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Synaptic localization of the Amyloid Precursor Protein in the rat hippocampus

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Paula Agostinho (Universidade de Coimbra, Faculdade de Medicina) e do Doutor Rodrigo Cunha (Universidade de Coimbra, Faculdade de Medicina) , com supervisão institucional do Doutor Ângelo Tomé (Universidade de Coimbra, Faculdade de Ciências e Tecnologia).

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Abbreviation list

Aβ	β- amyloid peptide
AD	Alzheimer´s disease
APP	Amyloid precursor protein
APLP1	Amyloid precursor-like protein 1
APLP2	Amyloid precursor-like protein 2
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CLAP	Cocktail of proteases inhibitors
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ECF	Enhanced chemifluorescence
EDTA	Ethylenediaminetetraacetic acid
GFAP	Glial fibrillary acidic protein
HBM	HEPES buffered medium
IB	Isolation buffer
KPI	Kunitz serinic protease inhibitor
NFT	Neurofibrillary tangles
NHS	Normal horse serum
PFA	Paraformaldehyde
PBS	Phosphate buffer saline
PVDF	Polyvinilidene fluoride
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	Standard error of the mean
TGN	Trans-Golgi-network
TEMED	Tris(hydroxymethyl)aminomethane
TBS	Trizma buffered saline
TBS-T	Trizma buffered saline with tween
vAChT	Vesicular acetylcholine transporter
vGAT	Vesicular GABA transporter
vGLUT1	Vesicular glutamate transporter 1

Abstract

Amyloid precursor protein (APP) is a transmembrane protein that is highly expressed in the brain. This protein is highly trafficked and processed in the neurons and its cleavage by β- and γ-secretases results in the production of β-amyloid, an important peptide in the pathophysiology of Alzheimer's disease (AD). Although the real functions of APP are not known, many synaptic functions have been linked to it, such as synaptogenesis and regulation of pre-synaptic morphology. The synaptic presence of APP is not well defined, as is a possible differential distribution between different types of nerve terminals. The objectives of this study were to evaluate whether APP is enriched in synaptosomes, and if it is preferentially located in pre-synaptic, post-synaptic or extra-synaptic fractions of the synapse, as well as to determine if APP is differentially present in glutamatergic, GABAergic and cholinergic terminals in the rat hippocampus.

Comparing the immunoreactivity of APP in synaptosomes and total membranes, we observed that this protein was not enriched in synaptosomes; however, it is present in significant amount in nerve terminals. Using a procedure of synaptic fractioning that allowed us to separate the pre-, post- and non-synaptic fractions, with a good degree of confidence, we observed that APP is principally localized in the pre-synaptic fraction, and that a small part of this protein was present in the post-synaptic fraction. These results are in accordance with the possible synaptic functions of APP and with the current knowledge of the traffic of this protein that is constitutively released in the synapse. Using immunocytochemistry in a preparation of purified nerve terminals (enriched in pre-synaptic fractions) from rat hippocampus, we observed that APP was more located in terminals positives for a glutamatergic marker, than in terminals positives for GABAergic or cholinergic markers. We evaluated if some

of the non-synaptic APP was present in astrocytes or in microglia, and did not find any evidences of its presence in these glial cells.

Overall, this study shows that APP is present in hippocampal synapses, mainly in the pre-synaptic compartment. This could justify some of the synaptic functions of APP, and this “pool” of synaptic APP could also be involved in the production of β-amyloid peptide (Aβ) at the synapse. The accumulation of Aβ peptide at the synapses might contribute to the synaptotoxicity that occurs in early phases of AD, and is believed to contribute to the cognitive deficits associated with this neurodegenerative disorder.

Resumo

A proteína precursora amilóide (APP) é uma proteína transmembranar bastante expressa no cérebro. Esta proteína é altamente processada e transportada nos neurónios e é clivada por β- e γ- secretases resultando na produção de β-amilóide, um péptido importante na patofisiologia da doença de Alzheimer (AD). Embora as verdadeiras funções da APP não estejam ainda esclarecidas, várias funções sinápticas têm sido atribuídas a esta proteína, como sinaptogénese e regulação da morfologia pré-sináptica. A presença sináptica da APP não está bem definida, e não é conhecido se a esta proteína está distribuída de maneira diferente entre os diversos tipos de terminais nervosos. Os objectivos deste estudo foram avaliar se a APP está enriquecida em sinaptossomas, se está preferencialmente localizada nas suas fracções pré-sinápticas, pós-sinápticas ou não sinápticas, e determinar se a APP está igualmente localizada em terminais glutamatérgicos, GABAérgicos e colinérgicos no hipocampo de rato.

Comparando a imunorreactividade da APP em sinaptossomas e membranas totais, observamos que esta proteína não se encontra enriquecida em sinaptossomas; no entanto ela existe em quantidade considerável nos terminais nervosos. Usando um procedimento de fraccionamento sináptico que nos permite separar fracções pré-, pós- e extra- sinápticas com um bom grau de pureza, observámos que a APP está principalmente localizada na fracção pré-sináptica. Estes resultados estão de acordo com as possíveis funções sinápticas da APP e com o actual conhecimento do transporte desta proteína, que é constitutivamente libertada na sinapse. Através de técnicas de imunocitoquímca numa preparação de terminais nervosos purificados (enriquecidos em fracções pré-sinápticas) de hipocampo de rato, observámos que a APP está mais presente em terminais nervosos glutamatérgicos, do que em terminais GABAérgicos e colinérgicos. Avaliámos também se alguma parte do “pool” da APP

não sináptica estava presente em astrócitos ou células de microglia, e não encontrámos evidências da presença desta proteína nestas células gliais.

No geral, este estudo mostra que a APP está presente em sinapses de hipocampo, principalmente no compartimento pré-sináptico, o que pode justificar algumas das funções sinápticas da APP. Esta fracção sináptica de APP pode também estar envolvida na produção de péptido β-amilóide (Aβ) nas sinapses. A acumulação de péptido Aβ nas sinapses pode contribuir para a sinaptotoxicidade que ocorre nas fases iniciais da AD, e que se pensa contribuir para os défices cognitivos observados nesta doença neurodegenerativa.

1. Introduction

1. Introduction

1.1. What is Amyloid Precursor Protein and why is it important?

Amyloid Precursor Protein (APP) is a ubiquitous protein in the body that is expressed throughout the development (Turner et al., 2003). It is an extremely complex molecule that may be functionally important in its full-length configuration, and is also the source of numerous fragments with varying effects on neuronal function. However the exact functions of this protein are unknown and may include be a receptor or a trophic factor. Involvement in synaptogenesis, protein traffic control and cell adhesion are also suggested functions of this protein (Turner et al., 2003; O'Brien and Wong, 2011). Being mostly known for giving origin to a very important peptide in Alzheimer's disease (the β-amyloid peptide – Aβ), it is also studied for its functions as full length protein and for the functions of other peptides that arise from its cleavage (Turner et al., 2003; O'Brien and Wong, 2011; Zhang et al., 2011). For example, a cleavage fragment of APP is augmented in patients with severe forms of autism (Ray et al., 2011). Outside the brain, APP has been found to be enhanced in some malignancies, like prostate and thyroid cancer (Hansel et al., 2003; Krause et al., 2008; Takayama et al., 2009). In these diseases the actions of this protein and its cleavage products may contribute to an increase of trophic conditions that leads to an overgrowth (Hansel et al., 2003; Ray et al., 2011).

Independently of the functions of APP, immunohistochemistry of APP is widely used for detecting diffuse traumatic axonal injury. This protein travels from the neuronal cell body to the axonal periphery via a fast transport mechanism, and if the axon is disrupted, APP accumulates at the point of injury (Reichard et al., 2005).

Although the specific functions of this protein are not known, there are evidences pointing out to an important role of APP, sustaining the importance of further

studies about this protein (O'Brien and Wong, 2011; Zhang et al., 2011; Zhou et al., 2011).

1.2. Cell biology of APP

APP is a type I transmembrane protein of approximately 120 kDa, ubiquitously expressed in mammalian cells (Selkoe et al., 1988; Tanaka et al., 1989; Sisodia and Price, 1995; Turner et al., 2003), that possesses a large aminic extracellular domain (N-terminal) and a short intracellular carboxylic terminal (C-terminal). In the extracellular domain this protein has one cysteine rich subdomain, close to the N-terminal, followed by an acidic subdomain and two others subdomains, one of whom is thought to have a neuroprotective role (Figure 1). This protein also displays subdomains that bind to heparin, copper, zinc, and collagen. The neurotrophic RERMS (APP 328-332 pentapeptide) sequence and adhesion related RHDS sequence are found in two of the domains binding to heparin (Turner et al., 2003).

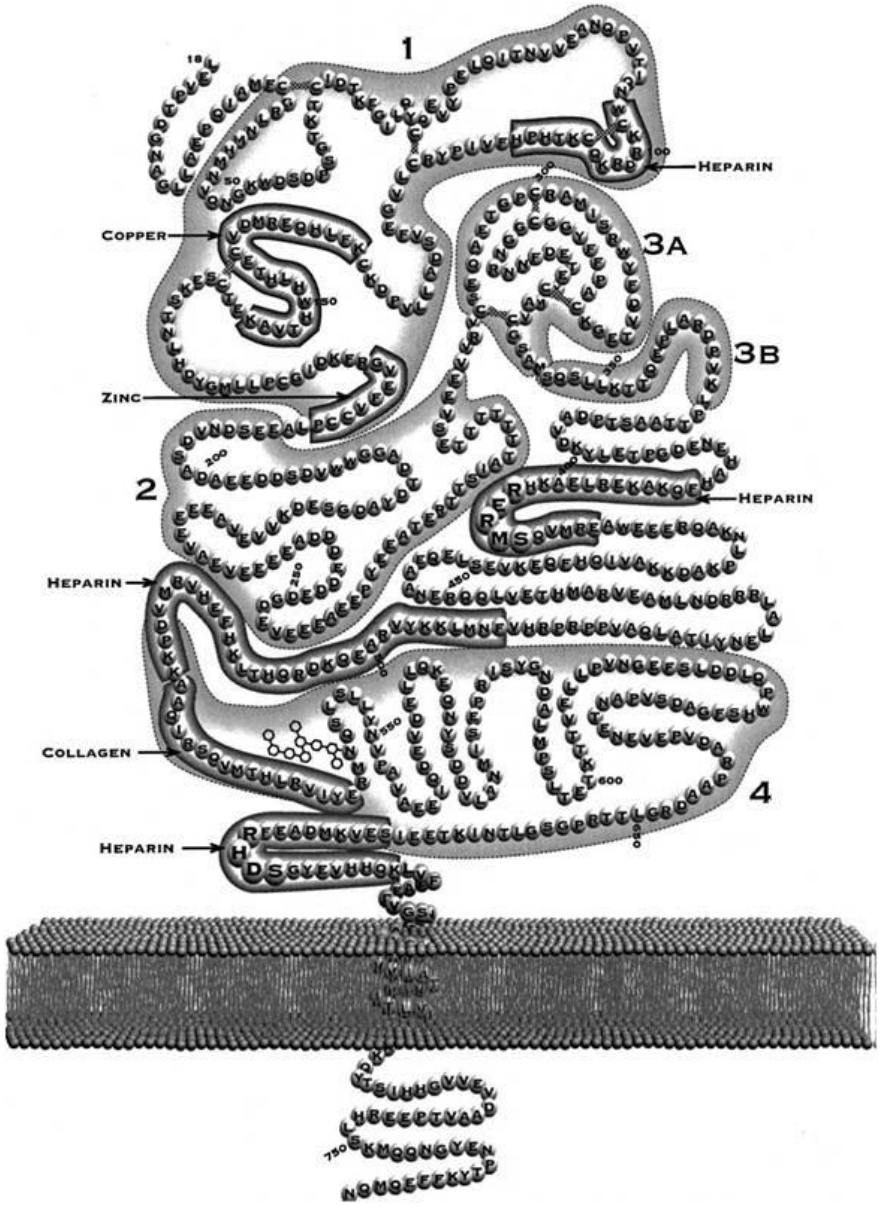

Figure 1: Schematic representation of APP770. The extracellular domains identified are: (1) Cysteine-rich domain, (2) anionic domain, (3A) exon 7/ kunitz protease inhibitor domain, (3B) exon 8, (4) neuroprotective domain. Heparin, copper, zinc and collagen binding domains are also indicated in the figure. Neurotrophic RERMS sequence and the adhesion related RHDS sequence are shown as larger spheres in two of the heparin binding domains. Adapted from Turner et al*,* 2003.

The human gene of APP was identified in 1987, by many independent authors, and it is located in chromosome 21 (Thinakaran and Koo, 2008). The gene possesses

18 exons (Hattori et al., 1997) and alternate splicing of its transcript generates eight isoforms of which three are most common, namely APP770, APP751 and APP695 (Bayer et al., 1999). The isoform APP695 lacks an extracellular domain of 56 amino acids named Kunitz Protease Inhibitor (KPI), whose functional relevance is not completely known (Ponte et al., 1988; Turner et al., 2003).

In the brain the predominant isoform produced is APP695. In cerebral cortex the ratio for mRNA levels of APP770:751:695 is 1:10:20 (Tanaka et al., 1989). However the mRNA of APP695 is reduced and the mRNA of APP 770 is augmented in Alzheimer´s disease (AD), suggesting that the differential expression of APP isoforms that have the KPI domain could play a role in the pathogenesis of this neurodegenerative disease (Rockenstein et al., 1995; Preece et al., 2004). Some studies showed that the expression of APP 695 is higher in adult than in old rodents, and in these last ones the quantity of mRNA of total APP (all isoforms) is higher than in the adult ones, in both sexes (Thakur and Mani, 2005; Sivanandam and Thakur, 2010). The expression of APP´s mRNA is regulated by sex steroids, which suggests that this protein could be present in different amounts in males and females (Thakur and Mani, 2005; Sivanandam and Thakur, 2010).

The gene that codifies APP belongs to a small family of evolutionary conserved genes, that include APLP1 and APLP2 (in mammals), Appl (in *Drosophila*) and apl-1 in *Caenorhabditis elegans*), but only the APP gene contains the sequence that encodes the Aβ domain (Thinakaran and Koo, 2008; O'Brien and Wong, 2011). APP and APLPs belongs to one group of transmembrane proteins that includes the Notch receptor and Sortilins triage receptors (SorCS1B and SorLA), which participates in highly conserved processing pathways, like development processes and cell bounding (Brunkan and Goate, 2005; Nyborg et al., 2006). APLP1 and APLP2 seem to work in a similar way to APP in many biologic pathways (Heber et al., 2000).

