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ASTROCYTIC CHANGES IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

ASTROCYTÁLNÍ ZMĚNY V MYŠÍM MODELU ALZHEIMEROVI CHOROBY

PHD THESIS

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS:

$[Ca^{2+}]_i$	intracellular calcium
$[K^+]_o$	extracellular potassium
129/C57BL6	hybrid mice generated from 129/Sv and C57BL6 wild-type strains
192 IgG Saporin	12 clone monoclonal antibody to the nerve growth factor receptor
20-HETE	20-hydroxyeicosatetraenoic acid
3xTg-AD	triple transgenic mouse model expressing the APPSwedish, PS1M146L and TauP301L mutations
4R/2N	single transgenic mouse model expressing the human Tau R406W mutation
7TauTg	single transgenic mouse model overexpressing tau protein without the L neurofilament subunit
AA	arachidonic acid
Aβ	amyloid beta-peptide aggregates
AC	anterior cingulate cortex
AD	Alzheimer's disease
ADDL	amyloid beta derived diffusible ligand
ADnnPP7 x tauP301S	double transgenic animal model expressing the Danish APP mutation and the tau P301S mutation
AF64A	etylcholine mustard aziridinium ion
AGm	agranular cortex
ALS	amyotrophic lateral sclerosis
ALZ7	transgenic animal model which expresses the longest human four-repeat (4R) tau isoform (htau40) under thecontrol of the human Thy-1 promoter
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
apoE	apolipoprotein E
APP	amyloid precursor protein
APP23	transgenic animal model which overexpresses APP751 with the Swedish mutation under the Thy1 promoter
APP23 x JNPL3 on C57BL8	double transgenic animal model which overexpresses APP751 with the Swedish mutation and the tau P301L mutation, generated on wild-type mouse background
APP23 x PR5	double transgenic animal model which overexpresses APP751 with the Swedish mutation and the tau P301L mutation
APP_{751SL}	transgenic animal model expressing the APP751SL mutation
$APP_{751SL}/PS1_{M146L}$	transgenic animal model expressing the APP751SL and PS1M146 mutations
APP_{Swe}	double transgenic animal model expressing the APP Swedish K670N/M671L mutations
APP_{Swe} and Indiana V717F	double transgenic animal model expressing the APP

	Swedish K670N/M671L and Indiana V717F mutations
APP_{Swe} and PS1M146L	double transgenic animal model expressing the APP Swe (K595N, M596L) and PS1 (A246E) mutations
APP_{Swe}/PS1dE9	double transgenic animal model expressing the APP Swe (KM593/594NL) and PS1dE9 mutations
APP_{V717F}	double transgenic animal model expressing the Swedish and Indiana (D664A) mutations
APP-V717l x tauP301L	double transgenic animal model expressing the APP (V717I) and tau (P301L) mutations
APS	ammonium persulfate
AQPs	aquaporins
ARs	adrenergic receptors
ATP	adenosine triphosphate
BACE1	beta-site amyloid precursor protein clearing enzyme 1
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BF	basal forebrain
BSA	bovine serum
C83	C-terminal fragment of 83 amino acid residues
C99	C-terminal fragment of 99 amino acid residues
CA	cornu ammonis
$[Ca^{2+}]i$	intracellular calcium concentration
cAMP	cyclic adenosine monophosphate
CBD	corticobasal degeneration
CEPB1	cytoplasmic polyadenylation element (CEP)-binding protein
ChEIs	cholinesterase inhibitors
CJD	Creutzfeld-Jakob disease
CLA	claustrum
CNS	central nervous system
COX	cyclooxygenase
CSPG-4	chondroitin sulfate proteoglycan 4
CYP450	cytochrome 450
D1; D2	dopamine receptor 1; 2
DAAO	D-amino acid oxidase
DAB	3,3'-diaminobenzidine
DAG	diacylglycerol
DG	dentate gyrus
DLPFC	dorsolateral prefrontal cortex
DMPFC	dorsomedial prefrontal cortex
DMSO	dimethyl sulfoxide
D-ser	D-serine
E693G	transgenic animal expressing the APP Arctic mutation
EAAC1	excitatory amino acid carrier-1
EAATs	excitatory amino acid transporters
EC	entorhinal cortex
ECL	enhanced chemiluminescence substrate
EDTA	ethylenediaminetetraacetic acid

EETs	epoxyeicosatrienoic acids
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FPm	medial frontal polar cortex
FTD	frontotemporal dementia
G272V	single transgenic animal model expressing the tau G272V mutation
G272V, P301L, R406W	triple transgenic animal model expressing the G272V, P301L and R406W mutations
GABA	γ-aminobutyric acid
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate-aspartate transporter
Gln	glutamine
GLT-1	glutamate transporter 1
Glu	glutamate
GLUT	glucose transporter
GS	glutamine synthetase
HAD	HIV-associated dementia
HC	hippocampus
HDL	high-density lipoproteins
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IF	intermediate filament
IgG	immunoglobulin G
iGluR	ionotropic glutamate receptor
IL	infralimbic cortex
Ins(1,2,3)P_3	inositol-1,4,5-triphosphate
IP_3	inositol triphosphate
IR	immunoreactive
JNPL3	transgenic animal model which expresses P301L tau and gtau43 mutations under the murine PrP promoter
$[K^+]_o$	extracellular potassium concentration
K670M/N671L	single transgenic animal model (rat) expressing the APP Swedish K670N/M671L mutation
K670N/M671L and V717F	double transgenic animal model expressing the APP Swedish K670N/M671L and Indiana V717F mutations
Kir	inwardly rectifying potassium channel
KO	knockout
LDH	lactate dehydrogenase
LDL	low-density lipoproteins
LTP	long-term potentiation
mAChRs	muscarinic acetylcholine receptors
MBP	myelin basic protein

MDD	major depressive disorder
mGluR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex
MS	multiple sclerosis
MSCs	mesenchymal stem cells
MTB	microtubule-binding
MTC1; 2	monocarboxylase transporter 1; 2
nAChRs	nicotinic acetylcholine receptors
nbM	nucleus basalis of Meynert
$[Na^+]i$	intracellular sodium concentration
NF200	neurofilament 200
NFTs	neurofibrillary tangles
NG2	neuron-glial antigen 2
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
NOS	nitric oxide synthase
NSCs	neural stem cells
Nv	numerical density (cell number/mm^3)
OD	optical density
OFC	orbitofrontal cortex
OMIM 104760	Online Mendelian Inheritance in Man p.104760
OPCs	oligodendrocyte progenitor cells
P301L	single transgenic animal model expressing the P301L tau mutation
P301L TET-off	single transgenic animal model expressing the P301L tau mutation regulated by tetracycline responsive promoters
P301S	single transgenic animal model expressing the P301S tau mutation
P301S/G272V	double transgenic model expressing the P301S and G272V tau mutations
PB	phosphate buffer
PDAPP	transgenic animal model expressing V717E mutant APP under the control of the human platelet derived growth factor beta (PDGFβ) promoter
PET	positron emission tomography
PFC	prefrontal cortex
PGE_2	prostaglandin E_2
PiD	Pick's disease
PIR	piriform cortex
PL	prelimbic cortex
PLA_2	phospholipase A_2
PLC	phospholipase C
PLP	proteolipid protein
PPF	paired pulse facilitation
PR5	transgenic animal model expressing the P301L tau mutation with the htau40 isoform and under the control

	of the mThy1.2 promoter
PRC	perirhinal cortex
PS1; PS2	presenilin1; presenilin 2
PS1M146L	transgenic animal model expressing the PS1M146L mutation
PS1M156V	transgenic animal model expressing the PS1M156V mutation
PS2APP(PS2/APP_{Swe})	double transgenic animal model expressing the APP (K670N, M671L) and PS2 (N141I) mutations
PSEN1; PSEN2	presenilin1; presenilin2 genes
PSP	progressive supranuclear palsy
RE	nucleus reuniens of the thalamus
ROS	reactive oxygen species
RT	room temperature
sAPPα	soluble N-terminal fragment of amyloid precursor protein α
sAPPβ	soluble N-terminal fragment of amyloid precursor protein β
SDS	sodium doddecyl sulfate
SEM	standard error of the mean
SGZ	subgranular zone
SLC1	solute carrier family 1
SON	supraoptic nucleus
SR	serine racemase
STAT3	signal transducer and activator of transcription 3
STEN	lysis (Salt, Tris, EDTA, NP40) buffer
SVZ	subventricular zone
synthetic $A\beta_{1-42}$ x PR5	double transgenic animal model expressing the P301L tau mutation with the htau40 isoform and injected synthetic Aβ1-42 fibrils
Tau_{P301L}	single transgenic animal model expressing the P301L tau mutation
Tau_{P301L}(4R, 2-, 3-)	single transgenic animal model expressing the tau P301L mutation
TauPS2APP(APP152xPR5)	double transgenic animal model expressing the APP152 and tau P301L mutations
TBST	Tris Buffered Saline Tween 20
TEMED	tetramethylethylene-diamine
Tg1116	transgenic animal model (rat) expressing the APP V717l mutation
TG23	transgenic animal model which expresses the shortest human tau isoform (htau 44) under the murine 3-hydroxy-methyl-glutaryl CoA reductase promoter
Tg2576	transgenic animal model expressing human APP (1-695) with the Swedish mutation under the hamster PrP promoter
Tg2576 and VLW	triple transgenic animal model expressing the APP Swedish (KM670/671NL), V717F and triple tau

	(G272V, P301L and R406W) mutations
Tg2576xJNPL3(APP_{Swe})	triple transgenic animal model expressing the APP Swedish (KM670/671NL), V717F and tau P301L mutations
Tg478	transgenic animal model (rat) expressing the APP Swedish K670N/M671L mutation
Tg478/Tg1116	double transgenic animal model (rat) expressing the APP Swedish K670M/N671L and V717I mutations
TGF-α	transforming growth factor α
TNF-α	tumor necrosis factor α
TRITC	tetramethylrhodamine isothiocyanate
TS	Trizma base saline
TSPs	thrombospondins
TT	tania tecta
V337M	transgenic animal model expressing the human tau V337M mutation
V717F	transgenic animal model expressing the APP V717F mutation
V717l	transgenic animal model expressing the APP V717I (London) mutation
VEGF	vascular endothelial growth factor
Vim	vimentin
VLPFC	ventrolateral prefrontal cortex
VMPFC	ventromedial prefrontal cortex
VO	ventral orbital cortex
WB	Western blot

1. INTRODUCTION:

1.1. ASTROCYTES:

1.1.1. Historical background and types of astroglial cells:

The brain consists of two cooperating major classes of cells – excitable neurons and nonexcitable glia (Verkhratsky et al., 2011). The first concept of glia was introduced by the 19th century pathologist Rudolf Ludwig Karl Virchow in his commentary (Virchow, 1858). The term "glia" was adopted from the Greek word "γλία" and is translated as "glue" – meaning something with sticky features. Virchow claimed that glia have a fully connective function, literally gluing together. Carl Ludwig Schleich (Schleich, 1894) proposed more active interactions between neurons and glia, important for proper brain function such as the control of excitatory or inhibitory transmission. The name "astrocyte", as a construction of two Greek words meaning star ("astro") and cell ("cyte"), was finally introduced by Michael von Lenhossek (Lenhossek, 1893). Two broad groups of "classic" astrocytes are: protoplasmic astrocytes residing in the grey matter and fibrous astrocytes of the white matter. Additionally, interlaminal astrocytes are located in the cerebral cortex of higher primates (Colombo and Reisin, 2004). All of the mentioned cells are common in the mature brain, whereas radial glia are the source and first-in-place during development, also acting as a platform in the process of neuronal migration (Gotz et al., 2002). After reaching maturation, cells of radial appearance can still be found in the retina and cerebellum as Müller and Bergmann glia, respectively (Lewis and Fisher, 2000; Bellamy, 2006). In the cerebellum velate astrocytes also reside, where they ensheath granule neurons. Among other astroglial cells tanycytes, pituicytes, perivascular and marginal astrocytes have to be mentioned, as well as ependymocytes found in the ventricles, cells of the choroid plexus and retinal pigment epithelial cells (Verkhratsky and Butt, 2007). Also, stem cells were identified as astrocyte-like cells from two neurogenic regions of the mature brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) (Ganat et al., 2006). The SVZ of the lateral ventricles is the place where new neurons are generated and migrate through the rostral migratory stream (RMS) to reach mainly the olfactory bulb, while the SGZ is located in the dentate gyrus

of the hippocampus, where granule cells are produced (Gage, 2000; Ming and Song, 2011).

1.1.2. Morphology and organization of astrocytes:

The image of astrocytes as star-like cells is still valid and commonly found in the literature. However, together with developing novel techniques to reconstruct their shape, some researchers claim that they exhibit a more cubic, rounded or spongiform appearance instead (Bushong et al., 2002; Nedergaard et al., 2003). Generally, the protoplasmic astrocytes show a very complex arrangement of their main processes, likewise the very fine ones that appear at the last stage of the maturation period (Wilhelmsson et al., 2006). This type of astrocyte establishes their own microanatomical domains, with strict boundaries within the limits of their processes (Bushong et al., 2002; Oberheim et al., 2009). Those domains are the special workplaces of astrocytes, where they cover synapses and neuronal membranes and send processes towards the blood vessels in their close proximity to establish, isolate and metabolically support their functional units (Verkhratsky et al., 2011; Nedergaard and Verkhratsky, 2012). In contrast, fibrous astrocytes are less intricate, with rather straight and not so branched processes. These processes are not isolated in separate domains, instead they overlap with the extensions of neighboring astrocytes (Oberheim et al., 2006). While the processes of protoplasmic astrocytes form perivascular endfeet, the fibrous astrocytes establish contacts with neuronal axons at the nodes of Ranvier (Verkhratsky and Butt, 2007).

The total density of protoplasmic astrocytes within the cortex equals around 10,000 to 30,000 per mm^3, while all of their processes can cover a surface area of even 80,000 μm^2 (Verkhratsky and Butt, 2007). In the adult mouse cortex, a single astrocyte can closely interact with 4 to 8 neurons and approximately 300 to 600 neuronal dendrites (Halassa et al., 2007), whereas in humans and rodents the density of synaptic contacts varies between 1,100 and 1,300 million per mm^3 (DeFelipe et al., 2002). Also, the astrocyte-neuron ratio increases together with advances in evolution and brain development, thus while in the leech just one astrocyte coexists with 25-30 neurons, in the human cortex this ratio equals 1.4:1 (Bass et al., 1971). Astrocytes in humans outnumber the astrocytes found in lower mammals by tenfold and are far more complex. As an example, human protoplasmic astrocytes posses about 40 main processes, compared to barely 3-4 main ones in murine

astrocytes (Oberheim et al., 2006) (Fig.1). Such correlations in numbers between neurons and astrocytes evoke strong interest among scientists to investigate why and how astroglial cells contribute to specific brain activities such as higher cognitive functions.

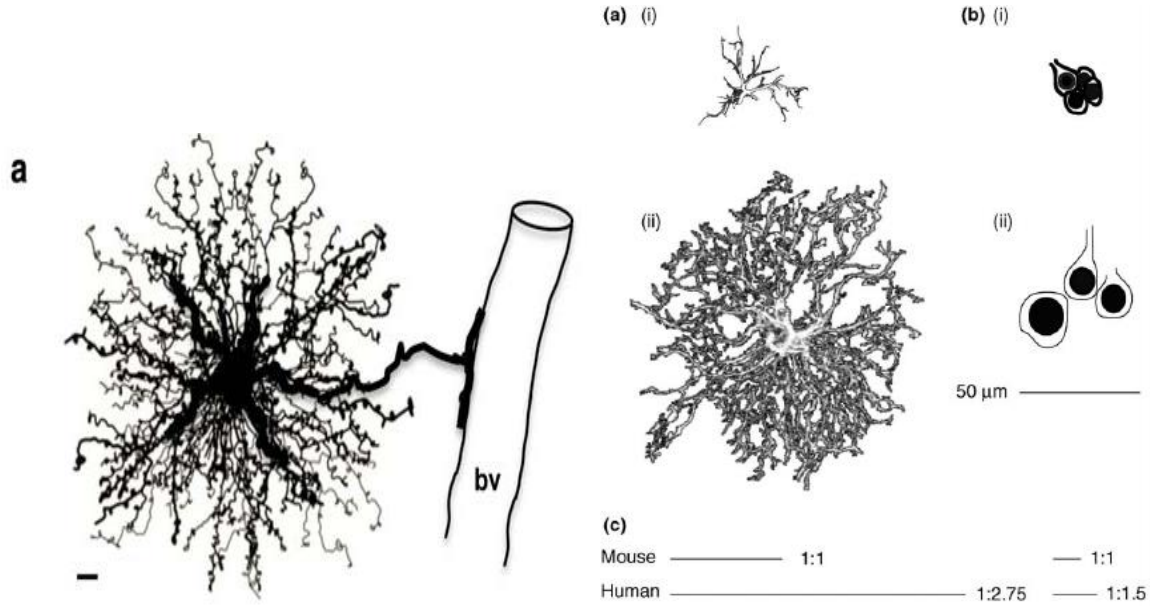

Fig.1 *(Right) Evolution of astrocytes and neurons.(a) Graphics of (i) mouse and (ii) human cortical astrocytes as well as (b) (i) mouse and (ii) human cortical neurons; (c) corresponding bars representing the sizes of the cells (Oberheim et al, 2006). (Left) Representation of a protoplasmic astrocyte with an established connection with a blood vessel via the astrocytic foot; scale bar 3µm (Sofroniew et al, 2010).*

1.1.2.a. Astrocytic intermediate filaments:

Intermediate filaments (IF) serve as a main component of the astrocytic cytoskeleton. They create a network that assures the flexibility and integrity of the cell. Among major astrocytic IF proteins are (i) predominant glial fibrillary acidic protein (GFAP), (ii) vimentin, (iii) nestin and (iv) synemin (Middeldorp and Hol, 2011). All of them seem to mingle and interact with each other in various ways, depending on the developmental stage and pathophysiological conditions. Intermediate filaments (with a diameter of 8-12 nm) are not alone in creating the cytoskeleton of each eucaryotic cell, they also require close collaboration with larger microtubules (diameter 25 nm) and small actin microfilaments (diameter of 7 nm) (Lepekhin et al., 2001). Up to now, knowledge about the functions of astrocytic IF remains incomplete; however, certain presumptions and hypotheses have been made.

