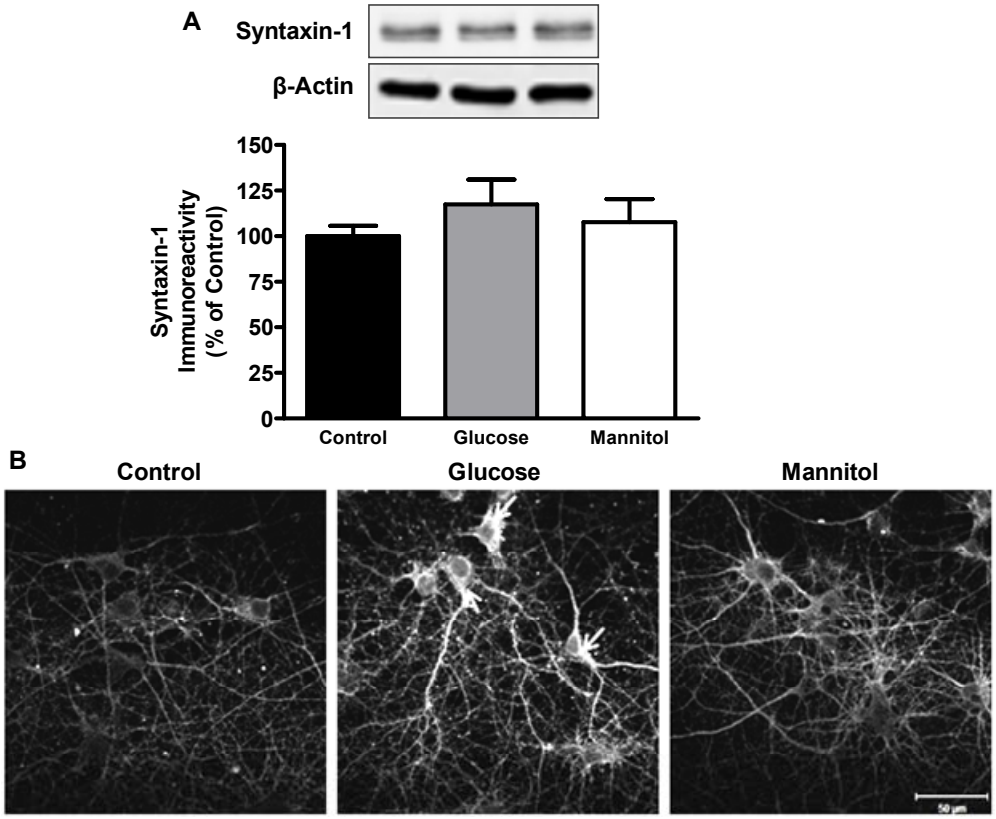


Figure 3.3: High glucose induces an accumulation of syntaxin-1 in cell bodies. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of syntaxin-1 were analyzed by Western blotting. Representative images of syntaxin-1 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least six independent experiments. (B) The immunoreactivity and distribution of syntaxin-1 was analyzed by immunocytochemistry using an antibody against syntaxin-1. Arrows point to cells with accumulation of syntaxin-1 in cell bodies. Magnification 400x; Scale bar 50 µm.

Contrarily to VAMP-2 and syntaxin-1, the content of SNAP-25 significantly decreased in high glucose-treated cells (54.9 ± 9.4% of control; Figure 3.4A). This effect was not due to the increase in osmolarity since in mannitol-treated cells no significant effect was found. By immunocytochemistry, a reduction in SNAP-25 immunoreactivity was evident in high glucose-treated cells, consistent with Western blotting data (Figure 3.4B).

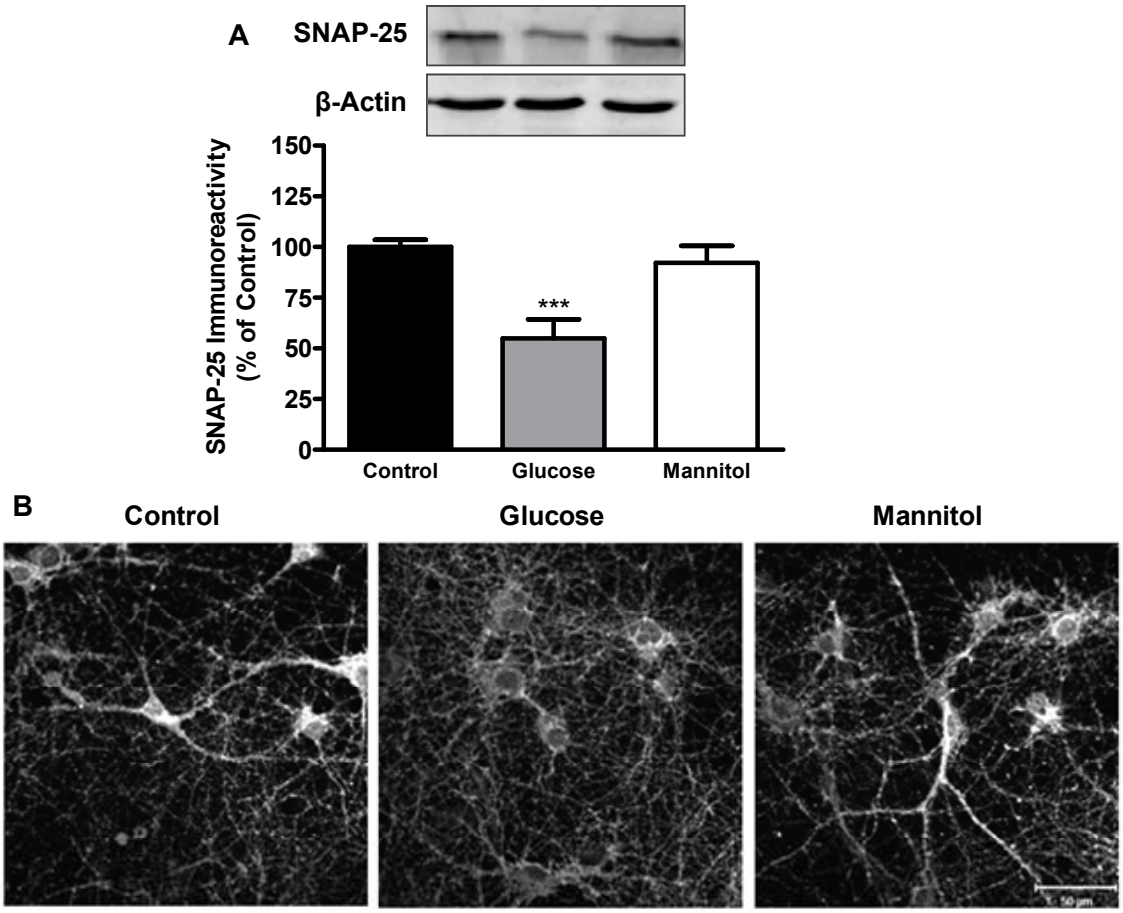


Figure 3.4: High glucose decreases the protein levels of SNAP-25. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of SNAP-25 were analyzed by Western blotting. Representative images of SNAP-25 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least five independent experiments. ****p*<0.001, significantly different from control. (B) The immunoreactivity and distribution of SNAP-25 was analyzed by immunocytochemistry using an antibody against SNAP-25. Magnification 400x; Scale bar 50 μm.

3.3.3 High glucose does not affect the content and localization of synapsin-1, synaptophysin and rabphilin 3a

Exposure of hippocampal neurons to high glucose, or mannitol, did not affect the total protein content of synapsin-1 (Figure 3.5A), synaptophysin (Figure 3.6A) and rabphilin 3a (Figure 3.7A). The immunoreactivity of these proteins also revealed that high glucose and mannitol did not induce any change in their localization in hippocampal neurons when compared to control cells (Figure 3.5B, 3.6B and 3.7B).

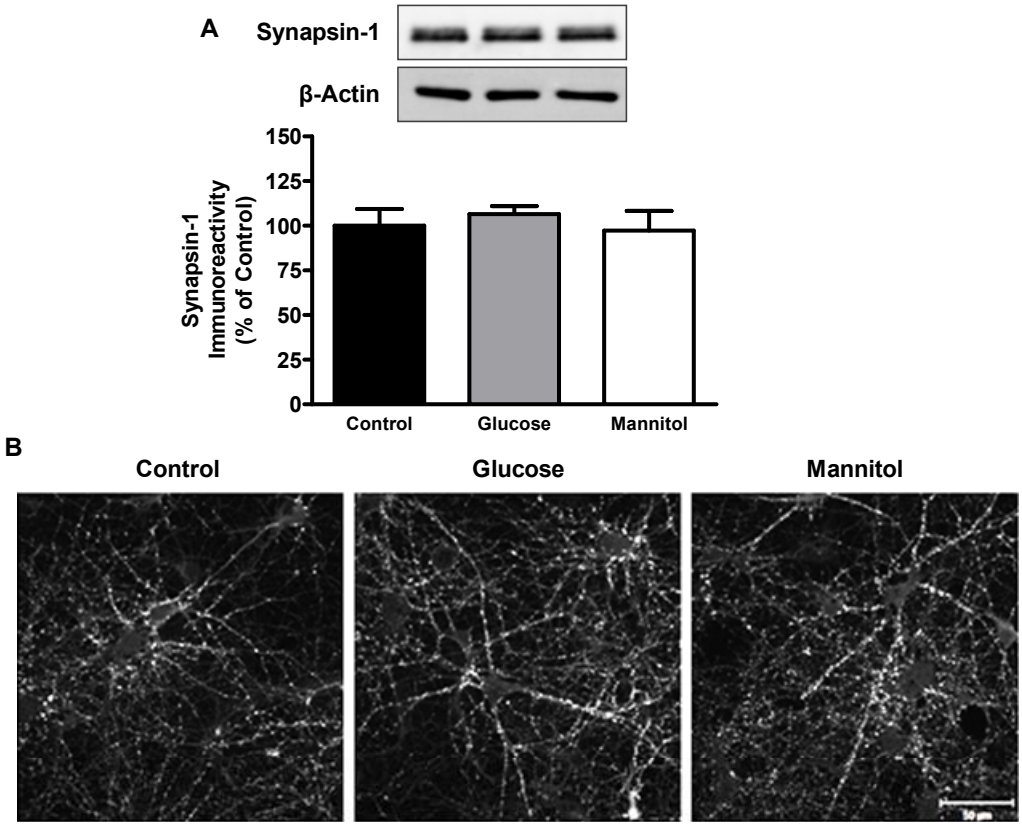


Figure 3.5: High glucose does not affect the content and distribution of synapsin-1. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of synapsin-1 were analyzed by Western blotting. Representative images of synapsin-1 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least six independent experiments. (B) The immunoreactivity and distribution of synapsin-1 was analyzed by immunocytochemistry using an antibody against synapsin-1. Magnification 400x; Scale bar 50 μm.

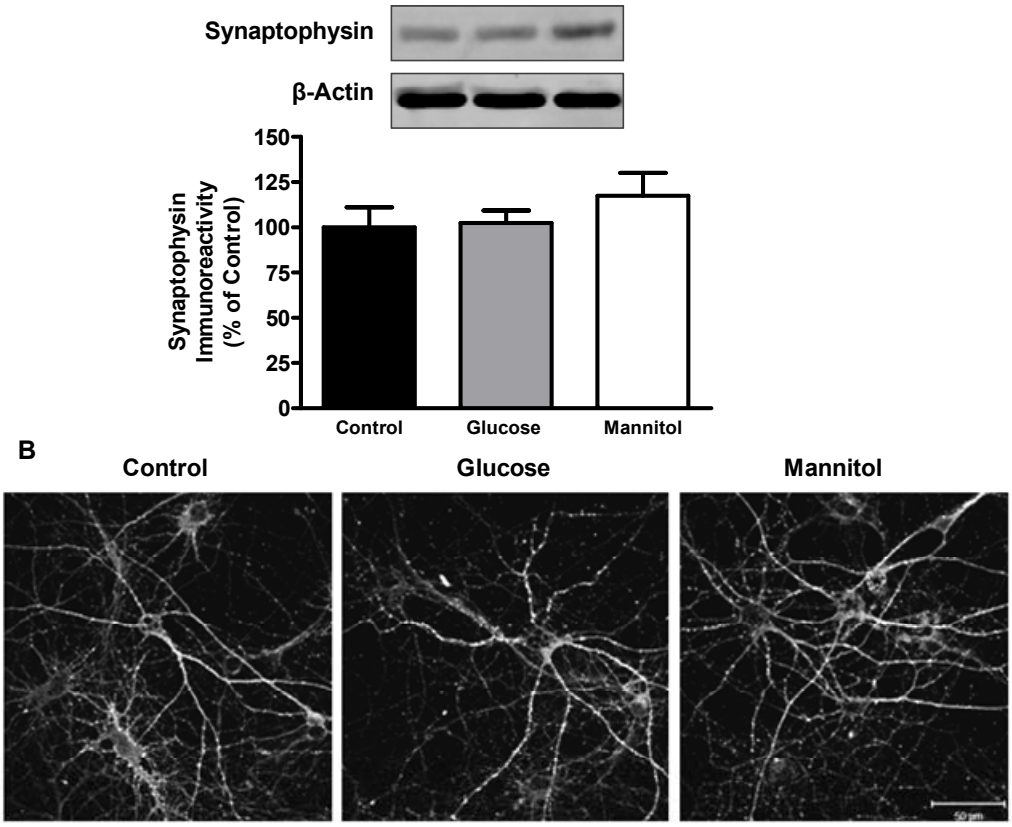


Figure 3.6: High glucose does not affect the content and distribution of synaptophysin. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of synaptophysin were analyzed by Western blotting. Representative images of synaptophysin immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least five independent experiments. (B) The immunoreactivity and distribution of synaptophysin was analyzed by immunocytochemistry using an antibody against synaptophysin. Magnification 400x; Scale bar 50 µm.

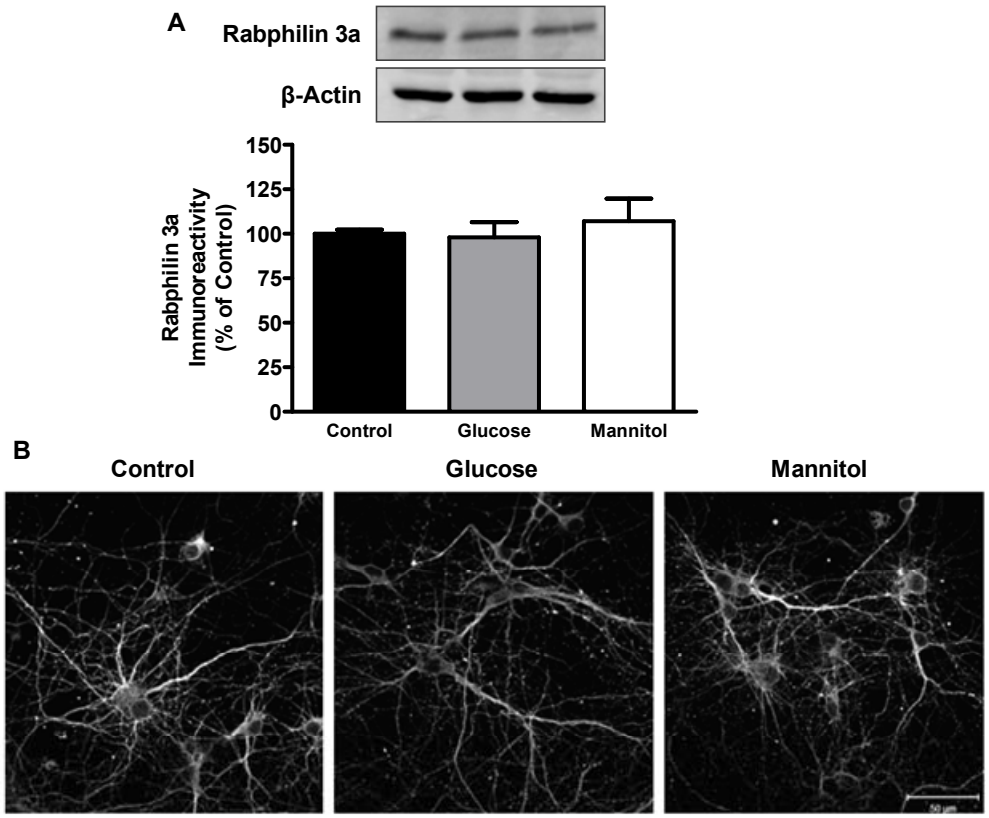


Figure 3.7: High glucose does not affect the content and distribution of rabphilin 3a. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of rabphilin 3a were analyzed by Western blotting. Representative images of rabphilin 3a immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least six independent experiments. (B) The immunoreactivity and distribution of rabphilin 3a was analyzed by immunocytochemistry using an antibody against rabphilin 3a. Magnification 400x; Scale bar 50 µm.

3.3.4 High glucose increases the content of synaptotagmin-1 and induces its accumulation in cell body

The content of synaptotagmin-1, a calcium sensor protein, significantly increased in hippocampal neurons exposed to elevated glucose for seven days (132.3 ± 7.8% of control; Figure 3.8A). This effect was not due to the increase in osmolarity since no changes were detected in cells exposed to mannitol. An increase in the immunoreactivity of synaptotagmin-1 was also found by immunocytochemistry in hippocampal neurons

exposed to elevated glucose, but not in mannitol-treated neurons (Figure 3.8B). Also evident was the accumulation of synaptotagmin-1 in some cell bodies.

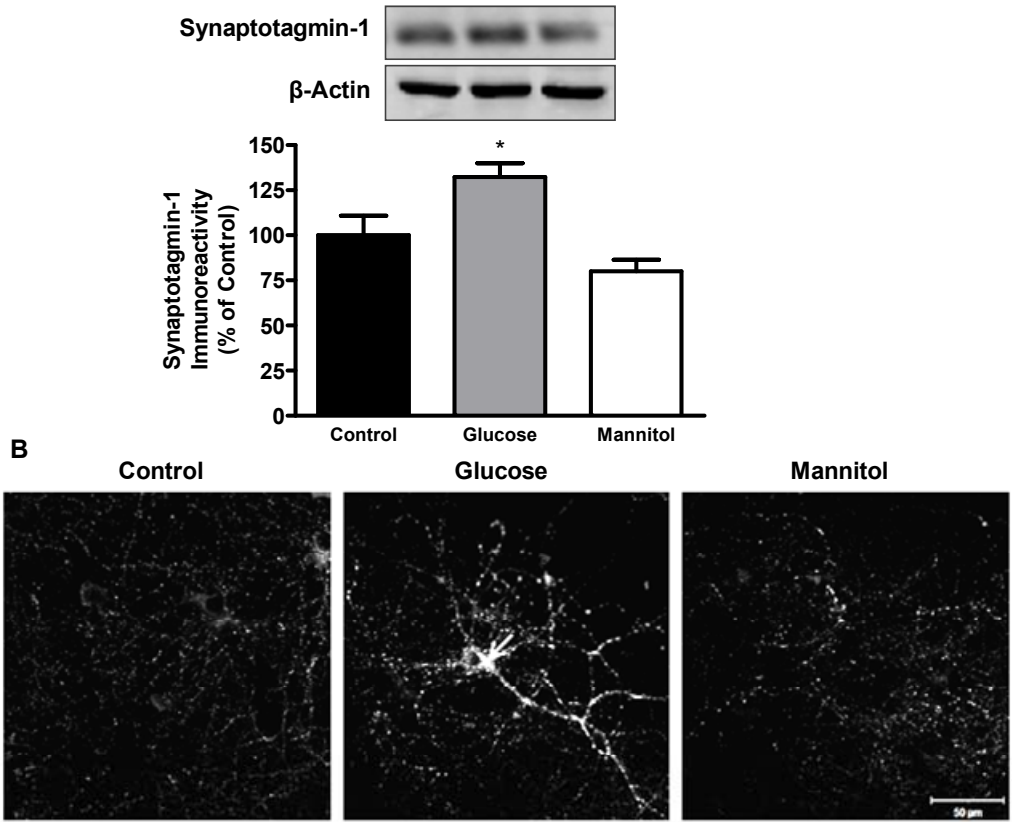


Figure 3.8: High glucose increases the content of synaptotagmin-1. Cultured hippocampal neurons were incubated with 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of synaptotagmin-1 were analyzed by Western blotting. Representative images of synaptotagmin immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least five independent experiments. *p<0.05, significantly different from control. (B) The immunoreactivity and distribution of synaptotagmin-1 was analyzed by immunocytochemistry using an antibody against synaptotagmin-1. Arrow points to a cell with accumulation of synaptotagmin-1 in cell body. Magnification 400x; Scale bar 50 μm.