A recent study reports a different location of APP, APLP1 and APLP2 at the cellular level. The APLP1 is mainly located in the cellular surface, while APP and APLP2 are principally present in intracellular compartments (Kaden et al., 2009). Although it was thought that APP and APLPs exist in the form of monomers, recent evidences from biochemical and structural analyses have shown the existence of APP molecules as dimers and as more complex oligomers (Wang and Ha, 2004; Chen et al., 2006). It was also observed that APP dimerization influences its processing, and this could prevent its cleavage by secretases (Kaden et al., 2008; Kaden et al., 2011).

Moreover, the dimerization and different cellular location of APLPs could “mask” APP characteristics, and some of the APP antibodies used in various studies can also bind to APLPs, making the study of the location, function and processing of APP rather difficult.

1.3. Cellular location of APP

APP is highly expressed in the brain. This protein can be found in many membranous structures in the cell, like the Endoplasmatic Reticulum (ER), Golgi compartments, early endosomes, and cell membrane (Turner et al., 2003). The location of APP in biosynthetic organelles is partly explained by the very high rate of synthesis and turnover of this protein (t ½ =1 hour). Immature APP is localized exclusively at the ER, and only the mature APP that has been N- and O-glycosylated leaves the ER/Golgi compartments (Tomita et al., 1998). In the brain, APP is mainly present in the olfactory bulb, cerebral cortex, globus pallidus, cerebellum and hippocampus (Bendotti et al., 1988; Card et al., 1988; Shivers et al., 1988).

In the last decade APP has been found in dendrites, and mainly, in the cellular body and axons of cultured neurons of hippocampus and cortex from rat brain

(Schubert et al., 1991; Shigematsu et al., 1992; Ferreira et al., 1993; Allinquant et al., 1994; Bouillot et al., 1996). Moreover, the presence of APP was also shown in synaptic membranes preparations of rat brain (Huber et al., 1997; Kirazov et al., 2001; Groemer et al., 2011). Using different experimental approaches, including immunofluorescence and electron microscopy, the presence of APP was shown in neuromuscular junctions (Schubert et al., 1991; Shigematsu et al., 1992; Akaaboune et al., 2000; Wang et al., 2007), as well as in synaptosomes from rodent`s brain (Caputi et al., 1997; Huber et al., 1997; Huber et al., 1999; Sabo et al., 2003). This protein was found in post-synaptic fractions, where it co-immunoprecipitates with NMDA type glutamate receptors (Shigematsu et al., 1992; Hoe et al., 2009); as well as in pre-synaptic fractions and in synaptic vesicles of human and rodent brain tissues (Sabo et al., 2003; Groemer et al., 2011).

Furthermore, APP is enriched in cell adhesion sites, in the proximity of proteins such as β-integrins (Storey et al., 1999), and it is also present in growing cones in primary cultures of hippocampal neurons (Ferreira et al., 1993; Sabo et al., 2003). At the ontogenic level, it was observed that APP levels are augmented during synaptogenesis, and that the levels of this protein are higher in nerve terminals during the firsts post-natal days in rodents (Kirazov et al., 2001). There is some evidence that APP can be located in glial cells, namely in astrocytes (von Bernhardi et al., 2003; Marksteiner and Humpel, 2008; Schmidt et al., 2008).

Although APP is generally thought as a synaptic protein, its presence in this structure, a possible enrichment in some synaptic fractions and its distribution in different regions of the brain still remain to be clearly defined. It is also unclear if the location and density of APP are affected in the initial phases of AD.

1.4. APP processing and trafficking

APP can be extensively processed by glycosylation and by specific proteolytic cleavage. During its transportation from ER to plasmatic membrane, APP suffers post-translational modifications, such as N- and O-glycosylation, tyrosine sulfation and phosphorylation in its cytoplasmatic domain and ectodomain (Turner et al., 2003; Thinakaran and Koo, 2008). Concerning proteolytic cleavage, there are two cleavage sites near the plasma membrane and one in the transmembrane domain of APP, named α, β and γ kcleavage sites. The responsible enzymes for the proteolysis of each one of them are named α-, β- and γ- secretases, respectively. APP is first cleaved in the extracellular domain by α- and β- secretases (two mutually exclusive events) resulting in the splitting of one large extracellular domain from the C-terminal fragments, sAPPα or sAPPβ depending on the secretase, and then is cleaved in the transmembrane domain by γ- secretase, releasing C-terminal fragments. The processing pathway of APP that involves the cleavage by α- and γ- secretases is designated non-amyloidogenic pathway, resulting in the release to the extracellular medium of sAPPα and a peptide fragment, p3 (3 kDa). The other APP processing pathway it's the amyloidogenic one, involving the cleavage by β- and γ- secretases and generates the sAPPβ fragment and the Aβ1-42 (4 kDa) peptide, which is the predominant fragment. Besides these peptides, the APP cleavage by γ- secretases can also generate a cytoplasmatic polypeptide, named as APP intracellular domain or AICD (Turner et al., 2003; Thinakaran and Koo, 2008; O'Brien and Wong, 2011; Zhang et al., 2011).

Both pathways are important for the normal brain function. All cleavage fragments of APP are related to or interact directly with other proteins in a diversity of processes in the brain. Changes in the normal processing of APP are associated with various diseases. In Alzheimer´s disease there is a shift to the amyloidogenic pathway favouring the production of Aβ (Turner et al., 2003; O'Brien and Wong, 2011; Zhang et

al., 2011). In severe forms of autisms the levels of sAPPα are generally elevated, suggesting that in this disorder there may be an aberrant non-amyloidogenic processing of APP (Sokol et al., 2006; Ray et al., 2011).

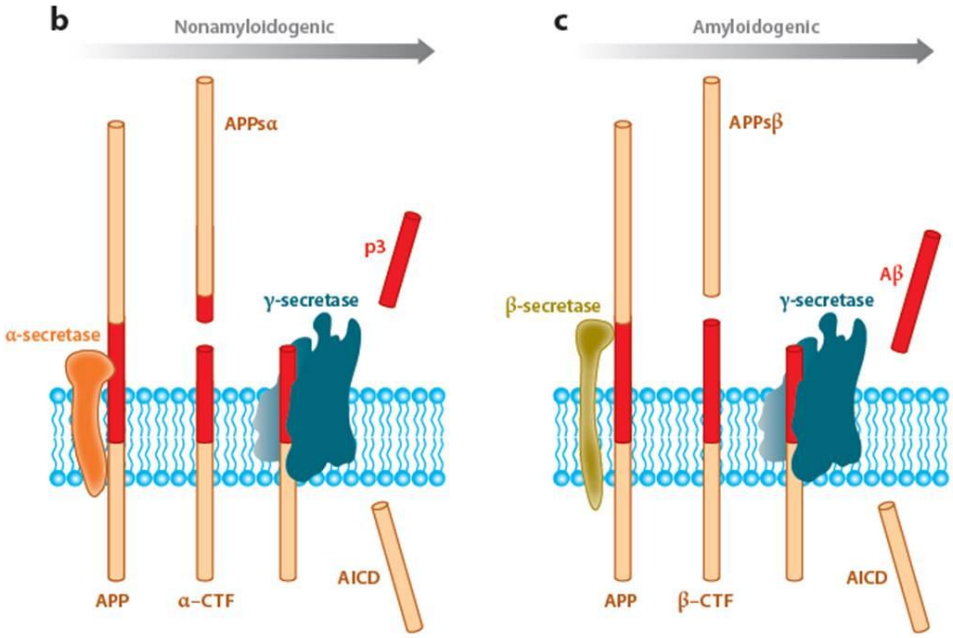

Figure 2: APP processing through non-amyloidogenic (A) and amyloidogenic (B) pathways. (A) Non-amyloidogenic processing of APP involving α- secretase followed by γ- secretase. (B) Amyloidogenic processing of APP involving β- secretase followed by γ- secretase. Both processes generate soluble ectodomains (sAPP α and sAPP β), an identical intracellular C-terminal fragments (AICD), and small peptide fragments (p3 in the non-amyloidogenic pathway and Aβ in the amyloidogenic pathway). Adapted from O´Brian et al 2011.

The processing of APP occurs during its intracellular traffic from the ER to the plasmatic membrane. Nascent APP molecules mature throughout the constitutive secretory pathway. APP is synthesized in the ER and then transported through the Golgi apparatus to the Trans-Golgi-Network (TGN), where the highest concentration of APP is found in native neurons. Once APP reaches the cell surface, it is rapidly internalized and, subsequently, trafficked by endocytic and recycling compartments back to the cell surface or degraded in lysosomes (Figure 3). Non-amyloidogenic

processing of APP occurs mainly at the cell surface, where α-secretases are present, whereas the amyloidogenic processing involves the transport of APP through the endocytic organelles, where this protein encounters β- and γ- secretases (Turner et al., 2003; Thinakaran and Koo, 2008; Groemer et al., 2011; O'Brien and Wong, 2011; Zhang et al., 2011).

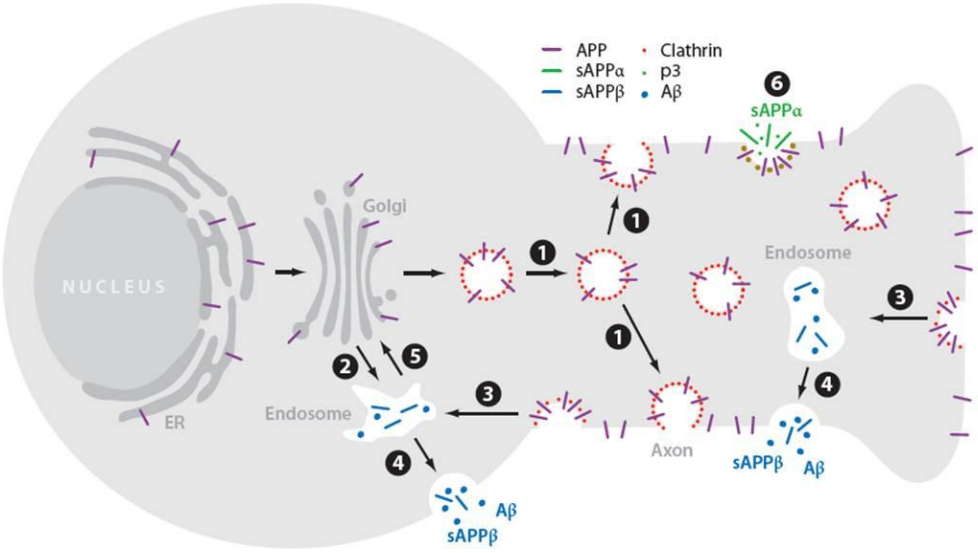

Figure 3: APP trafficking in neurons. Newly synthesized APP (purple rows) is transported from the Golgi down the axon (1) or into a cell body endosomal compartment (2). After insertion into the cell surface, some APP is cleaved by α-secretase generating sAPPα fragment, which diffuses away (green rows), and some is re-internalized into endosomes (3), where Aβ is generated (blue dots). After APP proteolysis, the endosome recycles to the cell surface (4), releasing Aβ (blue dots) and sAPPβ (blue rows). Retrograde transportation from the endosomes to the Golgi prior to APP cleavage can also occur. Adapted from O´Brian et al 2011.

Although the subcellular localization of Aβ in brain tissue was shown to be mainly endosomal (Takahashi et al., 2002; Cataldo et al., 2004), it cannot be ruled out that a pool of Aβ was produced in another intracellular compartment and/or

endocytosed from the extracellular space. The presenilins (PS1 and PS2) were the first γ-secretase components to be discovered and their subcellular localization has been determined in brain, and also it was found their presence in synaptic compartments (Frykman et al., 2010). However, it remains to be defined whether APP and γ-secretase components are present in the same places at the synapse and in synaptic vesicles. If APP and amyloidogenic pathway secretases were present in the same places in the synapse, this could explain the production of Aβ pool outside the endosomal pathway.

1.5. APP functions

Many physiologic functions have been attributed to APP since its discovery, but the precise physiological function of this protein is not known and remains a controversial issue in the field (O'Brien and Wong, 2011). One of the functional roles of this protein is a trophic function. Indeed, it was shown that APP has one RERMS domain in the extracellular domain, and some studies in which APP was added to cultured fibroblasts have showed an increase on them growing (Saitoh et al., 1989; Ninomiya et al., 1994). In other studies it was observed that the administration of APP in the brain of rodents augments the synaptic density and improves the memory retention by these animals, and that the increase in APP levels seems to be related with the improvement of learning capacity (Roch et al., 1994; Huber et al., 1997; Meziane et al., 1998). It was also shown that APP stimulates the growing of neuritis and synaptogenesis, playing a role in synaptic physiology, regulating synaptic scaling and synaptic vesicle release (Kamenetz et al., 2003; Priller et al., 2006; Abramov et al., 2009). It was also shown that APP could regulate the pre-synaptic expression and activity of the high affinity choline transporter (Wang et al., 2007). The data obtained

with APP knockout mice, reinforce the idea that APP may modulate synapses formation and function (Wang et al., 2005; Wang et al., 2007).

Although the most consistent role of APP is that of a trophic factor, this function could be only related to the sAPPα that contains the RERMS domain and is constitutively released. A function of cellular adhesion has also being suggested to APP, due to the presence of RHDS sequence that seems to promote cellular adhesion. Moreover, APP colocalizes with integrins at sites of cellular adhesion in the surface of axons (Turner et al., 2003; Thinakaran and Koo, 2008; O'Brien and Wong, 2011).

Among the first functions pointed out to APP is its interaction with G protein-coupled receptors (GPCRs) (Turner et al., 2003). Although the first evidences that supported this idea were not convincing enough, various ligand candidates have been proposed for APP, such as Aβ (Lorenzo et al., 2000), nectrin-1 (Lourenco et al., 2009) and F-spondin (Ho and Sudhof, 2004), being the last one more promising as a real ligand (O'Brien and Wong, 2011). Some data suggests that F-spondin, a signalling molecule secreted a neuronal level, can bind to the extracellular domain of APP, as well to APLPs (Ho and Sudhof, 2004). The binding of this molecule reduces the cleavage of APP by β-secretase and the trans-activation of the AICD peptide, suggesting that F-spondin could be a ligand of APP that regulates its processing (Turner et al., 2003; Ho and Sudhof, 2004; Thinakaran and Koo, 2008; O'Brien and Wong, 2011).

Although many functions have being pointed out to APP, it remains to be clearly defined the role of this protein, and even the studies performed with APP knockout mice are not very conclusive regarding to its function. It is a bit disappointing that genetic deletion of APP in mice produces only few phenotype alterations, such as reduced locomotor activity and gliosis (Zheng et al., 1995), deficits in synaptic plasticity, learning and memory (Dawson et al., 1999), without profoundly affecting the

adult animal. Triple knockouts mice, involving APP, APLP1 and APLP2, show scattered cortical migration abnormalities (Herms et al., 2004), whereas the double knockout mice lacking APP and APLP2 exhibit a mismatch between the pre-synaptic and post-synaptic markers at the neuromuscular junction, and excessive nerve terminal sprouting (Wang et al., 2005). However, none of these studies suggest a role for APP in the mature Central Nervous System, in which APP production is known to continue at very high rate (O'Brien and Wong, 2011).