GFAP was defined, purified and described from the specific plaques made of fibrous astrocytes and demyelinated axons, found in the brains of multiple sclerosis (MS) patients (Eng et al., 1971; Eng et al., 2000). This protein is assumed to be a standard astrocytic marker used worldwide in various studies and in the context of many diseases, such as Alexander's disease, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), depression and autism (Miguel-Hidalgo et al., 2000; Brenner et al., 2001; Laurence and Fatemi, 2005; Middeldorp and Hol, 2011). Among other functions, GFAP is known to be involved in the motility of astrocytes, as well as in the structural stability of their processes (Weinstein et al., 1991; Lepekhin et al., 2001).
All types of intermediate filaments proteins consist of three main domains: the amino-terminal "head", central helical "rod" and carboxy-terminal "tail" (Reeves et al., 1989) (Fig.2).

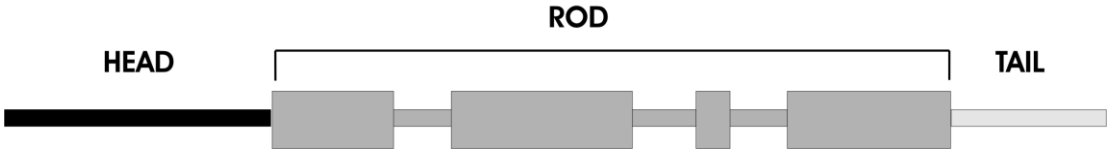

Fig.2 *General structure of intermediate filaments.*

The process of proper IF protein assembly, especially GFAP, is regulated by phosphorylation and depends on a complete head domain as well as on proper protein concentration. In case this concentration is too high, the cytoskeleton will break down and remains as IF aggregate (Eng et al., 1998; Sihag et al., 2007; Middeldorp and Hol, 2011). GFAP exists in many different isoforms, among which the best known and studied is GFAPα (Middeldorp and Hol, 2011). During several pathological events within the brain such as injury and CNS degeneration, GFAP expression increases excessively, resulting in a process called reactive astrogliosis (see chapter 1.1.4.a) (Pekny and Pekna, 2004). A close partner of GFAP is vimentin, also a type III intermediate filament. Vimentin is normally present in radial glia, potential precursors in adult neurogenesis, as well as in immature astrocytes, in which during the process of differentiation it is replaced by GFAP (Dahl et al., 1981; Gotz et al., 2002). All in all, GFAP is the main intermediate filament in the adult brain, expressed as a norm by adult astrocytes, where it

replaces vimentin, dominant in the neonatal brain (Middeldorp and Hol, 2011). In addition to the switch from vimentin to GFAP during the maturation of astrocytes, an increase in GFAP expression is also observed also during aging, in both humans and laboratory rodents (Nichols et al., 1993; Eng et al., 1998; Hinman and Abraham, 2007). This process is most probably connected with the ongoing accummulation of oxidized proteins in the body (Morgan et al., 1997).

Several studies on rodents knockouts (GFAP-/-, Vim-/- and double KO) have demonstrated substantial interrelations between intermediate filaments and their specificity. For example, mice without GFAP (GFAP-/-) are able to develop and reproduce in a normal way, but they are unable to compensate for the lack of this protein by the upregulation of the others, what leads to fully IF-deficient astrocytes in the hippocampus and the white matter of the spinal cord (Pekny et al., 1995; Faulkner et al., 2004; Pekny and Nilsson, 2005). When GFAP or vimentin is not present, the mean cell speed of astrocytes is restricted, and this restriction worsens when both proteins are eliminated (Lepekhin et al., 2001). Furthermore, IF proteins are strongly involved in the recovery process after CNS damage, when their action can have both positive and also negative effects (see chapter 1.1.4.a).

GFAP is a key player in many cellular processes involving astrocytes-neuron regulatory interactions, such as synaptic plasticity, glutamate homeostasis, neurites outgrowth and axonal myelination. Taking into consideration the protein's importance, every change in its expression can lead to significant malfunctions at the level of the synapses as well as the glutamate-glutamine cycle (Olabarria et al., 2010; Middeldorp and Hol, 2011; Yeh et al., 2011; Kulijewicz-Nawrot et al., 2012). As an example, Tanaka and his colleagues (Tanaka et al., 2002) found out that after transient ischemia in GFAP-/- mice, both hippocampal long-term potentiation (LTP) and paired pulse facilitation (PPF) are significantly depressed compared to controls. Together with the observed lower immunoreactivity for NF200 (neurofilament 200) in the GFAP-/- post-ischemic hippocampus, which indicates loss of axonal branches, the researchers concluded that GFAP was highly relevant for neuronal survival and synaptic plasticity.

Other IF proteins, nestin and synemin are expressed by undifferentiated astrocytes (Luna et al., 2010). Nestin, a type VI IF protein is also assumed to be a widely accepted marker

for multipotent neural stem cells (NSCs), and its expression is used in monitoring the proliferation, migration and differentiation of NSCs (Park et al., 2010). Similar to vimentin, during development nestin gradually becomes downregulated and is replaced in astrocytes by GFAP. After an injury of the adult CNS, nestin is detected again, indicating its regenerative potential (Pekny and Nilsson, 2005; Park et al., 2010). Nevertheless, the presence of nestin is not vital for the integrity of the cytoskeleton, and this protein is not able to polymerize by itself or to form IF network (it can just incorporate into such a network), neither alone nor with GFAP (Eliasson et al., 1999; Park et al., 2010). As was shown on primary astrocytic cultures, nestin requires a partnership to organize itself into a network composition (Marvin et al., 1998). The same tendency and needs are displayed by synemin, which means that not GFAP, but rather vimentin is the proper partner for polymerization (Titeux et al., 2001). Synemin is one of the most interesting IF proteins. Sultana and her group (Sultana et al., 2000) showed that during the development of the rat cortex, synemin is present only in a specific astrocytic subpopulation that expresses all three proteins: GFAP, vimentin and nestin. Surprisingly, synemin is not found before the appearance of GFAP, in contrast to the other two proteins typical of immature astrocytes/radial glia. It can be concluded that synemin serves as a transient and unique protein, expressed during the subtle biochemical and morpohological changes that radial glia cells undergo in order to become mature astrocytes.

1.1.2.b. S100β:

Another protein and growth factor typical for astrocytes is S100β (or S100B), isolated for the first time from bovine brain and named "S100" due to its solubility in a 100% saturated solution of ammonium sulfate (Moore, 1965; Van Eldik and Wainwright, 2003). This protein is a member of the broad family of S100 proteins, which because of their structure are able to detect Ca^{2+} and upon activation by this molecule interact with intracellular target proteins, influencing their activity (Steiner et al., 2011). S100β protein is engaged in many processes including the most important neuron-glia interactions within the brain. Among the beneficial effects of S100β are those connected with neuronal development and maintenance, together with the induction of neurite outgrowth, neuronal guidance during development and neuronal survival (Haglid et al., 1997; Mrak and Griffinbc, 2001; Van Eldik and Wainwright, 2003). S100β protein was shown to be

an important potential neuromodulator in the nervous system because of its effects on neuronal electrical activity by increasing or decreasing concrete potassium currents (Kubista et al., 1999).

It is well known that reactive astrocytes overexpress S100β (Mrak and Griffinbc, 2001). Elevated levels of S100β were reported in various pathological conditions, including Alzheimer's disease (Van Eldik and Wainwright, 2003; Steiner et al., 2011). This increased level can be directly neurotoxic or can provoke astrocytes and microglia to generate an inflammatory response manifested by the production of cytokines and NO, which can result in the dysfunction or even the death of neurons (Steiner et al., 2011). In Alzheimer's disease, the vast majority of overexpressing S100β reactive astrocytes are detected around Aβ plaques, where the degree of the increased expression of this protein is correlated with the stage of plaque formation from non-fibrillar deposits to their final form (Mrak and Griffinbc, 2001).

S100β has been used in many studies as a potential biomarker of the disease progression; however, due to certain limitation and inconsistent data, the usefulness of the protein is still under debate and needs more attention together with careful experimental conditions (Tumani et al., 2008; Steiner et al., 2011).

1.1.3. Role of astrocytes in the physiology of the CNS:

Astrocytes are omnipresent glial cells occupying 25-50% of brain volume (Kimelberg and Norenberg, 1989; Magistretti and Ransom, 2002). They are key players in providing metabolic and structural support, as well as contributing to BBB formation, controlling the microenvironment and signaling within the CNS (Verkhratsky and Butt, 2007; Sofroniew and Vinters, 2010). Specific kinds of stem cell like-astrocytes residing in the hippocampal subgranular zone and the subventricular zone actively participate in adult neurogenesis and gliogenesis (Gotz and Huttner, 2005; Verkhratsky and Butt, 2007; Rodriguez et al., 2008; Rodriguez et al., 2009a; Rodriguez and Verkhratsky, 2011b). Astrocytes are joined by gap junctions to create characteristic complexes called syncytia, which are formed from connexons (Giaume and Venance, 1998). Connexons shape pores that are permeable to ions and small molecules, such as nucleotides, sugars, amino acids, small peptides, cAMP, inositol triphosphate (IP_3) and Ca^{2+} (Magistretti and Ransom, 2002). Glial Ca^{2+} signaling is a crucial form of communication within syncytia and

allows coordination between certain adjoining cells (Cornell-Bell et al., 1990; Magistretti and Ransom, 2002; Scemes and Giaume, 2006). The important role of astrocytes is connected with controlling homeostasis in the brain, which means the concentrations of ions, metabolites and neurotransmitters (including the most neurotoxic one, glutamate; see chapter 1.1.3.a) as well as regulating water flow (Danbolt, 2001; Simard and Nedergaard, 2004; Verkhratsky and Butt, 2007). In terms of ion concentration regulation, one of huge importance is K^+, the presence of which increases extracellularly as a result of neuronal activity and which can significantly alter neuronal activity when unregulated (Kofuji and Newman, 2004). Astrocytes possess important mechanisms to buffer $[K^+]_o$. One of these is spatial K^+ buffering, where K^+ is tranferred from region of a higher $[K^+]_o$ to areas with lower $[K^+]_o$ within a syncytium or single cell (Kofuji and Newman, 2004; Benarroch, 2005). In another mechanism, astrocytes buffer ion concentration via K^+ uptake by inwardly rectifying K^+ channels (Kir) (Newman et al., 1984). Both ways of regulating of K^+ concentration are connected with water transport and the activation of astroglial water channels called aquaporins (AQPs), localized in perisynaptic and astrocytic endfeet processes (Amiry-Moghaddam and Ottersen, 2003; Simard and Nedergaard, 2004).

Astrocytes are known to express a variety of neurotransmitter receptors (both forms: ionotropic and metabotropic), including those for such important molecules as glutamate, purines or GABA (Lalo et al., 2006; Verkhratsky and Butt, 2007; Lalo et al., 2008). Various types of correlation between neuronal and astrocytic receptors exist, meaning that both classes of cells present a similar set of neurotransmitter receptors in their close proximity (Verkhratsky and Shmigol, 1996; Verkhratsky et al., 1998). Astrocytes also express receptors for glycine, mainly in the spinal cord (Kirchhoff et al., 1996), adrenergic receptors (αARs and βARs), dopamine receptors D1 and D2 in the cortex (Khan et al., 2001), acetylcholine receptors (muscarinic mAChRs and nicotinic nAChRs) and many others (for review see (Verkhratsky, 2009)). Astrocytic nAChR receptors have begun to be suspected of involvement in Alzheimer's disease pathology after an increased number of astrocytes positive for the α7 subunit of this receptor was found in AD patients with Aβ plaques (Teaktong et al., 2003; Yu et al., 2005).

The vast majority of other regulatory and metabolic functions of astrocytes along with the tight interplay between neurons and astroglia will be introduced in details in the following chapters.

1.1.3.a. Role of astrocytes in glutamate metabolism:

Glutamate is the major excitatory amino acid in the mammalian CNS and is involved in many important brain functions, including cognition, memory and learning – in the last two glutamate is involved mainly via long-term potentiation (LTP) (Fonnum, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990; Baudry and Lynch, 2001; Danbolt, 2001). This excitatory neurotransmitter is a key player when it comes to the development of the CNS, which means synapse formation and removal, as well as managing the life and death of the cells. Glutamate acts through glutamate receptors, expressed by both neurons and glial cells (Verkhratsky and Butt, 2007). For its proper function and metabolism, this excitatory neurotransmitter needs specific proteins and transporters.

1.1.3.a.1. Glutamine synthetase (GS):

Astrocytes are able to prevent excitotoxicity by clearing excess amounts of glutamate from the extracellular space. They do that through astrocytic glutamate transporters, which are assumed to play the main role in glutamate clearance (Dringen et al., 2000; Maragakis et al., 2004; Zou et al., 2010). Subsequently in astrocytes, glutamate is converted to nontoxic glutamine, which is then hydrolyzed in neurons to glutamate by the enzyme glutaminase (Zou et al., 2010). The cycle of the constant flow and conversion of glutamate and glutamine between astrocytes and neurons is considered to be the main pathway of glutamate recycling and strictly requires the astrocytic enzyme glutamine synthetase (GS) (Danbolt, 2001; Shaked et al., 2002). GS is an omnipresent enzyme, encoded by a single gene, which uses ATP to convert glutamate and ammonium to glutamine (Matthews et al., 2005). This enzyme is present in almost all tissues, among them in kidney, liver, skeletal muscle, spleen, heart and brain (Meister, 1985; Rowe, 1985; Sihag et al., 2007). In vertebrates GS is present throughout the brain, playing a crucial role in the detoxification of brain ammonia and the metabolic regulation of glutamate (Kaneko et al., 1988; Suarez et al., 2002). Glutamine synthetase is found

primarily in astrocytes, in vivo as well as in vitro, but is also expressed to some extent by Müller glial cells of the retina and oligodendrocytes (Martinez-Hernandez et al., 1977; Riepe and Norenburg, 1977; Norenberg, 1979; Linser and Moscona, 1983; Yamamoto et al., 1987; Fages et al., 1988; D'Amelio et al., 1990; Derouiche and Frotscher, 1991; Prada et al., 1998). The activity of GS is dependent on the developmental stage: during maturation its expression is increasing, in rodents reaching adult levels at around 20 days of postnatal life, while during aging GS expression declines (Caldani et al., 1982; Smith et al., 1991; Takahashi et al., 2002). It is widely accepted that the increase in GS expression during development is connected with astrocytic differentiation, not necessarily with proliferation and that in the adult CNS GS-immunoreactivity is detected in the majority of brain regions (Caldani et al., 1982; Derouiche and Frotscher, 1991). Because GS possesses a divalent cation site, the enzyme is highly sensitive to oxidation, so that changes of GS activity are usually used for estimating oxidative damage limits of brain tissue (Schor, 1988). Also, considering the central role of astrocytic GS in the metabolism of glutamate, an imbalance of which is well known to be involved in many neurological disorders, it is not an overstatement to say that glutamine synthetase plays an important role in brain pathological states (Vardimon, 2000; Suarez et al., 2002). In many brain injuries and disorders, including ischemia, hypoxia, schizophrenia, depression, hepatic encephalopathy, spinocerebellar atrophy and Alzheimer's disease, up- or downregulations of the enzyme has been described (Lavoie et al., 1987; Sher and Hu, 1990; Smith et al., 1991; Kish et al., 1994; Le Prince et al., 1995; Tumani et al., 1999; Robinson, 2000; Burbaeva et al., 2003; Burbaeva et al., 2005; Hoshi et al., 2006; Steffek et al., 2008; Miguel-Hidalgo et al., 2010). GS activity is also found to be reduced in the case of glucose deprivation (Rosier et al., 1996). Additionally, some researchers claim that GS is expressed by neurons when deprived of glutamine or interactions with astrocytes, but until now definitive information is scarce (Fernandes et al., 2010). However, as in the case of Alzheimer's disease, taking into consideration some discrepancies in results between certain groups and the still limited number of studies, a deeper insight into glutamate converting enzymes in AD is needed (Burbaeva et al., 2005; Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2013; Yeh et al., 2013).