3.3.5 High glucose increases the content of VGluT-1 and induces its accumulation in the cell body

We also analyzed the effect of elevated glucose on the content of the vesicular glutamate transporter, VGluT-1. The total amount of VGluT-1 in cells exposed to high glucose significantly increased as compared to control cells (123.9 ± 6.9% of control;

Figure 3.9A). This effect was not due to the increase in osmolarity since no changes were detected in cells exposed to mannitol. Moreover, in high glucose-treated cells occurred an accumulation of the protein in cell bodies (Figure 3.9B).

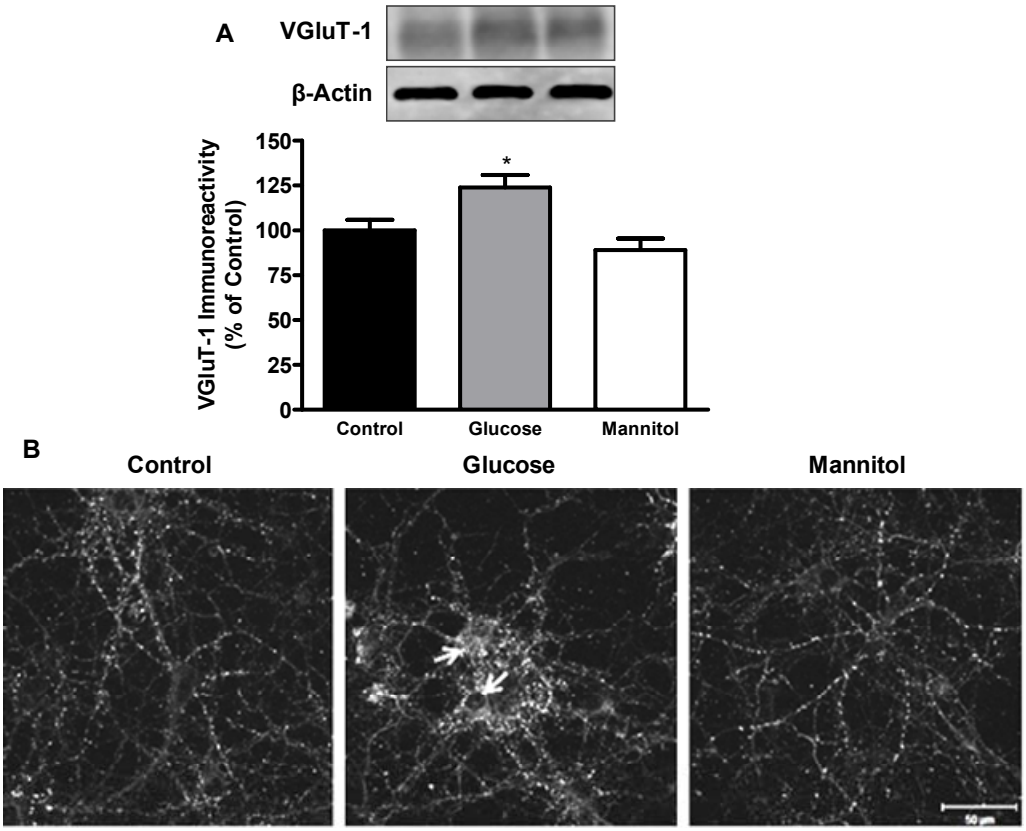


Figure 3.9: High glucose induces an accumulation of VGluT-1 in cell bodies. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of VGluT-1 were analyzed by Western blotting. Representative images of VGluT-1 immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least five independent experiments. (B) The immunoreactivity and distribution of VGluT-1 was analyzed by immunocytochemistry using an antibody against VGluT-1. Arrows point to cells with accumulation of VGluT-1 in cell bodies. Magnification 400x; Scale bar 50 µm.

3.3.6 High glucose does not affect the content and localization of VGAT

Exposure of hippocampal cells to elevated glucose, or mannitol, did not cause any change in the content of the vesicular GABA transporter, VGAT (Fig. 3.10A). Also, no

changes were detected in the immunoreactivity and localization of VGAT in cells exposed to high glucose or mannitol when compared to control cells (Fig. 3.10B).

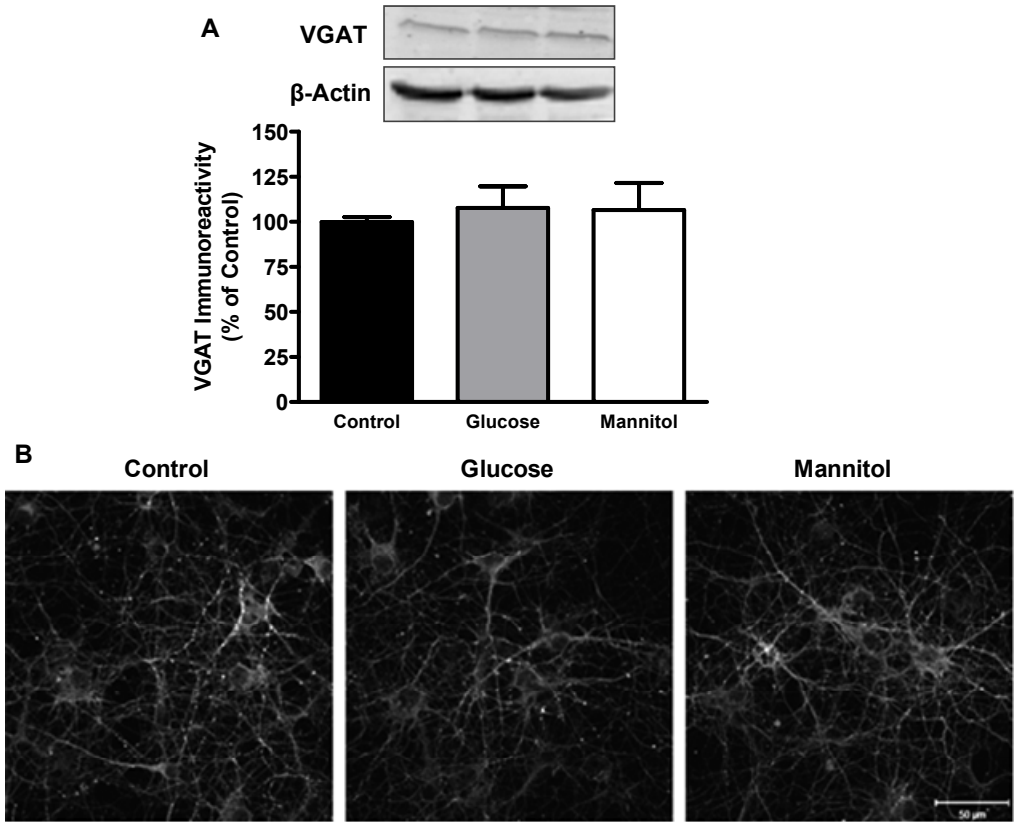


Figure 3.10: High glucose does not affect the content and distribution of VGAT. Cultured hippocampal neurons were exposed to 25 mM glucose (Control), 50 mM glucose (Glucose) and 25 mM mannitol (Mannitol) for 7 days. (A) The protein levels of VGAT were analyzed by Western blotting. Representative images of VGAT immunoreactive bands are presented above the graphs, with the respective loading control (β-actin). The densitometry of each band was analyzed and the results are expressed as percentage of control ± SEM, of at least four independent experiments. (B) The immunoreactivity and distribution of VGAT was analyzed by immunocytochemistry using an antibody against VGAT. Magnification 400x; Scale bar 50 µm.

3.4 Discussion

In this study, we found that long-term exposure to elevated glucose induces changes in the protein content of SNAP-25, synaptotagmin-1 and VGluT-1 in hippocampal cell cultures, and also changes in the cellular localization of syntaxin-1, synaptotagmin-1 and VGluT-1, inducing their accumulation in the cell bodies of hippocampal neurons. These

effects were not due to an increase in the osmolarity since exposure to mannitol (osmotic control) had no effect in both protein levels and cellular localization of synaptic proteins.
Several evidences have shown that diabetes affects neurotransmitter release (Yamato et al., 2004; Satoh and Takahashi, 2008) and the content of some exocytotic proteins (Grillo et al., 2005; Duarte et al., 2009) in hippocampus. In a previous work, we have demonstrated that diabetes induces significant changes in the content of several synaptic proteins involved in exocytosis in rat hippocampal and retinal nerve terminals (Gaspar et al., 2010).

Since hyperglycemia is considered the primary pathogenic factor for the development of diabetic complications, which include memory and cognitive impairments, we investigated whether chronic elevated glucose *per se*, which mimics a chronic hyperglycemic condition, could affect the content and localization of exocytotic proteins in cultured hippocampal neurons.

A small increase in cell death was detected in hippocampal neurons exposed to high glucose, which is consistent with previous studies where apoptosis was found in neural cells exposed to high glucose (Santiago et al., 2007; Rackova et al., 2009; Sharifi et al., 2009) and in the hippocampus of diabetic animals (Sima and Li, 2005; Jafari Anarkooli et al., 2008). Diabetic animals also present reduced dendritic branching and spine density in cortical neurons (Martínez-Tellez et al., 2005; Malone et al., 2008). However, this toxic effect of glucose does not mean a widespread neuronal degeneration. In our in vitro model, the morphology of hippocampal neurons exposed to high glucose is similar to the morphology of control cultures, as assessed by immunocytochemistry. Probably, the exposure for seven days to elevated glucose is not long enough to significantly affect dendrite branching.

Our hypothesis was that chronic hyperglycemia is the main cause for the previous detected changes in the content of exocytotic proteins (Vanguilder et al., 2008; Duarte et al., 2009; Gaspar et al., 2010). However, among nine proteins studied, we only detected changes in the content of three proteins, SNAP-25, synaptotagmin-1 and VGluT-1. There was a modest increase in the content of synaptotagmin-1 and VGluT-1, and a significant decrease in the content of SNAP-25, which was reduced by about 50%. The content of SNAP-25 was also significantly reduced in hippocampal nerve terminals (50% reduction)

and in total hippocampal extracts (25% reduction) from diabetic rats (Gaspar et al., 2010). 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). In diabetic animals, there are changes in synaptic transmission (Kamal et al., 2006) and plasticity (Biessels et al., 1998) in hippocampus, and these alterations might be related to pre- and post-synaptic alterations. The decrease in SNAP-25 content might account for changes in synaptic transmission caused by diabetes.

The increase in the content of synaptotagmin-1 and VGluT-1 was accompanied by an increase in the immunoreactivity of both proteins in cell bodies, suggesting that the transport of these proteins to nerve terminals might be affected. Probably, cells upregulate the expression of these proteins because they try to compensate a potential decrease in the content of synaptotagmin-1 and VGluT-1 in nerve terminals. Syntaxin-1 also accumulated in cell bodies when hippocampal neurons were exposed to high glucose. When exposed to elevated glucose, β-cells tend to have fewer granules near the plasma membrane and an accumulation of syntaxin-1A in the interior of the cell occurs (Somanath et al., 2009).

As mentioned above, the accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in cell bodies in hippocampal cultures exposed to high glucose can be due to an impairment of axonal transport to the synapse. Evidences show that hyperglycemia contribute to the modification of major axonal cytoskeletal proteins such as tubulin and actin, that can result in axonal atrophy/degeneration and impaired axonal transport (Sugimoto et al., 2008). A few studies with diabetic animals reported an impairment of retrograde axonal transport in retinal ganglion cells (Zhang et al., 2000) and axonal transport in sciatic nerve (Mayer et al., 1984). However, it is not clear why the other proteins studied did not accumulate in cell bodies. This issue needs to be further explored and clarified.

The results presented in this paper suggest that hyperglycemia *per se* contributes to changes in the content and distribution of some synaptic proteins. However, since the effects observed in this *in vitro* model were limited only to a few proteins, and other

exocytotic proteins were found to be affected in the hippocampus of diabetic animals (Grillo et al., 2005; Duarte et al., 2009; Gaspar et al., 2010), it is likely that other factors, such as the hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis and the loss of insulin (Wrighten et al., 2009), will certainly contribute to those changes. In diabetic patients occurs hyperactivation of the HPA axis, which results in elevated circulating cortisol levels (Couch, 1992). The exposure to corticosteroids induces changes in the expression and distribution of synaptophysin in the hippocampus, which are similar to those found in the STZ animal model of diabetes (Grillo et al., 2005). Corticosterone treatment and diabetes induce depletion and clustering of synaptic vesicles in hippocampal mossy fiber terminals (Magarinos and McEwen, 2000). Stranahan and colleagues (2008) demonstrated that high levels of glucocorticoids contribute to the impairment of synaptic plasticity and neurogenesis in the hippocampus, as well as to associated learning and memory deficits in rodent models of diabetes.

In addition to corticosteroids, the loss of insulin, a potent trophic factor, can also contribute to changes in hippocampal function (Li et al., 2002; Li et al., 2005; Sima and Li, 2005). Short-term replacement of insulin since the onset of diabetes in type I diabetic rats prevents cognitive deficits and protects against hippocampal deficits (Biessels et al., 1998). Moreover, morphological changes induced by diabetes in hippocampal CA3 subregion are reversed with insulin replacement (Magarinos et al., 2001). Altogether, our data and these evidences suggest that several factors underlie the changes detected in exocytotic proteins induced by diabetes. Hyperglycemia appears to be an important determinant of those changes, but the lack or reduced levels of insulin and the increase in the levels of corticosteroids are certainly involved in those changes. Although each of these factors is individually able to cause synaptic dysfunction, they probably act together potentiating the individual effects. Moreover, functional studies will be required to assess the physiological impairment induced by high glucose on the exocytosis in hippocampal neurons.

In conclusion, we show that long-term exposure to high glucose induces changes in the content and/or distribution of some synaptic proteins of the exocytotic machinery in cultured hippocampal neurons, although the majority of the proteins analyzed were not affected by high glucose. Together with previous findings, these observations suggest that, in addition to hyperglycemia, other factors such as the lack of insulin and the increase in

glucocorticoids levels might underlie the changes in synaptic proteins in hippocampal nerve terminals induced by diabetes. Those factors probably act in a synergistic way thus leading to the impairment of synaptic function.

Acknowledgments

The authors acknowledge Luísa Cortes for the technical support in confocal microscopy. This work was supported by Project GAPI 17/08 (Faculty of Medicine, University of Coimbra, Portugal). Joana M. Gaspar is supported by a fellowship (SFRH/BD/32949/2006) from Fundação para a Ciência e a Tecnologia (FCT), Portugal.

CHAPTER 4

Diabetes induces changes in vesicular transporters and neurotransmitter release in hippocampus

Joana M. Gaspar; Filipa I. Baptista; Armando Cristóvão; Paulo F. Santos; Attila Köfalvi;

António F. Ambrósio

This chapter is part of an original research manuscript, submitted to Brain Research (2010). 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 hippocampus and retina.

Abstract

Diabetes induces alterations in neurotransmitter release in central nervous system that depend on the type of neurotransmitter and region studied. In this study, we evaluated the effect of diabetes (two and eight weeks duration) in the basal and evoked release of [^{14}C]glutamate and [^{3}H]GABA in hippocampal nerve terminals. We also analyzed by Western blotting 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.

No changes were detected in the protein levels of vesicular glutamate transporters in hippocampal nerve terminals of diabetic animals. The content of the vesicular GABA transporter and the α_{1A} subunit decreased at two weeks of diabetes, being similar to control at eight weeks of diabetes. In total extracts, no significant changes were detected. Basal and evoked GABA release was similar to control at two and eight weeks of diabetes. Similarly, at two weeks of diabetes, the release of glutamate was similar to control. However, after eight weeks of diabetes, the basal release of glutamate increased in hippocampus.

In conclusion, diabetes induced changes in the content of the GABA vesicular transporter and in the basal release of glutamate in hippocampal nerve terminals. These changes at presynaptic level may underlie alterations in synaptic transmission and consequently memory impairments detected in diabetic animals and humans.

Key words: Diabetes; Hippocampus; Neurotransmitter Release; Glutamate; GABA.

4.1 Introduction

Diabetes mellitus is a metabolic disease that results from the impairment in insulin secretion and/or insulin resistance leading to hyperglycemia. Diabetes is associated with several complications, such as diabetic encephalopathy and retinopathy (ADA, 2010).

Several studies have demonstrated that diabetes impairs synaptic structure and function in the hippocampus at presynaptic (Grillo et al., 2005; Gaspar et al., 2010) and postsynaptic components (Biessels et al., 1996; Kamal et al., 1999), which can contribute to the development of cognitive impairments. In diabetic animals, changes in the content of exocytotic proteins and neuromodulator receptors in hippocampal nerve terminals (Duarte et al., 2006; Duarte et al., 2007; Duarte et al., 2009; Gaspar et al., 2010) and the depletion of synaptic vesicles in hippocampal mossy fiber terminals (Magarinos and McEwen, 2000) have been detected. At the postsynaptic component, impairments in synaptic plasticity (Biessels et al., 1996; Artola et al., 2005) and in NMDA receptor subunit composition (Trudeau et al., 2004) were observed. Furthermore, Chabot and colleagues (1997) reported a deficit in calcium-dependent processes modulating postsynaptic AMPA receptors during synaptic potentiation. Diabetes also triggers changes in neurotransmitter release in various brain regions, which depend on neurotransmitter and brain region studied (Guyot et al., 2001; Morris and Pavia, 2004; Miyata et al., 2007; Satoh and Takahashi, 2008). For instance, diabetes decreases the basal release levels of serotonin and dopamine in the hippocampus (Yamato et al., 2004) and of glutamate in the dentate gyrus, but basal γ-aminobutyric acid (GABA) release is not affected (Reisi et al., 2009).

The synaptic level of GABA and glutamate is determined by two 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 CNS, and several studies evidence their role in neurological disorders (Vemuganti, 2005; Benarroch, 2010).

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 tissue (Evans and Zamponi, 2006), and P/Q type calcium channels have a predominant role in the release of glutamate in hippocampal synaptosomes (Ambrósio et al., 1997).

Thus, our previous observations showing that diabetes changes the protein content of several exocytotic proteins in rat hippocampal nerve terminals (Gaspar et al., 2010) has prompted us mapping the effect of diabetes on basal and evoked glutamate and GABA release in hippocampal nerve terminals, as well as on the content of vesicular glutamate and GABA transporters.

4.2 Methods

Materials

Reagents were purchased 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 hippocampal synaptosomal extracts

Percoll purified synaptosomes were isolated as previously described (Köfalvi et al., 2007), with minor changes. Hippocampi were dissected and homogenized in a sucrose-HEPES solution (0.32 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mg/mL BSA, pH 7.4). The homogenate was centrifuged at 3,000 x *g* for 10 min at 4ºC. The supernatant was collected and centrifuged at 14,000 x *g* for 12 min at 4ºC, and the resulting pellet was

resuspended in 45% (v/v) Percoll solution prepared in Krebs–Henseleit Ringer (KHR) solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose, pH 7.4). After centrifugation at 16,100 x *g* for 2 min at 4ºC, the top layer was removed (synaptosomal fraction). For release experiments, the synaptosomal fraction was collected and stored in a sealed container on ice until use. For synaptosomal extracts, pellet was resuspended in lysis buffer [RIPA: 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland) and 1 mM dithiothreitol (DTT)]. The samples were stored at -80ºC until use.

Preparation of total hippocampal extracts

After dissection, hippocampi were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, supplemented with complete mini protease 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 done at 4ºC. The supernatant was stored at -80ºC until use.

[^{3}H]GABA and [^{14}C]glutamate release assays for hippocampal synaptosomes

Dual-label [^{3}H]GABA / [^{14}C]glutamate release experiments were performed as described by Köfalvi and colleagues (2007), with some modifications. The synaptosomal pellet was ressuspended in 2 mL of Krebs solution [(in mM): 113 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, oxygenated with 95% O_2 and 5% CO_2, pH 7.4]. The radiolabeled compounds [1 µCi/mL [^{14}C]glutamate and 2.3 µCi/mL [^{3}H]GABA (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] were added to the synaptosomes for 10 min at 37ºC. All solutions contained the GABA transaminase/ glutamate decarboxylase inhibitor, aminooxyacetic acid (100 µM). Aliquots (400 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. All experimental procedures

were performed at 37ºC. At the 4th and the 12th min of the sample collection period, the release of transmitters was evoked with 20 mM KCl for 30 seconds each time.

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 95ºC. 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 antibodies, VGluT-1 and VGluT-2 (1:10,000 and 1:5,000, respectively; 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 4ºC. 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 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 [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 $p<0.05$.

4.3 Results

4.3.1 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 264.6 ± 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 4.1. 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 4.1).