Although the exact function of APP is not known, some evidences from this study points to a possible role of APP in the synapse, either by acting as full-length protein or due to some of its cleavage fragments. Either overexpression or deletion of these proteins affects the normal function and morphology of synapses. These observations corroborate the necessity and importance of the study of synaptic roles of APP.

1.6. Alzheimer's disease and the importance of the synaptic study of APP

Alzheimer's disease (AD) is the most common neurodegenerative disorder affecting the aged population. Neurologically, AD is initially manifested as a mild cognitive impairment, deficits in short term memory and loss of spatial memory. As the disease progresses these symptoms become severe, and ultimately result in total loss of executive functions (Pimplikar, 2009; Perl, 2010). The neuropathological features of this disease include the deposition of extracellular β-amyloid (Aβ) plaques, neurofibrillary tangles (NFTs) and synaptic and neuronal loss. The principal components of amyloid plaques (also known as senile plaques) are 40 and 42 amino acid Aβ peptides derived from the APP, surrounded by abnormally configured neuronal processes or neuritis. NFTs consist of abnormal accumulations of hyperphosphorylated

microtubule associated protein tau within the cytoplasm of some neurons (Pimplikar, 2009; Perl, 2010). The loss of synapses in this neurodegenerative disorders is closely associated with the duration and severity of cognitive impairment in AD patients, and it is now well established that this feature is the initial morphological trait in AD (Wang et al., 2005; Scheff et al., 2007). Genetic, biochemical, and behavioural studies suggest that Aβ peptides, derived from amyloid precursor protein (APP), are the root cause of AD (Pimplikar, 2009).

Currently it is accepted that the soluble Aβ oligomers, rather than the insoluble Aβ fibrils, are the main culprit of AD and are responsible for the observed synaptic dysfunction in the brains of AD patients. Thus, over the last years, the idea that synapses are particularly vulnerable to Aβ oligomers has been gaining support (Selkoe, 2002; Walsh and Selkoe, 2007). Aβ peptides affect mainly glutamatergic synapses (Kamenetz et al., 2003; Bell et al., 2006), as well as cholinergic synapses at the neuromuscular junctions (Bartus et al., 1982; Moller, 1999). Results from our group showed that in an AD animal model, consisting of Aβ intracerebroventricular injection, there is loss of glutamatergic and cholinergic, but not of GABAergic synapses, together with memory dysfunction (Cunha et al., 2008).

These evidences suggesting that there are nerve terminals that are more susceptible to Aβ, lead us to investigate which are the characteristics making them more susceptible. One hypothesis could be that susceptible terminals might have higher quantities of APP, leading to the production of higher amounts of Aβ and the subsequent degeneration of those nerve terminals. The location of APP in the synapses, particularly in different nerve terminals, is a preeminent question that still needs to be clarified. Because of the pivotal role of APP in AD pathogenesis, it is essential to understand its physiological function, particularly its potential activity in synaptic regulation.

1.7. Objectives

There are many reports referring the presence of APP in the synapse, however, it is not very clear if APP is enriched in synapses. It is also unknown if APP is differentially distributed in different nerve terminals. The objectives of this study are:

- To define if APP is enriched in synaptic fractions of the rat hippocampus,
- To define if APP is mainly located pre-synaptically, post-synaptically or non-synaptically in rat hippocampus
- To determine if it is a widespread synaptic protein or it is restricted to a particular type of synapses, namely glutamatergic, GABAergic or cholinergic synapses in rat hippocampus
- To analyze if APP is only localized in synapses, or if it is present in other part of the neuron and/or in glial cells

2. Material and Methods

2. Material and methods

2.1. Material

2.1.1. Reagents

Table 1: Reagents used and respective suppliers.

Reagent	Supplier
30% Acrylamide/Bis solution	Bio Rad (Portugal)
Ammonium persulfate (APS)	Sigma-Aldrich (Portugal)
Bicine	Sigma-Aldrich (Portugal)
BCA Kit	Thermo scientific (USA)
Boric acid	Sigma-Aldrich (Portugal)
Bovine serum albumin (BSA)	Sigma-Aldrich (Portugal)
Bromophenol blue	Sigma-Aldrich (Portugal)
Calcium chloride (CaCl2)	Sigma-Aldrich (Portugal)
CAPS ([3-(cyclohexylamino)-1-propane-sulfonic acid)	Sigma-Aldrich (Portugal)
Citric acid	Sigma-Aldrich (Portugal)
CLAP (cocktail of proteases inhibitors)	Sigma-Aldrich (Portugal)
DAKO Fluorescence Mounting Medium	DAKO (Denmark)
DAPI	Sigma-Aldrich (Portugal)
Dithiothreitol (DTT)	Sigma-Aldrich (Portugal)
ECF	GE Healthcare (United Kingdom)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Portugal)
Gelatin	Sigma-Aldrich (Portugal)
Glucose	Sigma-Aldrich (Portugal)
Glycerol	Sigma-Aldrich (Portugal)
Halothane	Sigma-Aldrich (Portugal)
HEPES	Sigma-Aldrich (Portugal)
Hydrochloric acid (HCl)	Sigma-Aldrich (Portugal)
Magnesium Chloride (MgCl)	Sigma-Aldrich (Portugal)
Metanol	Sigma-Aldrich (Portugal)
Normal Horse Serum (NHS)	Invitrogen (United Kingdom)
Paraformaldehyde	Sigma-Aldrich (Portugal)
Percoll	GE Healthcare (United Kingdom)
Penylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich (Portugal)
Poli-D-Lysine	Sigma-Aldrich (Portugal)
Potassium chloride (KCl)	Sigma-Aldrich (Portugal)
ProLong Gold Antifade	Invitrogen (United Kingdom)
Sodium dodecyl sulfate (SDS)	Bio Rad (Portugal)
Sodium azide	Sigma-Aldrich (Portugal)
Sodium Bicarbonate (NaHCO3)	Sigma-Aldrich (Portugal)
Sodium Chloride (NaCl)	Sigma-Aldrich (Portugal)
Sodium phosphate monobasic (NaH2PO4)	Sigma-Aldrich (Portugal)

Table 1 (cont.): Reagents used and respective suppliers.

Reagent	Supplier
Sodium thiopental	B.Braun Medical (Portugal)
Sucrose	Sigma-Aldrich (Portugal)
Tissue-Tek	(Sakura-Americas, USA)
TMED	Sigma-Aldrich (Portugal)
Triton - x -100	Sigma-Aldrich (Portugal)
Trizma base	Sigma-Aldrich (Portugal)
Tween	Sigma-Aldrich (Portugal)

2.1.2. Antibodies

Table 2: Primary and secondary antibodies for Western blot.

Antibody	Supplier	Host	Type	Dilution
APP C-terminal	Sigma	Rabbit	Polyclonal	1:8000
APP N-terminal 22C11	Millipore	Mouse	Monoclonal	1:1000
Synaptophysin	Millipore	Rabbit	Polyclonal	1:20000
SNAP-25	Sigma	Mouse	Monoclonal	1:40000
Syntaxin	Sigma	Mouse	Monoclonal	1:40000
PSD-95	Sigma	Mouse	Monoclonal	1:20000
β-Actin	Sigma	Mouse	Monoclonal	1.20000
Anti-Mouse alkaline phosphatase conjugated (AP)	GE Healthcare	Goat	IgG + IgM (H+L)	1:20000
Anti–Rabbit (AP)	GE Healthcare	Goat	IgG (H+L)	1:20000

Table 3: Primary and secondary antibodies for immunocytochemistry.

Antibody	Supplier	Host	Type	Dilution
APP C-terminal	Sigma	Rabbit	Polyclonal	1:1000
APP N-terminal 22C11	Millipore	Mouse	Monoclonal	1:100
Synaptophysin	Millipore	Rabbit	Polyclonal	1:200
Synaptophysin	Sigma	Mouse	Monoclonal	1:200
SNAP-25	Sigma	Mouse	Monoclonal	1:400
PSD-95	Sigma	Mouse	Monoclonal	1:200
GFAP	Dako	Rabbit	Polyclonal	1.200
vGLUT1	Synaptic Systems	Guinea pig	Polyclonal	1:1000
vGAT	Synaptic Systems	Guinea pig	Polyclonal	1:500
vAChT	AbCam	Rabbit	Polyclonal	1:300
Anti–Mouse Alexa Fluor 488	Invitrogen	Donkey	IgG (H+L)	1:200
Anti – Mouse Alexa Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:200
Anti-Rabbit Alexa Fluor 488	Invitrogen	Donkey	IgG (H+L)	1:200
Anti-Rabbit Alexa Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:200
Anti – Guinea pig Alexa Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:200

Table 4: Primary and secondary antibodies for immunohistochemistry.

Antibody	Supplier	Host	Type	Dilution
APP C-terminal	Sigma	Rabbit	Polyclonal	1:1000
APP N-terminal 22C11	Millipore	Mouse	Monoclonal	1:100
Neu N	Millipore	Mouse	Monoclonal	1:100
CD11b	ABD Serotec	Mouse	Monoclonal	1:100
GFAP	Dako	Rabbit	Polyclonal	1.1000
GFAP	Sigma	Mouse	Monoclonal	1:1000
Anti – Mouse Alexa Fluor 488	Invitrogen	Donkey	IgG (H+L)	1:400
Anti-Rabbit Alexa Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:400

2.2. Animals

We used male Wistar rats with 8-10 weeks of age that were obtained from Charles River (Barcelona, Spain). For synaptosomes isolation the animals were anesthetized under halothane atmosphere before being sacrificed by decapitation; and the hippocampi were rapidly isolated from the rat brain. For purified nerve terminals the left hippocampus was used (tissue was homogenized just after the dissection), whereas to prepare the synaptosomes and total membranes we used the right hippocampus from the same animal (tissue was stored at -80ºC until use), wielding 4 structures for both procedures. To prepare pre- post- and extra-synaptic fractions we used 5 or 6 pairs of hippocampi per procedure. This material was collected during one year from spearing hippocampus from animals used in other experiments (tissue was stored at 80ºC until use). For immunohistochemical studies we used 3 rats with 8 weeks old.

2.3. Synaptic preparations

Nervous tissue is composed of neurons and their supporting cells, the glia. Neurons do not last intact to homogenization, and the cell bodies are sheared from their processes that break up into discrete fragments. The plasma membrane of these cell fragments may reseal to form osmotically active particles and when such particles contain the organelles of the synapse they are known as synaptosomes (Figure 4). Subcellular fractions enriched in synaptosomes are sufficiently pure to allow the study of certain physiological and pharmacological aspects of synaptic function and in this respect they have been very useful to study the synaptic morphology and function (Phelan, 1997).

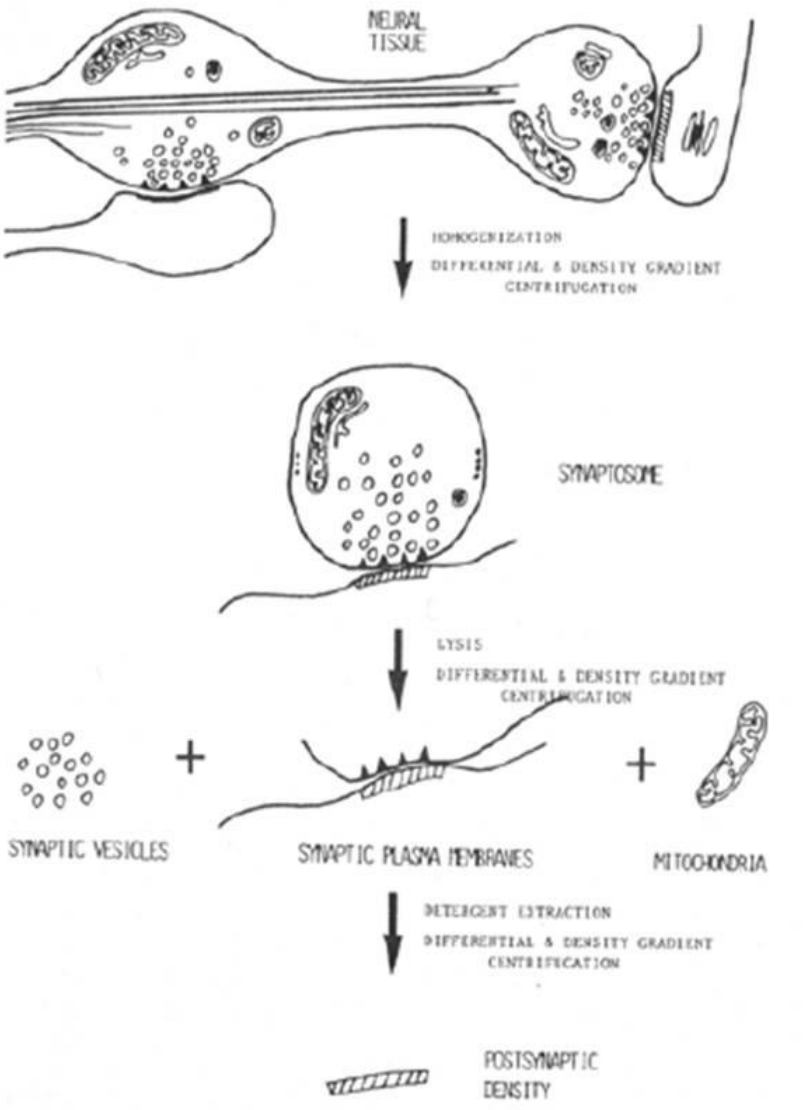

Figure 4: Diagram illustrating the formation of synaptosomes and their subcellular fractions from homogenate of neural tissue. Adapted from Turner et al 1997.

Synapses represent about 1-2% of the total hippocampal volume and have a huge amount of proteins levels, mainly adhesion and cytoskeletal proteins that are responsible for maintaining neuronal architecture and connections. Therefore, the biochemical study of synapses in native brain preparations has a poor signal-to-noise ratio and the accessibility of antibodies to epitopes is expected to be limited. One good way to overcome these limitations is to use synaptosomes (Cunha, 1998).

2.3.1. Synaptosomes and total membranes

In order to compare the density of proteins of interest in the synaptosomal and in total membranes fractions of the same animal, half of the volume of the supernatant resulting from the first centrifugation (common to both protocols, see below) was separated to isolate total membranes and the other half to isolate synaptosomes.