1.1.3.a.2. Glutamate transporters:

Glutamate transporters (EAATs – Excitatory Amino Acid Transporters), by high activity cellular uptake, which assures the fast removal of excitatory amino acid from the extracellular space, and the ending of receptor activation, are essential for maintaining glutamate homeostasis in the mammalian brain (Walton and Dodd, 2007). Until now, five different Na^+-dependent high-affinity glutamate transporters have been described (EAAT1-5), which for the transfer of one molecule of glutamate require the symport of three Na^+ and one H^+ ions and the countertransport of one K^+ ion (Zerangue and Kavanaugh, 1996; Kanai and Hediger, 2003; Walton and Dodd, 2007). The transporters are localized on the plasma membrane of miscellaneous cells in the CNS, belong to the solute carrier family 1 (SLC1) and share 50-60% sequence homology (Kanai and Hediger, 2003, 2004; Sheldon and Robinson, 2007). Two of them, EAAT1 (rodent analog – GLAST) and EAAT2 (rodent analog – GLT-1), are found primarily on astrocytes, while others two, EAAT3 (rodent analog – EAAC1) and EAAT4, are assumed to be expressed mainly by neurons (Kanai and Hediger, 1992; Arriza et al., 1994; Rothstein et al., 1994; Lehre et al., 1995) (Fig. 3). The last type of glutamate transporter, EAAT5, is colocalized particularly with rod photoreceptors and bipolar cells of the retina, where it controls excitatory neurotransmitter release by hyperpolarizing the presynaptic nerve terminal (Arriza et al., 1997; Hasegawa et al., 2006; Veruki et al., 2006).

The two astrocytic transporters, EAAT1/GLAST and EAAT2/GLT-1, are fundamentally responsible for extracellular glutamate uptake and maintaining glutamate metabolic equilibrium in the CNS (Rothstein et al., 1996; Tanaka et al., 1997; Danbolt, 2001).

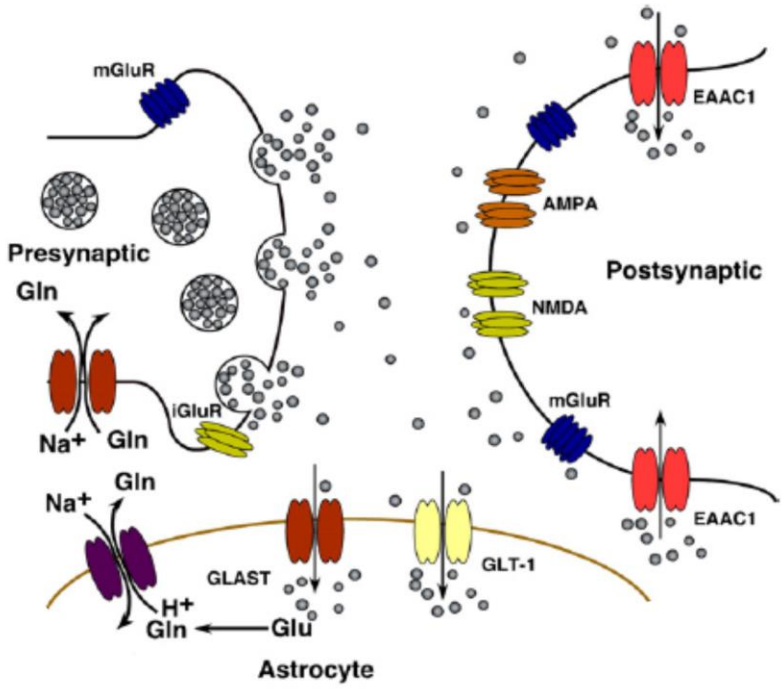

Fig.3 *Drawing showing an excitatory synapse. Excitatory neurotransmitter glutamate released from pre-synaptic terminals activates its ionotropic and metabotropic receptors. Glutamate is taken up by certain glutamate transporters. In astrocytes glutamate is converted to nontoxic glutamine by a specific enzyme, glutamine synthetase. Subsequently, glutamine is shuttled and taken up by neurons.* ***Glu*** *-glutamate;* ***Gln*** *– glutamine;* ***mGluR*** *–metabotropic glutamate receptors;* ***iGluR*** *–ionotropic glutamate receptors;* ***NMDA*** *-N-methyl-D-aspartate;* ***AMPA*** *-α-amino-3-hydroxy-5-methyl-4-isoxazole propionate;* ***GLAST*** *–excitatory amino transporter 1;* ***GLT-1*** *–excitatory amino transporter 2;* ***EAAC1*** *–excitatory amino acid transporter 3 (Sheldon et al, 2007).*

*** EAAT1/GLAST glutamate transporter:**

EAAT1/GLAST is expressed in many regions of the brain and spinal cord, with different intensities depending on the region. It serves as the main glutamate transporter in the cerebellum, the inner ear, the retina and the circumventricular organs close to the BBB (Furness and Lehre, 1997; Lehre and Danbolt, 1998; Rauen et al., 1999; Berger and Hediger, 2000; Cummings, 2004). GLAST is a specific astrocytic transporter, also found in other astroglial cells such as a certain subpopulation of radial glia, the cerebellar Bergmann glia, supporting glia in the vestibular organ and glia-like Müller cells of the retina (Robinson, 2006). It is hardly expressed in nonastrocytic cells, while occasional studies report the presence of GLAST in oligodendrocytes (for example in the rat optic nerve) (Domercq et al., 1999; Regan et al., 2007).

*** EAAT2/GLT-1 glutamate transporter:**

Among all glutamate transporters, EAAT2/GLT-1 is the most common one throughout the CNS, expressed mainly in the forebrain, striatum, hippocampus and spinal cord (Lehre et al., 1995; Furuta et al., 1997; Yang and Rothstein, 2009). It is presumed that at the protein level GLT-1 expression can account for 1% of total brain protein (Danbolt, 2001). GLT-1 protein is found in protoplasmic as well as fibrous astrocytes from the grey and white matter, with no described presence in the pituitary gland or sensory circumventricular organs: the subfornical organ, the vascular organ of the lamina terminalis and the area postrema (Berger and Hediger, 2000; Yang and Rothstein, 2009). Based on a variety of studies, GLT-1 is an astrocyte-specific transporter with no evidence that this protein is expressed by any other nonastrocytic glial cells in vivo and no definite and clearly convincing functional presence in neurons (Danbolt, 2001; Yang and Rothstein, 2009). When deprived of neuronal influence, astrocytes express very low levels of GLT-1 protein, which suggests that certain soluble factors released by neurons in vivo (not defined yet) play an important activating role in GLT-1 expression (Yang and Rothstein, 2009). However, some molecules such as EGF, TGF-α, estrogen, glucocorticoids, pituitary adenylate cyclase-activating polypeptide, and dibutyryl cAMP were reported to activate astrocytic GLT-1 expression in vitro (Schlag et al., 1998; Figiel and Engele, 2000; Zschocke et al., 2005; Yang and Rothstein, 2009). As evidence of the high importance of neuronal influence, membrane GLT-1 shows a dependency on neuronal activity, as demonstrated by changes in the localization of this glutamate transporter to the close proximity of neurons releasing glutamate at the moment (Poitry-Yamate et al., 2002).

According to some studies, in general both astrocytic glutamate transporters, GLAST and GLT-1, are coexpressed in the same cells as separate homooligomeric complexes on the astrocytic membrane at sites of dense glutamatergic innervation (mainly the hippocampus and cerebellum), with a higher proportion of one or the other depending on the brain region (Lehre et al., 1995; Haugeto et al., 1996). Nonetheless, more recent studies on transgenic mice have shown that GLAST and GLT-1 glutamate transporters function in different, nonoverlapping subpopulations of astrocytes (Regan et al., 2007).

As already mentioned astrocytes play an important role in the modulation of glutamatergic synaptic transmission. A high density of EAAT1 and EAAT2 is found in the membranes of astrocytes that enwrap the synapses, meaning that their regulation is dependent on the level of synaptic coverage (Ventura and Harris, 1999; Huang et al., 2004).

Similarly to glutamine synthetase (GS), glutamate transporters, as crucial part of the glutamatergic system within the brain, are implicated (by a decrease or increase in their expression) in various neurological and neuropsychiatric disorders, including Alzheimer's disease, ALS, Parkinson's disease, Huntington's disease, HIV-associated dementia (HAD), schizophrenia, bipolar disorder, brain glioma growth, retinal diseases and glaucoma (Doble, 1995; Rothstein et al., 1995; Wang et al., 2003; Maragakis et al., 2004; Beart and O'Shea, 2007; Sheldon and Robinson, 2007). What is known for sure is that the downregulation of astrocytic glutamate transporter expression can lead to a much greater susceptibility of the CNS to glutamate excitotoxicity, which was first shown in an animal model in GLT-1 -/- rats and subsequently found in ALS patients (Rothstein et al., 1992; Rothstein et al., 1996; Tanaka et al., 1997). When it comes to AD, the mechanism responsible for the altered expression of EAAT1 and EAAT2 has not been fully revealed. However, applying a non-toxic dosage of Aβ in vitro was shown to aid neuronal survival by increasing the expression of EAAT1/GLAST as well as EAAT2/GLT-1 (Abe and Misawa, 2003; Rodriguez-Kern et al., 2003). Similar results were obtained with amyloid precursor protein, APP (Masliah, 1997; Mattson et al., 1999). Nevertheless, the metabolic interactions within the CNS between neurons and astrocytes are very complex, and it is still under debate which was first, the chicken or the egg, meaning whether altered glutamate transporter expression is the primary cause of the diseases or their consequence.

1.1.3.b. Role of astrocytes in glucose metabolism:

Neurons receive metabolic support by the glucose-lactate shuttle working within astrocytic domains (Danbolt, 2001). When glucose enters the brain, it is first transported by the endothelial cells and taken up by astrocytic glucose transporter GLUT1, the most abundant type of glucose transporters in the brain (Waagepetersen and Sonnewald, 2009).

Neurons can be provided directly with glucose by the GLUT3 transporter (Chih et al., 2001). GLUT1 in astrocytes is localized on the processes that enclose blood vessels and those close to synapses, while neuronal GLUT3 is found all over the cell surface (Morgello et al., 1995; Vannucci et al., 1997). The principal difference between the mentioned isoforms is their rate of transport, which is seven times faster for GLUT3 in comparison to GLUT1 (Vannucci et al., 1997). An increase of Na^+ concentration in the cytosol of astrocytes, as a result of increased neuronal activity and accelerated glutamate release, initiates glycolysis. During this process, glucose is converted into pyruvate and then to lactate by lactate dehydrogenase (LDH) isoenzyme LDH5, expressed exclusively by astrocytes. Subsequently, lactate is transported to neurons with the help of monocarboxylase transporters 1 (MTC-1, neuronal) and 2 (MTC-2, astrocytic) (Magistretti, 2006; Pellerin et al., 2007). The mechanism of the astrocyte-neuron lactate shuttle is asssumed to be of high importance for neuronal metabolic support during synaptic activity (Benarroch, 2005). Astrocytes are also equipped with a system of brain energy reserves based on glycogen storage of up to 50% of the glucose entering the brain and accumulated by astroglial cells (Magistretti and Ransom, 2002). Apart from assuring energy supply, glycogen is also a source of carbon for glutamine synthesis, which takes place in astrocytes through pyruvate carboxylation and for subsequent glutamate/GABA synthesis in adjoining neurons (Hertz et al., 2003; Gibbs et al., 2006).

1.1.3.c. Neuron-glia vascular unit:

Considering the anatomic location of astrocytes in the brain, they can serve as a major transfer station from neurons to the vessels, passing information about the extracellular environment.

The processes of protoplasmic astrocytes create a specific form of contact (called endfeet) that is essential from the point of view of the neuron-astrocyte-blood vessel unit (Oberheim et al., 2009). Also, a single astrocyte by its numerous processes can contact many synapses, as well as stay in touch with other cells within the astrocytic syncytium (Fischer and Kettenmann, 1985; Ventura and Harris, 1999).

According to actual research findings, astrocytes are vital partners in the regulation of cerebral blood vessels diameter, by releasing a variety of vasoactive substances, such as

adenosine-5'-triphosphate (ATP), adenosine, nitric oxide (NO), prostaglandin E_2 (PGE_2) and epoxyeicosatrienoic acids (EETs) (Amruthesh et al., 1993; Murphy et al., 1993; Guthrie et al., 1999; Zhang et al., 2003). The endfeet by themselves are a region that expresses noradrenergic receptors and soluble phospholipase A_2 (PLA_2), produces arachidonic acid (AA) and nitric oxide synthase (NOS) and posseses hemichannels (Paspalas and Papadopoulos, 1996; Farooqui et al., 1997; Simard et al., 2003). Special features of this astrocytic compartment are its high concentration of water channels, K^+ channels activated by Ca^{2+} and purinergic receptors, which taken together are postulated to be strongly involved in the regulation of the cerebrovasculature (Nicchia et al., 2000; Price et al., 2002; Simard et al., 2003; Gordon et al., 2007). To stimulate the release of the mentioned substances, an increase of Ca^{2+} in the astrocytic endfeet is needed, as a result of neuronal activity. An increase in Ca^{2+} concentration can lead to either vasoconstriction or vasodilation (Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006) (Fig.4.).

Neurovascular dysfunctions in Alzheimer's disease are the subject of broad research. It was shown that AD can occur together with cerebrovascular disease and atherosclerosis (Gorelick, 2004; Roher et al., 2004). Also, several pathologies connected with Aβ presence in the AD brain were found, including cerebral microvascular pathology, cognitive impairment related to amyloid angiopathy and inadequate Aβ clearance across the BBB (Farkas and Luiten, 2001; Greenberg et al., 2004; Zlokovic, 2004). However, it is still under debate if the neurovascular impairments are the genesis or the result of the disease (Zlokovic, 2005).