Table 4.1: 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±16.6	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 animal death. ***p <0.001

4.3.2 Diabetes does not changes the protein content of vesicular glutamate transporters in hippocampal nerve terminals

The protein levels of vesicular glutamate transporters were evaluated by immunoblotting in nerve terminals and total extracts of hippocampus from both diabetic and age-matched control animals. In hippocampal nerve terminals, the content of VGluT-1 and VGluT-2 was not affected at two and eight weeks of diabetes (Figure 4.1A and 4.1B, respectively). Similarly, in total extracts from hippocampus (Figure 4.1C and 4.1D) no significant differences were detected in the protein content of both vesicular transporters between diabetic and age-matched control animals.

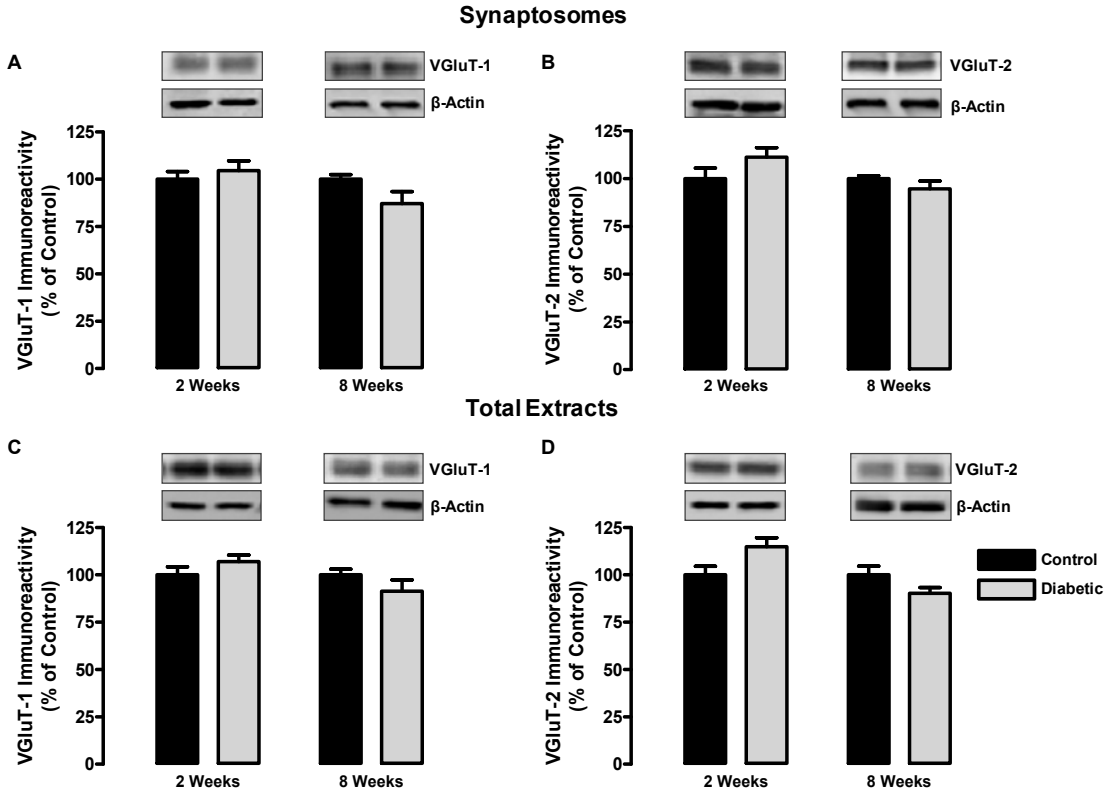


Figure 4.1: Diabetes does not affect the protein content of vesicular glutamate transporters in the hippocampus. The protein levels of VGluT-1 and VGluT-2 were analyzed by immunoblotting in extracts of hippocampal nerve terminals (A and B) and in total hippocampal extracts (C and D) isolated from control 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 in the gel. The results are expressed as percentage of age-matched controls, and data are presented as mean ± SEM, of at least 5 animals.

4.3.3 Diabetes changes the protein content of vesicular GABA transporter in hippocampal nerve terminals

The protein content of VGAT significantly decreased at two weeks of diabetes in hippocampal nerve terminals (reduction to 75.8 ± 6.4% of the control; Figure 4.2A). However, eight weeks after the onset of diabetes, the protein levels of VGAT in hippocampal nerve terminals were similar to those found in age-matched controls. In total extracts, no significant differences were observed in the protein levels of VGAT between diabetic animals and age-matched controls (Figure 4.2B).

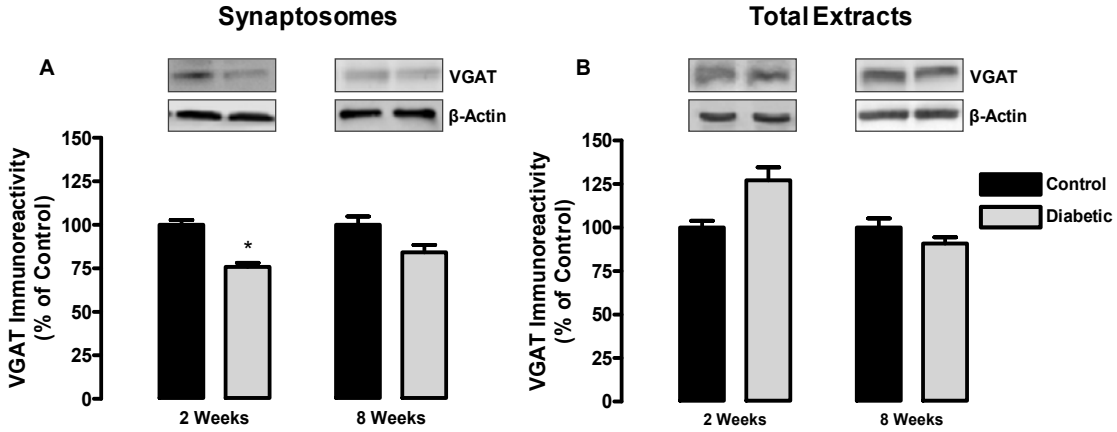


Figure 4.2: Diabetes changes the protein content of vesicular GABA transporter in hippocampal nerve terminals. The protein levels of VGAT were analyzed by immunoblotting in hippocampal synaptosomes (A) and also in total hippocampal extracts (B), from diabetic animals and aged-matched controls. Representative 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 mean ± SEM, of at least 4 animals. * $p < 0.05$, compared to age-matched control animals.

4.3.4 Diabetes changes the protein content of the α_{1A} subunit of P/Q calcium channels in hippocampal nerve terminals

The protein content of α_{1A} subunit of P/Q type calcium channels significantly decreased in hippocampal nerve terminals (reduction to 77.6 ± 6.4% of age-matched controls) after two weeks of diabetes (Figure 4.3A). Conversely, eight weeks after the onset of diabetes, the protein levels of α_{1A} subunit in nerve terminals were similar to those found in controls. In total extracts no significant changes were observed in the protein levels of this subunit (Figure 4.3B).

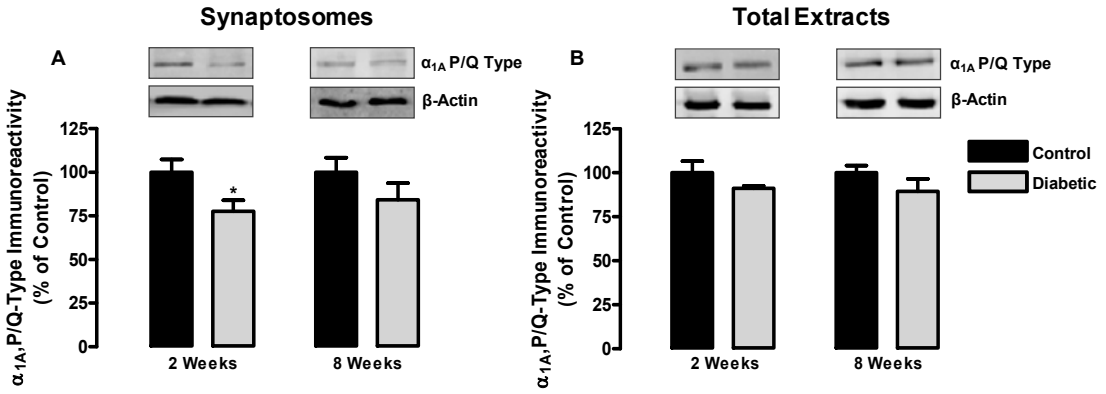


Figure 4.3: Diabetes changes the protein content of the α_{1A} subunit of P/Q calcium channels in hippocampal nerve terminals. The protein levels of α_{1A} subunit of P/Q type calcium channels were analyzed by immunoblotting in hippocampal synaptosomes (A), and also in total hippocampal extracts (B), 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 mean ± SEM, of at least 4 animals. * $p < 0.05$, compared to age-matched control animals.

4.3.5 The basal release of [^{14}C]glutamate increased in hippocampal nerve terminals after eight weeks of diabetes, but [^{3}H]GABA release was not affected by diabetes

The effect of diabetes on [^{14}C]glutamate and [^{3}H]GABA release from hippocampal nerve terminals was also evaluated. At two weeks of diabetes, no differences were found in the basal release of [^{14}C]glutamate between diabetic and age-matched control animals (Figure 4.4A). However, after eight weeks, the basal release of [^{14}C]glutamate was significantly higher in diabetic animals (2.42 ± 0.02 FR% for control and 2.74 ± 0.02 FR% for diabetic animals; Figure 4.4B). The amplitude of the repeated KCl-evoked release of [^{14}C]glutamate (S1 and S2) in synaptosomes isolated from two and eight weeks diabetic and control animals was not significantly different (Figures 4.4C and 4.4D). When the Ca^{2+} concentration of the extracellular medium was reduced to 100 nM Ca^{2+}, there was a 30% reduction of the evoked [^{14}C]glutamate release (data not shown), indicating that only about 30% of the KCl-evoked release is Ca^{2+}-dependent. Under these conditions no differences were observed between control and diabetic animals as well. The S2/S1 ratio was also determined, but no differences were found between diabetic and control animals for both time points (Figure 4.4E).

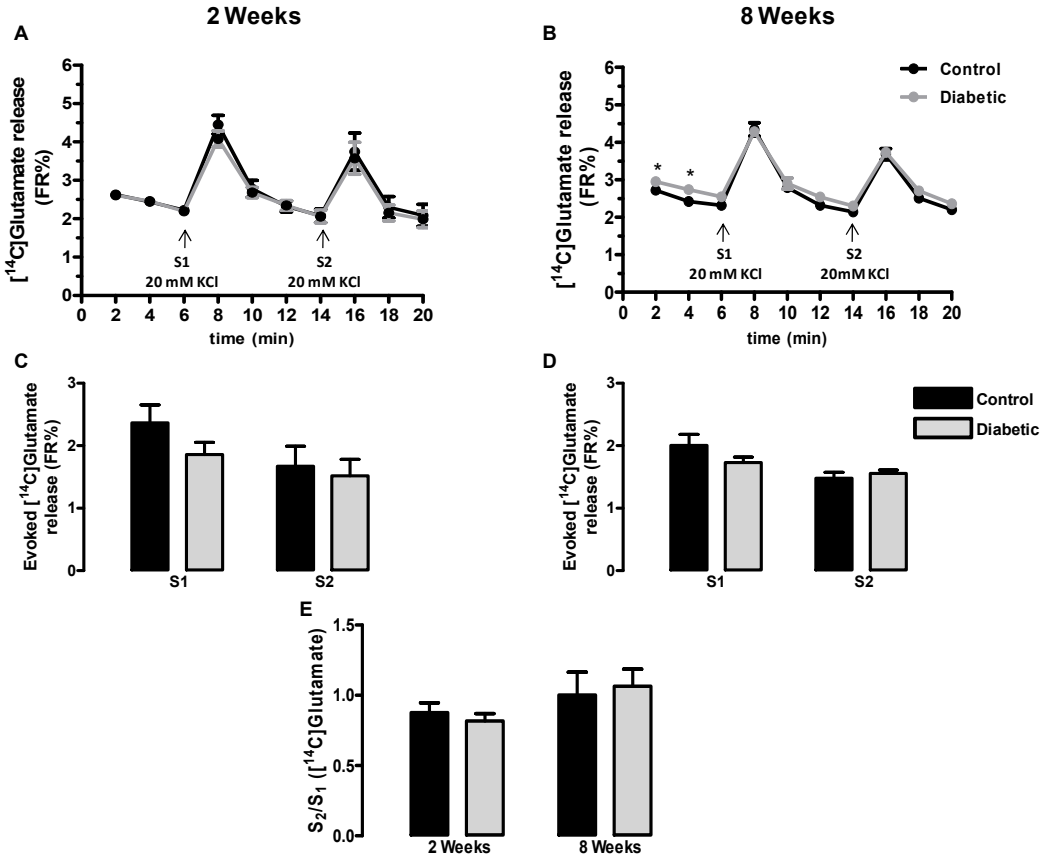


Figure 4.4: Diabetes increases the basal release of glutamate after eight weeks of diabetes. Synaptosomes were simultaneous loaded with [^{14}C]glutamate and [^{3}H]GABA, and after 15 min of washout, 2 min samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 20 mM KCl for 30 sec each. (A) Glutamate release at two weeks of diabetes. (B) Glutamate 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 at least 8 animals. * $p < 0.05$, compared to age-matched control animals.

Regarding [^{3}H]GABA release, no changes were observed in diabetic animals, either in basal or evoked release, as well as for S2/S1 ratio, at two and eight weeks of diabetes (Figure 4.5). As for glutamate, the reduction of extracellular Ca^{2+} concentration from the extracellular medium to 100 nM Ca^{2+}, caused 30% decrease in [^{3}H]GABA release (data not shown), indicating that only 30% of the KCl-evoked [^{3}H]GABA release is Ca^{2+}-dependent.

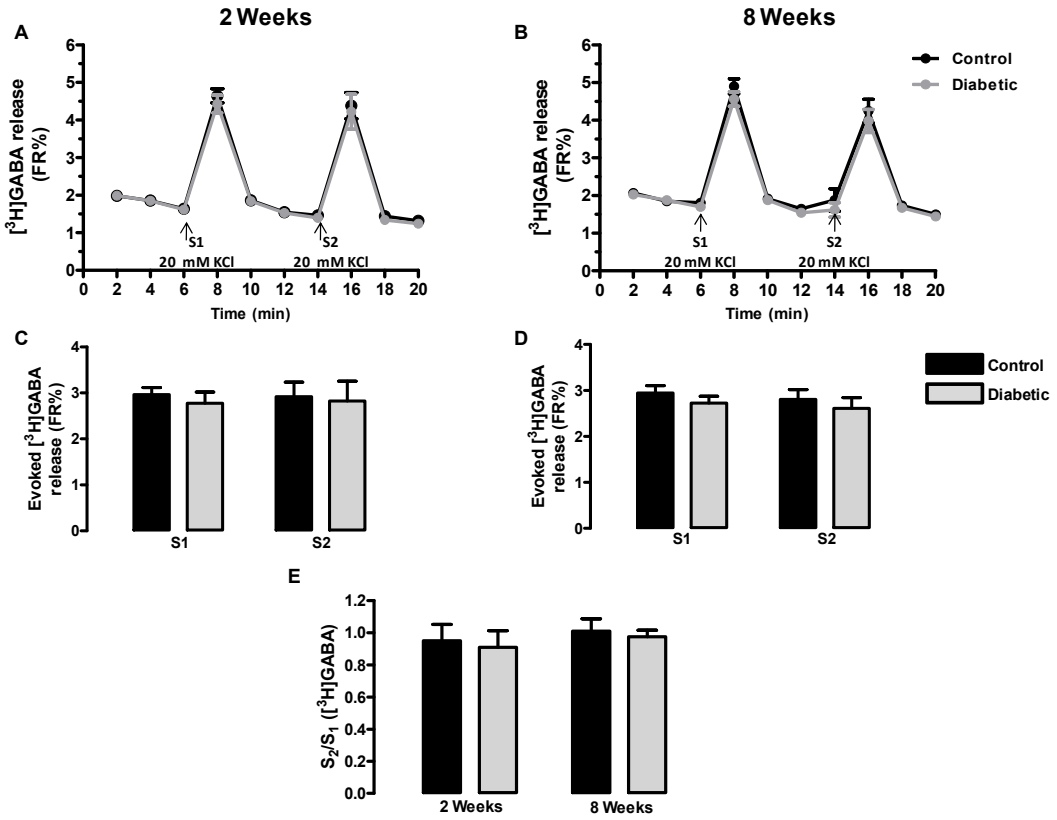


Figure 4.5: Diabetes does not affect GABA release from hippocampal synaptosomes. Results were obtained from the same experiments shown in Figure 4.4. Synaptosomes were simultaneously loaded with [^{3}H]GABA and [^{14}C]glutamate, and after 15 min of washout, 2 min samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 20 mM KCl for 30 sec 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 at least 8 animals.

4.4 Discussion

In the present study, we demonstrated that diabetes induces significant changes in the protein content of vesicular GABA transporter and also in the α_{1A} subunit of P/Q type calcium channels in hippocampal nerve terminals, but not in total extracts. However vesicular glutamate transporters were not changed either in nerve terminals or in total extracts. Moreover, we also showed that the basal release of glutamate increased in hippocampal nerve terminals after eight weeks of diabetes. A fine balance between GABA and glutamate is essential for a proper brain function and any imbalance may lead to

physiological alterations or even to neurotoxicity. This study shows that this balance can be early affected in hippocampus under diabetic conditions.

Alterations in memory and cognitive deficits (Biessels et al., 1996) 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 hippocampal and retinal nerve terminals (Gaspar et al., 2010), suggesting that diabetes might impair neurotransmitter release early in the course of the disease. However, in the hippocampus there are only a few studies addressing this issue. In STZ-injected and in spontaneously diabetic rats (WBN/Kob rats) the basal release of monoamines is impaired (Yamato et al., 2004; Ramakrishnan et al., 2005). The other shows that the basal release of glutamate in dentate gyrus is decreased after twelve weeks of diabetes (Reisi et al., 2009). 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). No changes in VGluT-1 and -2 were detected in hippocampal nerve terminals. However, the content of VGAT decreased in nerve terminals at two weeks of diabetes. The decrease in VGAT in nerve terminals could lead to a slower packaging of GABA leading to changes in synaptic transmission. However, this decrease was transient, at least in the early stages of diabetes, suggesting that after the initial stress conditions induced by diabetes, hippocampus is somehow able to react against diabetes-induced stress.

The P/Q type voltage-gated calcium channels are abundant in nerve terminals and play a predominant role in neurotransmitter release at central synapses (Ambrósio 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, hippocampus recovered the levels of α_{1A} subunit after eight weeks of diabetes, supporting the fact that hippocampus is able to recover from changes occurring in synaptic proteins induced by diabetes, at least temporarily. Since impaired Ca^{2+} regulation may result in synaptic dysfunction, impaired plasticity and neuronal degeneration (Mattson, 2007), the observed decrease in the levels of α_{1A} subunit in diabetic animals might be considered a protective strategy against Ca^{2+} overload.

A few studies have demonstrated that diabetes induces changes in neurotransmitter release. In this study, at two weeks of diabetes, no differences were found in neurotransmitter release in the hippocampus, but after eight weeks the basal release of glutamate increased in nerve terminals. We are aware of only a few studies reporting changes in transmitter release in the diabetic hippocampus: while (Yamato et al., 2004) found a decrease in the basal levels of serotonin and dopamine, (Ramakrishnan et al., 2005) reported an increase in the levels of dopamine. The apparent contradiction may be explained by the different time-points analyzed. Reisi and colleagues (2009) also reported that the basal glutamate release decreases in the dentate gyrus of diabetic animals. Altogether, a growing body of evidence suggests that diabetes impairs hippocampal neurotransmission. Since we previously observed a significant decrease in the content of several proteins involved in neurotransmitter release (Gaspar et al., 2010), we were expecting to observe more significant alterations in both glutamate and GABA release. This was not the case, probably because the release was mostly calcium-independent (not involving exocytosis).