2.3.1.1. Rapid isolation of synaptosomes

Membranes from Percoll-purified hippocampal synaptosomes were prepared as previously described (Canas et al., 2009). One hippocampus from one animal was homogenized at 4ºC in sucrose solution (0.32 M) containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml BSA (pH7.4), centrifuged at 3,000 x*g* for 10 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatants collected were further centrifuged at 14000 x*g* for 12 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatants were discarded and the pellet resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs-HEPES medium (composition: 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 20,800 x*g* for 2 min at 4ºC (in an eppendorf centrifuge), the top layer was collected (synaptosomal fraction), resuspended in 1 ml Krebs solution, and further centrifuged at 20,800 x*g* for 2 min at 4ºC (in an eppendorf centrifuge). The supernatants were discarded and the pellet was resuspended in a lysis solution composed by 5% (w/v) SDS, 2 µM PMSF, 1% CLAP (a cocktail of proteases inhibitors) and then stored at -20ºC.

2.3.1.2. Total membranes preparation

The hippocampal tissue was homogenised in a 0.32 M sucrose solution containing 1 mM EDTA, 10 mM HEPES and 1 mg/ml BSA, pH7.4 at 4ºC. Then, the

homogenates were centrifuged at 3,000 x*g* for 10 minutes, 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatant was further centrifuged at 25,000 x*g* for 60 minutes at 4ºC (Avanti J-26X centrifuge, rotor JA-22-50). The supernatants were discarded and the pellet, corresponding essentially to total cytoplasmatic membranes, was resuspended in a lysis solution composed with 5% (w/v) SDS, 2 µM PMSF, 1% CLAP, and then stored at -20ºC.

2.3.2. Isolation of synaptosomes using a discontinuous Percoll gradient

The preparation of purified nerve terminals was carried out as described previously by our group (Rodrigues et al., 2005). This procedure for preparation of synaptosomes is crucial to reduce the amount of post-synaptic density material.

Animals were sacrificed and the tissue was homogenised in a medium containing 0.25 M sucrose and 10 mM HEPES (pH 7.4) and centrifuged at 2,000 x*g* for 5 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatant was further centrifuged at 9,500 x*g* for 13 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatant was discarded and the pellet was resuspended in 2 ml of 0.25 M sucrose and 10 mM HEPES medium (pH 7.4), and was placed on the top of a Percoll discontinuous gradient. For each sample in a centrifuge tube, the gradient was built as follows (from top to bottom): 2 ml of the resuspended pellet, 3 ml of a 3% (v/v) Percoll solution, 4 ml of a 10% (v/v) Percoll solution and a 23% (v/v) Percoll solution. The Percoll solutions were prepared in a 0.32 M sucrose solution with 1 mM EDTA and 0.25 mM DTT, pH 7.4 at 4ºC. The gradients were centrifuged at 25,000 x*g* for 11 minutes at 4ºC (Avanti J-26X centrifuge, rotor JA-22-50), without deceleration (to prevent the disruption of the gradients). Synaptosomes were collected in the interface between the 10% (v/v) and 23% (v/v) Percoll bands (Figure 5 A) and further diluted in 10 ml of HEPES Buffered Medium (HBM) without calcium (140 mM NaCl, 5 mM KCl,

1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4, at 4ºC).

A centrifugation of 22,000 x*g* of 11 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H) was then performed, without deceleration and the resulting freely-moving pellet was collected. For immunocytochemistry studies the pellet was resuspended in 2 ml of HBM.

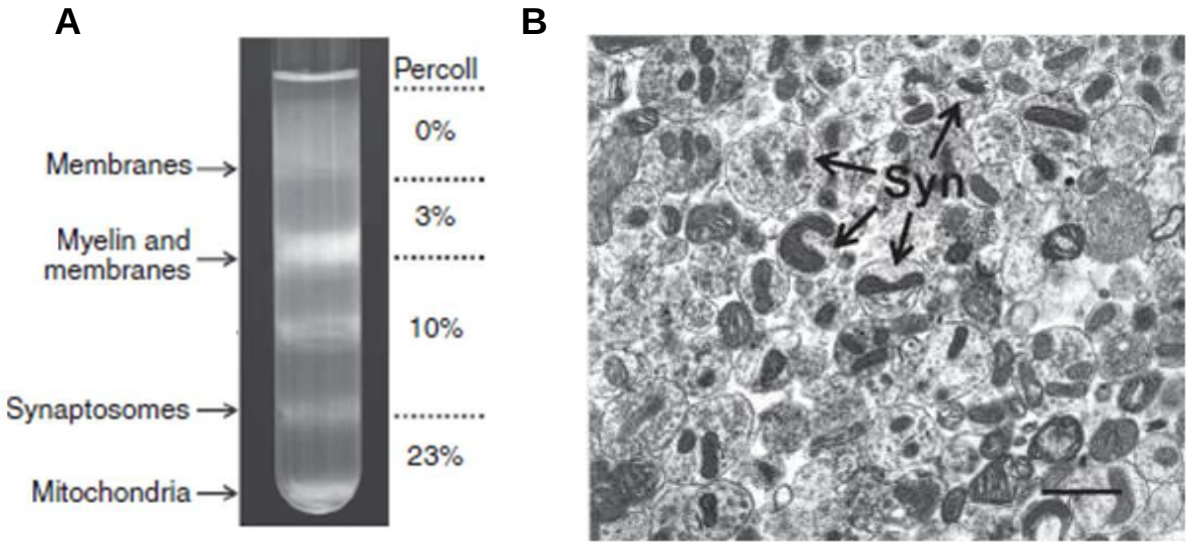

Figure 5: (A) Picture of the discontinuous Percoll gradient. (B) Electron microscopy image of the synaptic fraction obtained using the Percoll gradient. Arrows indicate the synaptosomes (Syn). Adapted from Dunkley et al 2008.

2.3.3. Fractioning of synaptic membranes (Pre, Post, Extra)

To isolate the subcellular components of synaptosomes, such as the pre-synaptic, post-synaptic and extra-synaptic fractions, from rat hippocampal synaptosomes, we used a methodology previously described by our group (Rebola et al., 2005). This subsynaptic fractionation method allows an over 90% effective separation of the pre-synaptic active zone (enriched in SNAP-25 protein), post-synaptic density (enriched in PSD95 protein) and non-active zone fraction or extra-synaptic fraction (enriched in

synaptophysin protein). The use of antibodies against SNAP-25, syntaxin, PSD95 and synaptophysin, which are markers of different synaptic fractions, were used to assess the subsynaptic distribution of proteins (see Figure 6) (Pinheiro et al., 2003; Rebola et al., 2003).

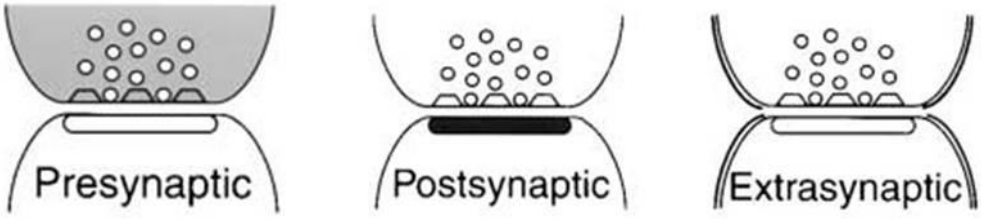

Figure 6: Scheme illustrating the synaptic components that are expected to be enriched in each fraction isolated in this procedure. Adapted from Phillips et al 2001.

For synaptosomes preparation, the hippocampi (for a relatively good yield it is recommended to use 10 hippocampi of rats - for mice should be 20 - per procedure) were homogenised in 2.5 ml of Isolation Buffer (IB) (constituted by 0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% CLAP and 1 mM PMSF). The homogenate was transferred to 50 ml centrifuge tubes and resuspended in 2 M sucrose and 0.1 mM $CaCl_2$. The mixture was gently agitated at 4ºC giving a 1.25 M sucrose solution. This solution was divided into 2 tubes Ultraclear™ and 2.5 ml (per tube) of a 1 M sucrose solution (containing 0.1 mM $CaCl_{2)}$) was carefully added to allow the formation of a gradient. The tubes were filled and equilibrated with IB and then centrifuged at 100,000 x*g*, 4ºC, for 3 hours (Beckman Coulter - Optima CL-100XP DU ultracentrifuge, rotor SW41Ti). The IB and the myelin layer present at the interface IB/1 M sucrose were aspirated. The synaptosomes were collected at the interface 1.25/1 M sucrose and then were diluted 10 times in IB and centrifuged at 15,000 x*g* during 30 minutes (Avanti J-26X centrifuge, rotor JA-22-50). The resulting pellet was resuspended in 1.1 ml IB

[100 µl of the supernatant (synaptosomes fraction) was kept at -80ºC for control analysis], and diluted 10 times in cooled 0.1 mM $CaCl_2$. A similar volume (10 ml) of 2x solubilization buffer pH 6.0 (40 mM Tris, 2% (v/v) Triton X-100, pH 6.0 precisely adjusted at 4ºC) was added. The mixture was softly stirred during 30 minutes on ice and divided into 2 Ultraclear™ tubes for a centrifugation at 40,000 x*g* for 30 minutes, 4ºC (Avanti J-26X centrifuge, rotor JA-22-50). The pellet corresponds to synaptic junctions and the supernatant to extra-synaptic proteins. The supernatants were kept on ice while the pellet was washed in 1x solubilization buffer pH 6.0 (20 mM Tris, 1% (v/v) Triton X-100, pH 6.0 precisely adjusted at 4ºC) and resuspended in 5 ml of solubilization buffer pH 8.0 (20 mM Tris, 1% (v/v) Triton X-100, pH 8.0 precisely adjusted at 4ºC). This mixture was stirred softly for 30 minutes on ice and centrifuged at 40,000 x*g* for 30 minutes at 4ºC (Avanti J-26X centrifuge, rotor JA-22-50). The pellet corresponds to the post-synaptic density and the supernatant to pre-synaptic proteins. The supernatant was transferred to centrifuge tubes and the pellet resuspended in a minimal volume of 5% SDS solution with 0.1 mM PMSF and kept at -80ºC. To concentrate the extra-synaptic and pre-synaptic proteins, a maximum volume of cold acetone (-20ºC) was added to the supernatants and kept overnight at -20ºC. Both fractions were pelleted by centrifugation at 18,000 x*g* for 30 minutes at -15ºC (Sorvall RC6, rotor SS34). Both pellets were resuspended in a minimal of 5% SDS solution with 0.1 mM PMSF, sonicated and kept at -80ºC.

2.4. Protein quantification and Western Blot

2.4.1. Protein quantification by the BCA method and preparation of the samples

Protein quantification was carried using the Bicinchoninic acid (BCA) protein assay reagent kit, a colorimetric method compatible with high concentrations of most

components of lysis solution used. A standard curve was prepared in milliQ water, using 2; 1; 0.5; 0.25; 0.125; 0.0625 and 0 µg/µl of BSA. All the samples and the solution used to lyse the samples were diluted 10 times. In a 96 well dish, the standard curve was prepared by pipetting 25 µl of each concentration of BSA, in triplicates, for different wells. To each well, 25 µl of the diluted lysis buffer was added, as well as 200 µl of the BCA reagent. Triplicates of the diluted samples were prepared in the same way, but 25 µl of milliQ water were added to each well instead of the diluted lysis buffer. The dish was protected from light and placed in a 37ºC incubator for 30 minutes. Finally, the protein was read at 570 nm in a spectrophotometer.

For Western blot analysis, the samples were normalized to 1 µg/µl, by adding 1/6 volume of 6x SDS sample buffer (composed of 4x Tris HCl/SDS solution (0.5 M Tris and 0.4% SDS, pH 6.8 corrected with HCl and filtered with 0.45 µm pore filters, 30% glycerol (v/v), 10% (w/v) SDS, 0.6 M DTT and 0.012% (w/v) of bromophenol blue) and correcting with milliQ water. The samples were finally boiled at 95ºC during 5 minutes.

2.4.2. Western Blot

Western blot analysis was performed using the Bio-Rad system. The samples diluted in Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) buffer and the pre-stained molecular weight markers were loaded and separated by SDS-PAGE electrophoresis (in 7.5% polyacrylamide resolving gels with 4% polyacrylamide stacking gels) under denaturing, reducing conditions and using a bicine buffered solution (20 mM Tris, 192 mM Bicine and 0.1% SDS, pH 8.3).

Table 5: Gel formulation

Gel formulation (1 Gel)	4% (Stacking gel)	7,5% (Resolving gel)
Water	6.1 ml	3.45 ml
Tris – HCl 1.5 M pH 8.8	----------	3.022 ml
Tris – HCl 0.5 M pH 6.8	2.5 ml	----------
Bis – Acrylamide (30%)	1.3 ml	2.25 ml
SDS 10%	100 µl	195 µl
APS (freshly prepared, diluted in water)	50 µl	6 µl
TEMED	10 µl	90 µl

The electrophoresis was carried out applying a voltage of 90-110 mV for 1 hour. The proteins were then electro-transferred (with 1 A current, for 90 minutes at 4ºC under regular agitation) to previously activated Polyvinylidene Difluoride (PVDF) membranes, using a CAPS [3-(cyclohexylamino)-1-propane-sulfonic acid] buffered solution with methanol [10mM CAPS, 10% (v/v) methanol, pH 11]. Membranes were then blocked for 1 hour at room temperature (RT) with 3% (w/v) BSA in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.6) with 0,1% (v/v) Tween 20 (TBS-T). After that membranes were incubated with the primary antibodies diluted in TBS-T with 3% BSA, overnight, at 4ºC. After being washed 3 times, 15 minutes each, in TBS-T, the membranes were incubated with phosphatase-linked secondary antibodies, also diluted in TBS-T with 3% BSA for 2 hours at RT. Membranes were washed 3 times, 15 minutes each, in TBS-T and then incubated with enhanced chemi-fluorescence substrate (ECF) for different times in a maximum of 1 minute. Finally, proteins were detected and analysed with Molecular Imager VersaDoc 3000 and Quantity One software (Bio Rad, USA).

Re-probing of the membranes with a different antibody was achieved by washing the ECF in 40% methanol for 20 minutes and stripping the previous antibodies in a mild stripping solution of 0.2 M glycine with 0.1% SDS and 1% (v/v) Tween 20, pH 2.2, for 1 hour. The membranes were washed 3 times, 15 minutes each, in TBS-T with 3% BSA, between different solutions. Finally, before incubation with new antibodies, the membranes were again blocked for 1 hour at RT with TBS-T 3% BSA.