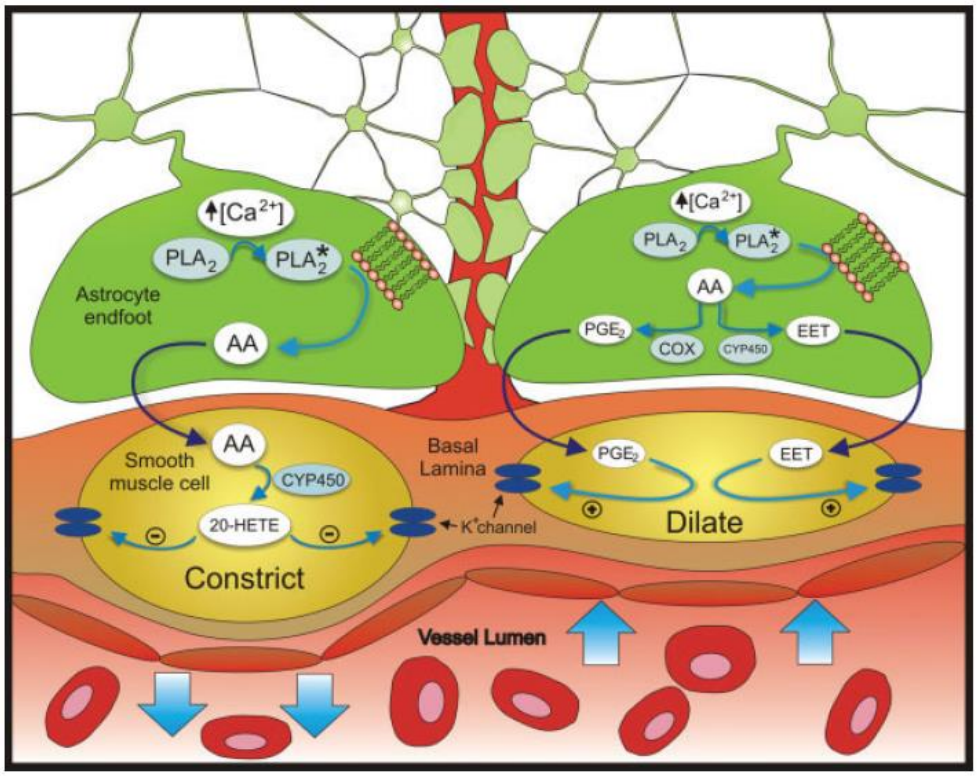

Fig.4 *Drawing illustrating two opposite mechanisms of controlling blood vessel diameter by vasoactive substances synthesized and released by astrocytes.* ***PLA_2*** *-phospholipase A_2;* ***AA*** *-arachidonic acid;* ***COX*** *-cyclooxygenase;* ***CYP450*** *–cytochrome 450;* ***20-HETE*** *-20-hydroxyeicosatetraenoic acid;* ***EET*** *-epoxyeicosatrienoic acid (Gordon et al, 2007).*

1.1.3.d. Astrocytic involvement in synaptic transmission:

Astrocytes have the capability to release several chemical substances, called gliotransmitters. Gliotransmitters, similarly to neurotransmitters released by neurons, need to meet certain criteria. This means that a substance called a gliotransmitter needs to be synthesized and stored in a glial cell, its release needs to be elicited by physiological or pathological stimuli, it has to trigger an immediate response in neighbouring cells and it has to be involved in (patho)physiological processes (Volterra and Meldolesi, 2005; Parpura and Zorec, 2010). Also, there are different mechanisms by which a gliotransmitter can be released, including (i) channel opening triggered by cell swelling, hemichannels on the cell surface or purinergic receptors; (ii) transporters, such as plasma membrane excitatory amino acid transporters, or exchange mediated by the cystine-glutamate antiporter as well as organic anion transporters; and (iii) Ca^{2+} dependent exocytosis (Bezzi et al., 2004; Parpura and Zorec, 2010). Among the broad repertoire of gliotransmitters released by astrocytes are glutamate, ATP, adenosine, GABA, tumor

necrosis factor alpha (TNF-α), brain derived neurotrophic factor (BDNF), cholesterol and thrombospondins (TSPs) (Araque et al., 1998; Mauch et al., 2001; Beattie et al., 2002; Zhang et al., 2003; Elmariah et al., 2004; Christopherson et al., 2005; Volterra and Meldolesi, 2005; Angulo et al., 2008). One of the most interesting neuroactive substances released by astrocytes is D-serine (Wolosker et al., 2002). D-serine is formed from L-serine by serine racemase (pyridoxal-5'-phosphate-dependent enzyme) present in protoplasmic astrocytes and is assumed to be degraded by D-amino acid oxidase (DAAO) (Snyder and Ferris, 2000; Snyder and Kim, 2000; Wolosker et al., 2002; Pollegioni and Sacchi, 2010). The level of D-serine varies within the brain, with the highest amount observed in forebrain areas, such as the cortex, hippocampus and striatum, where NMDA-type glutamate receptors are abundant (Hashimoto et al., 1993; Hashimoto and Oka, 1997). This specific glial neuromodulator has been proven to be an endogenous ligand for the glycine site of the NMDA receptor, being three-fold more effective even than glycine (Matsui et al., 1995; Schell et al., 1995; Snyder and Ferris, 2000; Wolosker et al., 2002). It is believed that glutamate released from a presynaptic neuron triggers the release of D-serine from neighbouring astrocytes to coactivate the NMDA receptors on nearby postsynaptic neurons (Snyder and Ferris, 2000) (Fig.5.). The possible role of D-serine in pathology of Alzheimer's disease was described in chapter 1.2.5.

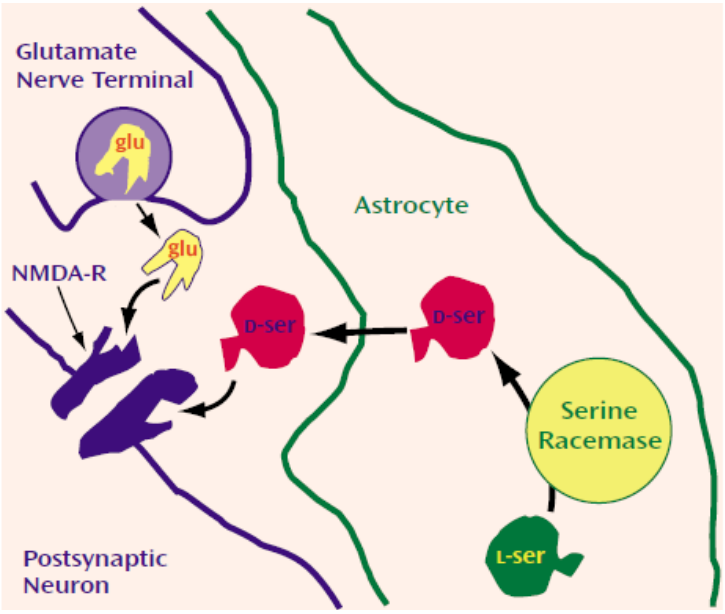

Fig.5 *D-serine activity in glutamatergic neurotransmission. Astrocytes synthesize specific D-amino acid (from L-serine by serine racemase) and release it close to NMDA receptors, where D-serine binds to its glycine site, allowing glutamate to bind as well. In that way D-serine can influence glutamatergic neurotransmission.* ***Glu*** *-glutamate;* ***D-ser*** *-D-serine;* ***NMDA-R*** *-N-methyl-D-aspartate receptor (Snyder et al, 2000).*

Astrocytes are well known to be unable to generate action potentials, a key feature of neuronal excitation, but they show a response to stimuli (such as glutamate) by producing $[Ca^{2+}]_i$ transients and oscillations. Also, an increase of $[Ca^{2+}]_i$ can expand within glial networks in the form of so-called calcium waves (Cornell-Bell et al., 1990; Charles et al., 1991; Bezzi et al., 2001). Set of actions standing behind an increase of Ca^{2+} in astrocytes include the activation of G-coupled-receptors, which activates phospholipase C (PLC), which in turn causes an elevation of the second messenger inositol-1,4,5-triphosphate ($Ins(1,2,3)P_3$) and the release of Ca^{2+} from intracellular stores (Haydon, 2001) (Fig.6).

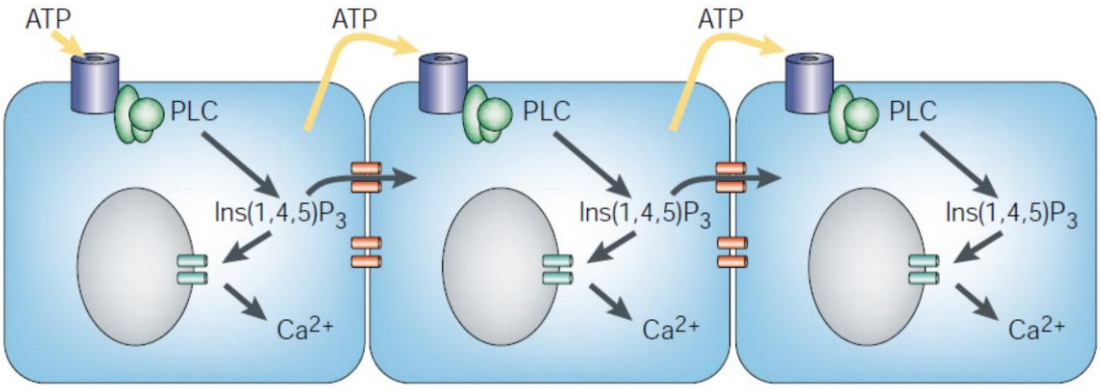

Fig.6 *Possible mechanism of glial Ca^{2+} wave generation and propagation. Ca^{2+} is released from intracellular stores as a result of an increased concentration of internal $Ins(1,4,5)P_3$, which simultaneously can spread out to adjoining cells through gap junctions (mechanism of* ***short-range signaling****). To achieve* ***longer-range signaling****, the release of ATP is needed, which leads to the regenerative production of $Ins(1,4,5)P_3$ and even more ATP release from astrocytes in the close vicinity.* ***$Ins(1,4,5)P_3$*** *- inositol-1,4,5-triphosphate;* ***ATP*** *–adenosine triphosphate (Haydon, 2001).*

The described astrocytic excitation can be neuron-dependent, when it is created as a response to chemical signals within neuronal networks, in addition to spontaneous excitation, which arises without neuronal impact. Hence, astrocytes can (i) detect and consolidate synaptic activity and appropriately respond to neurons by the release of gliotransmitters; (ii) by spontaneous excitation elicit increased activity on adjacent neuronal cells, what makes astrocytes important executives in the whole process of the excitation within the CNS (Parri et al., 2001; Volterra and Meldolesi, 2005). Considering the crucial function of astrocytes in modulating synaptic activity, the tripartite synapse model was proposed (Araque et al., 1999). In agreement with this model, the tripartite synapse, formed between the astroglial perisynaptic processes, the presynaptic neuronal terminal and the postsynaptic neuronal membrane, is a common type of synapse in the central nervous system (Araque et al., 1999; Halassa et al., 2007; Heneka et al., 2010). Therefore, failure in any astrocytic metabolic function and released transmitters can lead to a disruption of synaptic performance, plasticity and neuronal response (Halassa et al., 2007). (Fig.8) Recently, a new concept of astroglial involvement in synaptic transmission was proposed, named the astroglial cradle. According to this, the processes of perisynaptic astrocytes form a coat around the synapse to protect it from the influence of adjacent ones, creating a separate functional unit. In this model the gliotransmitters do not need to be released – the most important aspect is the physical barrier formed by

astrocytes within which they control synaptic performance by active ions shuttling and metabolic support (Nedergaard and Verkhratsky, 2012) (Fig.7).

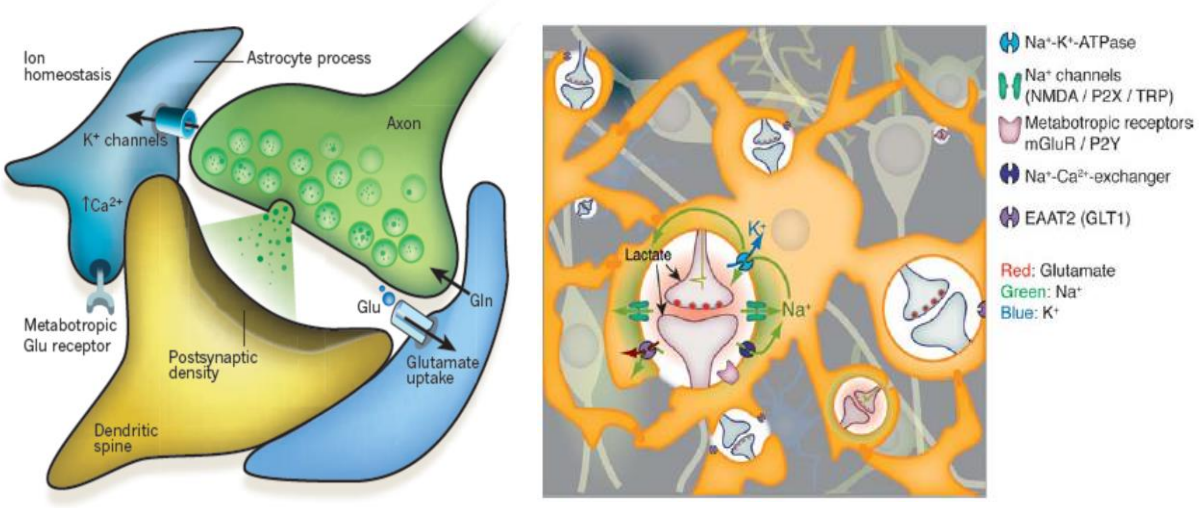

Fig.7 *(Left) Scheme of the tripartite synapse;* ***Glu*** *–glutamate;* ***Gln*** *–glutamine (Eroglu et al, 2010). (Right) Model of the astroglial cradle (Nedergaard et al, 2012).*

1.1.4. Astrocytes in pathological states:

As has already been mentioned several times, astrocytes are implicated in CNS pathologies (Seifert et al., 2006; Giaume et al., 2007; Rossi and Volterra, 2009). Their abilities to serve neurons and to assure proper performance as well as homeostasis within the brain, altered under pathological conditions, can account for the cascade of events leading to brain damage. This subtle balance between physiology and pathology, in addition to the two faces of the astrocytic response triggered by such damage as injury or neurodegeneration, is an important issue to consider when thinking about therapeutic approaches, such as genetic manipulation or drug administration.

1.1.4.a. Reactive astrogliosis:

While astrocytes are active partners within the CNS, they naturally react to all kinds of insults, including infection, trauma, ischemia and neurodegenerative diseases. The form in which they respond is called reactive astrogliosis, a process that involves a set of advancing morphological and molecular changes regulated by various complicated intra- and intercellular signaling (Rossi and Volterra, 2009; Sofroniew and Vinters, 2010).

Reactive astrogliosis is not a straightforward event, and there are many discrepancies and misunderstandings in this field. The most cautious and accurate definition is based on a few hallmarks: (i) reactive astrogliosis is a range of changes in astrocytes that appear as a response to miscellaneous types of the insult within the CNS of different severities; (ii) the molecular alterations of reactive astrocytes diverge depending on the nature of the insult and its level of severity, from subtle changes in the expression of molecules (such as an initial increase in GFAP, vimentin and nestin levels), through progressive hypertrophy, in severe cases resulting in proliferation and scar formation; (iii) the changes are strictly regulated by certain signaling cascades and molecules with the ability to control the nature and level of the changes (i.e. structural changes, astrocytic hypertrophy and astrocyte scar formation are under the regulation of STAT3, while astrocyte migration is controlled by CEPB1); (iv) the changes during reactive astrogliosis influence astrocytic features (which are lost or gained), so that their influence on neighbouring cells can also be altered in a detrimental or advantageous way (Sofroniew, 2009). Changes from the resting state to the reactive one also involve the upregulation of the gene repertoire and the release of cytokines, eicosanoids (such as prostaglandins), reactive oxygen species, nitric oxide and excitatory amino acids (Perry et al., 1995; Rossi and Volterra, 2009). Scar formation demands the triggering of specific molecules, not fully known yet, but definitely including epidermal growth factor (EGF), fibroblast growth factor (FGF), endothelin 1 and ATP (Gadea et al., 2008; Sofroniew, 2009).

In healthy tissue astrocytic domains are separate entities: they do not overlap and remain preserved with hypertrophic changes during mild and moderate states (Bushong et al., 2002; Nedergaard et al., 2003). However, in a severe level of reactive gliosis, astrocytes proliferate, their processes start to overlap and these events lead to scar formation (Bush et al., 1999; Faulkner et al., 2004; Wilhelmsson et al., 2006). The glial scar is a result of the close collaboration of mainly reactive astrocytes, fibromeningeal cells, microglia and invading macrophages (Silver and Miller, 2004; Sofroniew and Vinters, 2010). During reactive gliosis the expression of many astrocytic intermediate filaments (IF) increases (including vimentin and nestin), but the upregulation of GFAP is assumed to be the key feature of this pathophysiological reaction involving reactive astrocytes (Wilhelmsson et al., 2006). Reasonably, this shows a special role for GFAP, a major IF protein of adult

astrocytes in the intact CNS. Also, the upregulation of GFAP, vimentin and nestin, which reappear under reactive gliosis conditions, accounts for the formation of an IF network (Pekny and Nilsson, 2005). Important is then the relation between each of the intermediate filament network components and their necessity for astrocytes. It has been proven using Vim-/- and GFAP-/- mice that GFAP can form IF without vimentin (even if they have a defective organization), but vimentin cannot form IF without GFAP. In mice lacking both proteins, IF are not created at all (Pekny et al., 1995; Eliasson et al., 1999; Pekny et al., 1999). Also, a partnership of intermediate filament proteins depending just on nestin or nestin-GFAP is not attainable from a proper IF formation point of view (Eliasson et al., 1999). What is more, scar formation in mice lacking vimentin or GFAP is quite normal, whereas in double knockout (GFAP-/- and Vim-/-) animals it is significantly reduced and accompanied by slower healing of the wound (Pekny et al., 1999). Logically, the upregulation of particular IF in reactive astrocytes plays a key role in the post-traumatic process of recovery (Pekny and Pekna, 2004; Pekny and Nilsson, 2005).

1.1.4.b. Excitotoxicity:

As was already discussed earlier in detail (see chapter 1.1.3.a and 1.1.3.d), astrocytes have several transporters and enzymes to prevent excitotoxicity by taking up an excess of glutamate (via glutamate transporters) and converting it to non-toxic glutamine (by glutamine synthetase, GS) or by modulating neuronal NMDA receptor activity (Wolosker et al., 1999; Danbolt, 2001). However, when this complicated astrocytic machinery fails, the neurotoxic effect of an excess of glutamate starts its lethal action. Glutamate excitotoxicity is known to be involved in many brain diseases and pathologies, such as ischemia, trauma, epilepsy, ALS, Huntington's disease and Alzheimer's disease (Rothstein et al., 1995; Won et al., 2002; Hynd et al., 2004; Tannenberg et al., 2004; Fujikawa, 2005; Rego and de Almeida, 2005; Yi and Hazell, 2006). During continuous exposure to increased extracellular glutamate, the relevant glutamate receptors, mainly NMDA, are tonically activated, which causes the constant local depolarization of neurons. This triggers a series of intracellular actions leading to an influx of Na^+ and Ca^{2+} accompanied by the further exocytosis of glutamate. The influx of Ca^{2+} is a root cause of neuronal necrosis and an activator of apoptotic pathways (Walton and Dodd, 2007).