The accumulation of glutamate in the synaptic cleft can lead to excitotoxic neuronal damage due to excessive activation of glutamate receptors (Dong et al., 2009). The increase in the basal release of glutamate can be responsible for glutamate accumulation in the synaptic cleft which can progressively conduct to neuronal cell dysfunction and death, as observed in the hippocampus of diabetic animals (Li et al., 2002; Jafari Anarkooli et al., 2008). Recently was reported that the basal glutamate release decreases in the dentate gyrus of STZ-induced diabetic animals (Reisi et al., 2009), however we found an increase in whole hippocampus. This discrepancy may be due to the experimental approaches used. While we measured the release of glutamate and GABA in whole hippocampal nerve terminals in a superfusion system, they measured the release using microdialysis and only in dentate gyrus. However, concerning GABA release, no differences were found, similarly to what was observed by Reisi and colleagues. Notwithstanding, it was also shown that hyperglycemia exacerbates the ischemia-triggered increase of extracellular glutamate concentration in the hippocampus and cortex (Li et al., 2000). This is important because the removal of the neurotransmitters by the transporter system is highly energy dependent. Under the impairment of glucose homeostasis the

energy supply may not meet the demands of the transport processes. Some studies also suggest that the impairments in glutamatergic neurotransmission underlie the functional changes in synaptic plasticity that occur in hippocampus of diabetic animals, but at postsynaptic level, involving the reorganization of receptors (Di Luca et al., 1999; Gardoni et al., 2002). Together, our findings clearly indicate that diabetes elicits alterations in excitatory neurotransmission, at presynaptic level, but additional experiments are needed to establish a clear correlation with changes in glutamatergic transmission and cell dysfunction or death in hippocampal neurons.

Taken together, these results demonstrate that diabetes induces alterations in hippocampal nerve terminals, namely on the density of the vesicular GABA transporter and α_{1A} subunit of P/Q type calcium channels, and also in neurotransmitter release, suggesting a potential imbalance between excitation and inhibition, which can contribute to neuronal dysfunction under diabetes. Moreover, this study points the importance of presynaptic changes to alterations in synaptic transmission, which may contribute to memory impairments detected in diabetic animals and humans.

Acknowledgments

This work was supported by Faculty of Medicine, University of Coimbra, Portugal (GAPI 07/09). Joana M. Gaspar acknowledges a fellowship from Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/32949/2006).

CHAPTER 5

Type 1 diabetes induces neural changes in rat hippocampus

Joana M. Gaspar; Filipa I. Baptista; Áurea F. Castilho; Joana Liberal; Joana Gonçalves; António F. Ambrósio

Submitted to *Diabetologia*

Abstract

Cognitive impairments induced by diabetes have been associated with modifications in hippocampal structure and function, including changes in neuronal and glial cells. Therefore, the aim of this work was to further evaluate and identify changes in hippocampal neural cells induced by diabetes along the early stages of the disease (two, four and eight weeks).

Neurodegeneration and apoptosis was evaluated by cresyl violet staining and detection of caspase-3. Neuronal changes were assessed by evaluating the immunoreactivity of microtubule associated protein-2 (MAP-2) and synaptophysin, as well as the protein content of Tuj-1, calbindin D28k and tau. Astrocyte reactivity was analyzed by measuring the content and immunoreactivity of glial fibrillary acidic protein (GFAP). Changes in microglial cells were evaluated with CD11b and ED1 markers.

No signs of neurodegeneration or caspase-3 activation were detected in the hippocampus. However, the protein levels of all neuronal markers decreased after eight weeks of diabetes. MAP-2 immunoreactivity decreased in DG at two and four weeks of diabetes and after eight weeks decreased in all subregions. Synaptophysin immunoreactivity increased in CA3 at all time points. No changes were detected in astrocyte reactivity, but the number of activated microglia increased in DG and CA3 subregion at eight weeks of diabetes.

Diabetes does not induce neurodegeneration in hippocampus during the early stages of the disease. However, neuronal changes can occur, being CA3 subregion the most affected. Moreover, diabetes does not induce astrocyte reactivity, at least for the time points studied, but activates microglia, suggesting the existence of a pro-inflammatory response. Altogether, these changes might affect cognitive performance.

Keywords: Diabetes; Hippocampus; Neurons; Astrocytes; Microglia.

5.1 Introduction

Diabetes *mellitus* is a metabolic disorder characterized by deficiency in insulin synthesis or secretion and/or resistance to insulin, and consequently hyperglycemia (ADA, 2010). Long-term diabetes can result in a variety of subtle cerebral disorders with manifestations demonstrated at neurochemical, electrophysiological, structural and neurobehavioural level (Biessels et al., 1994). Impairments in memory and learning are more common in diabetic patients than in non-diabetics (Jafari Anarkooli et al., 2008), and patients with diabetes have an increased risk of cognitive dysfunction (Gispen and Biessels, 2000). The functional changes caused by diabetes are accompanied by neurochemical and structural abnormalities, as well as by degenerative changes in the brain. Diabetes induces structural alterations in several brain regions (Hernandez-Fonseca et al., 2009) and reduces the length and ramification of neuronal dendrites (Martínez-Tellez et al., 2005; Malone et al., 2008). Several evidences have also suggested that diabetes increases the production of reactive oxygen and nitrogen species, with a subsequent induction of oxidative (Guglielmotto et al., 2010; Wang et al., 2010) and nitrosative stress (Kuhad et al., 2009), and the accumulation of extracellular glutamate (Li et al., 2000), which can contribute to neuronal damage and cognitive deficits. For instance, cell death has been detected in the hippocampus of diabetic animal models (Li et al., 2002; Li et al., 2005; Jafari Anarkooli et al., 2008).

Astrocytes have important roles in energy metabolism, in the regulation of extracellular glutamate levels and against reactive oxygen species. Astrocytes can also regulate synaptic activity (Newman, 2003). Hence, alterations in astrocyte activity might contribute to central nervous system (CNS) pathologies. Some studies have evaluated the reactivity of astrocytes under diabetic conditions, but the results are contradictory. The number of cells positive for glial fibrillary acidic protein (GFAP) decrease three days after the induction of diabetes with streptozotocin (STZ). However, that number increases after seven days, and after fourteen days is similar to control (Lebed et al., 2008). In another work, after four weeks of diabetes, no differences were detected in GFAP immunoreactivity in hippocampus, cortex and cerebellum (Guven et al., 2009). However, recently, in the same animal model, it was demonstrated that GFAP expression decreases in

hippocampus, cerebral cortex, and cerebellum at four and eight weeks after diabetes onset, and this effect was rescued by insulin treatment (Coleman et al., 2010).

Hyperglycemia triggers the production of advanced glycation end products (AGEs) that interact with AGEs receptor (RAGE) in microglial cells, which can lead to the expression of pro-inflammatory genes (Schmidt et al., 1999). The expression of pro-inflammatory factors is increased in the hippocampus of diabetic animals (Kuhad et al., 2009; Sima et al., 2009), suggesting that microglial cells are activated, but there are no studies showing that diabetes induces microglia activation in the hippocampus.

Evidences have shown that hippocampus is susceptible to changes caused by diabetes. However, the morphological changes occurring in hippocampus induced by diabetes, particularly the alterations occurring in different cell types, are not well characterized yet. Therefore, in this work we aimed to further evaluate and clarify the effect of type 1 diabetes, at different time points, on rat hippocampal neuronal and glial cells. For that, we evaluated neurodegeneration, the protein levels of several neuronal markers (Tuj-1, tau and calbindin D28k), and the immunoreactivity of microtubule associated protein-2 (MAP-2; as a dendritic marker) and synaptophysin (presynaptic marker). Furthermore, astrocyte activation was analyzed by measuring the protein levels and immunoreactivity of GFAP. Changes in microglia activation were analyzed using CD11b and ED1 markers.

5.2 Methods

Materials

Reagents were purchased 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). The minimum possible number of animals was used and all efforts were made to minimize animal suffering. 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 killing the rats, blood samples were collected for measurement of glucose levels and rats were weighted. Diabetic rats and age-matched controls were anesthetized with sodium thiopental (Emivete, Veterinary Products, Portugal) and then killed at two, four and eight weeks after the onset of diabetes.

Brain Slices Preparation

Rats from each experimental group were deeply anesthetized with sodium thiopental 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 on a freezing microtome and collected in 0.1 M PBS with 0.01% sodium azide. Slices were used for free-floating immunohistochemistry and cresyl violet staining experiments. In this case, slices were mounted on gelatin-coated slides.

Cresyl Violet Staining

The slices used for cresyl violet staining obtained from diabetic and age-matched control animals were from the same hippocampal area. One set of sections was washed three times in distilled water, followed by staining with 0.25% cresyl violet for 3 min and then washed again. Slices were dehydrated with 70%, 96% and 99% ethanol for 3 min each, followed by rehydration with 96% and 70% ethanol for 2 min each. Slices were washed in distilled water for 2 min, stained with 0.25% cresyl violet for 3 min and washed again. Slices were dehydrated once more through a series of alcohols, cleared in xylene, and afterwards coverslipped.

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 5.1) for 24 h at room

temperature. Incubation with primary antibodies was followed by incubation with an Alexa Fluor 594-conjugated secondary antibody (goat anti-mouse IgG, 1:250) 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 in Dako fluorescent mounting medium. Sections were examined with a LSM 510 Meta Confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Table 5. 1: List of primary antibodies used in Western blotting experiments.

Primary antibody	MW (kDa)	WB	IH	protein (µg)	Company
Rabbit anti-caspase-3	33/17 (pro/active)	1:1,000	_____	60	Cell Signaling Technology, Danvers, MA, USA
Mouse anti-tuj-1	50	1:1,000	_____	10	Covance, Emeryville, CA, USA
Rabbit anti-calbindin D28k	28	1:1,000	_____	50	Cell Signaling Technology, Danvers, MA, USA
Mouse anti-tau	50	1:1,000	_____	30	Cell Signaling Technology, Danvers, MA, USA
Mouse anti-MAP-2	_____	_____	1:1,000	_____	Sigma, St. Louis, MO, USA
Mouse anti-synaptophysin	_____	_____	1:500	_____	Sigma, St. Louis, MO, USA
Mouse anti-GFAP	55	1:5,000	1:500	10	Oncogene Science, Cambridge, MA, USA
Mouse anti-CD11b	_____	_____	1:50	_____	Serotec, Oxford, UK
Mouse anti-ED-1	108	1:200	1:50	65	Serotec, Oxford, UK

IH: immunohistochemistry; WE: western blotting

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 mini protease 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 done at 4ºC. The supernatant was stored at -80ºC 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 95ºC. 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 polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). Afterwards, 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, followed by incubation with the primary antibody (Listed in Table 5.1) overnight at 4ºC. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated with the respective alkaline phosphatase-linked secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK), prepared in TBS-T with 1% low-fat milk for 1 h at room temperature. For protein detection, the membranes were processed using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare, Buckinghamshire, UK) and a Storm device (Molecular Dynamics, GE Healthcare, Buckinghamshire, UK). The membranes were reprobed and tested for β-actin immunoreactivity (1:20,000) to prove that similar amounts of protein were applied in the gels. Digital quantification of band intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA).

Statistical Analysis

Results are presented as mean ± SEM. Statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Mann–Whitney *U* test. Gels were always loaded with samples from age-matched animals and not from animals with different ages. Differences were considered significant for $p<0.05$.

5.3 Results

5.3.1 Animals

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (255.3 ± 8.1 g for control animals and 258.8 ± 3.8 g for diabetic animals). The glucose levels were also similar in both groups (95.8 ± 2.0 mg/dL for controls and 95.1 ± 3.9 mg/dL for diabetic animals). The average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table 5.2. A marked impairment in weight gain occurred in diabetic rats comparing with age-matched controls in the time points analyzed (two, four and eight weeks of diabetes). Furthermore, diabetic animals presented significantly higher blood glucose levels comparing to age-matched controls.

Table 5.2: Average weight and blood glucose levels of diabetic and aged-matched control rats.

Diabetes duration		Weight (g)	Blood glucose (mg/dL)
2 Weeks	Control	317.1 ± 10.7	88.5 ± 3.7
	Diabetic	257.1 ± 8.7***	414.0 ± 12.4***
4 Weeks	Control	334.3 ± 13.1	90.2 ± 2.3
	Diabetic	264.3 ± 11.2***	416.6 ± 29.7***
8 Weeks	Control	363.1 ± 9.9	105.8 ± 5.3
	Diabetic	279.9 ± 13.2***	441.6 ± 24.1***

Measurements were made immediately before animal death. ***p <0.001

5.3.2 Diabetes induces neuronal changes in hippocampus

Hippocampal neurodegeneration, which could be potentially induced by diabetes, was morphologically evaluated by cresyl violet staining. No signs of neurodegeneration were observed in the hippocampus of diabetic animals, at least at the time points studied (Figure 5.1A). These results were confirmed by Fluoro-Jade C staining (data not show). In order to investigate if diabetes induces cell death by apoptosis in the hippocampus,

caspase-3 protein levels were assessed in total hippocampal lysates. Diabetes had no significant effect on the protein levels of pro-caspase-3 (Figure 5.1B). Moreover, the p17 subunit that corresponds to the activated caspase-3 was not detected by Western blot analysis. Altogether these observations indicate that diabetes does not induce cell death, at least for the time points studied.

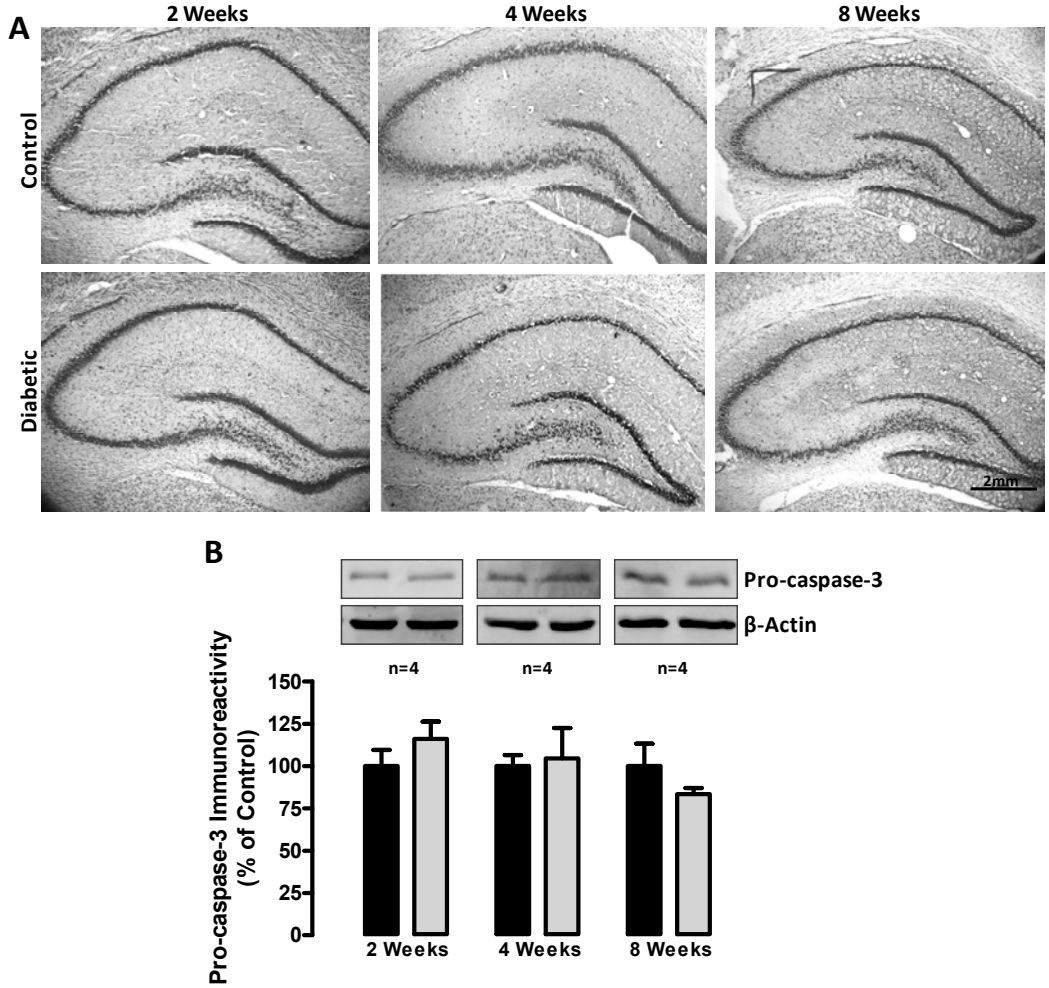


Figure 5.1: Diabetes does not induce cell death in rat hippocampus. (A) Representative images of hippocampal slices (30 µm thickness) stained with cresyl violet obtained from diabetic (two, four and eight weeks diabetes duration) and age-matched control animals. Scale bar: 2 mm. (B) Western blot analysis showing that diabetes does not change the protein levels of pro-caspase-3 (33 kDa). The bands corresponding to the activated caspase-3 (17 kDa) were not detected. Representative Western blotting images of pro-caspase-3 and β-actin (loading control) are shown above the graph. The results represent the mean ± SEM and are expressed as percentage of age-matched controls.

To evaluate if diabetes induces neuronal changes, the immunoreactivity of MAP-2, a specific dendritic marker, and synaptophysin, a presynaptic marker, was analyzed. At two and four weeks of diabetes there was a decrease in MAP-2 immunoreactivity in the dentate gyrus (DG) of diabetic animals comparing to age-matched controls, but no differences were detected in CA1 and CA3 subregions (Figure 5.2A and B). Changes in MAP-2 immunoreactivity were more evident after eight weeks of the onset of diabetes, being observed an intense decrease in all hippocampal subregions in diabetic animals comparing to control animals (Figure 5.2C). Regarding synaptophysin immunoreactivity, no alterations were detected in DG and CA1 subregions after two, four and eight weeks of the onset of diabetes (Figure 5.3A-C). However, in CA3 subregion, there was an increase in synaptophysin immunoreactivity in diabetic rats comparing to age-matched control animals at all time points.

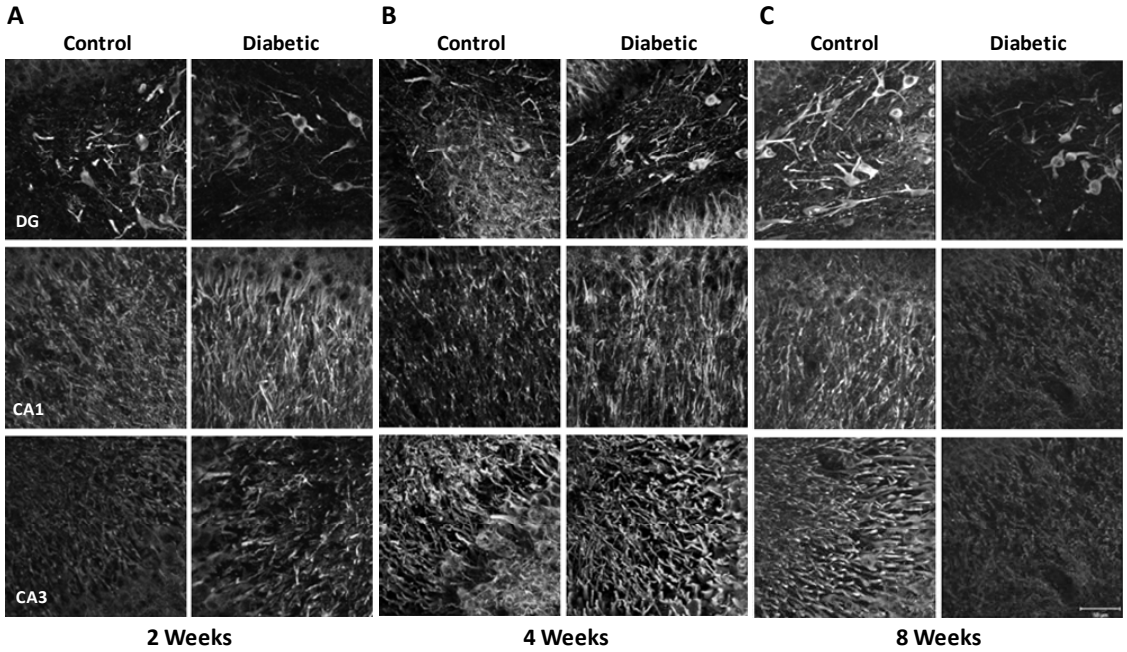


Figure 5.2: Diabetes decreases the immunoreactivity of MAP-2 in rat hippocampus. Representative confocal images of MAP-2 immunoreactivity obtained from hippocampal slices (30 µm thickness) in control and diabetic animals at different time points: two weeks (A), four weeks (B) and eight weeks (C). Images depicting DG, CA1 and CA3 subregions are shown; n=4 for each time point. Scale bar: 50 µm.