2.5. Immunofluorescence

2.5.1. Immunocytochemistry in synaptosomes

For immunocytochemical analysis of purified nerve terminals rat hippocampal synaptosomes were obtained through a discontinuous Percoll gradient, as described above. The procedure was followed as previously (Rodrigues et al., 2005). Glass sterilized coverslips of 16 mm were covered with poli-D-lysine (0.1 mg/mL, in borate buffer 150 mM, pH 8.2), for 1 hour at 37ºC. Then, the coverslips were rinsed two times with milliQ water and left to dry completely. The synaptosomes were put in the coverslips and left to adhere at RT for 1 hour. They were then fixed with 4% (w/v) paraformaldehyde (PFA) [prepared in a solution of 0.9% NaCl with 4% sucrose (w/v)] for 15 minutes at RT and rinsed twice with Phosphate Buffer Solution (PBS). The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 minutes, rinsed twice with PBS, and then blocked for 1 hour in PBS with 3% BSA (w/v) and 5% normal horse serum (v/v). After that they were rinsed two times with PBS 3% BSA (w/v) and incubated with primary antibodies: diverse synaptic markers and markers for vesicular transporters, diluted in PBS with 3% BSA for 1 hour at RT. The antibodies used and the dilutions used are described in table 3. Then they were rinsed tree times in PBS 3% BSA (w/v) and incubated for 1 hour at RT with the respective secondary antibodies labelled with a fluorescent dye (see table 3). After being rinsed tree times

coverslips were mounted on slides with Prolong Gold Antifade. The preparations were then visualized in a Zeiss Imager Z2 fluorescence microscope equipped with a AxioCam HRm and 63x Plan-ApoChromat objective (1.4 numerical aperture), with Axiovision SE64 4.8.2 software. Five images were randomly taken from each coverslip (two or three per experience /marking).

It was confirmed that none of the secondary antibodies produced any signal in preparations by using preparations where primary antibodies were omitted. It was also confirmed that individual signals in double-labelled fields are not enhanced over the signals in single-labelling conditions. A bright field channel was acquired in each image, so we could better distinguish synaptosomes from false positives (antibody precipitates) and synaptosomal aggregates, when defining the size of synaptosomes to be counted.

The quantification of the images was done in Image J (NIH, USA) using a customised macro. In this macro the synaptosomes were defined between 2-15 pixels of size.

2.5.2. Preparation of fixed brain slices

Perfusion of rats with PFA was carried as previously described in Canas et al 2009. Wistar male rats with 8 weeks old were anaesthetised with thiopental (180 mg/kg), the heart was exposed, the descending aorta was clamped (to spare solution and time) and then a catheter was inserted into the ascending aorta. The right atrium was opened to allow the outflow of the perfusate. The rat was then perfused with 200 ml of a saline solution of 0.9% NaCl with 4% sucrose (w/v), followed by 200 ml of 4% PFA solution (prepared in saline solution). After this procedure the brain of the rat was removed and maintained in 4% PFA solution overnight at 4ºC. The brains were transferred to PBS with 30% sucrose and kept in this solution until they descended

(normally 24h or 48h). After that the brains were embedded in Tissue-Tek, frozen at -20ºC and cut into 30 µm coronal sections using a cryostat (Leica CM3050 S). Each series of brain sections comprised slices 300 µm apart, allowing representative sections of different areas of the brain structures. Slices were store at 4ºC in Walter´s antifreeze solution (30% glycerol (v/v), 30% ethyleneglycol (v/v) in 0.5 m phosphate buffer).

2.5.3 Immunohistochemistry

Immunohistochemistry was performed on free floating slices as previously described (Rebola et al 2011). Selected brain sections comprising most of the hippocampus were placed into wells containing PBS. The sections were washed 3 times (5 minutes each) under gentle agitation. The slices were then exposed to a 10 mM citric acid solution (prepared in PBS, pH 6.0) during 20 minutes at 60ºC for antigen retrieval. Next they were rinsed three times PBS (5 minutes), followed by blocking for 2 hours at RT with PBS with 0.25% (v/v) Triton-X-100 and 5% (v/v) NHS. After that the incubation with the primary antibody was performed overnight at 4ºC (the antibodies were diluted in the blocking solution – dilutions in table 4). The slices were then rinsed one time in PBS and twice in PBS 2% (v/v) NHS (10 minutes each). Secondary antibodies (dilutions in table 4) were incubated for 2 hours at RT, in a 2% NHS (v/v) and 0.25% Triton X-100 in PBS solution (in some double immunostaining cases, to avoid cross reaction between some secondary antibodies, the antibodies were sequentially incubated). Slices were then rinsed three times (10 minutes), incubated with DAPI (diluted in PBS 1:5000) for 15 minutes, and finally, rinsed more three times in PBS (5 minutes) and mounted onto 2% gelatine-subbed microscope slide. The slides dried at RT and were covered with DAKO mounting medium. Images were acquired in a Zeiss Imager Z2 fluorescence microscope equipped with 20x (Plan Neofluar

objective, 0.4 numerical aperture) and 40x (Plan Neofluar objective, 0.6 numerical aperture) objectives and Axiovision SE64 4.8.2 software. Some preparations were further analysed in a Zeiss LSM510 META confocal laser-scanning microscope using a 63x Plan-ApoChromat objective (1.4 numerical aperture) with LSM510 software. It was confirmed that none of the secondary antibodies produced any signal in slices by using slices that were not incubated with primary antibodies.

2.6 Data presentation

Whenever possible, the data is presented as mean ± standard error of the mean (SEM) of the number (n) of experiments indicated in figure legends.

3. Results and Discussion

3. Results and Discussion

3.1. The antibodies against APP

One of the best options to study the localization of mature APP without changing its expression (which can affect its location and properties) is the use of antibody based techniques (Groemer et al., 2011). Since it is still unknown if APP is a receptor and there is no globally accepted ligand (Turner et al., 2003; O'Brien and Wong, 2011), more quantifiable techniques like binding and autoradiography are not applicable. *In situ* hybridization only gives information about where APP is being expressed. However, the antibodies of APP face up some important problems. Some of them can also recognize the APLPs. APP has a high rate of processing, and the antibodies may recognise different cleavage fragments of this protein. This is at the same time an advantage, allowing the retrieving of more information from each technique. So it becomes very important to use more than one antibody against APP, namely against different epitopes of this protein.

In the present study we used two different antibodies against APP, one specific for an epitope located at the carboxi-terminal (C-terminal; APP C-term) end and another against the amino-terminal (N-terminal, APP N-term). The N-terminal antibody recognises the three isoforms of APP, sAPP, mature and immature forms of APP (Hoffmann et al., 2000), and the C- terminal antibody also reacts with the three isoforms. By immunobloting analysis it was observed that the two antibodies displayed the same immunoreactivity pattern, 2 bands around 100-120 kDa in synaptosomes and in total membranes from rat hippocampus (Figure 7 A), indicating that in our experimental conditions and in this type of preparation we are observing mainly the full-length APP. In plated purified synaptosomes it was observed by immunocytochemistry that the two antibodies colocalized almost totally (Figure 7 B), which allowed us to use

them as each other substitute to combine in double immunocytochemistry with the different synaptic markers antibodies that were only available in one type of host species. In immunohistochemistry studies it was observed that the APP N-term and the APP C-term displayed different patterns of immunoreactivity. The APP C-term antibody immunoreactivity was present mainly in the cellular body, whereas the APP N-terminal antibody immunoreactivity was found in neuronal extensions similar to axons (Figure 7 C, D), although both antibodies showed similar immunoreactivity pattern and colocalization in the neuronal extensions. Interestingly, another immunoreactivity pattern of both antibodies was observed in structures similar to synaptic buttons (Figure 7 D).

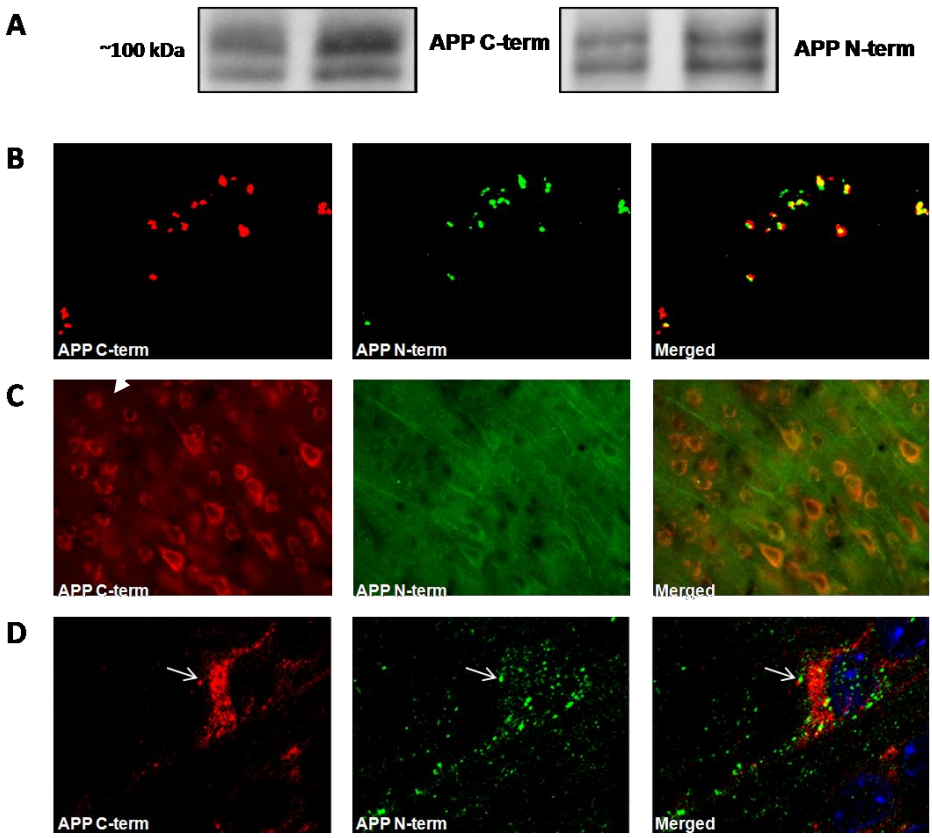

Figure 7: Verification of APP antibodies immunoreactivity by Western blot and immunofluorescence assays. (A) Western blot analysis of APP N-tem and APP C-term antibodies immunoreactivity in rat hippocampal synaptosomes (SYN) and total membranes (TM). (B) The immunocytochemistry analysis of plated purified synaptosomes of rat hippocampus showed that the two antibodies colocalized almost totally. (C) Immunohistochemical studies performed in rat brain slices (30µm) showed that the pattern of immunoreactivity of APP N-term and APP C-term antibodies did not overlap completely. (D) The images of confocal microscopy (with higher magnification), confirmed that the APP C-term immunoreactivity was present for the most part in the cellular body, whereas the APP N-term staining was more concentrated in neuronal processes. It was observed a synaptic-button type immunoreactivity pattern with both antibodies (white arrows). Magnification: B and D 630x; C 200x).

3.2. APP in Synaptosomes and Total membranes

One of the main goals of this study was to access if APP is enriched at synapses relatively to other neuronal sites. There were a considerable amount of studies reporting that APP is present at synapses (Schubert et al., 1991; Caputi et al., 1997; Huber et al., 1997; Huber et al., 1999; Kirazov et al., 2001). However, the data reported by these results are quite variable and the experimental approaches fairly specific, and sometimes the presence or the levels of APP are determined only in total brain homogenates or in synaptic fractions, and sometimes compare the levels of APP in synaptosomes and total membranes from different animals or brain regions (Kim et al., 1995; Caputi et al., 1997; Kirazov et al., 2001) .

In the present study we aimed to compare the levels of APP in synaptosomes with the levels of this protein in total membranes of hippocampi of the same animal by Western blot analysis. We focused the study in the hippocampus, because this is one of the mainly affected areas in AD (Rosenblum, 1999; Uylings and de Brabander, 2002; Perl, 2010), and the AD model that we use in our laboratory presents mainly synaptic impairment in the hippocampus (Cunha et al., 2008). The synaptosomes were isolated using a 45% Percoll solution procedure, and are considered to be total synaptosomes, composed of both pre- and post-synaptic compartments. The purity degree of synaptosomal and total membranes preparations was verified by determining the proportion levels of synaptic markers, like PSD-95 and SNAP-25, which are proteins present at high levels in post- and pre-synaptic terminals, respectively (Figure 8).

We analysed the density of APP in synaptosomes (Syn) and total membranes (TM) of rat hippocampi of the same animal in the same gel by Western blot analysis (10 mg of protein samples were loaded in each gel lane). We used two different APP antibodies, one against the N-terminal (APP N-term) and other against the C-terminal (APP C-term) of this protein. We re-probed the membranes with β-actin to normalize

the blots. To assess if APP is mostly present in synaptosomes we have determined the ratio of APP immunoreactivity in synaptosomes fraction vs total membrane fraction. If the resulting ratio value is above 1, it means that APP is mostly synaptic, whereas for ratio values under 1 it can be concluded that APP is mainly present in the bulk of cellular membranes.

Our data show that the ratio between APP immunoreactivity in synaptosomes and total membrane fraction (normalized with β-actin) was 0.64 ± 0.1 (n=4) in rat hippocampus (Figure 8). These data suggest that APP is less localized in synapses than in of the bulk of total membranes. We also observed APP immunoreactivity in synaptosomes and total membranes in rat striatum, as a control to the hippocampus synaptic location of the protein, because it helps us to see if the protein distribution is the same between different brain regions, and because this structure is not very affected in the animal models of AD used in our group (being this way used as a control in such experiments). The ratio of APP immunoreactivity in synaptosomes and total membrane fraction (normalized with β-actin) was 0.96 ± 0.12 in rat striatum (n=4) (Figure 9). The quantification was performed in the experiments using the APP C-term antibody. Although these results suggest that APP is not enriched in the synaptosomes of rat striatum, the distribution of this protein is more homogenous in synaptosomes and total membranes of striatum than of hippocampus. The smaller quantity of APP in the hippocampal synaptosomes can be a special characteristic of this brain region.