NMDA receptor dysfunction is believed to be of crucial significance in the pathophysiology of many neurologic and psychiatric disorders (Fossat et al., 2011). NMDAR hyperactivity can be a cause of cell death in stroke and neurodegenerative diseases, such as Parkinson's and HIV-associated dementia (Kemp and McKernan, 2002; Hardingham and Bading, 2003). On the other hand, hypoactivity of the receptors causes apoptosis during brain development and can account for psychotic and cognitive symptoms observed, for example, in schizophrenia (Millan, 2005).

When it comes to AD, as was suggested earlier in this work, glutamate-related alterations are most probably not the origin of the disease, nevertheless they can account for the observed neuronal pathology (Miguel-Hidalgo et al., 2002). Therefore, knowing that NMDA receptors are in general major mediators of excitotoxicity due to an excess of glutamate, significant efforts have been made to find a way to regulate its activity pharmacologically (Greenamyre et al., 1988; Santangelo et al., 2012).

1.1.4.c. Dementia:

Dementia is a highly deteriorating disease, which leads to a brutal loss of global cognitive abilities and takes away the core of humanity together with the self identity of every person affected. Among the wide variety of causes of dementia are numerous types of traumatic injuries, viral infections, as well as genetic predispositions or other undefined genetic malfunctions. However, dementia is strongly connected with increasing life expectancy, due to higher living standards nowadays, especially in developing countries. In this case, if age is the greatest risk factor, the number of people with dementia will increase drastically in long-living societies and can reach around 115.4 million in 2050 (Rodriguez et al., 2009b; Abbott, 2011).

Astrocytes are significantly involved in many types of dementia, including Alzheimer's disease. The reaction of astrocytes to these pathological conditions can take the form of (i) astrogliosis; (ii) astroglial atrophy; or (iii) both of them, depending on the stage of the disease (Heneka et al., 2010). In the case of frontotemporal dementia, astrogliosis can be accompanied by degenerating astrocytes or directly followed by the rapid and profound apoptosis of astrocytes (Martin et al., 2001; Broe et al., 2004). Prominent astrogliosis is also found in such diseases as thalamic dementia, Creutzfeld-Jakob disease (CJD) and

analogous prion dieases (Potts and Leech, 2005; Kovacs and Budka, 2008). Basically the same type of glial pathology is seen in HIV infected individuals (Sabri et al., 2003).
Considering the recent research findings, astrocytic activation together with altered glutamate uptake mechanisms are of the high importance for neuronal degeneration and the progression of dementias (Sheldon and Robinson, 2007; Rossi and Volterra, 2009; Sofroniew and Vinters, 2010).

Among other astrocytic alterations found in dementias, worthy of mention are tau-positive inclusions of different appearance (widely accepted as a feature of neuronal pathology in Alzheimer's diease) as hallmarks of such brain diseases as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and Pick's disease (PiD) (Komori, 1999). The collection of changes that astrocytes undergo signals their active participation, if not leadership, in the progression of the disease, and probably in many cases such changes lie behind its strict genesis.

1.1.5. Other glial cells of the CNS:

Apart from astroglial cells, the CNS is occupied by other non-neuronal cells: (i) microglia, (ii) NG2 glia and (iii) oligodendrocytes. Microglial cells are specialized resident macrophages of the central nervous system, which comprise 5-20% of total glial cells, depending on the brain region (Perry and Gordon, 1991; Saijo and Glass, 2011). They are of haematopoietic origin and act first in the case of brain injury or infection with pathogens (Ransohoff and Perry, 2009). The term "microglia" was used for the first time by Pio del Rio Hortega in his work from 1919 (Del Rio-Hortega, 1919), while in 1932 he described these cells in detail, including their origin and morphology, their method of migration within the brain, their anatomical localization, changes in their appearance upon activation and their phagocytotic role (Del Rio-Hortega, 1932; Kettenmann et al., 2011). In the ramified state, the morphological phenotype of microglia is characterized by small cell somata with extensively ramified thin processes, by which constant surveillance of the surroundings is performed (Nimmerjahn et al., 2005). After receiving a signal to defend the CNS, microglia change from their resting phenotype to an "ameboid" like activated one (Kreutzberg, 1996). Activated microglia produce and

release pro- inflammatory substances, including various cytokines, reactive oxygen species (ROS), complement factors and nitric oxide (Saijo and Glass, 2011). To some extent those factors are helpful for the clearance of infections by pathogens within the CNS; however, prolonged microglia activation can lead to pathological forms of inflammation, neuronal dysfunction and cell death, contributing to the progression of neurodegenerative diseases (Heneka et al., 2010; Saijo and Glass, 2011).

NG2-expressing cells (NG2; also known as polydendrocytes) are a rather newly discovered (in the 1980s) type of glial population (Verkhratsky and Butt, 2007). Polydendrocytes were discovered by chance in the Stallcup laboratory, when using a novel antibody in search of the cell surface molecules involved in the differentiation of neurons and glia (Stallcup, 1981; Nishiyama et al., 2009). The NG2 proteoglycan (also known as chondroitin sulfate proteoglycan 4; CSPG-4) is a transmembrane protein, expressed by many cells within and outside the CNS. In the CNS cells expressing NG2 constitute 5-10% of all glia cells and are located in both grey and white matter, where they are able to proliferate even in the adult brain and contribute to the turnover of the cell population (Trotter et al., 2010). It is well known that NG2 cells are oligodendrocyte progenitor cells (OPCs), which generate oligodendrocytes in the CNS. Also, it is speculated that they can generate neurons and astrocytes; however, this hypothesis is still under debate (Nishiyama et al., 2009). Nevertheless, NG2 cells establish close functional synaptic contacts with neurons in such structures as the cerebellum, cortex and hippocampus as well as the corpus callosum (Trotter et al., 2010). When it comes to morphology, NG2 cells of the white matter send processes in various directions, which gives them a symmetrical radial appearance, while those from the grey matter are more polarized with processes following the axons of neurons. Apart from being able to generate other cells of the CNS, polydendrocytes are assumed to be an astrocytic companion in glial scar formation as a response to ongoing insult and disturbance within neuronal networks (Verkhratsky and Butt, 2007).

Oligodendrocytes have the task of producing the myelin sheaths of axons in the CNS. Generally four types of oligodendrocytes are known (I-IV), and this separation was made based on their different morphology, number of processes and size of the fibers in close association (Verkhratsky and Butt, 2007; Bradl and Lassmann, 2010). After completing the migration process, oligodendrocytes start to differentiate, mature and initiate the

production of the myelin sheath by myelin proteins, such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin associated protein (MAG) (Heneka et al., 2010).

1.2. ALZHEIMER'S DISEASE:

1.2.1. Historical background and general facts about the disease:

The person who first discovered and described Alzheimer's disease (AD) at the beginning of the twentieth century was Alois Alzheimer in 1906. In his pioneer work published in 1907 he sketched the case of a patient, known as Auguste D., who until the time of death, at the age of 55, had lost all cognitive functions. After the patient's death, Alzheimer could observe in the affected brain several pathological alterations, which he described as "striking changes in the neurofibrils" and "minute miliary foci caused by deposition of a particular substance in the cortex". The novel disease was presented to the public by Alzheimer under the name of "Dementia Praecox" (Alzheimer, 1907). For a long time it was believed that Alzheimer's disease (AD) is a "presenile dementia", affecting younger elderly (before the age of 65). Just in the 1970s it became clear for neuropathologists that "senile dementia" and the disease described by Alzheimer do not differ (Terry and Katzman, 1983; Gandy, 2011). Within that time (1960s, 1970s and early 1980s) valuable observation were made and milestones reached in terms of recognizing and understanding Alzheimer's disease as the common basis for senile dementia. First of all, two main histopathological hallmarks were colocalized and connected with AD: senile plaques ("miliary foci"; Aβ deposits) and neurofibrillary tangles (a hyperphosphorylated and aggregated form of tau protein). What is more, pathologists established that apart from degenerating cholinergic neurons, no other neurotransmitter system is so clearly deteriorating, which suggests the multifarious character of AD (Selkoe, 2001). This special fact, together with increased life expectancy nowadays, makes AD a favorite target of doctors and scientists, being one of the most studied neurodegenerative diseases.

Alzheimer's disease is a chronic, progressive and irreversible neurodegenerative disease, affecting more than 35 million people worldwide, with a trend to roughly double by 2050. Alzheimer's disease can be "early-onset", when the patient starts showing

symptoms at an age under 65, or “late-onset”, when AD develops at an age of 65 or more (Alzheimer's, 2010). This insidious ailment leads to death within 3 to 9 years after diagnosis, at the stage of complete detachment from the patient’s identity and conscious contact with the surrounding world (Querfurth and LaFerla, 2010; Ittner and Gotz, 2011). AD is characterized by three main groups of symptoms. The first refers to cognitive dysfunctions, such as the loss of memory, language difficulties and executive dysfunctions (loss of higher level planning and intellectual coordination skills). In the second group (non-cognitive symptoms) are included psychiatric signs and behavioral disruptions, such as depression, hallucinations, delusions and agitation. All kinds of problems with “daily routine”, generally described as “instrumental”, such as with independent eating, dressing, driving or shopping constitue the third group of symptoms. The severity of the symptoms varies during the progression of the disease (Burns and Iliffe, 2009).

1.2.2. Forms of Alzheimer’s disease – familial (FAD) and sporadic:

Several risk factors have been connected with Alzheimer’s disease onset. Among them are familial and genetic factors, such as apoE genotype, Down’s syndrome (trisomy 21) together with other diseases causing mutations on chromosomes 1 and 14, family history as well as low intelectual activity. When it comes to non-genetic risk factors, the leading one is advancing age, followed by closed head trauma, hypertension, diabetes, high cholesterol, atrial fibrillation or the presence of cerebral emboli (Mayeux et al., 1993; Burns and Iliffe, 2009). The prevalence of Alzheimer’s disease differs depending on the criteria used for diagnosis, the age of group of the people studied, even geography and ethnicity (Nussbaum and Ellis, 2003). In general, it affects 1 person in 8 over 65 years old and nearly 1 in 2 over 85 (Alzheimer's, 2010). The prevalence of its familial (“early-onset” disease; FAD) form can fluctuate from 5-10% to an impressive 50% or more and there is still much uncertainty in this matter (Selkoe, 2001). The familial form of AD is inherited in an autosomal dominant manner and is of high importance when it comes to understanding the mechanisms of the disease (Nussbaum and Ellis, 2003). Interestingly, concerning the phenotype of the disease (such as clinical manifestations and histopathological hallmarks: plaques and tangles), the familial form resembles to a large extent the sporadic, non-familial form of AD, which gives a privileged position to

detailed knowledge about the amyloid precursor protein (APP) and the presenilin genes (*PSEN1* and *PSEN2*) from around 100 candidate AD genes and their mutations for understanding the pathogenesis of this neurodegenerative disease (Selkoe, 2001) (Fig.8). Also, not directly involved, but responsible for a significant increase in susceptibility to AD is the gene for apolipoprotein E (apoE) and especially its form apoE4 (Bertram and Tanzi, 2004).

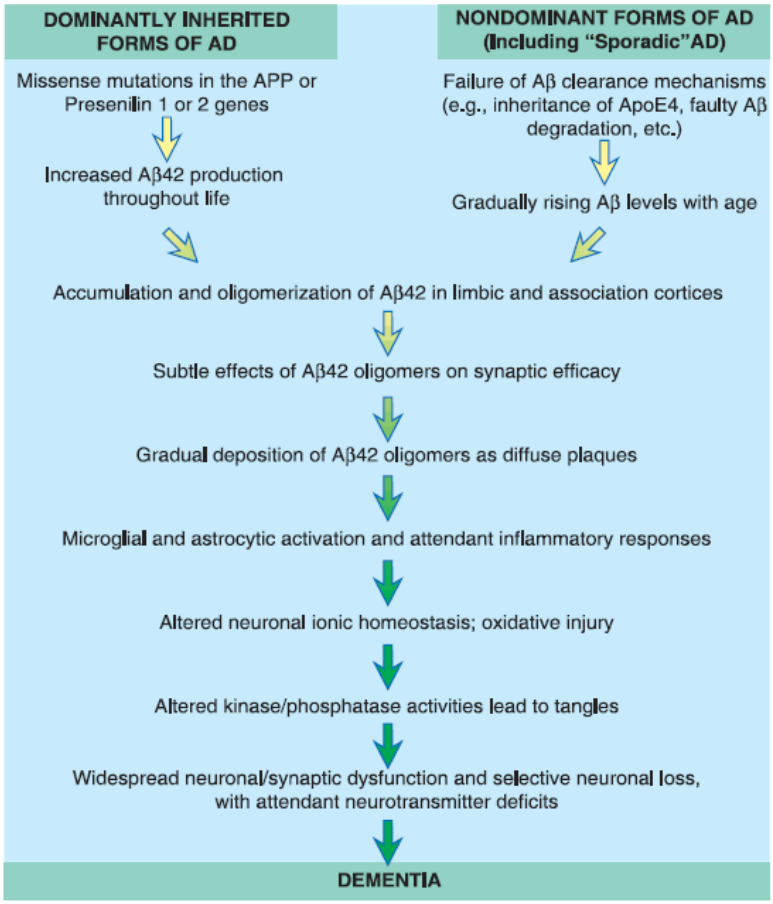

Fig.8 *Schematic diagram of the hypothetical sequence of pathogenetic steps in AD (Selkoe, 2001).*

1.2.3. Genetics and histopathology of the disease:

1.2.3.a. Presenilins:

The first of missense mutations altering a single amino acid in presenilin genes were identified in *PSEN1* (on chromosome 14), encoding presenilin 1 (PS1) – a conserved

membrane protein. Not long after, the second gene *PSEN2* (on chromosome 1) encoding presenilin 2 (PS2) was found to be a serious suspect causing the disease. Both presenilin genes are very similar to each other; however, mutations on *PSEN1* are more frequent than on *PSEN2* (Nussbaum and Ellis, 2003; Bertram and Tanzi, 2004). Presenilins are serpentine integral membrane proteins, generally with eight transmembrane domains and without a signal peptide (Li and Greenwald, 1998). When it comes to their localization within the cell, in neurons presenilins abide in the endoplasmic reticulum (ER, both smooth and rough), the ER-Golgi intermediate compartment (ERGIC) and to some extent in the cis-Golgi (Annaert et al., 1997). Considering PS mutations, it is believed that they do not cause significant alterations or malfunctions of the proteins' physiological functions, but they can add new toxic features (Haass and De Strooper, 1999). Mutations connected with Alzheimer's disease in *PSEN1*, *PSEN2* or the gene encoding APP lead to increased production of the toxic form of amyloid β ($A\beta_{42}$) by about 1.2- to 3-fold by shifting the cleavage site towards γ-secretase (Citron et al., 1997; Nussbaum and Ellis, 2003). To underline the importance of the presence of presenilins within the cell, it is worth mentioning that even mutant genes encoding PS1 are able to "recover" a lethal phenotype in mice knockedout for these genes, which means rescuing them from embryonic lethality, abnormal somitogenesis, axial skeletal malformations and accompanying CNS defects (Qian et al., 1998).

1.2.3.b. Amyloid precursor protein (APP) and amyloid beta (Aβ):

Amyloid precursor protein (APP) is a single transmembrane polypeptide, translocated into the endoplasmic reticulum via its signal peptide while undergoing the process of translation. After that, APP goes through posttranslational modification, called maturation, through the secretory pathway. While being modified, the precursor protein can be cleaved in a proteolityc manner in various ways and release its derivatives (Selkoe, 2001). One of those derivatives is amyloid β (Aβ), a product of the consecutive enzymatic activity of a series of proteases: α-, β- and γ-secretases (Nussbaum and Ellis, 2003). Generally, amyloid β is a normal metabolite, 38-43 residue fragment of APP (Shoji et al., 1992). It is abundantly present within the brain in its more common, 40 amino acid non-toxic form ($A\beta_{40}$). The other, toxic product of the amyloidogenic pathway in APP processing is hydrophobic $A\beta_{42}$, which displays a tendency towards self-

association and is connected directly with AD pathology (Ferreira and Klein, 2011) (Fig.9). An elevated level of both forms of Aβ protein can be observed in the case of a double mutation in the two amino acids directly preceding the β-secretase (also called BACE-1) cleavage site, which leads to the induced activity of the secretase and produces larger amounts of $A\beta_{40}$ and $A\beta_{42}$. This type of mutation is called "Swedish" APP mutation, from the ethnic roots of the investigated family (Selkoe, 2001).