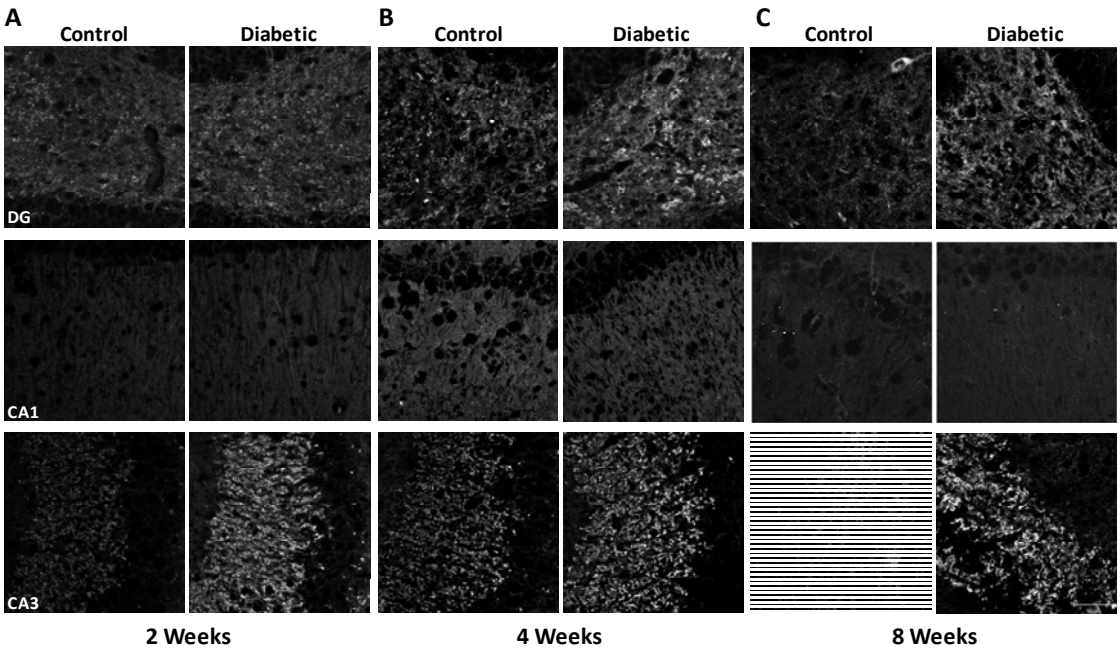


Figure 5.3: Diabetes increases the immunoreactivity of synaptophysin in hippocampal CA3 subregion. Representative confocal images of synaptophysin immunoreactivity obtained from hippocampal slices (30 µm thickness) in control and diabetic animals at different time points: two weeks (A), four weeks (B) and eight weeks (C). Images depicting DG, CA1 and CA3 subregions are shown; n=5 for each time point. Scale bar: 50 µm.

To further analyze whether diabetes induces neuronal changes in hippocampus, the protein levels of Tuj-1, a cytoskeletal neuronal marker, calbindin D28k, a calcium binding protein, and tau, a microtubule-associated protein involved in axonal transport and neurite outgrowth, were also evaluated by western blotting. Although no changes were detected in the protein levels of Tuj-1 at two weeks of diabetes, after four and eight weeks there was a significant decrease (68.8 ± 7.7% and 63.5 ± 6.8% of the control, respectively, Figure 5.4A). A significant decrease in the protein levels of calbindin D28k (85.2 ± 2.9% of the control, Figure 4B) and tau (75.1 ± 7.7% of the control, Figure 5.4C) was also found, but only after eight weeks of diabetes. Taken together these results suggest that diabetes induces neuronal changes, mainly after eight weeks of diabetes onset.

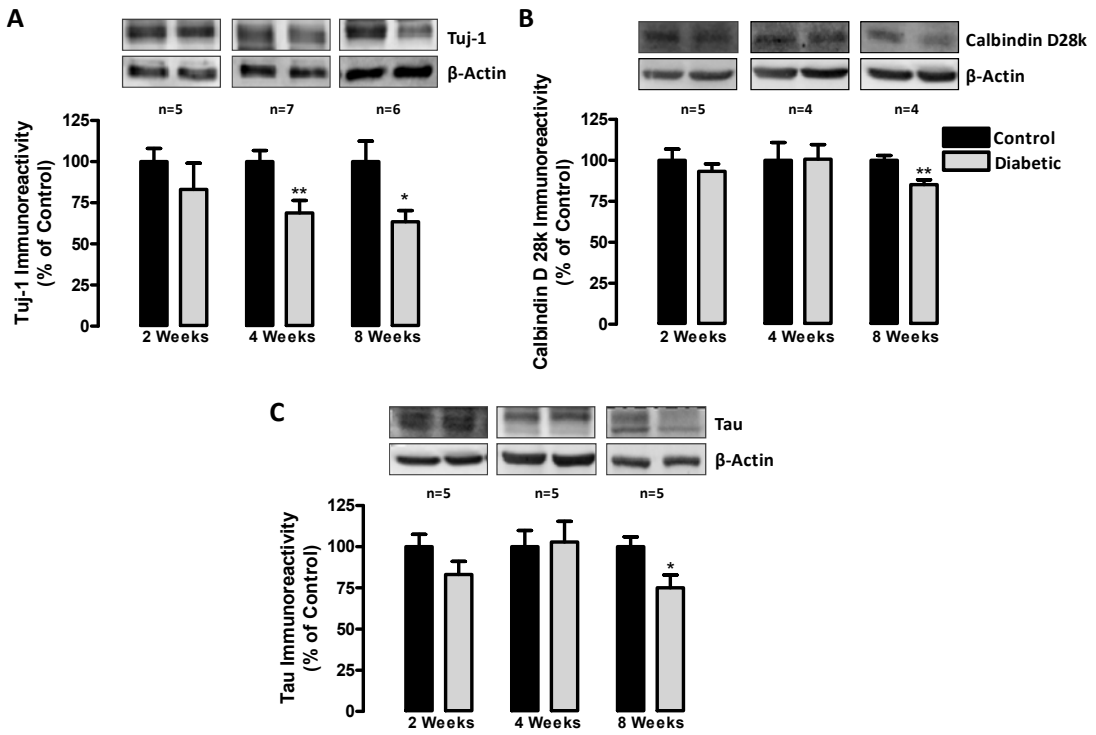


Figure 5.4: Diabetes decreases the protein levels of neuronal-specific class III beta-tubulin (Tuj-1), calbindin D28k and tau. The protein levels of Tuj-1 (A), calbindin D28k (B) and tau (C) were analyzed by Western blotting in total hippocampal extracts from diabetic and age-matched control animals. 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. *p<0.05, **p<0.01, compared to age-matched controls using Mann-Whitney test.

5.3.3 Effect of diabetes on astrocyte and microglia reactivity

Astrocytes and microglial cells are the main source of pro-inflammatory cytokines in the brain (Mandrekar-Colucci and Landreth, 2010). Several reports have suggested that gliosis frequently accompanies brain toxicity (Minghetti et al., 2005; Leonard, 2007). In order to evaluate the effect of diabetes on astrocyte reactivity, potential changes in GFAP protein levels and immunoreactivity were evaluated. By Western blotting analysis, no significant differences were detected in the protein levels of GFAP in hippocampal extracts between diabetic and age-matched control animals at any time point of the study (Figure 5.5A). This result was corroborated by immunohistochemistry, where no changes were observed in astrocyte reactivity in diabetic conditions (Figure 5.5B-D).

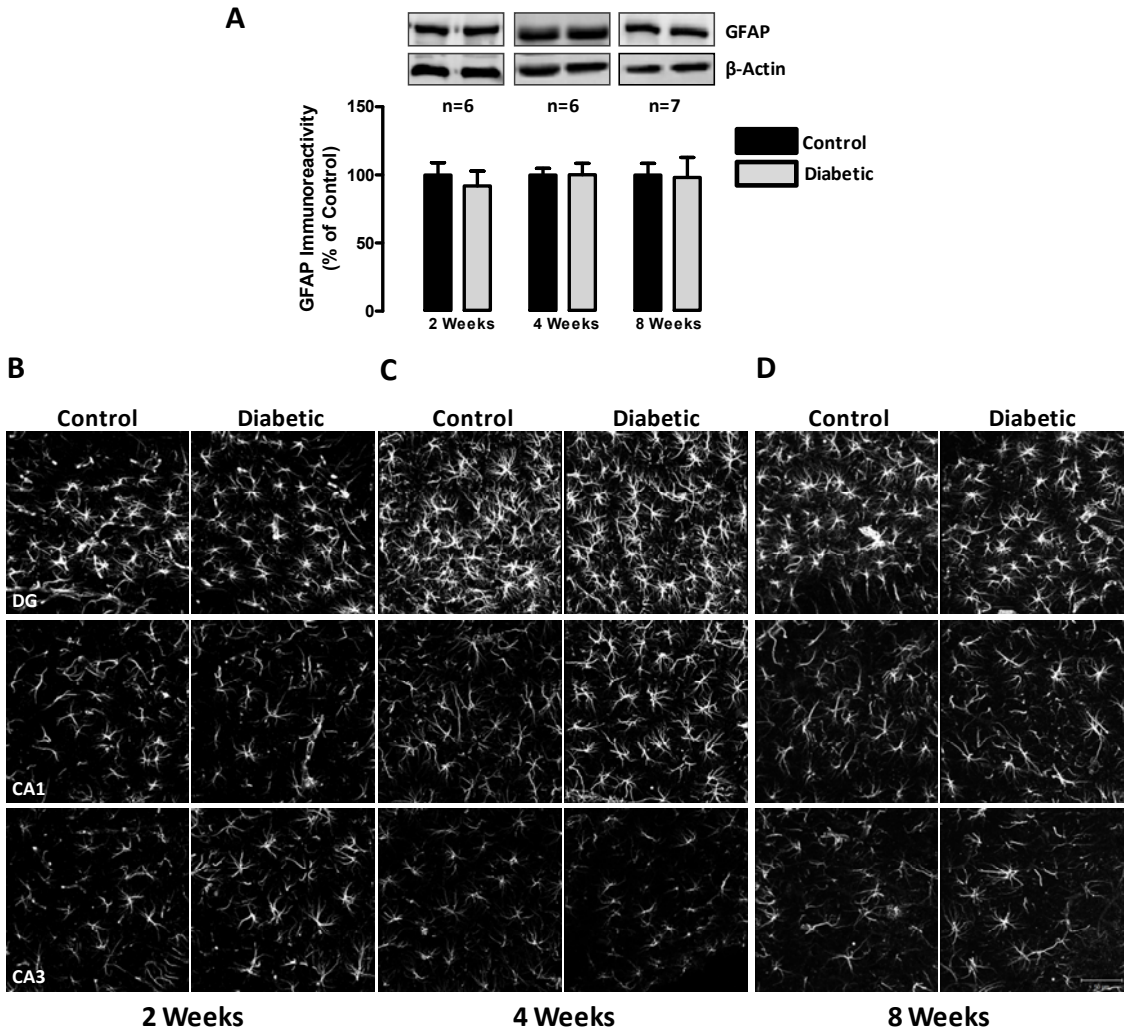


Figure 5.5: Diabetes does not induce astrogliosis in rat hippocampus. (A) The protein levels of GFAP were analyzed by immunoblotting in hippocampal total extracts isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading control (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. (B, C, D) Representative confocal images of GFAP immunoreactivity obtained from hippocampal slices (30 μm thickness) in control and diabetic animals at different time points: two weeks (B), four weeks (C) and eight weeks (D). Images depicting DG, CA1 and CA3 subregions are shown; n=5 for each time point. Scale bar: 50 μm.

We also evaluated whether microglial cells became activated in the hippocampus of diabetic animals. No changes were observed in CD11b immunoreactivity and microglial cells morphology in the hippocampus of diabetic animals comparing to controls, at all time

points (Figure 5.6A-C). Analyzing the protein levels of ED1, a specific marker for activated microglial cells, in total hippocampal extracts, again no significant differences were found between control and diabetic animals at two and four weeks of diabetes (Figure 5.7A). At eight weeks of diabetes there was a tendency to an increase in the protein levels of ED1, although not significant (Figure 5.7A). This result was somehow confirmed by immunohistochemistry experiments, where no changes in ED1 immunoreactivity were observed after two and four weeks of diabetes (Figures 5.7B and C), but after eight weeks there was an increase in the number of ED1 positive cells specifically in DG and CA3 subregions of diabetic animals (Figure 5.7D). This indicates that microglia activation induced by is region-specific.

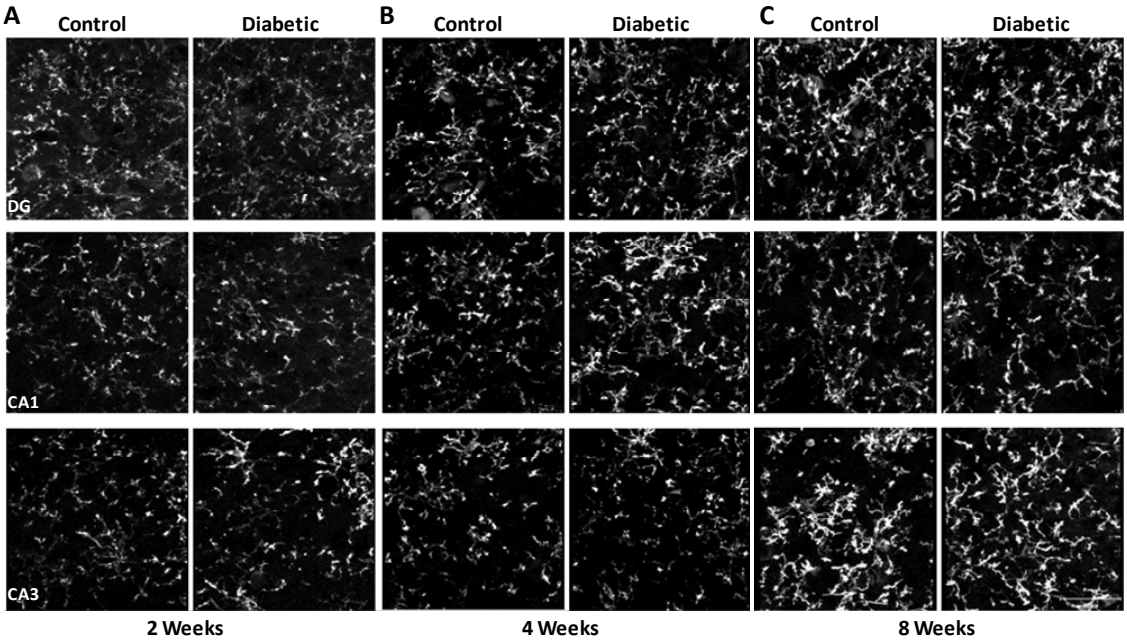


Figure 5.6: Diabetes does not change CD11b immunoreactivity in rat hippocampus. Representative confocal images of CD11b immunoreactivity obtained from hippocampal slices (30 µm thickness) in control and diabetic animals, at different time points: two weeks (A), four weeks (B) and eight weeks (C). Images depicting DG, CA1 and CA3 subregions are shown; n=3 for each time point. Scale bar: 50 µm.

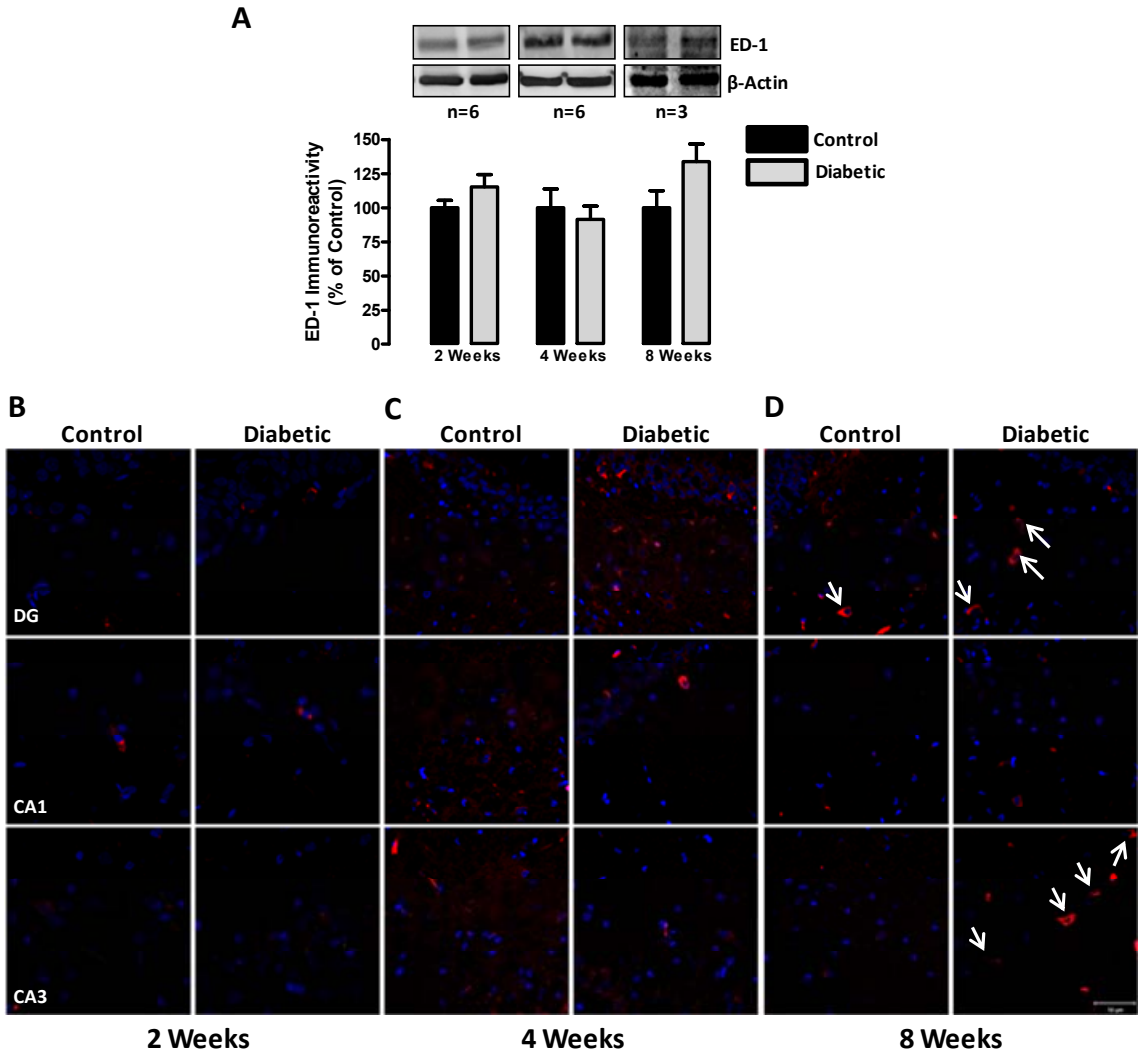


Figure 5.7: Diabetes induces the activation of microglial cells in rat hippocampus. (A) The protein levels of ED-1 were analyzed by immunoblotting in total hippocampal extracts isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the 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. (A, B, C) Representative confocal images of ED1 positive cells obtained from hippocampal slices (30 µm thickness) in control and diabetic animals at different time points: two weeks (B), four weeks (C) and eight weeks (D). Images depicting DG, CA1 and CA3 subregions are shown; n=3 for each time point. Scale bar: 50 µm.

5.4 Discussion

In this work, using an animal model of type I diabetes, we demonstrated that although diabetes does not induce neuronal degeneration in rat hippocampus, at least

during the early stages of the disease, neuronal changes occur. The hippocampal subregions are differentially affected, being CA3 the most affected. Moreover, astrocytes did not become reactive with diabetes, at least for the time points studied, but an activation of microglial cells occur, suggesting the existence of a pro-inflammatory reaction.

Diabetic patients typically display deficits in a wide range of cognitive functions (Ryan, 1988; Ryan et al., 2006). These cognitive impairments have been associated with structural alterations (Hernandez-Fonseca et al., 2009) or even with brain atrophy (Ferguson et al., 2005; Manschot et al., 2006), and with changes in electrophysiological properties (Biessels et al., 1996). Hippocampus has a key role in memory and associative learning, and alterations in hippocampus structure and function can lead to cognitive dysfunction (Budson, 2009). In fact, diabetes elicits neuroanatomical, electrophysiological and neurochemical changes in the rat hippocampus that have been associated with impairments in hippocampal-dependent behavior (Gispen and Biessels, 2000). Some of these alterations implicate the existence of changes at synaptic level that may impair synaptic transmission. We and others reported alterations in exocytotic proteins in hippocampal nerve terminals (Duarte et al., 2009; Gaspar et al., 2010) and depletion and dispersion of synaptic vesicles in the hippocampus of diabetic animals (Magarinos and McEwen, 2000). Despite all these evidences, little is known about neural morphological changes caused by diabetes in the hippocampus.