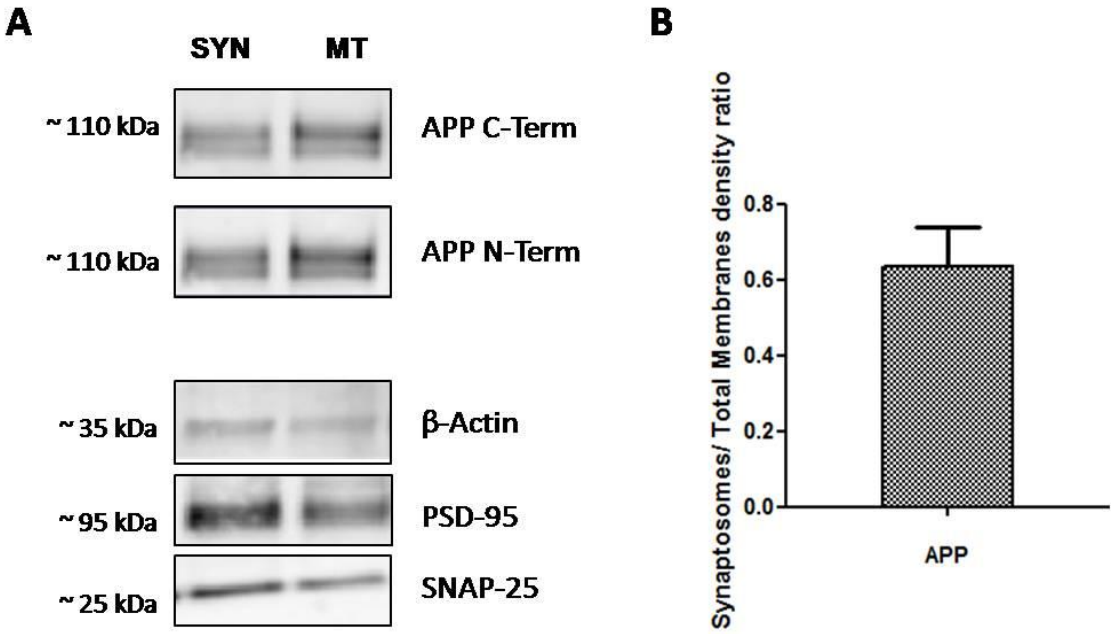

Figure 8: Levels of APP in synaptosomes and total membranes of rat hippocampus. The density of APP, using APP N-term and APP C-term antibodies, were evaluated in synaptosomes (Syn) and total membranes (TM) by Western blot analysis. The immunoreactivity PSD-95 and SNAP-25 (enriched in the synaptosomal fraction), allows to assess the purity degree of our preparations. The immunoreactive bands were quantified and the data were normalized in relation to β-actin density. The graphic bar represents the ratio of APP levels in synaptosomes and in total membranes, as mean ± SEM of 4 independent experiments (4 different animals). The quantification was performed in the experiments using the APP C-term antibody.

The distribution pattern of APP in the synaptosomes/total membranes of rat hippocampus was similar for APP N-term and APP C-term antibodies (the results we observed were in account only for full-length APP), and are in accordance with the idea that APP is localized, mainly in endoplasmic reticulum, Golgi apparatus and early endosomes (Turner et al., 2003). However, the results obtained also indicated that APP is present in synaptosomes of rat hippocampus in a significant amount (Fig. 8).

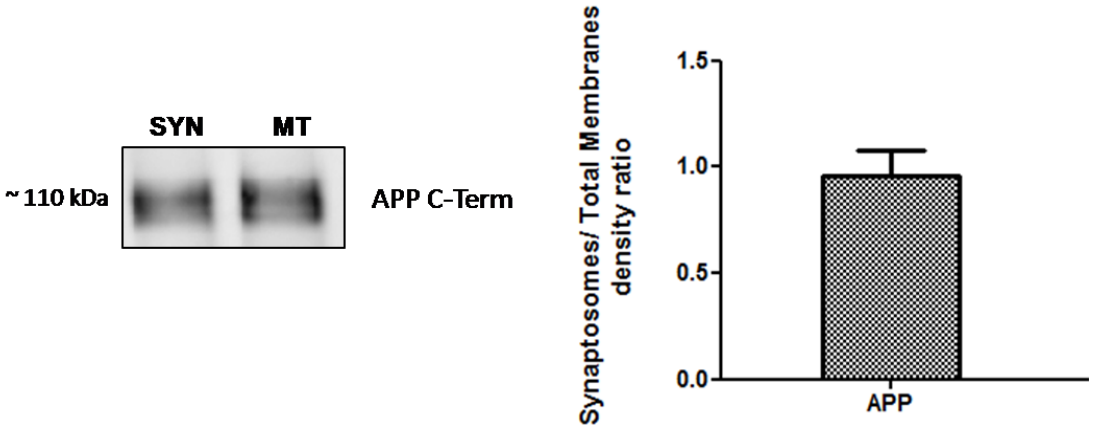

Figure 9: Levels of APP in synaptosomes and total membranes of rat striatum. The density of APP, using APP C-term antibody, was evaluated in synaptosomes (Syn) and total membranes (TM) by Western blot analysis. The immunoreactive bands were quantified and the data were normalized in relation to β-actin density. The graphic bar represents the ratio of APP levels in synaptosomes and in total membranes, as mean ± SEM of 4 independent experiments (4 different animals).

3.3. Subsynaptic location of APP

It is relatively well established that APP is transported through vesicles to the synapse, where it is released and cleaved by secretases (Thinakaran and Koo, 2008; O'Brien and Wong, 2011). Some reports have shown that APP increases in synapses in neonatal rats and in developing cultured neurons of hippocampal neurons (Ferreira et al., 1993; Kirazov et al., 2001; Sabo et al., 2003) . Our data also showed that APP is present in synapses of hippocampus of adult rats. This is in accordance with evidences that point out to an important role of APP at the synapse, and it was suggested that APP can be involved in synaptogenesis (Priller et al., 2006).

In this part of the study we analysed if APP was differently distributed in the synapse. We used a fractioning method that allows the separation of the subsynaptic fractions of the synapse: the active pre-synaptic fraction, the post-synaptic density and

the non synaptic zone (extra-synaptic fraction) (Phillips et al., 2001). This technique for separation of the pre-synaptic active zone from the post-synaptic density and from other pre-synaptic proteins not located in synapses was previously validated by our group and allows an over 90% efficiency of separation of these fractions (Pinheiro et al., 2003; Rebola et al., 2003). We verified the purity of ours preparations by Western blot using antibodies against pre-synaptic proteins (anti-syntaxin), extra-synaptic markers (anti-synaptophysin) or post-synaptic markers (anti-PSD-95).

We used rat hippocampus, and because this procedure has a low yield it was necessary to use a considerable amount of material, therefore, we joined the hippocampi of 5 or 6 animals that were considered as a n=1. It should be referred that the gender of the animals did not affect the results (data not shown). We had also the opportunity to access the APP synaptic distribution in rat striatum (n=2). In rat hippocampus, we observed that APP immunoreactivity was present: 59.9 ± 4.3% (n=3) in the pre-synaptic fraction, 29.6 ± 4.7% (n=3) in the post-synaptic fraction and 10.4 ± 2.5 % (n=3) in the extra-synaptic fraction (Figure 10). The quantification was performed in the experiments using the APP C-term antibody. These results were confirmed with the two APP antibodies (APP N-term and APP C-term). The same distribution pattern of immunoreactivity as observed in rat striatum (n=2) (Figure 11). The purity of the striatum preparations was similar to the hippocampal preparations (data not shown).

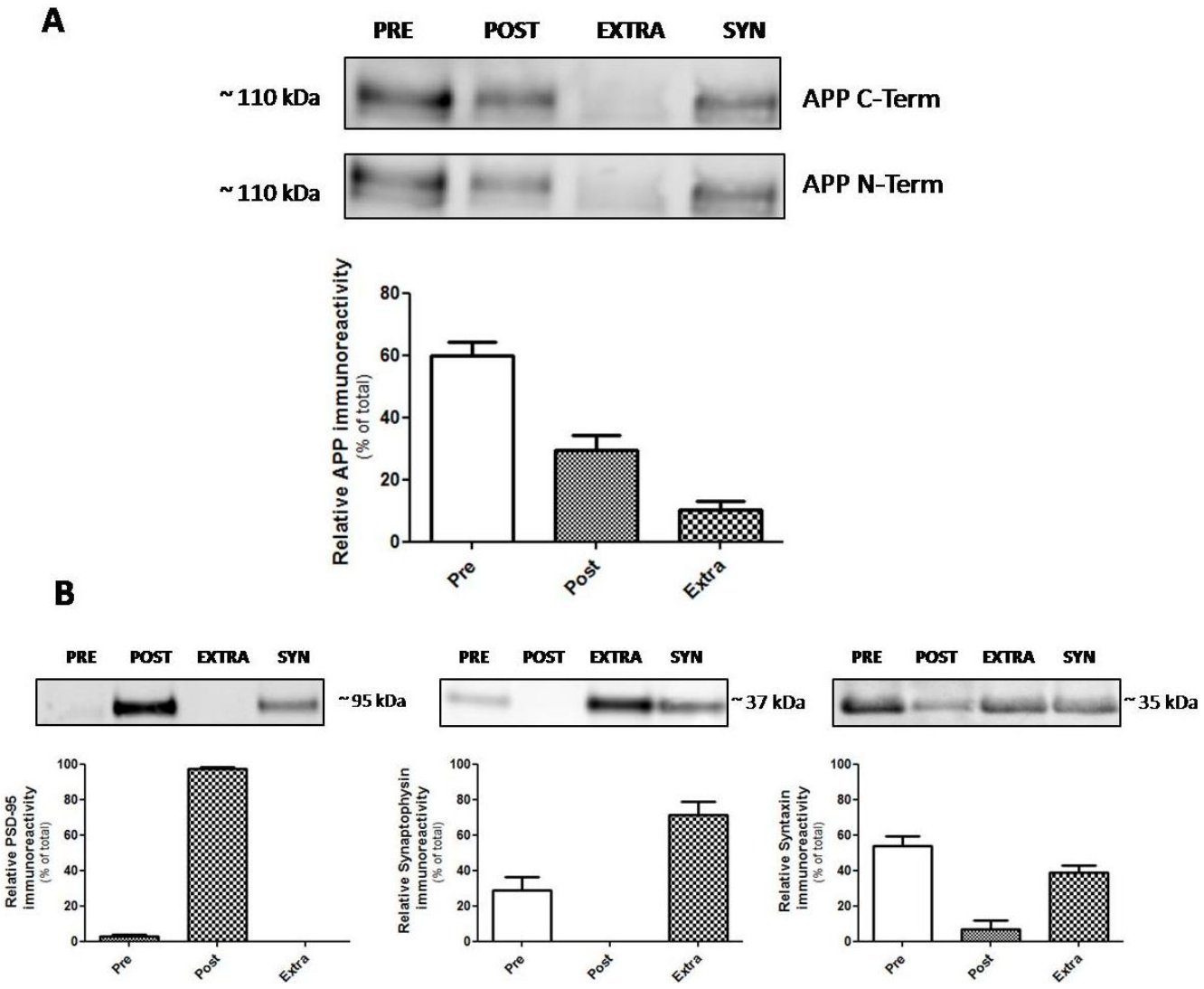

Figure 10: APP is enriched in pre-synaptic zone in rat hippocampus. Analysis of APP levels in pre, post and extra synaptic fractions of rat hippocampus. The levels of this protein where assessed by immunoblot using two antibodies for APP (APP C-Term and APP N-term) (A). The percentage of immunoreactivity was calculated relative to the maximum reactivity of each membrane and a representative image is shown above the graphic (% of the total). The quantification was performed in the experiments using the APP C-term antibody. (B) Controls of sub-synaptic preparations where it is expected an enrichment of sub-synaptic proteins in their respective membrane fraction. Thus, syntaxin is enriched in the pre-synaptic fraction relative to all other fractions, including total synaptosomes membranes; PSD-95 is enriched in the post-synaptic fraction and synaptophysin is enriched in the extra-synaptic fraction. Results are presented as mean ± SEM of n=3 independent experiments. (SYN) total synaptosomes fraction.

Figure 10 shows that the levels of APP are higher in the pre-synaptic fraction than in post-synaptic fraction in rat hippocampus. It was also observed a very small amount of APP in the extra-synaptic fractions. These results are in accordance with the data of literature about the distribution of APP, which state that APP is principally present in axons, and is transported by vesicles and secreted at the synapse (Groemer et al., 2011). Recently, the presence of APP in the synaptic vesicles has been shown (Groemer et al., 2011). Our results suggests that APP is restricted to a specific local in the nerve terminal and not spread indistinctly in the membranes of the synapse, axons and dendrites. The levels of APP in the pre-synaptic nerve terminals might be related with the high rate of its release at the synapse from synaptic vesicles, or with a putative specific function of full length APP in the synapse. This bulk of pre-synaptic APP could be involved in the production of Aβ at the synapse (Frykman et al., 2010).

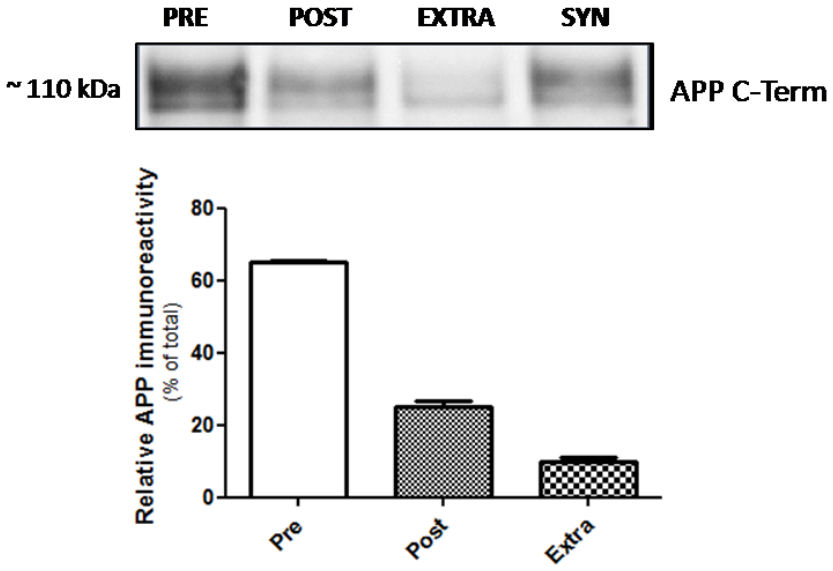

Figure 11: APP is more distributed in pre-synaptic zone in rat striatum. Analysis of APP levels in pre, post and extra synaptic fractions of rat striatum. The levels of this protein where assessed by immunoblot. The percentage of immunoreactivity was calculated relative to the maximum reactivity of each membrane (% of the total) and a representative image is shown above the graphic. In rat striatum, it was observed that APP immunoreactivity was present: 65.0 ± 0.3% (n=2) in the pre-synaptic fraction, 25.1 ± 1.5% (n=2) in the post-synaptic fraction and 9.9 ± 1.3% (n=2) in the extra-synaptic fraction. Results are presented as mean ± SEM of 2 independent experiments. (SYN) total synaptosomes fraction

An other interesting result is the amount of APP immunoreactivity in the post-synaptic fraction. Although previous reports have shown the presence of APP in the post-synaptic zone [and even co-precipitation with NMDAR2 subunit (Hoe et al., 2009)] in small quantities, the amount of APP present in the post-synaptic compartment was never compared to the others compartments where APP is present. Our results show for the first time that in rat hippocampus the APP is mainly present in pre-synaptic terminals, and that this protein also exists in post-synaptic fraction, accounting for almost one third of the amount of APP in the synapse (Fig.11).