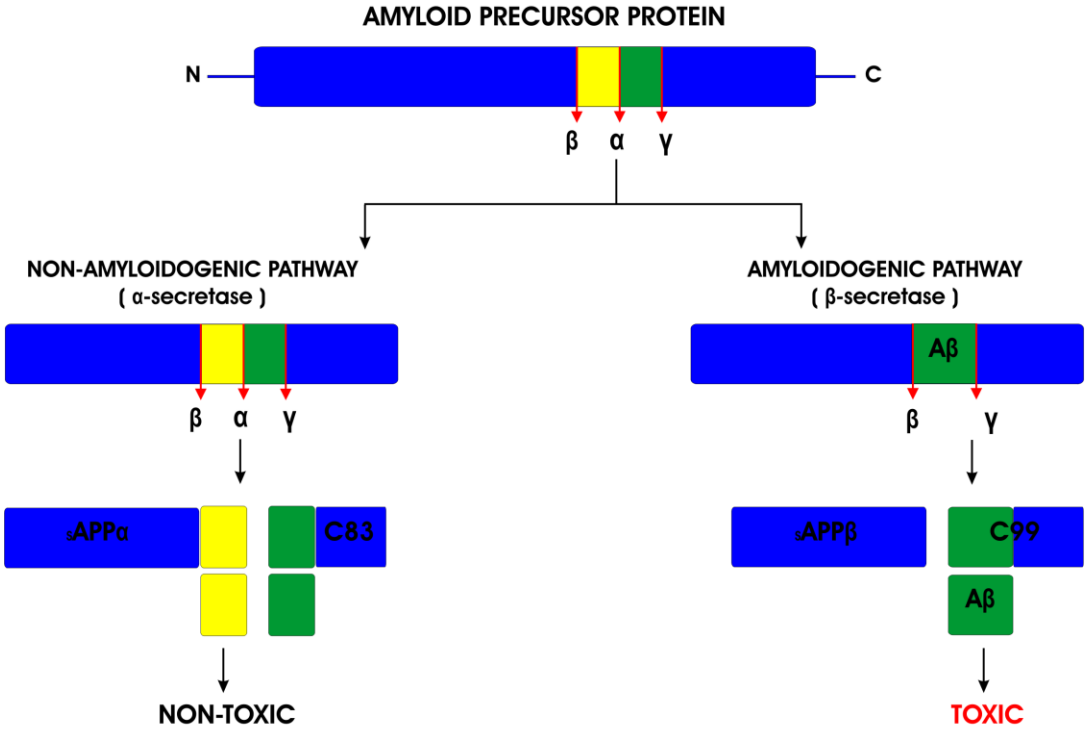

Fig.9 *Two pathways of APP processing. Adapted from Reddy et al, 2008.*

Apart from being expressed in large amounts in neurons, APP is also present also in many other brain cells, such as astrocytes, endothelial cells and smooth muscle cells, which together are responsible for generating a pool of Aβ which at later stages is accumulated extracellularly (Haass et al., 1992) (Fig.10). In contrast to the presenilins, deletion of the APP gene is not lethal; however, it results in cerebral gliosis, changes in locomotor behavior and changes in neurons observed in vitro: decreased viability and weak neurite outgrowth (Zheng et al., 1995; Perez et al., 1999; Selkoe, 2001).

A general hypothesis standing behind familial forms of Alzheimer's disease is called "Aβ hypothesis" or "amyloid cascade". According to this theory, a chronic imbalance between

the production and clearance of a small hydrophobic peptide (Aβ) with a tendency to misfold and aggregate, thus creating amyloid fibrils and plaques, leads to synaptic and neuritic failure combined with the activation of glial cells. Other pathological hallmarks, including neurofibrillary tangles of tau protein, are the consequence of this imbalance (Hardy and Selkoe, 2002; Selkoe, 2004). Nevertheless, even if toxic fibrils and amyloid plaques are known to be strongly correlated with AD progression, there is no clear evidence that they correlate with the deterioration of cognitive function (Ferreira and Klein, 2011). As a result of detailed studies, a new "oligomer hypothesis" was proposed, which suggests that both the memory loss observed early during AD progression and later neuronal degeneration are due to the negative effect of Aβ oligomers (Lambert et al., 1998). In their study, Lambert and colleagues showed that exposure to Aβ-derived diffusible ligand (ADDL) causes a loss of LTP in a way suggesting impaired signaling, not the direct degeneration and death of neurons. Currently, the concept of Aβ oligomers as the most toxic form of this protein is supported by hundreds of papers and still ongoing research on this subject (Ferreira and Klein, 2011).

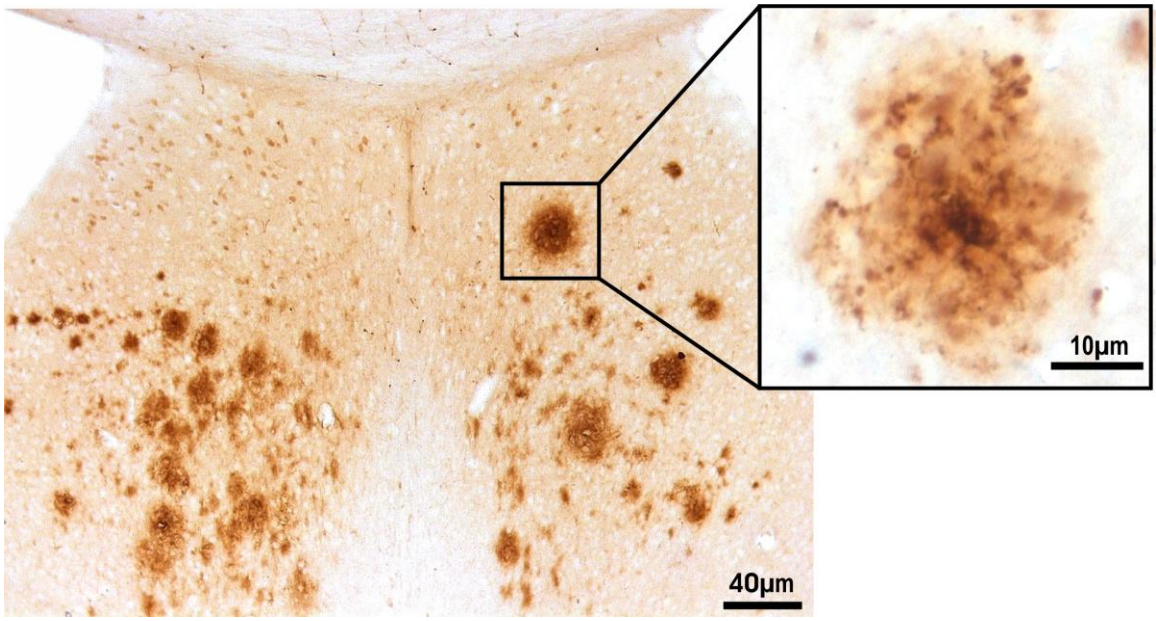

Fig.10 *Photomicrograph of robust plaque deposition in a 3xTg-AD mouse septum.*

1.2.3.c. Tau protein and neurofibrillary tangles:

Tau protein is constructed of three main domains: (i) an acidic amino-terminal projection domain (N-terminal), (ii) a proline-rich region and (iii) a carboxy-terminal domain of

microtubule-binding (MTB) regions (C-terminal) (Ballatore et al., 2007; Ittner and Gotz, 2011) (Fig.11).

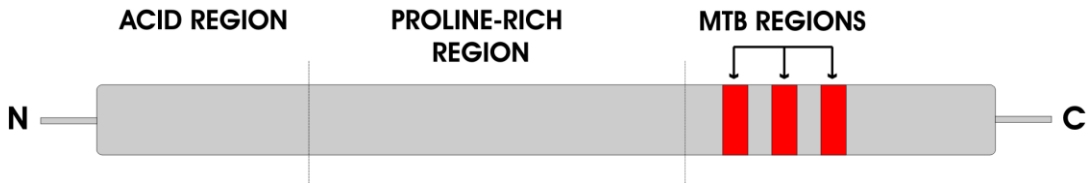

Fig.11 *The basic structure of tau protein.*

Under physiological conditions, the highest concentration of tau is found in neuronal axons as well as in dendrites, however in the latter location in much smaller amounts (Konzack et al., 2007). When it comes to its functions, tau is mainly responsible for the stabilization of molecules and the regulation of axonal transport (Gotz et al., 2006). Hyperphosphorylated and accumulated tau is a major element of neurofibrillary tangles – filamentous inclusions in pyramidal neurons observed in the brains of AD patients and in other neurodegenerative diseases, called by the common term tauopathies (Querfurth and LaFerla, 2010) (Fig.12).

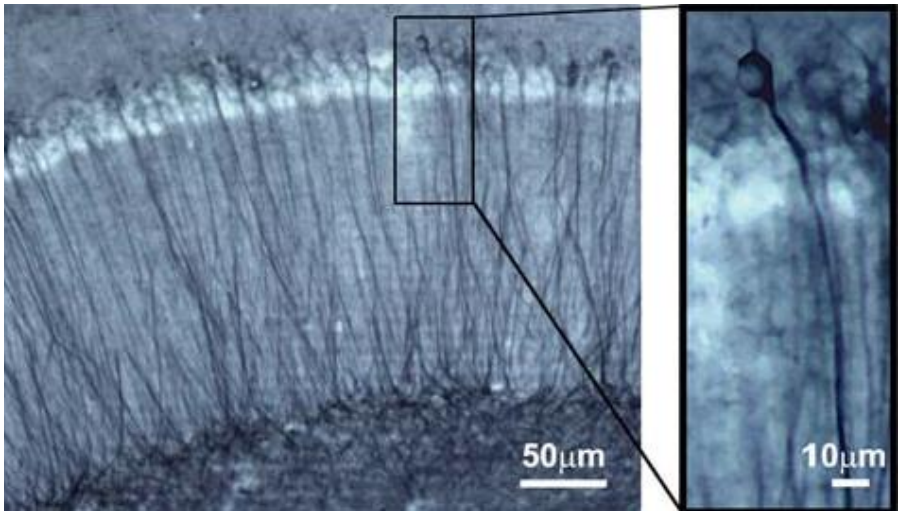

Fig.12 *Photomicrograph of phosphorylated tau protein within the CA1 pyramidal cell layer of a 3xTg-AD mouse hippocampus (Rodriguez et al, 2008).*

Tau is characterized by probably as many as 84 phosphorylation sites and undergoes phosphorylation on a higher level during development than in mature cells. It is well known that increased phosphorylation unables binding of tau to microtubules, leading to

altered axonal transport and mitochondrial respiration, however the detailed mechanism is still poorly understood (Ittner and Gotz, 2011). Hyperphosphorylated tau is aggregated in the somatodendritic compartment of neuron and forms neurofibrillary tangles (NFTs) (Gotz et al., 1995). Additionally, intermediate aggregates of abnormal tau show similar features as Aβ oligomers: toxicity to neurons and devastation for cognition (Oddo et al., 2006; Querfurth and LaFerla, 2010).

Generally, as observed in adult tau-/- mice, the lack of the tau gene is not lethal, and the alterations within the phenotype are rather frail and limited to delicate changes in tubulin spacing within axonal microtubules, a small delay in axonal outgrowth and increased aggression combined with memory deficits (Dawson et al., 2001; Ittner and Gotz, 2011). When it comes to tau gene mutations, in contrast to APP and the presenilins, no mutations have been found in cases of Alzheimer's disease, while they are common in some other neurodegenerative diseases, such as frontotemporal dementia (FTD) – the second most frequent form of early-onset dementia (Nussbaum and Ellis, 2003).

Nevertheless, its pathology in AD attracts great interest. Evidence from many studies indicates that an accumulation of Aβ is a primary hallmark of AD and works as a trigger for tau aggregation (Gotz et al., 2001; Oddo et al., 2003b; Querfurth and LaFerla, 2010). Even if this is the case, the assumption that tau has a more secondary role is no longer valid. This is connected with observations that tau-/- neurons do not undergo degeneration induced by Aβ; in other words, the presence of tau is indispensible for Aβ-mediated excitotoxicity (Ittner et al., 2010). These findings led to the formulation of the "tau axis hypothesis", which combines both pathological events of AD in a tightly cooperating set of actions. According to this hypothesis, everything begins classically with an accumulation of Aβ within the brain. At the initial stages of the disease, the levels of tau in dendrites are low, which makes neurons less sensitive to Aβ toxicity. With progression of the disease and increasing tau phosphorylation and its accumulation in the somatodendritic compartments due to the action of Aβ, dendritic levels of tau rapidly increase. At this stage neurons are much more vulnerable to the toxic effects of Aβ, which leads to even greater hyperphosphorylated tau accumulation and eventually synaptic malfunctions and cell death (Ittner and Gotz, 2011) (Fig.13). Considering the most recent data on the topic, in this case the most relevant animal models of AD should

combine Aβ and tau pathology in order to observe the full repertoire of correlations and cascades of actions and to find the best therapeutic strategy.

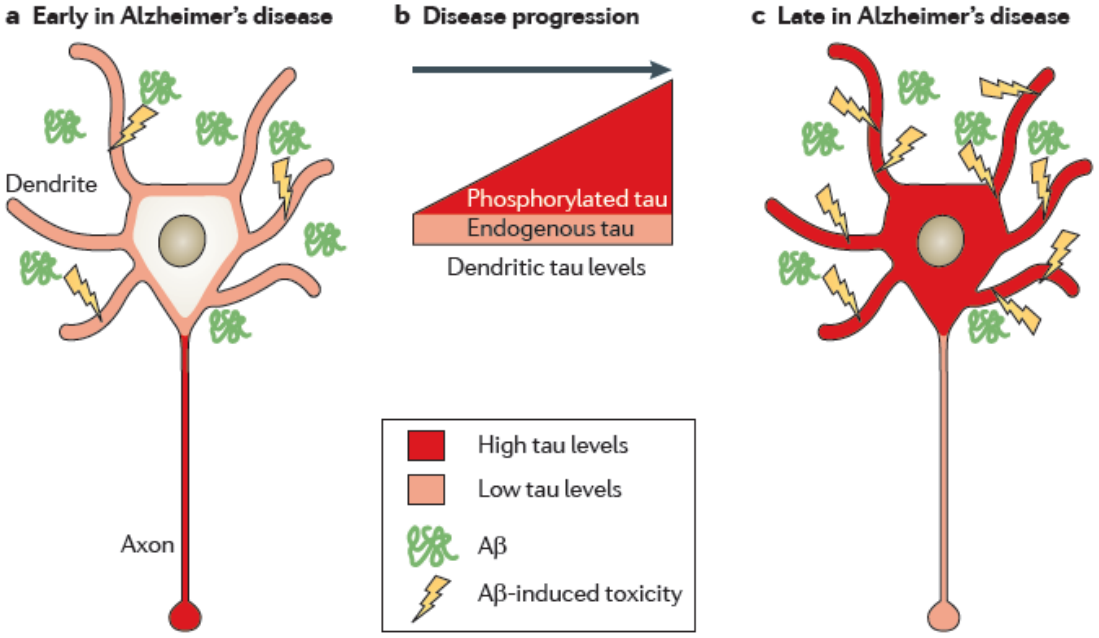

Fig.13 *Possible "tau axis hypothesis", in which due to the increasing level of tau neurons become susceptible to Aβ (Ittner et al, 2011).*

1.2.3.d. Apolipoprotein E (apoE):

Apolipoprotein E is an amino acid glycoprotein, present within the brain predominantly on high-density lipoproteins (HDL) (Fagan et al., 1999). It functions as a ligand in a process of receptor-mediated endocytosis of lipoprotein particles. Endocytosis of apoE-containing lipoprotein fragments is initiated by low-density lipoprotein (LDL) receptor family members. After the process is completed, apoE may undergo degradation or recycle to the cell surface (Rensen et al., 2000; Kim et al., 2009). Lipoprotein fragments with apoE contain cholesterol, which after its release participates in synaptogenesis and synaptic maintenance (Pfrieger, 2003). Among various organs in which apoE is expressed, the highest level in found in the liver and brain. Astrocytes are the cells that predominantly express apoE; however, it can also be found in microglia and even in neurons. When it comes to neuronal cells, low levels of apoE can be found only under non-physiological conditions, such as after excitotoxic injury to the CNS due to kainate acid treatment (Xu et al., 2006). Apart from apoE, other apolipoproteins such as apoA-I,

apoA-II, apoA-IV, apoD, apoE, apoH and apoJ are present within the brain (Ladu et al., 2000). Considering genetics, among all humans there are three versions of the apoE gene and of the protein itself: apoEε2, apoEε3 and apoEε4. These forms of apolipoproteins arise as a result of changes in a single amino acid within the apolipoprotein E sequence (Nussbaum and Ellis, 2003). It is assumed that the ε4 allele of apolipoprotein E predisposes the bearer to Alzheimer's disease by almost doubling the risk for developing the disease, while not having it decreases the risk by around 40%. To summarize all the numbers, analysis indicates that 70% of people without the apoEε4 allele are safe from the disease up to 80 years of age, while with the presence of one or two dangerous alleles the percentage drops to just 10% of non-AD patients at the age of 80 (Meyer et al., 1998; Nussbaum and Ellis, 2003). The reason why apoE and especially its ε4 allele is highly linked with AD is because of its possible stimulatory effect on the Aβ-dependent development of pathological changes and Aβ metabolism itself (Fagan et al., 2002). As was shown in APP transgenic mice with the human version of the APP gene (APP^{V717F} and APP_{Swe}) crossbred with apoE-/- mice, deletion of the apoE gene resulted in a major decrease of Aβ content, and this content was limited to just diffuse nonfibrillar forms of Aβ (Bales et al., 1997; Holtzman et al., 2000). According to the obtained data, it was proposed that apoE can be crucial for converting the soluble forms of Aβ to the classic fibrillar plaques observed with AD progression (Fagan et al., 2002). However, the detailed mechanism underlying apoE ε4- dependent pathology is not fully understood. It is still under debate if apoE has a negative effect due to its silencing of protective functions, adding some negative factor or something in between (Kim et al., 2009).