Some authors have reported the existence of apoptotic cell death in the hippocampus of spontaneously type 1 diabetic BB/Wor rats (Li et al., 2002; Li et al., 2005), type 2 BBZDR/Wor-rats (Li et al., 2007), and STZ-induced diabetic rats after eight weeks of diabetes (Jafari Anarkooli et al., 2008). In the present study, we did not detect cell death up to eight weeks of diabetes. It is possible that cell death can occur in longer time points. This result is different from the findings of (Jafari Anarkooli et al., 2008), despite the use of the same animal model and the analysis at the same time point, eight weeks of diabetes. This apparent contradictory result can be due to the use of different methodologies to access neurodegeneration. We used cresyl violet staining and evaluated the protein levels of caspase-3, and Jafari Anarkooli and colleagues (2008) measured caspase-3 activity and the ratio of mRNA and protein levels of Bax/Bcl2. The differences relatively to the studies

carried out by Li and colleagues, (Li et al., 2002; Li et al., 2005, 2007), can be related with the use of different animal models.

Although we did not find evidence of cell death in hippocampus after eight weeks of diabetes, there was evidence of neuronal changes. A decrease in MAP-2 immunoreactivity occurred after two and four weeks of diabetes in DG, and after eight weeks in all subregions (CA1, CA3 and DG). Since MAP-2 is a specific dendritic marker, this finding may be indicative of dendritic changes. In fact, in the same animal model of diabetes, it was demonstrated a decrease in the dendritic length and dendritic spine density in hippocampus (Magarinos and McEwen, 2000; Martínez-Tellez et al., 2005) and cortex (Malone et al., 2008).

There was also a significant decrease in the content of Tuj-1 in diabetic animals. This protein is a neuron-specific cytoskeletal protein, and its expression can provide insights into the neuronal responses to insults and alterations in the composition of neuronal cytoskeleton (Zhu et al., 2007). Actually, chronic treatment with glucocorticoids (stress condition), shows that the immunoreactivity of Tuj-1 decreases, suggesting the occurrence of morphological changes in neuronal cytoskeleton (Zhu et al., 2007). These changes can lead to vesicle detachment from cytoskeleton, contributing to the dispersion of synaptic vesicles that occur in the hippocampus of diabetic animals (Magarinos and McEwen, 2000).

The content of tau, an axonal protein, and calbindin D28k, a calcium binding protein, also decreased in the hippocampus after eight weeks of diabetes. Tau is a microtubule-associated protein that regulates neurotransmission. Excessive tau phosphorylation disrupts its binding to microtubules altering molecular trafficking, which can lead to synaptic dysfunction (Billingsley and Kincaid, 1997; Gendron and Petrucelli, 2009). Tau hyperphosphorylation and cleavage are common features of Alzheimer's disease (Iqbal et al., 2009), and seem to be a key factor for cognitive impairments in diabetic animals (Kim et al., 2009; Zhang et al., 2010). In this study, we evaluated only the total protein content of tau, and not tau phosphorylation. The decrease in the content of tau in the hippocampus of diabetic animals may result from an increase in its cleavage. Calbindin D28k buffers intracellular calcium concentration and its protein levels are also decreased in patients with Alzheimer's (Hof and Morrison, 1991) and in animal models of Alzheimer's disease (Lazarov et al., 2006). Our findings also associate diabetes with decreased calbindin D28k

levels in the hippocampus, which may underlie cell and synaptic dysfunction, and consequently memory and cognitive impairments.

We demonstrated recently that presynaptic proteins are affected by diabetes (Gaspar et al., 2010). In particular, we found an increase in the content of synaptophysin in hippocampal nerve terminals, without changes in total hippocampal extracts. Here, by immunocytochemistry, we show that synaptophysin immunoreactivity is increased in hippocampal slices at all time points of the study, but only in the CA3 subregion. Since we detected an increase in the content of synaptophysin in whole hippocampal nerve terminals (Gaspar te al., 2010), it is likely that the increase of synaptophysin content, specifically in nerve terminals, also occurs in CA1 and DG subregions, despite no significant differences were detected in the hippocampal slices in these subregions. Grillo and colleagues (2005) also reported an increase in synaptophysin immunoreactivity in CA3 subregion only. It is not clear why this subregion appears to be particularly affected, but the increase in synaptophysin content might be a compensatory mechanism to increase synaptic density, as a result of dendritic shortening (Malone et al., 2008). This compensatory mechanism may involve increased expression of synaptophysin in existing synapses and/or the generation of new synapses, but this needs to be further investigated.

Astrocytes regulate blood-brain barrier, glutamate levels, synaptic transmission, and protect against reactive oxygen species (Newman, 2003; Kimelberg, 2010). Alterations in astrocytes have been found in the hippocampus during stress, ageing and neurodegenerative diseases (Lambert et al., 2000; Pekny and Pekna, 2004). Therefore, it is likely that changes in their function could also contribute to brain dysfunction associated with diabetes. In this study, no significant changes in GFAP immunoreactivity were found in diabetic animals. Similar results were obtained after four weeks of diabetes in the same animal model (Guven et al., 2009). However, in another study, using the same animal model, there was a decrease in GFAP immunoreactivity in the hippocampus after four and eight weeks of diabetes (Coleman et al., 2010). Conversely, and increase in the number of GFAP-positive cells as well as in GFAP immunoreactivity in the hippocampus of diabetic mice and rats was also demonstrated (Saravia et al., 2002; Baydas et al., 2003). Moreover, Lebed and colleagues (2008) reported that GFAP immunoreactivity in diabetic animals depends on the duration of diabetes: decreases after three days of diabetes, increases

after seven days, and is similar to control after 14 days. Since different outcomes have been obtained by different groups regarding astrocyte reactivity, it is difficult to establish a correlation between astrocyte reactivity and structural and functional alterations occurring in the hippocampus of diabetic animals. Further studies are required to understand the contribution of astrocyte reactivity/dysfunction to brain deficits induced by diabetes.

When activated by an insult, microglial cells alter their morphology and produce and release high levels of pro-inflammatory cytokines (McGeer and McGeer, 2001; Piehl and Lidman, 2001). Recently, it was reported that pro-inflammatory cytokines, such as TNF-α and interleukins, are upregulated in the hippocampus of diabetic BB/Wor rats (Sima et al., 2009), suggesting that microglial cells are activated in the hippocampus of diabetic animals. However, these authors did not analyze the effect of diabetes on microglial cells activation. To our knowledge, our study is the first demonstrating that microglial cells are activated in the hippocampus, and CA3 and DG subregions appear to be the most affected regions.

In conclusion, the present study shows that diabetes induces neuronal changes, mostly at longer periods of diabetes, which include alterations in cytoskeleton, axonal and synaptic proteins. No changes in astrocyte reactivity were found, but diabetes activates microglial cells, suggesting the involvement of a pro-inflammatory response. Moreover, hippocampal subregions are differentially affected, being CA3 subregion the most affected. Altogether, neural changes may contribute to synaptic dysfunction occurring in diabetes. However, further studies are needed to better understand the factors underlying the increased vulnerability of the hippocampus under diabetic conditions.

Acknowledgments

The authors acknowledge Luísa Cortes for the technical support in confocal microscopy. Joana M. Gaspar is supported by a fellowship (SFRH/BD/32949/2006) from Fundação para a Ciência e a Tecnologia (FCT), Portugal. This work was supported by Faculty of Medicine, University of Coimbra, Portugal.

CHAPTER 6

General Discussion

6. General discussion

A growing body of evidence shows that diabetes is associated with cognitive deficits in humans. This condition, known as “diabetic encephalopathy”, impairs the daily performance of diabetic patients. The 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). In the last decade, important findings have improved our knowledge about the pathogenesis of diabetic encephalopathy. Despite this, the mechanisms by which cognitive abilities are impaired are still poorly understood and remain to be elucidated.

The cognitive impairments associated with diabetes have been linked to alterations in neurotransmission (Kamal et al., 1999; Artola et al., 2005) and synaptic plasticity (Biessels et al., 1998). It has been suggested that the impairments in hippocampal neurotransmission in diabetic animals underlying the functional changes in synaptic plasticity occur at postsynaptic level, involving the reorganization of glutamate receptors (Di Luca et al., 1999; Gardoni et al., 2002) and dendritic atrophy of CA3 pyramidal neurons (Reagan et al., 1999). However, some studies have demonstrated that presynaptic morphological changes also occur in the hippocampus of diabetic animals, including synaptic vesicle depletion in mossy fiber nerve terminals (Magarinos and McEwen, 2000), increased expression of the presynaptic marker synaptophysin (Grillo et al., 2005) and reduction in the content of SNAP-25 and syntaxin-1 in nerve terminals (Duarte et al., 2009). Additionally, several evidences have demonstrated that diabetes induces changes in neurotransmitter release in different brain regions (Guyot et al., 2001; Morris and Pavia, 2004; Yamato et al., 2004; Reisi et al., 2009). Together, these evidences strongly suggest that diabetes induces alterations at pre- and postsynaptic level that can contribute to the impairment of synaptic transmission. However, the presynaptic changes that might underlie the impairments in hippocampal neurotransmission are not fully explored and clarified. Therefore, the main aim of this work was to identify molecular and cellular changes in the hippocampus of diabetic animals, giving a particular attention to the alterations accorring at presynaptic level.

In the first part of this work (Chapters 2 and 3), we demonstrated that diabetes and high glucose induce alterations in the content and/or distribution of synaptic proteins

involved in exocytosis. All proteins analyzed have an important role in the exocytosis process, and alterations in their content or localization can have a negative effect on synaptic function. In diabetic animals, the protein content of SNAP-25, syntaxin-1 and synapsin-1 was strongly affected. Their levels decreased significantly in nerve terminals. A decrease in the content of SNAP-25 and syntaxin-1 was also detected in hippocampal total extracts, suggesting that changes can be occurring at the level of protein synthesis or degradation. Some studies using knockout (KO) animals have demonstrated the importance of the SNARE complex as a key structure for synaptic vesicle exocytosis (Sorensen et al., 2003; Fujiwara et al., 2006). The considerable decrease in the levels of these proteins in the hippocampus of diabetic animals, together with the significant decrease in synapsin-1, might impair neurotransmitter release. The content of synaptophysin in nerve terminals increased at all time points, but without changes in total extracts. Our results corroborate previous findings, where a decrease in the content of SNAP-25 and syntaxin-1 in hippocampal nerve terminals (Duarte et al., 2009) and an increase in synaptophysin levels in the hippocampus (Grillo et al., 2005) of diabetic animals were reported. Although the content of several proteins in nerve terminals was reduced, for the majority of proteins analyzed no alterations were detected in total hippocampal extracts. These findings may reflect an impairment of the trafficking of those proteins to the synapse (Sugimoto et al., 2008).

Hyperglycemia is considered the primary pathogenic factor for the development of diabetic complications. To test whether hyperglycemia per se was the main causative factor for the alterations in exocytotic proteins detected in the hippocampus of diabetic animals, we exposed cultured hippocampal neurons to elevated glucose for seven days. The long-term exposure to high glucose only altered the protein content of three proteins (SNAP-25, synaptotagmin-1 and VGluT-1), but also induced the accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in cell bodies of hippocampal neurons. These observations indicate that SNAP-25 appears to be particularly affected by diabetes and by hyperglycemic conditions in hippocampal neurons. However, the mechanisms underlying the alteration in the content and distribution of some exocytotic proteins need to be elucidated. The accumulation of syntaxin-1, synaptotagmin-1 and VGluT-1 in cell bodies of hippocampal neurons exposed to high glucose can occur due to an impairment of axonal

transport to the synapse, an alteration that was reported previously in diabetic animals (Mayer et al., 1984; Zhang et al., 2000; Lee et al., 2002). Hyperglycemia might contribute to the modification of major axonal cytoskeletal proteins, such as tubulin and actin, that can result in axonal atrophy/degeneration and impaired axonal transport (Sugimoto et al., 2008). Our results suggest that diabetes can impair the trafficking of exocytotic proteins to the synapse. On the other hand, the effects observed in this *in vitro* model were limited only to a few proteins, whereas other exocytotic proteins were found to be affected in the hippocampus of diabetic animals, thus suggesting that other factors, besides hyperglycemia, such as the hyperactivation of the HPA axis and the lack or low levels of insulin (Wrighten et al., 2009), may contribute to those changes.

Changes induced by diabetes in synaptic proteins involved in exocytosis can lead to changes in neurotransmitter release. Since diabetes strongly affected several proteins involved in exocytosis, to check whether those changes would imply changes in transmitter release, in Chapter 4 we investigated the impact of diabetes on glutamate and GABA release from hippocampal nerve terminals. Contrary to what was expected, no marked changes were detected in the basal or evoked transmitter release. The exception was for the basal release of glutamate, which was increased after eight weeks of diabetes. This may be due to the fact that only about 30% of glutamate and GABA release is exocytotic in our synaptosomal preparation. Since under these conditions the release is mainly non-exocytotic, alterations in exocytotic release might me somehow masked. Previous studies have shown that diabetes affects neurotransmitter release in hippocampus. Diabetes decreases the basal release of serotonin and dopamine (Yamato et al., 2004) and decreases the basal glutamate release in the dentate gyrus. In this case, basal GABA release was not also affected after twelve weeks of diabetes (Reisi et al., 2009). In another study, an increase in the levels of dopamine was reported (Ramakrishnan et al., 2005). The apparent contradiction could be explained by the different time-points analysed and also due to the experimental approaches used. Nevertheless, the increase in the basal release of glutamate can lead to an accumulation of glutamate in the synaptic cleft and consequently to the excessive activation of glutamate receptors (Dong et al., 2009), which can progressively conduct to neuronal cell dysfunction and death as observed in the hippocampus of diabetic animals (Li et al., 2002; Jafari Anarkooli et al., 2008).

Hippocampus is susceptible to changes induced by diabetes, and although diabetic encephalopathy shares some pathological features with brain aging and also with Alzheimer disease (Valente et al., 2010), the potential changes occurring in neural cells in the hippocampus caused by diabetes are not well characterized. Therefore, in the last part of this work (Chapter 5) we aimed to give further insight into the effect of diabetes on rat hippocampal neuronal and glial cells. Although no signs of degeneration were found in the hippocampus of diabetic animals, at least for the time points studied, diabetes induced neuronal changes, being the CA3 subregion the most affected. We found alterations in the protein content and immunoreactivity of several neuronal markers. The decrease in MAP-2 immunoreactivity may be indicative of alterations in dendritic length and spine density (Magarinos and McEwen, 2000; Martínez-Tellez et al., 2005). The increase in synaptophysin immunoreactivity in CA3 subregion, also detected by Grillo and colleagues, (2005). These authors suggested that this increase can occur as a compensatory mechanism to increase synapse density as a result of dendritic shortening (Grillo et al., 2005). The decrease in the content of tuj-1 (neuron specific cytoskeletal protein) and tau (axonal protein) can change the composition of the neuronal cytoskeleton (Zhu et al., 2007), which may alter molecular trafficking and axonal transport (Kim et al., 2009; Zhang et al., 2010) to the synapse, and also underlie the dispersion of synaptic vesicles that occur in the hippocampus of diabetic animals (Magarinos and McEwen, 2000).

Although some studies have reported changes in astrocytes (Saravia et al., 2002; Baydas et al., 2003; Lebed et al., 2008; Coleman et al., 2010), we and others (Guven et al., 2009) did not observe changes in astrocyte reactivity. It is therefore difficult to establish a clear correlation between astrocyte reactivity and structural and functional alterations in the hippocampus of diabetic animals. Further studies are needed to understand the contribution of astrocyte reactivity to brain deficits induced by diabetes.

An upregulation of pro-inflammatory factors, such as TNF-α and interleukins occurs in the hippocampus of diabetic animals (Kuhad et al., 2009; Sima et al., 2009). These observations suggest that microglial cells might be activated, but to our knowledge this was not demonstrated yet. We detected an increase in activated microglial cells in CA3 hippocampal subregion, which corroborates the existence of a pro-inflammatory response in the hippocampus of diabetic animals.

In summary, the results presented in this thesis provide a better insight into the molecular and cellular changes triggered by diabetes in the hippocampus. The alterations induced by diabetes in synaptic proteins involved in exocytosis, vesicular transporters and neurotransmitter release, together with neural alterations, may contribute to synaptic dysfunction, and consequently to the memory and cognitive impairments detected in diabetic animals and humans.

CHAPTER 7

Main Conclusions

7. Main Conclusions

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

- Diabetes strongly affects the content of several synaptic proteins involved in exocytosis in hippocampal nerve terminals. Sintaxin-1 and SNAP-25 were the most affected proteins.

- Long-term exposure to high glucose also changes the content and the localization of various synaptic proteins involved in exocytosis, inducing their accumulation in the cell bodies of cultured hippocampal neurons. These observations suggest that the trafficking of these proteins to the synapse may be compromised. Since the majority of the proteins studied was not affected by the exposure to elevated glucose, it is likely that other factors, such as the lack or decreased insulin levels, and the increase in corticosteroid levels, can also contribute to the changes detected in synaptic proteins in the hippocampus of diabetic animals.

- Diabetes increased the basal release of glutamate in hippocampal nerve terminals. This increase may account for an accumulation of glutamate in the synaptic cleft, and might lead to neuronal cell dysfunction and death.

- Diabetes induces neuronal changes in the hippocampus, and CA3 subregion appears to be the most affected. Astrocytes did not become reactive, at least during the early stages of the disease, but microglial cells became activated, suggesting the existence of a pro-inflammatory response.

In summary, these results show that diabetes can induce alterations in synaptic proteins in hippocampal nerve terminals and affect neurotransmitter release, which together with the existence of neural alterations can contribute to synaptic dysfunction. Moreover, this work highlights the importance of presynaptic changes in CNS caused by diabetes, which may contribute to memory and cognitive impairments detected in diabetic animals and humans.

CHAPTER 8

References

8. References

Abderrahmani A, Cheviet S, Ferdaoussi M, Coppola T, Waeber G, Regazzi R (ICER induced by hyperglycemia represses the expression of genes essential for insulin exocytosis. Embo J 25:977-986.2006a).

Abderrahmani A, Plaisance V, Lovis P, Regazzi R (Mechanisms controlling the expression of the components of the exocytotic apparatus under physiological and pathological conditions. Biochem Soc Trans 34:696-700.2006b).

ADA (Diagnosis and classification of diabetes mellitus. Diabetes Care 33 Suppl 1:S62-69.2010).

Allen NJ, Barres BA (Neuroscience: Glia - more than just brain glue. Nature 457:675-677.2009).

Amaral DG, Scharfman HE, Lavenex P (The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res 163:3-22.2007).

Amaral DG, Witter MP (The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience 31:571-591.1989).

Ambrósio AF, Malva JO, Carvalho AP, Carvalho CM (Inhibition of N-,P/Q- and other types of Ca2+ channels in rat hippocampal nerve terminals by the adenosine A1 receptor. Eur J Pharmacol 340:301-310.1997).

Amor S, Puentes F, Baker D, van der Valk P (Inflammation in neurodegenerative diseases. Immunology 129:154-169.2010).

Anderson P, Morris R, Amaral D, Bliss T, O´Keefe J (eds.) (2007) The Hippocampus Book. New York: Oxford University Press.

Aragno M, Mastrocola R, Medana C, Restivo F, Catalano MG, Pons N, Danni O, Boccuzzi G (Up-regulation of advanced glycated products receptors in the brain of diabetic rats is prevented by antioxidant treatment. Endocrinology 146:5561-5567.2005).

Artola A, Kamal A, Ramakers GM, Biessels GJ, Gispen WH (Diabetes mellitus concomitantly facilitates the induction of long-term depression and inhibits that of long-term potentiation in hippocampus. Eur J Neurosci 22:169-178.2005).

Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA (Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. Arch Neurol 61:661-666.2004).

Augustine GJ (2004) Synaptic Transmission. In: Neuroscience(Purves, D. et al., eds) Sunderland: Sinauer Associates.

Bahler M, Greengard P (Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704-707.1987).

Baines AJ, Bennett V (Synapsin I is a microtubule-bundling protein. Nature 319:145-147.1986).

Barber AJ (A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Prog Neuropsychopharmacol Biol Psychiatry 27:283-290.2003).

Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW (Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 102:783-791.1998).

Barclay JW, Craig TJ, Fisher RJ, Ciufo LF, Evans GJ, Morgan A, Burgoyne RD (Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. J Biol Chem 278:10538-10545.2003).

Barker AJ, Ullian EM (Astrocytes and synaptic plasticity. Neuroscientist 16:40-50.2010).

Baydas G, Nedzvetskii VS, Tuzcu M, Yasar A, Kirichenko SV (Increase of glial fibrillary acidic protein and S-100B in hippocampus and cortex of diabetic rats: effects of vitamin E. Eur J Pharmacol 462:67-71.2003).