3.4. Presence of APP in Glutamatergic, GABAergic and Cholinergic nerve terminals

Several studies have shown the presence and the distribution of the most known and studied cleavage fragment of APP, the β-amyloid peptide (Aβ), in different areas of the brain (Gouras et al., 2000). The Aβ oligomers are thought to be the culprit of AD, and it is know that the synapse is particularly vulnerable to Aβ oligomers (Selkoe, 2002). There are also evidences indicating that the glutamatergic and cholinergic terminals are the most affected by the Aβ oligomers (Moller, 1999; Kamenetz et al., 2003; Wang et al., 2005; Bell et al., 2006). However, it remains to be established whether APP is differentially distributed in the different types of brain nerve terminals; although there are reports about the location and distribution of APP in cultured neurons and in neuromuscular junctions (colocalization with cholinergic markers) (Wang et al., 2005; Wang et al., 2007). In this part of the study we aimed to define if APP is differentially distributed in glutamatergic, GABAergic and cholinergic nerve terminals. Our group has considerable experience in analysing single synaptosomes by fluorescence microscopy. The purification of nerve terminals allows their enrichment and enhances the accessibility of antibodies to epitopes located in synapses, thus this immunocytochemical approach has a higher sensitivity than immunohistochemical analysis of brain sections (Rebola et al., 2005). In this study we used a preparation of purified nerve terminals, enriched in pre-synaptic components, which are then spread and plated in glass coverslips and further used to perform immunocytochemistry analysis. Using these plated synaptosomes from rat hippocampus we have first analysed, by double immunolabelling the purity of ours nerve terminals preparations by determining the percentage of pre-synaptic markers (SNAP-25), post-synaptic markers (PSD-95) and glial contaminants (Glial Fibrillary Acidic Protein, GFAP). The overall marker for the nerve terminals to which we compare the percentage and colocalization of the others markers was synaptophysin.

The data obtained shows that more 80% of SNAP-25 immunoreactivity colocalized with synaptophysin, whereas less than 8% of synaptophysin positive terminals displayed PSD-95 immunoreactivity, and only a few structures (less than 1%) exhibited immunoreactivity for GFAP (Figure 12). These results indicate that our preparations of rat hippocampal synaptosomes are enriched in pre -synaptic nerve terminals.

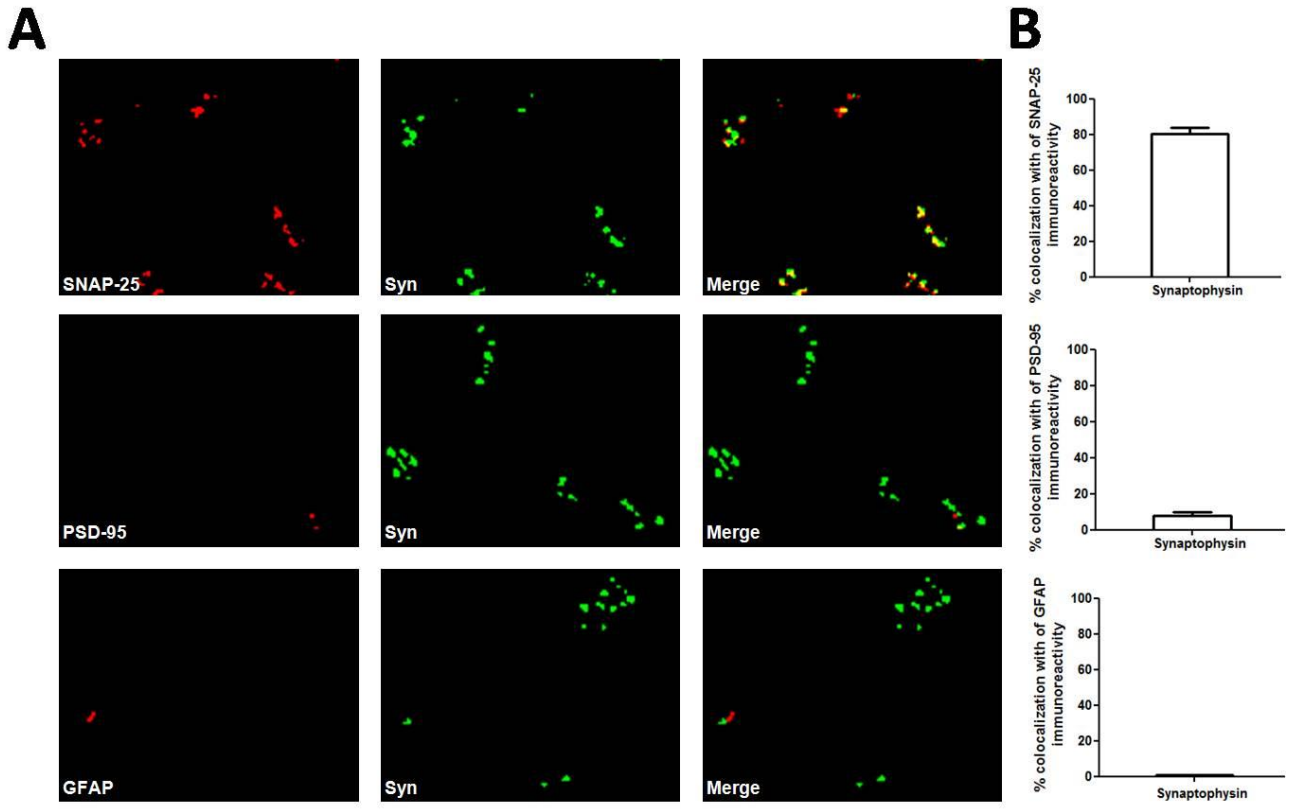

Figure 12: Characterization of plated purified nerve terminals preparations of rat hippocampus. Double immunocytochemistry analysis of synaptophysin and pre-synaptic (SNAP-25), post-synaptic (PSD--95 and glial markers (GFAP). (A) Representative images. (B) To assess the purity of the preparation the % of colocalization of synaptophysin with SNAP-25, PSD-95 or GFAP was quantified. Results are presented as mean ± SEM of 4 independent experiments. Magnification: 630x.

In order to assess if wether APP was equally distributed in the different types of nerve terminals or if it was more present in glutamatergic, GABAergic or cholinergic

nerve terminals; we determined the percentage of APP immunopositive terminals that colocalized with: i) the vesicular glutamate transporter (vGLUT1), a protein specific of glutamatergic nerve terminals (Fremeau et al., 2001; Hisano and Nogami, 2002; Gabellec et al., 2007; Liguz-Lecznar and Skangiel-Kramska, 2007); ii) the vesicular GABA transporter (vGAT) specific of GABAergic neurons (Takamori et al., 2000)and iii) the vesicular acetylcholine transporter (vAChT) that specifically labels cholinergic nerve terminals (Bejanin et al., 1994; Woolf et al., 2001). Previous studies performed by our group have characterized the proportion of the different type of nerve terminal in purified nerve terminals of rat hippocampus. With some variability, glutamatergic nerve terminals represented about 40% (Rebola et al., 2005; Rodrigues et al., 2005), GABAergic terminals 30% (unpublished data), cholinergic terminals 7% (Degroot et al., 2006), and dopaminergic terminals 9% (Degroot et al., 2006) of total hippocampal nerve terminals.

First we studied the presence of APP in purified synaptosomes of rat hippocampus. The percentage of synaptophysin immunopositive elements that were endowed with APP was 38.3 ± 3.9% (n=4). Then, we assessed the colocalization of APP with the different markers for nerve terminals (vGLUT1, vGAT and vAChT). The data presented in Figure 13 show that APP colocalizes with 30.9 ± 4.3% (n=4) of vGlut1 immunopositive terminals and 16.1% ± 2.8% (n=4) of GABAergic terminals (vGAT immunopositive), whereas a colocalization of only 3.7 ± 1.0% (n=4) was observed for cholinergic terminals (vAChT staining). The high localization of APP in glutamatergic terminals is not a surprise, because glutamatergic neurons are very abundant in the brain. It is likely that the high APP levels in glutamatergic terminals might favour the production of Aβ, which is known to cause synaptotoxicity. In fact, the glutamatergic neurons are also one of the most affected in AD (Moller, 1999; Kamenetz et al., 2003; Bell et al., 2008). Surprisingly, we observed a low percentage of cholinergic terminals that exhibited APP immunoreactivity, because there are some

reports showing APP in cholinergic synapses (Wang et al., 2005; Wang et al., 2007) and it was reported that the cholinergic synapses are also affected in AD (Bartus et al., 1982; Moller, 1999). These could be a characteristic specific of the hippocampal cholinergic terminals. However, this nerve terminals preparation from rat hippocampus has only a very small fraction of cholinergic terminals (around 7%), and the results may be diluted, being more significant in preparations that have a greater percentage of cholinergic terminals.

Although the higher presence of APP in glutamatergic than in GABAergic synapses is somehow expected from the evidences obtained in AD models, these results are also surprising if we take in account some observations from APP knockouts animals (Dawson et al., 1999; Seabrook et al., 1999). Studies with these animals suggest that normal glutamatergic transmission in hippocampus is not altered by the lack of this protein and its fragments, but GABAergic inhibitory synaptic transmission is reduced (Dawson et al., 1999; Seabrook et al., 1999). It would probably be expected that APP was more present in GABAergic terminals. One would also expect that APP was present in a significant amount in cholinergic synapses, because the APP knockout mice have alterations in the synaptic morphology and in maintenance in cholinergic terminals (Wang et al., 2005; Wang et al., 2007). However, it is always necessary to look cautiously at results from APP knockout mice, because most of its functions may be compensated by APLP1 and 2 (Heber et al., 2000).

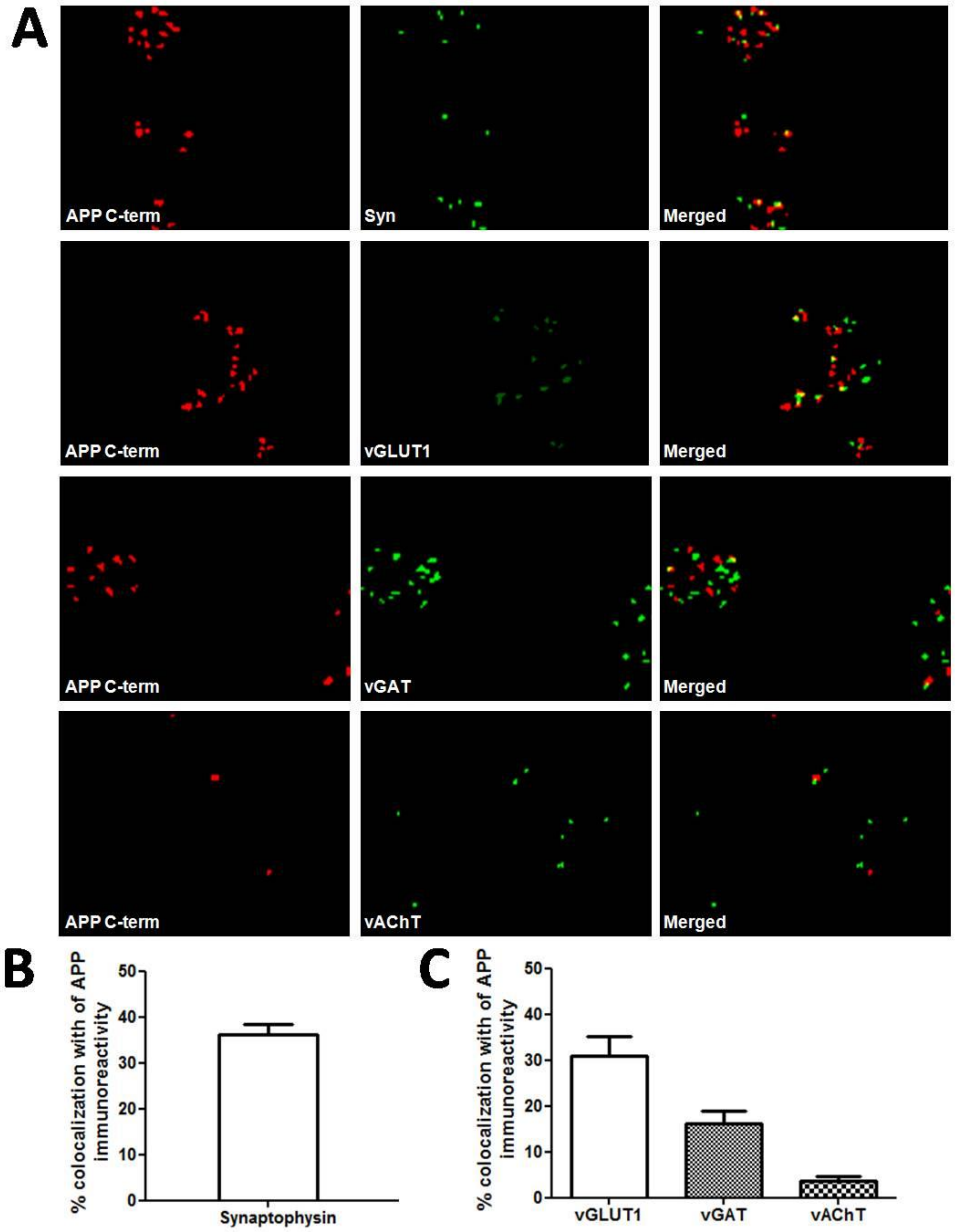

Figure 13: APP is highly localized in glutamatergic nerve terminals from rat hippocampus. Double immunocytochemistry analysis of APP with different markers of different types of synapses, mainly glutamatergic (vGLUT1), GABAergic (vGAT), and cholinergic (vAChT). (A) Representative images. (B) It was first determined the percentage of synaptophysin positive terminals that colocalized with APP. (C) The percentage of colocalization of the different nerve terminals markers with APP. Results are presented as mean ± SEM of 4 independent experiments. Magnification: 630x.

3.5. Is APP present in glial cells?

Since our data showed that APP is not present only in nerve terminals, we decided to investigate if that major bulk of non-synaptic APP was only neuronal or if this protein was also present in astrocytes and microglia. We focused on hippocampus of rat brain and performed double immunohistochemistry in rat hippocampal slices, using antibodies against APP and against proteins marker of mature neurons (Neu-N), astrocytes (GFAP) and microglia (CD11b). We decided to use the APP C-term antibody as a preferential antibody for these immunohistological analyses because it displays a greater immunoreactivity in the cellular bodies, which facilitates the observation of cells that have APP. We observed that APP immunoreactivity pattern was similar to the one of Neu-N (a neuronal marker), which points out for the possibility that hardly any APP is present in other cells in a significant amount (Figure 14).