1.2.4. Affected brain regions:

The reason why Alzheimer's disease is characterized by such a wide range of symptoms is because of the different structures affected. The various memory and other cognitive impairments in AD patiens are most probably connected with a loss of cholinergic neurons within the nucleus basalis of Meynert as well as ongoing severe changes in such structures as the entorhinal cortex (initial changes at Braak stage I/II), hippocampus and prefrontal cortex (changes from Braak stage III) (Bartus et al., 1982; Braak and Braak, 1991).

1.2.4.a. Nucleus basalis of Meynert:

The nucleus basalis of Meynert (nbM) is the grey matter of the substantia innominata of the forebrain, which is composed mostly of cholinergic neurons, projecting robustly to the hippocampus, amygdala, neocortex and other cortical regions (Schliebs and Arendt, 2006). The rodent equivalent of nbM is called the nucleus basalis magnocellularis (nbm) (Toledano and Alvarez, 2004). This structure is deeply involved in AD pathology, as basal cholinergic cell loss and altered cholinergic transmission are prominent features of Alzheimer's disease (Whitehouse et al., 1982; Schliebs and Arendt, 2006).

1.2.4.b. Entorhinal cortex:

The entorhinal cortex (EC) is located in the medial temporal lobe and serves as the main interface between the hippocampus and neocortex, being strongly implicated in mnesic processes (Yeh et al., 2011). Classically, the entorhinal cortex consists of six layers, both superficial (I-III) and deep ones (IV-VI), which are characterized by different anatomical organization as well as function (Suzuki and Amaral, 1994). When it comes to the superficial layers, they receive intracortical information and send efferent projections to the hippocampus, while the role of the deep layers is concentrated on projecting to cortical regions (Suzuki and Amaral, 1994; Yeh et al., 2011). The main afferents to the dentate gyrus (DG) come from layer II of the entorhinal cortex. The DG subsequently sends its collaterals to the CA2 and CA3 fields of the hippocampus (Witter et al., 1989; Suzuki and Amaral, 1994). Layer III neurons project to CA1 and the subiculum, from where projections return to layer V of the entorhinal cortex (Naber et al., 2001). Taking into consideration the broad connectivity and information flow from the EC, this region is assumed to be the quantitatively dominant deliverer of data to the hippocampus (Brodal, 2010). Also, the dorsolateral EC projects to layers I and II of the prefrontal cortex (prelimbic and infralimbic cortices), which further strengthen the functional loop of memory formation and consolidation (Delatour and Witter, 2002; Ranganath et al., 2003; Remondes and Schuman, 2004).

The EC is the first region affected in Alzheimer's disease (Braak and Braak, 1991). It undergoes disease-related neuronal loss and consequently atrophy (Gomez-Isla et al., 1996; Yeh et al., 2011). For unknown reasons, the most vulnerable to neurogedeneration during aging and AD are neurons of layer II of EC, which in cascading fashion contribute

to degenerative changes in the main interconnected region – the hippocampus (Mattson and Magnus, 2006; Stranahan and Mattson, 2010).

1.2.4.c. Hippocampus:

The hippocampus is an important part of the limbic system and plays crucial roles in the consolidation of information from short-term memory to long-term memory and spatial navigation (Brodal, 2010). It is divided into four main subfields: CA1, CA2 and CA3 (called together the hippocampus proper), where CA stands for *cornu ammonis,* and the dentate gyrus (DG) (Rapp and Gallagher, 1996). Compared to the neocortex, the hippocampus has a less complex organization; however, it is far from being simple. Several types of cells contribute to establishing strictly interconnected patterns inside the structure (Brodal, 2010). The hippocampus proper archicortex consists of three layers: the molecular, pyramidal and polymorphic ones. The molecular layer is the deepest layer, built of apical dendritic trees of pyramidal cells and axon terminals. The axon terminals are granule cells, which originate in the granule cell layer of the hippocampus. The middle layer, called the pyramidal layer, consists of pyramidal cells whose axon collateral branches (Schaffer collaterals) cross the polymorphic and pyramidal layers to establish synaptic contacts with the dendrites of pyramidal neurons. The last layer, a superficial one named the polymorphic layer, consists of interneurons, pyramidal cell dendrites and axon collateral branches (Patestas and Gartner, 2006). The dentate gyrus also has a laminar construction of molecular, granular and polymorphic layers and the hilus, which smoothly fuses with the hippocampus proper CA3 area (Amaral et al., 2007). The dendritic arborizations of the granule cells of the granule cell layer ramify in the molecular layer to form synapses with the terminals of the perforant pathway, the connective route arising in the EC.

EC, the first structure affected in AD, is the main projecting structure to the hippocampus. Also, many asymmetric, excitatory contacts are established in the hippocampus, having a high relevance for LTP and LTD formation, what means for cognition and memory. CA1 region shows the earliest pathological burden done by AD – it shows abberant Aβ load before other hippocampal areas (Olabarria et al., 2010). What is more, emerging evidence suggested that altered neurogenesis in the adult hippocampus represents an early clinical event in the AD progression (Mu and Gage, 2011).

1.2.4.d. Prefrontal cortex:

The prefrontal cortex (PFC) is situated in the anterior part of the frontal lobes of the brain. The primate PFC has been classically characterized and delineated using anatomical criteria, such as cytoarchitectonic features or connectivity into mainly the dorsolateral (DLPFC), ventrolateral (VLPFC), dorsomedial (DMPFC), ventromedial (VMPFC) and orbitofrontal (OFC) regions (Fuster, 2008). The prefrontal cortex of rodents is divided into medial, orbital and lateral parts (Ongur and Price, 2000). One of the most homologous structures to the primate PFC when it comes to function and organization is the rodent medial prefrontal cortex (mPFC), especially its ventral cortices: prelimbic (PL – equivalent of the DLPFC) and infralimbic (IL – equivalent of the orbital and medial PFC: OMPFC) (Uylings et al., 2003).

Apart from the PL and IL, the dorsal division of the mPFC consists of the agranular (AGm) and anterior cingulate (AC) cortices, involved in many motor actions (Ongur and Price, 2000; Heidbreder and Groenewegen, 2003). The infralimbic (IL) and prelimbic (PL) cortices are the ones anatomically and functionally linked with the limbic system, which makes them directly responsible for cognitive (including personality expression, decision making, active goal-directed behavior, response selection and implementation), mnemonic (working memory, selection of information) and emotional processes (Heidbreder and Groenewegen, 2003; Uylings et al., 2003; Hoover and Vertes, 2007). The mPFC receives a direct ipsilateral projection from the hippocampus, from the CA1 subfield and subiculum (Jay et al., 1989; Carr and Sesack, 1996; Vertes, 2004). The relationship between the hippocampus and PFC consists of two types of connective pathways: a direct hippocampal projection to the mPFC and an indirect return functional loop, which is crucial for long-term memory and as a consequence long-term potentiation (LTP) formation involving the active regulatory and relay role of the nucleus reuniens of the thalamus (RE) (Fig.14) (Jay et al., 1996; Buckner et al., 1999; Buckner et al., 2000; Vertes et al., 2007; Kulijewicz-Nawrot et al., 2012). Backward projections of CA1 neurons to the deep layers of the entorhinal as well as the perirhinal and postrhinal cortices strengthen the process of long-term potentiation due to the fact that the rhinal cortical region is a cardinal intermediary between the hippocampus and neocortex, being indispensible for the short term storage and consolidation of specific memory forms (Cousens and Otto, 1998; Kulijewicz-Nawrot et al., 2012). The PL and IL cortices

receive many projections from other structures, such as the perirhinal cortex (PRC), the entorhinal cortex (EC), the claustrum (CLA), the medial basal forebrain (BF) and the amygdala as well as sending various projections back to the dorsal and ventral tania tecta (TT), the piriform cortex (PIR), the medial frontal polar cortex (FPm) and the ventral orbital cortex (VO) (Vertes, 2004; Hoover and Vertes, 2007). Taking into consideration that the mentioned interconnections are just examples of a vast number of afferent and efferent communications, the highly complex nature of the PFC and its definite significance in managing all kinds of cognitive functions are not speculative (Goldman-Rakic, 1987; Miller, 2000). This information, together with the fact that cognitive function, meaning things that make a person a self-defined human being, are highly affected in AD, thus making the PFC the center of attention during investigations of astrocytic changes and their pathological/restorative potential presented in this thesis.

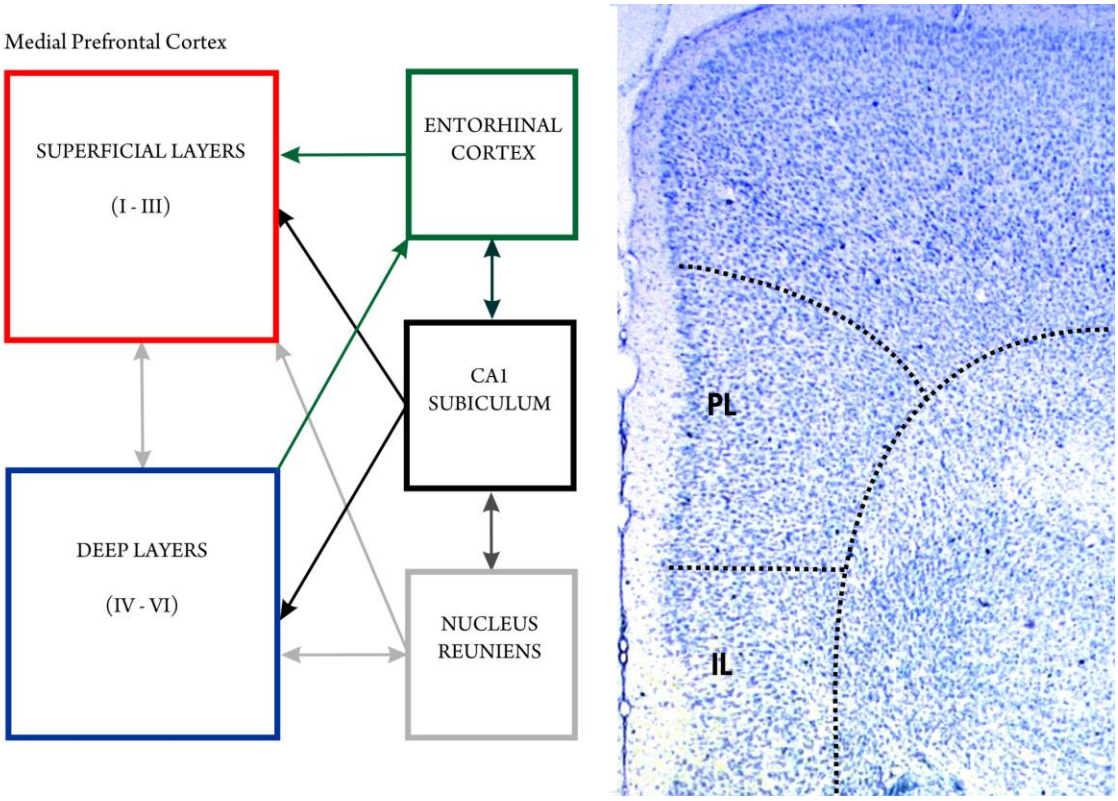

Fig.14 *(Right) Brightfield micrograph of a mPFC section showing the boarders of the PL and IL cortices. (Left) Afferent and efferent projections of the mPFC. Afferences from the limbic system are mainly received from the hippocampal formation, the entorhinal cortex and the thalamic nucleus reuniens, while the mPFC itself projects back indirectly to the*

hippocampus via the nucleus reuniens of the mid-line thalamus – an integrating part within this network.

1.2.5. Astroglia in AD:

Astrocytes are highly involved in Alzheimer's disease and the pathophysiology of other dementias due to their unique, indispensable functions for metabolism and the proper action of neurotransmitters, neuronal nutrition, control of neurovascular unit performance and much more. In Alzheimer's disease, the early occurrence of metabolic stress is a common response to ongoing pathology (Verkhratsky et al., 2010). Studies using positron emission tomography (PET) revealed altered glucose uptake, which is assumed to be due to astrocytic metabolic impairments (Alexander et al., 2002; Freemantle et al., 2006). Also, much effort has been made to establish the character of the relation between Aβ and glucose uptake. Some in vivo studies have shown that reduced glutamate metabolism can be an early marker of future Aβ plaques and neurobifrillary tangles, which is supported by in vitro studies in which exposure to Aβ decreased astroglial consumption of glucose (Parpura-Gill et al., 1997; Small et al., 2000). In contrast, according to other researchers, decreased glucose uptake in brain regions strongly affected by Aβ plaques is not necessarily the rule, and exposure to Aβ can even increase astroglial use of glucose (Edison et al., 2007; Allaman et al., 2011).

When it comes to astrocytes and their modifications linked to altered neurovascular unit performance in Alzheimer's disease, a reduction in astrocytic glucose transporter GLUT1 and lactate transporters and a retraction of endfeet from blood vessels were noticed before the appearance of Aβ plaques (Merlini et al., 2011).

In vitro studies show that glutamine synthetase (GS) can be oxidized by different forms of synthetic β-amyloid peptide, which is not the case in vivo, because of greatly limited interaction between glutamine synthetase inside astrocytes and β-amyloid produced in neurons (Hensley et al., 1994; Zhao et al., 1996; Aksenov et al., 1997). Nevertheless, as claimed by some researchers, such interaction is possible via astrocytosis (Vijayan et al., 1991; Ingelsson et al., 2004). Others do not find GS-immunoreactive astrocytes in the close proximity of Aβ plaques, nor any change in GS-positive astrocytes in regions with

astrogliosis (Robinson, 2000). The type of Aβ and GS synergy in AD and its significance are still unclear (Walton and Dodd, 2007).

Defects and failures of synapses are assumed to be early events in the progression of Alzheimer's disease (Coleman et al., 2004). Currently, synaptic dysfunction followed by synaptic loss serves as the most reliable indicator of the cognitive decline observed in AD (Terry et al., 1991; Masliah et al., 2001; Koffie et al., 2011). Astrocytic D-serine is thought to be involved in the mechanism of controlling NMDAR-mediated neurotoxicity, related also to Alzheimer's disease (Inoue et al., 2008). In patients diagnosed with AD, deficits in NMDA-dependent forms of neocortical LTP were observed and serve as proof of altered synaptic plasticity and the important correlation between AD and NMDAR performance (Battaglia et al., 2007). Considering receptor efficiency, Fisher and colleagues in their study (Fisher et al., 1998) found increase levels of D-serine in the cerebrospinal fluid (CSF) of AD patients compared to controls, which suggests an abnormally high activity of NMDA receptors. In contrast, Hashimoto and his group (Hashimoto et al., 2004) showed reduced levels of D-serine in the serum of AD patients, which can result from the reduced acitivity of serine racemase (SR), and supports the hypothesis of NMDA receptor hypofunction in the pathophysiology of Alzheimer's disease. However, the final contribution of endogenous D-serine to AD pathophysiology remains unclear and its clarification is of great interest. As was shown in the hypothalamic supraoptic nucleus (SON), where astrocytes reduce their overlay of neurons and synapses during lactation (Theodosis and Poulain, 1993; Hatton, 1997), the D-serine concentration available at the synaptic cleft, together with the control of the glycine site of synaptic NMDARs by D-serine, depend strongly on the extent of astrocytic coverage of the synapses (Panatier et al., 2006). It is a matter of wide debate whether this form of astrocytic-dependent metaplasticity can appear throughout the nervous system and be an important factor in several neuropsychiatric and neurodegenerative disorders (Halassa et al., 2007).