Beauquis J, Homo-Delarche F, Revsin Y, De Nicola AF, Saravia F (Brain alterations in autoimmune and pharmacological models of diabetes mellitus: focus on hypothalamic-pituitary-adrenocortical axis disturbances. Neuroimmunomodulation 15:61-67.2008).

Becherer U, Rettig J (Vesicle pools, docking, priming, and release. Cell Tissue Res 326:393-407.2006).

Benarroch EE (Glutamate transporters: diversity, function, and involvement in neurologic disease. Neurology 74:259-264.2010).

Benedict C, Hallschmid M, Schultes B, Born J, Kern W (Intranasal insulin to improve memory function in humans. Neuroendocrinology 86:136-142.2007).

Bennett MK, Calakos N, Scheller RH (Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255-259.1992).

Bernath S (Calcium-independent release of amino acid neurotransmitters: fact or artifact? Prog Neurobiol 38:57-91.1992).

Biessels G, Gispen WH (The calcium hypothesis of brain aging and neurodegenerative disorders: significance in diabetic neuropathy. Life Sci 59:379-387.1996).

Biessels GJ, Kamal A, Ramakers GM, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH (Place learning and hippocampal synaptic plasticity in streptozotocin-induced diabetic rats. Diabetes 45:1259-1266.1996).

Biessels GJ, Kamal A, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH (Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment. Brain Res 800:125-135.1998).

Biessels GJ, Kappelle AC, Bravenboer B, Erkelens DW, Gispen WH (Cerebral function in diabetes mellitus. Diabetologia 37:643-650.1994).

Biessels GJ, ter Laak MP, Hamers FP, Gispen WH (Neuronal Ca2+ disregulation in diabetes mellitus. Eur J Pharmacol 447:201-209.2002).

Billingsley ML, Kincaid RL (Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. Biochem J 323 (Pt 3):577-591.1997).

Bose M, Olivan B, Laferrere B (Stress and obesity: the role of the hypothalamic-pituitary-adrenal axis in metabolic disease. Curr Opin Endocrinol Diabetes Obes 16:340-346.2009).

Bradl M, Lassmann H (Oligodendrocytes: biology and pathology. Acta Neuropathol 119:37-53.2010).

Brewer GJ, Torricelli JR, Evege EK, Price PJ (Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567-576.1993).

Bronk P, Deak F, Wilson MC, Liu X, Sudhof TC, Kavalali ET (Differential effects of SNAP-25 deletion on Ca2+ -dependent and Ca2+ -independent neurotransmission. J Neurophysiol 98:794-806.2007).

Budson AE (Understanding memory dysfunction. Neurologist 15:71-79.2009).

Burkhardt P, Hattendorf DA, Weis WI, Fasshauer D (Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. Embo J 27:923-933.2008).

Byrne JH, Roberts JL (eds.) (2004) From Molecules to Networks: An Introduction to Cellular and Molecular Neuroscience. San Diego, California.

Calakos N, Bennett MK, Peterson KE, Scheller RH (Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. Science 263:1146-1149.1994).

Cases-Langhoff C, Voss B, Garner AM, Appeltauer U, Takei K, Kindler S, Veh RW, De Camilli P, Gundelfinger ED, Garner CC (Piccolo, a novel 420 kDa protein associated with the presynaptic cytomatrix. Eur J Cell Biol 69:214-223.1996).

Catterall WA (Interactions of presynaptic Ca2+ channels and snare proteins in neurotransmitter release. Ann N Y Acad Sci 868:144-159.1999).

Ceccaldi PE, Grohovaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F (Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: an analysis by videomicroscopy. J Cell Biol 128:905-912.1995).

Chabot C, Massicotte G, Milot M, Trudeau F, Gagne J (Impaired modulation of AMPA receptors by calcium-dependent processes in streptozotocin-induced diabetic rats. Brain Res 768:249-256.1997).

Chapman ER (How does synaptotagmin trigger neurotransmitter release? Annu Rev Biochem 77:615-641.2008).

Chi P, Greengard P, Ryan TA (Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4:1187-1193.2001).

Clarke DW, Mudd L, Boyd FT, Jr., Fields M, Raizada MK (Insulin is released from rat brain neuronal cells in culture. J Neurochem 47:831-836.1986).

Coleman ES, Dennis JC, Braden TD, Judd RL, Posner P (Insulin treatment prevents diabetes-induced alterations in astrocyte glutamate uptake and GFAP content in rats at 4 and 8 weeks of diabetes duration. Brain Res 1306:131-141.2010).

Coleman WL, Bill CA, Bykhovskaia M (Rab3a deletion reduces vesicle docking and transmitter release at the mouse diaphragm synapse. Neuroscience 148:1-6.2007).

Comin D, Gazarini L, Zanoni JN, Milani H, de Oliveira RM (Vitamin E improves learning performance and changes the expression of nitric oxide-producing neurons in the brains of diabetic rats. Behav Brain Res 210:38-45.2010).

Coppola T, Magnin-Luthi S, Perret-Menoud V, Gattesco S, Schiavo G, Regazzi R (Direct interaction of the Rab3 effector RIM with Ca2+ channels, SNAP-25, and synaptotagmin. J Biol Chem 276:32756-32762.2001).

Corradi A, Zanardi A, Giacomini C, Onofri F, Valtorta F, Zoli M, Benfenati F (Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive impairment. J Cell Sci 121:3042-3051.2008).

Costa G, Pereira T, Neto AM, Cristovao AJ, Ambrósio AF, Santos PF (High glucose changes extracellular adenosine triphosphate levels in rat retinal cultures. J Neurosci Res 87:1375-1380.2009).

Couch RM (Dissociation of cortisol and adrenal androgen secretion in poorly controlled insulin-dependent diabetes mellitus. Acta Endocrinol (Copenh) 127:115-117.1992).

Craft S, Newcomer J, Kanne S, Dagogo-Jack S, Cryer P, Sheline Y, Luby J, Dagogo-Jack A, Alderson A (Memory improvement following induced hyperinsulinemia in Alzheimer's disease. Neurobiol Aging 17:123-130.1996).

Cunha RA ([Caffeine, adenosine receptors, memory and Alzheimer disease]. Med Clin (Barc) 131:790-795.2008).

Deak F, Shin OH, Tang J, Hanson P, Ubach J, Jahn R, Rizo J, Kavalali ET, Sudhof TC (Rabphilin regulates SNARE-dependent re-priming of synaptic vesicles for fusion. Embo J 25:2856-2866.2006).

Dheen ST, Tay SS, Wong WC (Arginine vasopressin- and oxytocin-like immunoreactive neurons in the hypothalamic paraventricular and supraoptic nuclei of streptozotocin-induced diabetic rats. Arch Histol Cytol 57:461-472.1994).

Di Luca M, Ruts L, Gardoni F, Cattabeni F, Biessels GJ, Gispen WH (NMDA receptor subunits are modified transcriptionally and post-translationally in the brain of streptozotocin-diabetic rats. Diabetologia 42:693-701.1999).

Dias WB, Hart GW (O-GlcNAc modification in diabetes and Alzheimer's disease. Mol Biosyst 3:766-772.2007).

Dong XX, Wang Y, Qin ZH (Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin 30:379-387.2009).

Dore S, Kar S, Rowe W, Quirion R (Distribution and levels of [125I]IGF-I, [125I]IGF-II and [125I]insulin receptor binding sites in the hippocampus of aged memory-unimpaired and -impaired rats. Neuroscience 80:1033-1040.1997).

Dou JT, Chen M, Dufour F, Alkon DL, Zhao WQ (Insulin receptor signaling in long-term memory consolidation following spatial learning. Learn Mem 12:646-655.2005).

Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M (Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci U S A 97:12222-12226.2000).

Duarte JM, Carvalho RA, Cunha RA, Gruetter R (Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. J Neurochem 111:368-379.2009).

Duarte JM, Oliveira CR, Ambrósio AF, Cunha RA (Modification of adenosine A1 and A2A receptor density in the hippocampus of streptozotocin-induced diabetic rats. Neurochem Int 48:144-150.2006).

Duarte JM, Oses JP, Rodrigues RJ, Cunha RA (Modification of purinergic signaling in the hippocampus of streptozotocin-induced diabetic rats. Neuroscience 149:382-391.2007).

Dulubova I, Lou X, Lu J, Huryeva I, Alam A, Schneggenburger R, Sudhof TC, Rizo J (A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity? Embo J 24:2839-2850.2005).

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC, Rizo J (A conformational switch in syntaxin during exocytosis: role of munc18. Embo J 18:4372-4382.1999).

ECDCDM (Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 26 Suppl 1:S5-20.2003).

Evans RM, Zamponi GW (Presynaptic Ca2+ channels--integration centers for neuronal signaling pathways. Trends Neurosci 29:617-624.2006).

Fdez E, Hilfiker S (Vesicle pools and synapsins: new insights into old enigmas. Brain Cell Biol 35:107-115.2006).

Ferguson SC, Blane A, Wardlaw J, Frier BM, Perros P, McCrimmon RJ, Deary IJ (Influence of an early-onset age of type 1 diabetes on cerebral structure and cognitive function. Diabetes Care 28:1431-1437.2005).

Fields RD (Oligodendrocytes changing the rules: action potentials in glia and oligodendrocytes controlling action potentials. Neuroscientist 14:540-543.2008).

Fischer von Mollard G, Stahl B, Li C, Sudhof TC, Jahn R (Rab proteins in regulated exocytosis. Trends Biochem Sci 19:164-168.1994).

Fischer von Mollard G, Sudhof TC, Jahn R (A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. Nature 349:79-81.1991).

Francis GJ, Martinez JA, Liu WQ, Xu K, Ayer A, Fine J, Tuor UI, Glazner G, Hanson LR, Frey WH, 2nd, Toth C (Intranasal insulin prevents cognitive decline, cerebral atrophy and white matter changes in murine type I diabetic encephalopathy. Brain 131:3311-3334.2008).

Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, Sudhof TC, Scheller RH, Takai Y (Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. J Biol Chem 271:7265-7268.1996).

Fujiwara T, Mishima T, Kofuji T, Chiba T, Tanaka K, Yamamoto A, Akagawa K (Analysis of knock-out mice to determine the role of HPC-1/syntaxin 1A in expressing synaptic plasticity. J Neurosci 26:5767-5776.2006).

Fukui K, Onodera K, Shinkai T, Suzuki S, Urano S (Impairment of learning and memory in rats caused by oxidative stress and aging, and changes in antioxidative defense systems. Ann N Y Acad Sci 928:168-175.2001).

Gagne J, Milot M, Gelinas S, Lahsaini A, Trudeau F, Martinoli MG, Massicotte G (Binding properties of glutamate receptors in streptozotocin-induced diabetes in rats. Diabetes 46:841-846.1997).

Gardoni F, Kamal A, Bellone C, Biessels GJ, Ramakers GM, Cattabeni F, Gispent WH, Di Luca M (Effects of streptozotocin-diabetes on the hippocampal NMDA receptor complex in rats. J Neurochem 80:438-447.2002).

Garner CC, Kindler S, Gundelfinger ED (Molecular determinants of presynaptic active zones. Curr Opin Neurobiol 10:321-327.2000).

Gaspar JM, Baptista FI, Galvao J, Castilho AF, Cunha RA, Ambrosio AF (Diabetes differentially affects the content of exocytotic proteins in hippocampal and retinal nerve terminals. Neuroscience 169:1589-1600.2010).

Gendron TF, Petrucelli L (The role of tau in neurodegeneration. Mol Neurodegener 4:13.2009).

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC (Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. Cell 79:717-727.1994).

Gillon KR, Hawthorne JN, Tomlinson DR (Myo-inositol and sorbitol metabolism in relation to peripheral nerve function in experimental diabetes in the rat: the effect of aldose reductase inhibition. Diabetologia 25:365-371.1983).

Gispen WH, Biessels GJ (Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 23:542-549.2000).

Giugliano D, Ceriello A, Paolisso G (Oxidative stress and diabetic vascular complications. Diabetes Care 19:257-267.1996).

Greene DA, Lattimer SA, Sima AA (Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. N Engl J Med 316:599-606.1987).

Grillo CA, Piroli GG, Wood GE, Reznikov LR, McEwen BS, Reagan LP (Immunocytochemical analysis of synaptic proteins provides new insights into diabetes-mediated plasticity in the rat hippocampus. Neuroscience 136:477-486.2005).

Guglielmotto M, Aragno M, Tamagno E, Vercellinatto I, Visentin S, Medana C, Catalano MG, Smith MA, Perry G, Danni O, Boccuzzi G, Tabaton M (AGEs/RAGE complex upregulates BACE1 via NF-kappaB pathway activation. Neurobiol Aging.2010).

Guven A, Yavuz O, Cam M, Comunoglu C, Sevi'nc O (Central nervous system complications of diabetes in streptozotocin-induced diabetic rats: a histopathological and immunohistochemical examination. Int J Neurosci 119:1155-1169.2009).

Guyot LL, Diaz FG, O'Regan MH, Song D, Phillis JW (The effect of streptozotocin-induced diabetes on the release of excitotoxic and other amino acids from the ischemic rat cerebral cortex. Neurosurgery 48:385-390; discussion 390-381.2001).

Hall KE, Liu J, Sima AA, Wiley JW (Impaired inhibitory G-protein function contributes to increased calcium currents in rats with diabetic neuropathy. J Neurophysiol 86:760-770.2001).

Hernandez-Fonseca JP, Rincon J, Pedreanez A, Viera N, Arcaya JL, Carrizo E, Mosquera J (Structural and ultrastructural analysis of cerebral cortex, cerebellum, and hypothalamus from diabetic rats. Exp Diabetes Res 2009:329632.2009).

Hof PR, Morrison JH (Neocortical neuronal subpopulations labeled by a monoclonal antibody to calbindin exhibit differential vulnerability in Alzheimer's disease. Exp Neurol 111:293-301.1991).

Huttner WB, Schiebler W, Greengard P, De Camilli P (Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. J Cell Biol 96:1374-1388.1983).

Iqbal K, Liu F, Gong CX, Alonso Adel C, Grundke-Iqbal I (Mechanisms of tau-induced neurodegeneration. Acta Neuropathol 118:53-69.2009).

Jackman N, Ishii A, Bansal R (Oligodendrocyte development and myelin biogenesis: parsing out the roles of glycosphingolipids. Physiology (Bethesda) 24:290-297.2009).

Jafari Anarkooli I, Sankian M, Ahmadpour S, Varasteh AR, Haghir H (Evaluation of Bcl-2 family gene expression and Caspase-3 activity in hippocampus STZ-induced diabetic rats. Exp Diabetes Res 2008:638467.2008).

Jafari Anarkooli I, Sankian M, Vahedi F, Bonakdaran S, Varasteh AR, Haghir H (Evaluation of insulin and ascorbic acid effects on expression of Bcl-2 family proteins and caspase-3 activity in hippocampus of STZ-induced diabetic rats. Cell Mol Neurobiol 29:133-140.2009).

Jahn R, Scheller RH (SNAREs--engines for membrane fusion. Nat Rev Mol Cell Biol 7:631-643.2006).

Jaken S (Protein kinase C isozymes and substrates. Curr Opin Cell Biol 8:168-173.1996).

Johnston D, Amaral DG (2004) Hippocampus. In: The synaptic organization of the brain(Shepherd, G. M., ed), pp xiv, 719 p. Oxford ; New York: Oxford University Press.

Kamal A, Biessels GJ, Gispen WH, Ramakers GM (Synaptic transmission changes in the pyramidal cells of the hippocampus in streptozotocin-induced diabetes mellitus in rats. Brain Res 1073-1074:276-280.2006).

Kamal A, Biessels GJ, Urban IJ, Gispen WH (Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression. Neuroscience 90:737-745.1999).

Kamal A, Ramakers GM, Biessels GJ, Gispen WH (Effects of a phorbol ester and cyclosporin A on hippocampal synaptic plasticity in streptozotocin-induced-diabetic rats: reduced sensitivity to phorbol esters. Neurosci Lett 339:45-48.2003).

Kavalali ET (SNARE interactions in membrane trafficking: a perspective from mammalian central synapses. Bioessays 24:926-936.2002).

Kim B, Backus C, Oh S, Hayes JM, Feldman EL (Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. Endocrinology 150:5294-5301.2009).

Kimelberg HK (Functions of mature mammalian astrocytes: a current view. Neuroscientist 16:79-106.2010).

Klein JP, Waxman SG (The brain in diabetes: molecular changes in neurons and their implications for end-organ damage. Lancet Neurol 2:548-554.2003).

Klenchin VA, Martin TF (Priming in exocytosis: attaining fusion-competence after vesicle docking. Biochimie 82:399-407.2000).

Kloda A, Clements JD, Lewis RJ, Adams DJ (Adenosine triphosphate acts as both a competitive antagonist and a positive allosteric modulator at recombinant N-methyl-D-aspartate receptors. Mol Pharmacol 65:1386-1396.2004).

Köfalvi A, Pereira MF, Rebola N, Rodrigues RJ, Oliveira CR, Cunha RA (Anandamide and NADA bi-directionally modulate presynaptic Ca2+ levels and transmitter release in the hippocampus. Br J Pharmacol 151:551-563.2007).

Kuhad A, Bishnoi M, Tiwari V, Chopra K (Suppression of NF-kappabeta signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits. Pharmacol Biochem Behav 92:251-259.2009).

Kuhad A, Chopra K (Effect of sesamol on diabetes-associated cognitive decline in rats. Exp Brain Res 185:411-420.2008).

Kumar JS, Menon VP (Effect of diabetes on levels of lipid peroxides and glycolipids in rat brain. Metabolism 42:1435-1439.1993).

Lambert KG, Gerecke KM, Quadros PS, Doudera E, Jasnow AM, Kinsley CH (Activity-stress increases density of GFAP-immunoreactive astrocytes in the rat hippocampus. Stress 3:275-284.2000).

Lawson LJ, Perry VH, Dri P, Gordon S (Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151-170.1990).

Lazarov O, Peterson LD, Peterson DA, Sisodia SS (Expression of a familial Alzheimer's disease-linked presenilin-1 variant enhances perforant pathway lesion-induced neuronal loss in the entorhinal cortex. J Neurosci 26:429-434.2006).

Lebed YV, Orlovsky MA, Nikonenko AG, Ushakova GA, Skibo GG (Early reaction of astroglial cells in rat hippocampus to streptozotocin-induced diabetes. Neurosci Lett 444:181-185.2008).

Lee PG, Cai F, Helke CJ (Streptozotocin-induced diabetes reduces retrograde axonal transport in the afferent and efferent vagus nerve. Brain Res 941:127-136.2002).

Leenders AG, Sheng ZH (Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. Pharmacol Ther 105:69-84.2005).

Leonard BE (Inflammation, depression and dementia: are they connected? Neurochem Res 32:1749-1756.2007).

Leveque C, el Far O, Martin-Moutot N, Sato K, Kato R, Takahashi M, Seagar MJ (Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. J Biol Chem 269:6306-6312.1994).

Levy J (Abnormal cell calcium homeostasis in type 2 diabetes mellitus: a new look on old disease. Endocrine 10:1-6.1999).

Levy J, Gavin JR, 3rd, Sowers JR (Diabetes mellitus: a disease of abnormal cellular calcium metabolism? Am J Med 96:260-273.1994).

Li PA, Shuaib A, Miyashita H, He QP, Siesjo BK, Warner DS (Hyperglycemia enhances extracellular glutamate accumulation in rats subjected to forebrain ischemia. Stroke 31:183-192.2000).

Li ZG, Zhang W, Grunberger G, Sima AA (Hippocampal neuronal apoptosis in type 1 diabetes. Brain Res 946:221-231.2002).

Li ZG, Zhang W, Sima AA (The role of impaired insulin/IGF action in primary diabetic encephalopathy. Brain Res 1037:12-24.2005).

Li ZG, Zhang W, Sima AA (Alzheimer-like Changes in Rat Models of Spontaneous Diabetes. Diabetes.2007).

Lin RC, Scheller RH (Mechanisms of synaptic vesicle exocytosis. Annu Rev Cell Dev Biol 16:19-49.2000).

Lopes da Silva FH, Arnolds DE (Physiology of the hippocampus and related structures. Annu Rev Physiol 40:185-216.1978).

Lynch MA (Long-term potentiation and memory. Physiol Rev 84:87-136.2004).

Magarinos AM, Jain K, Blount ED, Reagan L, Smith BH, McEwen BS (Peritoneal implantation of macroencapsulated porcine pancreatic islets in diabetic rats ameliorates severe hyperglycemia and prevents retraction and simplification of hippocampal dendrites. Brain Res 902:282-287.2001).

Magarinos AM, McEwen BS (Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. Proc Natl Acad Sci U S A 97:11056-11061.2000).