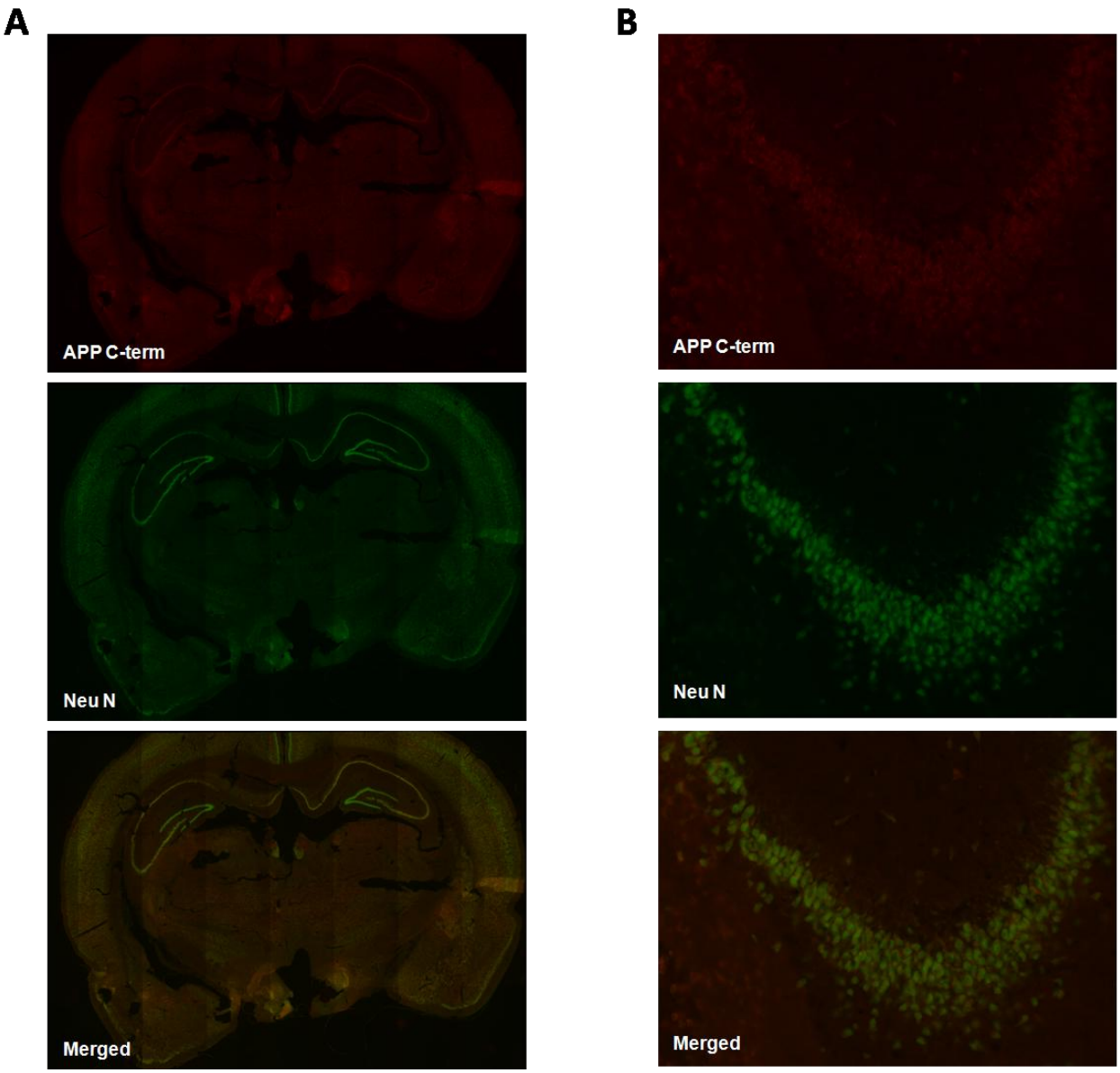

Figure 14: Double immunohistochemistry analysis of APP and Neu-N in rat brain slices, with focus on hippocampus (B). In (A) the image was acquired with Mosaic X application. The immunohistochemistry was performed in 30 µm slices of rat brain, which were labelled with APP C-term and Neu-N antibodies. Images are representative of 3 independent experiments. Magnification: A- 50x, B- 200x.

We further tried to assess the presence of APP in cells positive for GFAP or CD11b. In the images analysed in smaller magnifications we did not see APP immunoreactivity neither in GFAP positive cells nor in CD11b positive cells (Figure 16 A and 15 A respectively). The analysis of hippocampal slices by confocal microscopy

(with a higher magnification) was also performed to detect APP in glial cells; however, we still did not find evidences of APP presence in astrocytes or microglia (n=3) of rat hippocampus. The N-terminal APP antibody was tested in double immunohistochemistry with GFAP, but the results were also negative (Figure 15 C).

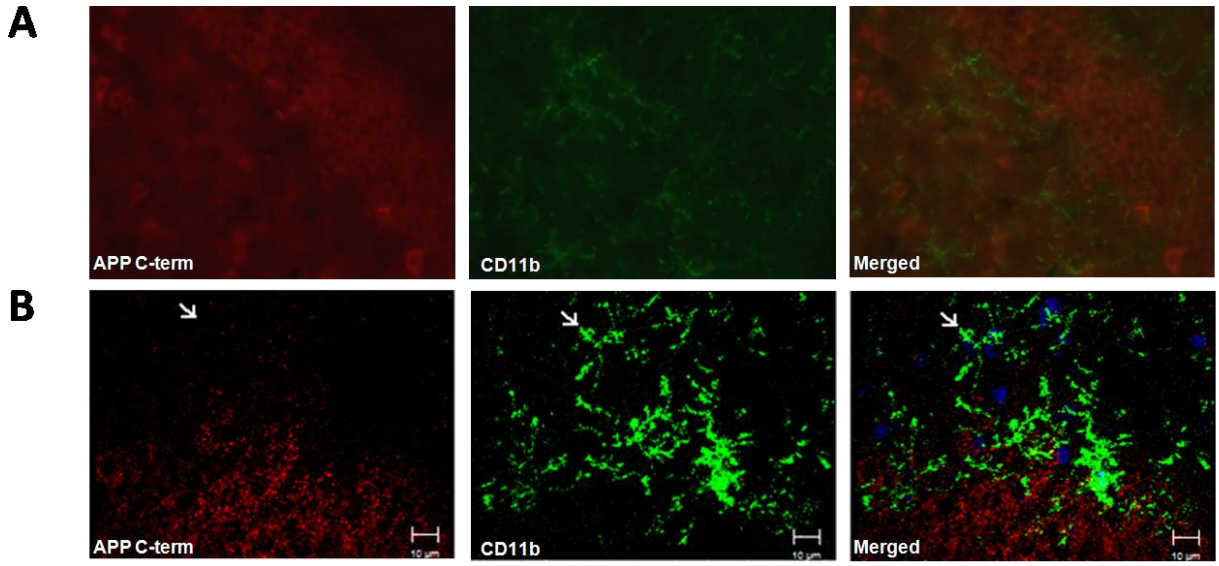

Figure 15: Double immunohistochemistry analyses of APP and CD11b in rat hippocampal brain slices. There is no evidence of presence of APP in CD11b positive cells. Arrows indicate a CD11b positive element that is not endowed with APP. The immunohistochemistry was performed in 30 µm slices of rat brain, which were labelled with APP C-term and CD11b antibodies. Images are representative of 3 independent experiments. Arrows indicate microglia that does not have APP immunoreactivity. Magnification: A -200x, B -630x (confocal image).

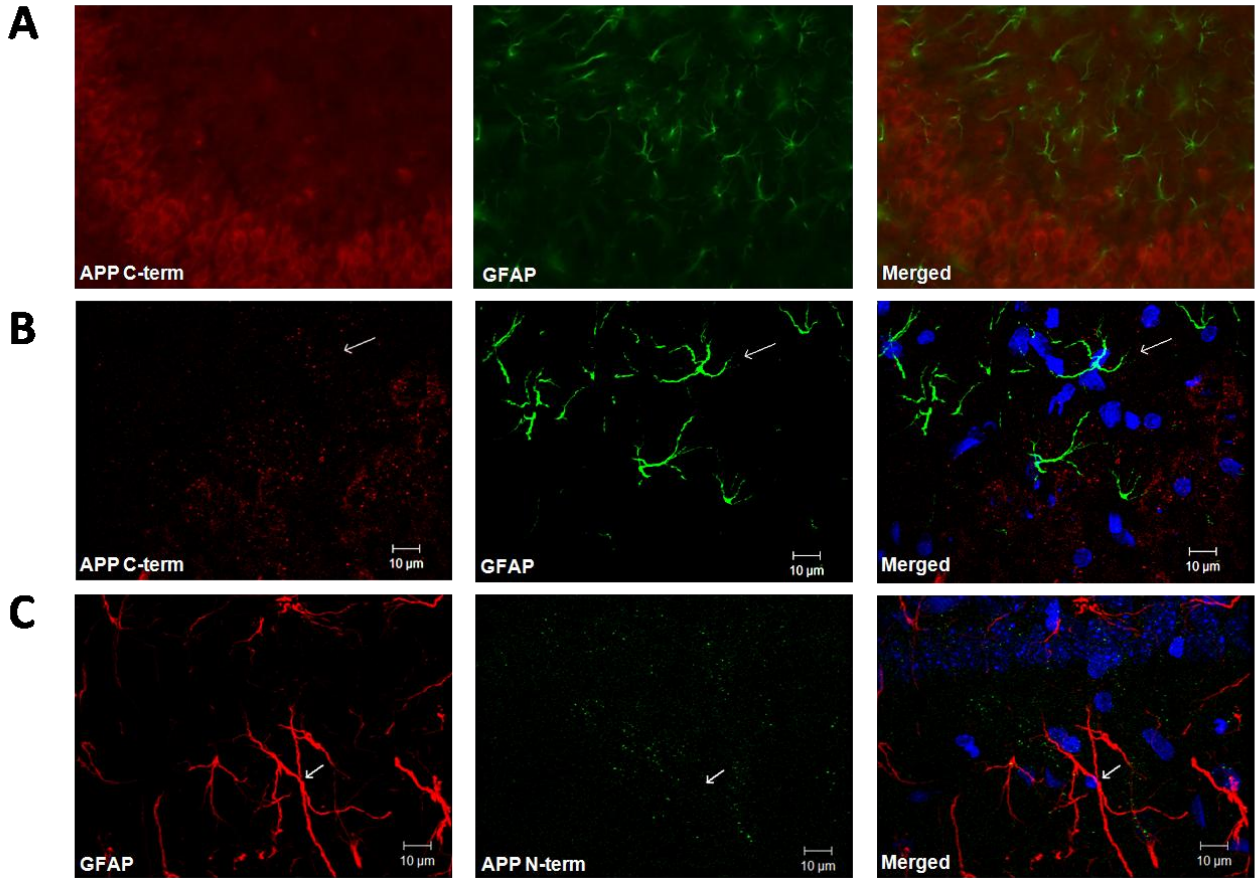

Figure 16: Double immunohistochemistry analyses of APP and GFAP in rat hippocampal brain slices. There is no evidence of presence of APP in GFAP positive cells. The immunocytochemistry with APP N-terminal antibody also did not show the presence of APP in astrocytes (C). The immunohistochemistry was performed in 30 µm slices of rat brain, which were labelled with APP C-term (A, B), APP N-term (C) and GFAP antibodies. Images are representative of 3 independent experiments. Arrows indicate astrocyte that does not have APP immunoreactivity. Magnification: A -200x, B and C-630x (confocal image).

In some studies the presence of APP in cultured astrocytes and microglia cells was reported. However, APP displayed very low immunoreactivity in these cells in normal conditions (Berkenbosch et al., 1990; von Bernhardi et al., 2003). Only when the glial cells were challenged with a noxious stimulus, did the amount of APP significantly raised (Berkenbosch et al., 1990). There are some articles referring the

presence of APP in astrocytes in rodent's brain (Ouimet et al., 1994; Marksteiner and Humpel, 2008; Schmidt et al., 2008). The discrepancy between these studies and our observations might be related with differences in the different method´s sensitivity, or because in our preparations the glial cells were not in a reactive state in our preparations.

4. Conclusions and Final remarks

4. Conclusions and Final remarks

4.1 Conclusions

The main goals of this study were to evaluate if APP is enriched in the synapses, whether it is preferentially located at the pre, post- or non-synaptic fraction, and if APP is differently distributed in glutamatergic, GABAergic and cholinergic synapses in the rat hippocampus.

We observed that APP is present in higher quantities in the bulk of neuronal membranes than in synaptosomes. Nevertheless, APP is present in significant amounts in the synapse. We also observed undoubtedly that in synapses, APP is preferentially located in the pre-synaptic fraction, whereas a small fraction of it is present in the post-synaptic fraction. The presence of APP in different nerve terminals was also evaluated; and we observed that glutamatergic terminals have a higher percentage of APP than GABAergic or cholinergic nerve terminals in the rat hippocampus. This is the first study showing differentially distribution of APP in different nerve terminals. Due to the great amount of APP that was present outside the nerve terminals, we assessed whether APP was present in astrocytes and microglia, but did not found any APP immunoreactivity in these cells of rat brain.

Overall, this study shows that in the rat hippocampus APP is present in synapses, mainly in the pre-synaptic compartment, and this could justify some of the synaptic functions of APP. We speculate that that this “pool” of synaptic APP could be involved in the production and release of Aβ peptide in the synaptic space This production of Aβ peptide in the synapses might contribute to the synaptotoxicity which occurs in early phases of AD, and is thought to contribute to cognitive deficits associated with this neurodegenerative disorder .

4.2 Final remarks

The conclusions of this study opens the “doors” for many questions and future work.

As we did not find evidence of the presence of APP in astrocytes and microglia, the presence of this protein could be evaluated in preparations that are more sensible and enriched in glial compartment, like gliosomes (fractions of astrocytes that can be isolated from tissue homogenate). Another experiment that can be done in this line, is the evaluation of APP immunoreactivity in brain slices obtained from animals models of diseases or treatments that are known to be associated with proliferation and activation of astrocytes and microglia (such as rodents models of AD, kainate, or LPS administration).

Another question that needs to be more deeply studied is the analysis of APP presence in cholinergic terminals. One way to achieve this it may be used purified nerve terminals preparations of brain regions with enriched in cholinergic neurons, such as striatum. An immunohistochemical approach to assess the presence of APP in cholinergic terminals in hippocampus may also be tried.

Besides hippocampus, the entorhinal cortex is a region quite affected in AD. It is of big interest to exploit the synaptic location of APP in this region.

It would be interesting to analyse if the secretases, involved in the APP processing (mainly γ- and β-secretases, that are involved in the generation of Aβ), have a distribution pattern similar to APP in the synapse. The evaluation of possible colocalization of the secretases and APP in different nerve terminals of hippocampus and entorhinal cortex should also be investigated.

The most preeminent experiments that could be carried out in the following of this study are the ones comparing the synaptic location of APP in animal models of AD,

to evaluate if the location of this protein is altered in those animals. These experiments could help to clarify some important questions about the processes that are involved in the beginning of AD pathology.

5. References

5. References

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