Malenka RC, Nicoll RA (Long-term potentiation--a decade of progress? Science 285:1870-1874.1999).

Malone JI, Hanna S, Saporta S, Mervis RF, Park CR, Chong L, Diamond DM (Hyperglycemia not hypoglycemia alters neuronal dendrites and impairs spatial memory. Pediatr Diabetes 9:531-539.2008).

Mandrekar-Colucci S, Landreth GE (Microglia and inflammation in Alzheimer's disease. CNS Neurol Disord Drug Targets 9:156-167.2010).

Manschot SM, Brands AM, van der Grond J, Kessels RP, Algra A, Kappelle LJ, Biessels GJ (Brain magnetic resonance imaging correlates of impaired cognition in patients with type 2 diabetes. Diabetes 55:1106-1113.2006).

Margeta MA, Shen K (Molecular mechanisms of synaptic specificity. Mol Cell Neurosci 43:261-267.2010).

Marks JL, Porte D, Jr., Stahl WL, Baskin DG (Localization of insulin receptor mRNA in rat brain by in situ hybridization. Endocrinology 127:3234-3236.1990).

Martínez-Tellez R, Gómez-Villalobosa MJ, G. F (Alteration in dendritic morphology of cortical neurons in rats with diabetes mellitus induced by streptozotocin. Brain Research 1048:108-115.2005).

Masson J, Sagne C, Hamon M, El Mestikawy S (Neurotransmitter transporters in the central nervous system. Pharmacol Rev 51:439-464.1999).

Mattson MP (Calcium and neurodegeneration. Aging Cell 6:337-350.2007).

Mayer JH, Tomlinson DR, McLean WG (Slow orthograde axonal transport of radiolabelled protein in sciatic motoneurones of rats with short-term experimental diabetes: effects of treatment with an aldose reductase inhibitor or myo-inositol. J Neurochem 43:1265-1270.1984).

McEwen BS, Magarinos AM, Reagan LP (Studies of hormone action in the hippocampal formation: possible relevance to depression and diabetes. J Psychosom Res 53:883-890.2002).

McGeer PL, McGeer EG (Inflammation, autotoxicity and Alzheimer disease. Neurobiol Aging 22:799-809.2001).

McGirr R, Ejbick CE, Carter DE, Andrews JD, Nie Y, Friedman TC, Dhanvantari S (Glucose dependence of the regulated secretory pathway in alphaTC1-6 cells. Endocrinology 146:4514-4523.2005).

Minghetti L, Ajmone-Cat MA, De Berardinis MA, De Simone R (Microglial activation in chronic neurodegenerative diseases: roles of apoptotic neurons and chronic stimulation. Brain Res Brain Res Rev 48:251-256.2005).

Misumi Y, Yamato T, Obata T, Aomine M (Effects of ion channel blockers on basal hippocampal monoamine levels in freely moving diabetic and non-diabetic rats. Int J Neurosci 118:761-780.2008).

Misura KM, Scheller RH, Weis WI (Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404:355-362.2000).

Miyata S, Yamada N, Hirano S, Tanaka S, Kamei J (Diabetes attenuates psychological stress-elicited 5-HT secretion in the prefrontal cortex but not in the amygdala of mice. Brain Res 1147:233-239.2007).

Mooradian AD (The antioxidative potential of cerebral microvessels in experimental diabetes mellitus. Brain Res 671:164-169.1995).

Mormede P, Andanson S, Auperin B, Beerda B, Guemene D, Malmkvist J, Manteca X, Manteuffel G, Prunet P, van Reenen CG, Richard S, Veissier I (Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol Behav 92:317-339.2007).

Morris MJ, Pavia JM (Increased endogenous noradrenaline and neuropeptide Y release from the hypothalamus of streptozotocin diabetic rats. Brain Res 1006:100-106.2004).

Newman EA (New roles for astrocytes: regulation of synaptic transmission. Trends Neurosci 26:536-542.2003).

Nimmerjahn A, Kirchhoff F, Helmchen F (Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.2005).

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M (Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787-790.2000).

Ortinau S, Laube B, Zimmermann H (ATP inhibits NMDA receptors after heterologous expression and in cultured hippocampal neurons and attenuates NMDA-mediated neurotoxicity. J Neurosci 23:4996-5003.2003).

Ostenson CG, Gaisano H, Sheu L, Tibell A, Bartfai T (Impaired gene and protein expression of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in pancreatic islets of type 2 diabetic patients. Diabetes 55:435-440.2006).

Pekiner C, Cullum NA, Hughes JN, Hargreaves AJ, Mahon J, Casson IF, McLean WG (Glycation of brain actin in experimental diabetes. J Neurochem 61:436-442.1993).

Pekny M, Pekna M (Astrocyte intermediate filaments in CNS pathologies and regeneration. J Pathol 204:428-437.2004).

Perry VH, Nicoll JA, Holmes C (Microglia in neurodegenerative disease. Nat Rev Neurol 6:193-201.2010).

Phillips MJ, Otteson DC, Sherry DM (Progression of neuronal and synaptic remodeling in the rd10 mouse model of retinitis pigmentosa. J Comp Neurol 518:2071-2089.2010).

Piehl F, Lidman O (Neuroinflammation in the rat--CNS cells and their role in the regulation of immune reactions. Immunol Rev 184:212-225.2001).

Popovic M, Biessels GJ, Isaacson RL, Gispen WH (Learning and memory in streptozotocin-induced diabetic rats in a novel spatial/object discrimination task. Behav Brain Res 122:201-207.2001).

Purves D (2004a) Plasticity of Mature Synapses and Circuits. In: Neuroscience (Purves, D. et al., eds) Sunderland: Sinauer Associates.

Purves D (2004b) Synaptic transmission. In: Neuroscience(Purves, D. et al., eds) Sunderland: Sinauer Associates.

Rabbani N, Thornalley PJ (Dicarbonyls linked to damage in the powerhouse: glycation of mitochondrial proteins and oxidative stress. Biochem Soc Trans 36:1045-1050.2008).

Rackova L, Snirc V, Jung T, Stefek M, Karasu C, Grune T (Metabolism-induced oxidative stress is a mediator of glucose toxicity in HT22 neuronal cells. Free Radic Res 43:876-886.2009).

Ramakrishnan R, Kempuraj D, Prabhakaran K, Jayakumar AR, Devi RS, Suthanthirarajan N, Namasivayam A (A short-term diabetes induced changes of catecholamines and p38-MAPK in discrete areas of rat brain. Life Sci 77:1825-1835.2005).

Ramakrishnan R, Sheeladevi R, Namasivayam A (Regulation of protein kinases and coregulatory interplay of S-100beta and serotonin transporter on serotonin levels in diabetic rat brain. J Neurosci Res 87:246-259.2009).

Ramakrishnan R, Sheeladevi R, Suthanthirarajan N (PKC-alpha mediated alterations of indoleamine contents in diabetic rat brain. Brain Res Bull 64:189-194.2004).

Reagan LP, Grillo CA, Piroli GG (The As and Ds of stress: metabolic, morphological and behavioral consequences. Eur J Pharmacol 585:64-75.2008).

Reagan LP, Magarinos AM, McEwen BS (Neurological changes induced by stress in streptozotocin diabetic rats. Ann N Y Acad Sci 893:126-137.1999).

Reger MA, Watson GS, Green PS, Baker LD, Cholerton B, Fishel MA, Plymate SR, Cherrier MM, Schellenberg GD, Frey WH, 2nd, Craft S (Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults. J Alzheimers Dis 13:323-331.2008).

Reisi P, Alaei H, Babri S, Sharifi MR, Mohaddes G, Soleimannejad E (Determination of the extracellular basal levels of glutamate and GABA at dentate gyrus of streptozotocin-induced diabetic rats. Pathophysiology.2009).

Richerson GB, Wu Y (Dynamic equilibrium of neurotransmitter transporters: not just for reuptake anymore. J Neurophysiol 90:1363-1374.2003).

Richmond J (Synaptic function. WormBook 1-14.2005).

Richmond JE, Weimer RM, Jorgensen EM (An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature 412:338-341.2001).

Rizzoli SO, Betz WJ (The structural organization of the readily releasable pool of synaptic vesicles. Science 303:2037-2039.2004).

Rizzoli SO, Betz WJ (Synaptic vesicle pools. Nat Rev Neurosci 6:57-69.2005).

Rodrigues RJ, Alfaro TM, Rebola N, Oliveira CR, Cunha RA (Co-localization and functional interaction between adenosine A(2A) and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum. J Neurochem 92:433-441.2005).

Roriz-Filho JS, Sa-Roriz TM, Rosset I, Camozzato AL, Santos AC, Chaves ML, Moriguti JC, Roriz-Cruz M ((Pre)diabetes, brain aging, and cognition. Biochim Biophys Acta 1792:432-443.2009).

Rosenmund C, Stevens CF (Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16:1197-1207.1996).

Rothwell NJ, Luheshi GN (Interleukin 1 in the brain: biology, pathology and therapeutic target. Trends Neurosci 23:618-625.2000).

Ryan CM (Neurobehavioral complications of type I diabetes. Examination of possible risk factors. Diabetes Care 11:86-93.1988).

Ryan CM, Freed MI, Rood JA, Cobitz AR, Waterhouse BR, Strachan MW (Improving metabolic control leads to better working memory in adults with type 2 diabetes. Diabetes Care 29:345-351.2006).

Sandi C (Stress, cognitive impairment and cell adhesion molecules. Nat Rev Neurosci 5:917-930.2004).

Santiago AR, Cristovao AJ, Santos PF, Carvalho CM, Ambrósio AF (High glucose induces caspase-independent cell death in retinal neural cells. Neurobiol Dis 25:464-472.2007).

Santiago AR, Pereira TS, Garrido MJ, Cristovao AJ, Santos PF, Ambrósio AF (High glucose and diabetes increase the release of [3H]-D-aspartate in retinal cell cultures and in rat retinas. Neurochem Int 48:453-458.2006a).

Santiago AR, Rosa SC, Santos PF, Cristovao AJ, Barber AJ, Ambrósio AF (Elevated glucose changes the expression of ionotropic glutamate receptor subunits and impairs calcium homeostasis in retinal neural cells. Invest Ophthalmol Vis Sci 47:4130-4137.2006b).

Saravia FE, Gonzalez SL, Roig P, Alves V, Homo-Delarche F, De Nicola AF (Diabetes increases the expression of hypothalamic neuropeptides in a spontaneous model of type I diabetes, the nonobese diabetic (NOD) mouse. Cell Mol Neurobiol 21:15-27.2001).

Saravia FE, Revsin Y, Gonzalez Deniselle MC, Gonzalez SL, Roig P, Lima A, Homo-Delarche F, De Nicola AF (Increased astrocyte reactivity in the hippocampus of murine models of type 1 diabetes: the nonobese diabetic (NOD) and streptozotocin-treated mice. Brain Res 957:345-353.2002).

Satoh E, Takahashi A (Experimental diabetes enhances Ca(2+) mobilization and glutamate exocytosis in cerebral synaptosomes from mice. Diabetes Res Clin Pract.2008).

Schmidt AM, Yan SD, Wautier JL, Stern D (Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ Res 84:489-497.1999).

Schoch S, Gundelfinger ED (Molecular organization of the presynaptic active zone. Cell Tissue Res 326:379-391.2006).

Seagar M, Leveque C, Charvin N, Marqueze B, Martin-Moutot N, Boudier JA, Boudier JL, Shoji-Kasai Y, Sato K, Takahashi M (Interactions between proteins implicated in exocytosis and voltage-gated calcium channels. Philos Trans R Soc Lond B Biol Sci 354:289-297.1999).

Sharifi AM, Eslami H, Larijani B, Davoodi J (Involvement of caspase-8, -9, and -3 in high glucose-induced apoptosis in PC12 cells. Neurosci Lett 459:47-51.2009).

Shen J, Rathore SS, Khandan L, Rothman JE (SNARE bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of membrane fusion. J Cell Biol 190:55-63.2010).

Sheng ZH, Rettig J, Takahashi M, Catterall WA (Identification of a syntaxin-binding site on N-type calcium channels. Neuron 13:1303-1313.1994).

Sima AA, Kamiya H, Li ZG (Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes. Eur J Pharmacol 490:187-197.2004).

Sima AA, Li ZG (The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. Diabetes 54:1497-1505.2005).

Sima AA, Zhang W, Kreipke CW, Rafols JA, Hoffman WH (Inflammation in Diabetic Encephalopathy is Prevented by C-Peptide. Rev Diabet Stud 6:37-42.2009).

Somanath S, Barg S, Marshall C, Silwood CJ, Turner MD (High extracellular glucose inhibits exocytosis through disruption of syntaxin 1A-containing lipid rafts. Biochem Biophys Res Commun 389:241-246.2009).

Somfai GM, Knippel B, Ruzicska E, Stadler K, Toth M, Salacz G, Magyar K, Somogyi A (Soluble semicarbazide-sensitive amine oxidase (SSAO) activity is related to oxidative stress and subchronic inflammation in streptozotocin-induced diabetic rats. Neurochem Int 48:746-752.2006).

Sorensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, Neher E (Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. Cell 114:75-86.2003).

Sorensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, Grubmuller H, Fasshauer D (Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. Embo J 25:955-966.2006).

Stahl B, Chou JH, Li C, Sudhof TC, Jahn R (Rab3 reversibly recruits rabphilin to synaptic vesicles by a mechanism analogous to raf recruitment by ras. Embo J 15:1799-1809.1996).

Stefanis L, Burke RE, Greene LA (Apoptosis in neurodegenerative disorders. Curr Opin Neurol 10:299-305.1997).

Stevens CF, Sullivan JM (Regulation of the readily releasable vesicle pool by protein kinase C. Neuron 21:885-893.1998).

Stranahan AM, Arumugam TV, Cutler RG, Lee K, Egan JM, Mattson MP (Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nat Neurosci 11:309-317.2008a).

Stranahan AM, Lee K, Pistell PJ, Nelson CM, Readal N, Miller MG, Spangler EL, Ingram DK, Mattson MP (Accelerated cognitive aging in diabetic rats is prevented by lowering corticosterone levels. Neurobiol Learn Mem 90:479-483.2008b).

Sudhof TC (Function of Rab3 GDP-GTP exchange. Neuron 18:519-522.1997).

Sudhof TC (The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.2004).

Sudhof TC (Neurotransmitter release. Handb Exp Pharmacol 1-21.2008).

Sugimoto K, Yasujima M, Yagihashi S (Role of advanced glycation end products in diabetic neuropathy. Curr Pharm Des 14:953-961.2008).

Sun MK, Alkon DL (Protein kinase C activators as synaptogenic and memory therapeutics. Arch Pharm (Weinheim) 342:689-698.2009).

Sutton RB, Fasshauer D, Jahn R, Brunger AT (Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395:347-353.1998).

Takei Y, Harada A, Takeda S, Kobayashi K, Terada S, Noda T, Takahashi T, Hirokawa N (Synapsin I deficiency results in the structural change in the presynaptic terminals in the murine nervous system. J Cell Biol 131:1789-1800.1995).

tom Dieck S, Sanmarti-Vila L, Langnaese K, Richter K, Kindler S, Soyke A, Wex H, Smalla KH, Kampf U, Franzer JT, Stumm M, Garner CC, Gundelfinger ED (Bassoon, a novel zinc-

finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. J Cell Biol 142:499-509.1998).

Tomlinson DR, Gardiner NJ (Glucose neurotoxicity. Nat Rev Neurosci 9:36-45.2008).

Trudeau F, Gagnon S, Massicotte G (Hippocampal synaptic plasticity and glutamate receptor regulation: influences of diabetes mellitus. Eur J Pharmacol 490:177-186.2004).

Tuzcu M, Baydas G (Effect of melatonin and vitamin E on diabetes-induced learning and memory impairment in rats. Eur J Pharmacol 537:106-110.2006).

Valente T, Gella A, Fernandez-Busquets X, Unzeta M, Durany N (Immunohistochemical analysis of human brain suggests pathological synergism of Alzheimer's disease and diabetes mellitus. Neurobiol Dis 37:67-76.2010).

Valtorta F, Benfenati F, Greengard P (Structure and function of the synapsins. J Biol Chem 267:7195-7198.1992).

Vanderklish P, Neve R, Bahr BA, Arai A, Hennegriff M, Larson J, Lynch G (Translational suppression of a glutamate receptor subunit impairs long-term potentiation. Synapse 12:333-337.1992).

Voitenko NV, Kruglikov IA, Kostyuk EP, Kostyuk PG (Effect of streptozotocin-induced diabetes on the activity of calcium channels in rat dorsal horn neurons. Neuroscience 95:519-524.2000).

Vanguilder HD, Brucklacher RM, Patel K, Ellis RW, Freeman WM, Barber AJ (Diabetes downregulates presynaptic proteins and reduces basal synapsin I phosphorylation in rat retina. Eur J Neurosci 28:1-11.2008).

Vemuganti R (Decreased expression of vesicular GABA transporter, but not vesicular glutamate, acetylcholine and monoamine transporters in rat brain following focal ischemia. Neurochem Int 47:136-142.2005).

Wang CF, Li DQ, Xue HY, Hu B (Oral supplementation of catalpol ameliorates diabetic encephalopathy in rats. Brain Res 1307:158-165.2010).

Wang SH, Sun ZL, Guo YJ, Yuan Y, Yang BQ (Diabetes impairs hippocampal function via advanced glycation end product mediated new neuron generation in animals with diabetes-related depression. Toxicol Sci 111:72-79.2009).

Wang X, Kibschull M, Laue MM, Lichte B, Petrasch-Parwez E, Kilimann MW (Aczonin, a 550-kD putative scaffolding protein of presynaptic active zones, shares homology regions with Rim and Bassoon and binds profilin. J Cell Biol 147:151-162.1999).

Westenbroek RE, Sakurai T, Elliott EM, Hell JW, Starr TV, Snutch TP, Catterall WA (Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. J Neurosci 15:6403-6418.1995).

Wild S, Roglic G, Green A, Sicree R, King H (Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047-1053.2004).

Williams SK, Howarth NL, Devenny JJ, Bitensky MW (Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. Proc Natl Acad Sci U S A 79:6546-6550.1982).

Wiser O, Bennett MK, Atlas D (Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca2+ channels. Embo J 15:4100-4110.1996).

Wrighten SA, Piroli GG, Grillo CA, Reagan LP (A look inside the diabetic brain: Contributors to diabetes-induced brain aging. Biochim Biophys Acta 1792:444-453.2009).

Yamagishi S, Imaizumi T (Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 11:2279-2299.2005).

Yamato T, Misumi Y, Yamasaki S, Kino M, Aomine M (Diabetes mellitus decreases hippocampal release of neurotransmitters: an in vivo microdialysis study of awake, freely moving rats. Diabetes Nutr Metab 17:128-136.2004).

Yeung CM, Lo AC, Cheung AK, Chung SS, Wong D, Chung SK (More severe type 2 diabetes-associated ischemic stroke injury is alleviated in aldose reductase-deficient mice. J Neurosci Res 88:2026-2034.2010).

Yusaf SP, Goodman J, Gonzalez IM, Bramwell S, Pinnock RD, Dixon AK, Lee K (Streptozocin-induced neuropathy is associated with altered expression of voltage-gated calcium channel subunit mRNAs in rat dorsal root ganglion neurones. Biochem Biophys Res Commun 289:402-406.2001).

Zamponi GW (Regulation of presynaptic calcium channels by synaptic proteins. J Pharmacol Sci 92:79-83.2003).

Zhai RG, Bellen HJ (The architecture of the active zone in the presynaptic nerve terminal. Physiology (Bethesda) 19:262-270.2004).

Zhang L, Ino-ue M, Dong K, Yamamoto M (Retrograde axonal transport impairment of large- and medium-sized retinal ganglion cells in diabetic rat. Curr Eye Res 20:131-136.2000).

Zhang T, Pan BS, Sun GC, Sun X, Sun FY (Diabetes synergistically exacerbates poststroke dementia and tau abnormality in brain. Neurochem Int 56:955-961.2010).

Zhang W, Khan A, Ostenson CG, Berggren PO, Efendic S, Meister B (Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. Biochem Biophys Res Commun 291:1038-1044.2002).

Zhao WQ, Alkon DL (Role of insulin and insulin receptor in learning and memory. Mol Cell Endocrinol 177:125-134.2001).

Zhu MY, Wang WP, Huang J, Regunathan S (Chronic treatment with glucocorticoids alters rat hippocampal and prefrontal cortical morphology in parallel with endogenous agmatine and arginine decarboxylase levels. J Neurochem 103:1811-1820.2007).

Zimmet P, Alberti KG, Shaw J (Global and societal implications of the diabetes epidemic. Nature 414:782-787.2001).