Regarding the main histological hallmarks of Alzheimer's disease, it is suggested that astrocytes are capable of accumulating great amounts of $A\beta_{42}$. However, at the end astrocytes become overloaded with Aβ, which severely influences their functionality,

most significantly their assistance to neurons (Nagele et al., 2004). Many in vitro experiments have been performed to understand the role of astrocytes in Aβ pathology. Recently, astrocytes have been proposed to be an important mediator of Aβ clearance, thus preventing plaque formation. It was shown that after plating adult astrocytes onto brain sections from AD transgenic mice, exogenous astrocytes were able to bind and degrade Aβ (Wyss-Coray et al., 2003). Similar astrocytic activation was observed by aggregated Aβ protein as well as by the intact cores of amyloid plaques isolated from human AD brain tissue (DeWitt et al., 1998). However, the mechanism underlying astrocytic Aβ uptake remains unknown (Nagele et al., 2003; Nielsen et al., 2010). What is also intriguing is that by administering Aβ peptide to cultured hippocampal neurons with astrocytes, atypical $[Ca^{2+}]_i$ transients and mitochondrial depolarization in astrocytes are obtained. These alterations are present before any symptoms of degeneration appear in neurons (Abramov et al., 2003, 2004; Rossi and Volterra, 2009). However, it was also shown that endogenous astrocytes are unable to remove Aβ from brain tissue, suggesting that during Alzheimer's disease their clearing abilities are highly altered (Wyss-Coray et al., 2003).

1.2.6. Animal models of AD:

To fully understand the pathology of the disease and the detailed mechanism of action of the involved factors as well as to introduce potential therapeutical approaches – this is why animal models have been designed. To develop relevant animal models of AD, that closely mimick the disease progression with a wide range of symptoms, different kinds of genetic, biochemical or dietary manipulations have been employed (Woodruff-Pak, 2008). Among all of the species used, from worms to polar bears, the greatest contribution to AD research has been made by mouse models of the disease, introduced in the 1990s (Games et al., 1995; Hsiao et al., 1996). Several features make the mouse the perfect model: (i) mice are easy to rear and reproduce efficiently, (ii) they have a comfortable size for the majority of research approaches, (iii) they have a rather short life span (around 30 months), which makes them convenient for aging studies, (iv) mice are suitable for studies focused on learning and memory, (v) the entire mouse genome has been mapped, which is of high importance for genetic manipulation and research purposes (Woodruff-Pak, 2008). A review of the vast majority of animal models of AD,

mainly murine models, together with the relevant neuropathology, can be found in **table 1**.

1.2.6.a. Types of animal models:

The damage of cholinergic neurons observed in Alzheimer's disease led to the first **lesion models** in the basal forebrain to mimic the pathogeny of specific neurodegeneration. The best examples of the lesion type were those performed in the nucleus basalis magnocellularis of rodents (nbm), which is the analogous structure to the human nucleus basalis of Meynert (nbM). Different lesions or dysfunctions induced by toxic substances led to miscellaneous models (Toledano and Alvarez, 2004). Electrolytic lesions efficiently produce neuronal death; however, they are not highly specific, causing damage to neighbouring fibers and influencing distant neurons (Vale-Martinez et al., 2002; Toledano and Alvarez, 2004). To obtain another type of lesion, excitoxic non-selective lesions of the forebrain cholinergic centers, substances acting on glutamatergic receptors were used: (i) NMDA, (ii) ibotenic acid, (iii) quisqualic acid, (iv) AMPA (Toledano and Alvarez, 2004).
The most relevant models when it comes to AD were thought to be those obtained by chemical and immunochemical cholinergic toxins, such as AF64A and 192IgG-saporin, respectively (Hanin, 1996; Schliebs et al., 1996). Those substances target only the cholinergic centers and do not harm other neurons or fibers passing through the target region (Toledano and Alvarez, 2004).

The intracellular accumulation of Aβ and amyloid plaques are key elements in the pathology of AD. That is why a major effort was made to develop relevant animal models (**single or double transgenic models**), that would express human beta-amyloid precursor protein (APP) in order to study the progression of the disease. Nowadays, a minimum of 16 mutations for APP (OMIM 104760) have been characterized, with a tendency to more commonly use in research a few distinct ones: K670N/M671L (Swedish), V717I (London), E693G (Arctic) and V717F (Indiana) (Trancikova et al., 2011).
Considering tau transgenic mice, the first model developed (ALZ7) expressed the longest human four-repeat (4R) tau isoform (htau40) under the control of the human Thy-1

promoter. Similarly to Alzheimer's disease cases, transgenic human tau protein was observed in the cell bodies of nerve cells, axons and dendrites in most brain regions. Within next years new models were developed, including TG23, JNPL3 or V337M. Generally, they are characterized by differential regional distribution and load of neurofibrillary tangles within the brain (Gotz et al., 1995; Brion et al., 1999; Lewis et al., 2000; Gotz et al., 2001).

1.2.6.b. Triple transgenic animal model (3xTg-AD):

The limitation of not having in the same model all of the main pathological hallmarks of AD: plaques (Aβ pathology) and tangles (tau pathology), was the stimuli for researchers to design combinatorial animal models, expressing both. One of the most relevant models used today is a triple transgenic mouse model (3xTg-AD), developed in Frank LaFerla's laboratory (Oddo et al., 2003b). 3xTg-AD animals harbour $PS1_{M156V}$, APP_{Swe} and tau_{P301L} transgenes. The genetic manipulations used to generate the model consist of comicroinjections of two independent transgenes encoding human APP_{Swe} and human tau_{P301L}, under the mouse Thy1.2 promoter, into single-cell embryos picked from homozygous mutant $PS1_{M146V}$ knockin (PS1-KI) mice (Oddo et al., 2003b). The PS1 knockin mice were generated as a hybrid 129/C57BL6 (Guo et al., 1999). This approach to generating the line has many advantages over classic crossbreeding of independent transgenic lines. First of all, the APP and tau transgenes co-integrated at the same genetic locus, which assures that they will be co-assorted in each future generation. Secondly, due to such strict connections also to the knockin of the PS1 mutation, the triple transgenic line can be breed as a single-transgenic line, without any additional genetic handling. Finally, the animals are homozygotes, which makes their breeding even more easy and efficient (Oddo et al., 2003b).

3xTg-AD mice develop extracellular Aβ deposits before the presence of neurofibrillary tangles. They arise in an age- and region-dependent manner. Intracellular Aβ is noticable in the neocortex already at early ages of 3-4 months, while in the hippocampus Aβ expression is apparent by 6 months of age. The initial location of extracellular Aβ deposits is the frontal cortex – aggregates starts to appear at 6 months and became broad at 12 months, when they can also be found in the hippocampus. However, Aβ deposits are not accompanied by altered tau until the age of around 12 months (in the

hippocampus progressing to the neocortex), which goes in line with the more common "amyloid cascade hypothesis" (Oddo et al., 2003a; Oddo et al., 2003b). Triple transgenic animals also develop cognitive deficits measured as deficits in LTP, paired-pulse facilitation (PPF) and/or spatial learning performance, which colocalize with the appearance of intracellular Aβ (Oddo et al., 2003b; Billings et al., 2005). At very early stages, 1-2 months of age, no sign of cognitive decline is observed, meaning that the mice are born with intact mental functions. The very first alteration of cognitive functions appears as retention, not learning problems, observed at 4 months of age and measured by a slight deterioration in long-term memory (Billings et al., 2005). All other changes become apparent at the age of 4-6 months, which is a strong point to conclude about intraneuronal Aβ deposition as a foe of proper synaptic performance (Oddo et al., 2003a; Billings et al., 2005).

All of the mentioned data support the notion that the 3xTg-AD mouse model is one of the most advanced AD models, mimicking the pathology observed in the human patiens in terms of the affected structures (hippocampus, amygdala and cerebral cortex) as well as the importance of Aβ and tau pathology in a combined version (Oddo et al., 2003b). This provides a huge opportunity for research to find a proper therapeutic approach and makes the triple transgenic animal model a great subject for various neuroanatomical, biochemical, neurochemical, neuroimaging, cognitive and behavioral studies.

Table 1. Neuropathology in the main AD animal models. Modified from (Ittner and Gotz, 2011; Rodriguez and Verkhratsky, 2011b).

Lesion and transgenic mouse, rat and primate models	Neuropathology	References
Aging	Cholinergic involution and amyloid deposition	(Sani et al, 2003) (Fischer et al, 1992) (Michalek et al, 1989)
Electrolytic lesion	Neuronal death	(Lescaudron et al, 1999) (Vale-Martinez et al, 2002)
Unspecific toxins (NMDA, Ibotenic acid, Quisalic acid, Quinolic acid, Colchicine, Alkaloids, Alcohol)	Neuronal death	(Dunnett et al, 1991) (Winkler et al, 1998) (Boegman et al, 1985) (Shaughnessy et al, 1994) (Di Patre et al, 1989) (Arendt, 1994)
Specific toxins (AF64A, 192Ig-G saporin)	Cholinergic neuronal death	(Waite et al, 1995) (Chrobak et al, 1988) (Hanin, 1996) (Wiley, 1992) (Wiley et al, 1991)
β-Amyloid	Cholinergic dysfunction	(Giovannini et al, 2002) (Pavia et al, 2000)
AMYLOID BETA (Aβ)		
PS1M146L	Diffuse plaques	(Blanchard et al, 2003)
PDAPP	Plaques	(Games et al, 1995)
APP_{Swe}	Plaques	(Hsiao et al, 1996)
APP23	Plaques	(Sturchler-Pierrat et al, 1997)
APP_{V717F}	Plaques	(Dodart et al, 2000)
APP_{751SL}	Plaques	(Blanchard et al, 2003)
K670M/N671L	Plaques	(Kloskowska et al, 2010)
Tg478/Tg1116	Plaques	(Flood et al, 2009)
APP_{Swe} and Indiana V717F	Plaques	(Chishti et al, 2001)
PS2APP (PS2/APP_{Swe})	Plaques	(Richards et al, 2003)
APP_{751SL}/$PS1_{M146L}$	Plaques	(Blanchard et al, 2003)
APP_{Swe}/$PS1_{dE9}$	Plaques	(Savonenko et al, 2005)
APP_{Swe} and $PS1_{M146L}$	Plaques	(Janus et al, 2000)
K670N/M671L and V717F	Plaques	(Janus et al, 2000)
TgAPPSwe and $PS1_{M146L}$	Plaques	(Takeuchi et al, 2000)
TAU		
ALZ7	Tangles	(Gotz et al, 1995)
Tau_{P301L}(4R,2-,3-)	Tangles	(Lewis et al, 2000)
P301L	Tangles	(Gotz et al, 2001)
7TauTg	Tangles	(Ishihara et al, 2001)
P301S	Tangles	(Allen et al, 2002)
V337M	Tangles	(Tanemura et al, 2002)
4R/2N	Tangles	(Tatebayashi et al, 2002)
Endogenous tau knockout	Tangles	(Andorfer et al, 2003)
Tau_{P301L}	Tangles	(Arendash et al, 2004)
P301L TET-off	Tangles	(Ramsden et al, 2005)
P301S/G272V	Tangles	(Schindowski et al, 2006)
G272V, P301L, R406W	Tangles	(Eriksen et al, 2007)
COMBINED Aβ AND TAU		
Tg2576 x JNPL3 (APP_{Swe})	Plaques and tangles	(Lewis et al, 2001)
Tg2576 and VLW	Plaques and tangles	(Ribe et al, 2005)
Synthetic $A\beta_{1-42}$ x pR5	Plaques and tangles	(Gotz et al, 2001)

APP23 x JNPL3 on C57BL/6	Plaques and tangles	(Bolmont et al, 2007)
APP-V717I x tau-P301L	Plaques and tangles	(Terwel et al, 2008)
TauPS2APP (APP152 x pR5)	Plaques and tangles	(Grueninger et al, 2010)
ADnnPP7 x TauP301S	Plaques and tangles	(Coomaraswamy et al, 2010)
APP23 x pR5	Plaques and tangles	(Ittner et al, 2010)
3xTg-AD	**Plaques and tangles**	**(Oddo et al, 2003)**
Tg478	None	(Flood et al, 2009)
Tg1116	None	(Flood et al, 2009)

1.2.7. Available treatments for AD and potential therapeutical targets:

Successful therapy for Alzheimer's disease is one of the urgent needs and dreams of today's medicine. Unfortunately, until now no real cure has been found. The only hope for patients are drugs and disease-modifying therapies, with an equal number of pros and cons (Pereira et al., 2005). When it comes to strategies to change the direction of the disease progression, three main lines of approach have been established: (i) a neurotrophic and neuroprotective one, (ii) a strategy to target a particular pathological feature of AD, (iii) an approach based on epidemiological observations. Great attention has been paid to anti-amyloid actions, as a prevalent hallmark of the disease. Scientists are focusing on several possibilities to act on Aβ, such as blocking its aggregation, anti-amyloid therapy or the modulation of Aβ production (Citron, 2004). In the case of blocking Aβ aggregation, zinc chelation with an antibiotic called clioquinol resulted in a decrease in β-amyloid accumulation (Cherny et al., 2001). Anti-amyloid immunotherapy based on active or passive immunization showed improvement in several transgenic mouse models (Schenk et al., 1999; Pfeifer et al., 2002; Oddo et al., 2006). An indirect approach with the administration of an antibody against Aβ is based on the assumption that the antibody binds to amyloid deposits and in this way activates microglia to phagocytize the amyloid (Bard et al., 2000). The therapeutic outcome is thought to be achieved also by the direct resolution of Aβ deposits or by the capture of its soluble form, which is an important factor affecting cognition (Dodart et al., 2002). Additionally, scientists focus on directly reducing $A\beta_{42}$ production via the stimulation of α-secretase or the inhibition of γ- and β-secretase. Although many paths have been explored in the hope that they will prove to be the correct one, none of them has been thoroughly tested for clinical application (Citron, 2004). In light of the uncertainty about Aβ-based therapy, attention has also been paid to tau protein and how to decrease its misfolding and thus

restitute tau function. This concept includes inhibiting tau hyperphosphorylation or fibril assembly, as well as improving intracellular tau aggregation (Brunden et al., 2009). However, until now none of these strategies have been approved for real AD treatment (Herrmann et al., 2011).

Considering drugs to slow down or improve the symptoms of Alzheimer's disease, there are a few already in use. The first group consists of cholinesterase inhibitors (ChEIs), as the loss of cholinergic neurons and disrupted cholinergic transmission are among the key elements of the pathology (Mangialasche et al., 2010). Improvement should be obtained by the modulation of acetylocholinesterase, the enzyme that hydrolyzes acetylcholine in the brain (Herrmann et al., 2011). The mechanism of action of ChEIs is connected with stopping the hydrolysis caused by acetylcholinesterase, so that the amount of acetylcholine available in the synaptic cleft is higher and cholinergic transmission works better (Seltzer, 2007). All ChEIs drugs available - donepezil, rivastigmine and galantamine - have been approved for AD treatment at different stages, mainly mild to moderate (with donepezil also included in 2006 for severe AD). These drugs work via non-competitive and reversible binding to acetylcholinesterase and further undergoing hydrolysis instead of acetylcholine (Herrmann et al., 2011). The inhibitory effect of rivastigmine has long-lasting action, even after the concentration of the drug in the plasma decreases (Polinsky, 1998). Similarly to donepezil, rivastigmine works in a dose-dependent manner and as such contributes to beneficial effects on cognitive functions; however, data about its efficacy differ in some cases (Herrmann et al., 2011). Galantamine acts in a reversible and competitive way and additionally modulates nicotinic acetylcholine receptors – improved nicotinic transmission is a bonus in this case (Shimohama, 2009). There are no evident data about its dose-dependent effect; nevetheless, it shows positive results demonstrated as a range of symptomatic improvements (Herrmann et al., 2011).

When it comes to non-cholinergic treatment, two drugs are on the market currently: memantine and cerebrolysin (Herrmann et al., 2011). Memantine (1-amino-3,5-dimethyladamantane) is a low affinity uncompetitive NMDA receptor antagonist, which achieve its effect by rapid, voltage-dependent interaction within the NMDAR channel. In other words, memantine preferentially blocks the receptor activity as a result of extended exposure to extracellular glutamate, but still enables its physiological activation (Parsons

et al., 1999; Hynd et al., 2004). Also, memantine was shown to prevent neurodegeneration induced by Aβ (Miguel-Hidalgo et al., 2002). From the clinical point of view, memantine is able to decrease neurotoxic devastation already at the concentrations which are not harmful for LTP induction and learning abilities (Muller et al., 1995; Chen et al., 1998). For this reason, it has started to be used worldwide as a therapeutic agent in many AD clinical trials, also in combination with acetylcholinesterase inhibitors (Jain, 2000). Though memantine is commonly used at all stages of Alzheimer's disease, from mild to severe as well as in patients suffering mild cognitive impairment, its beneficial role was found just in severe cases (Winblad and Poritis, 1999; Miguel-Hidalgo et al., 2002; Schneider et al., 2011). Effective therapy with memantine needs further attention and combined studies. Also, extended studies on the precise causes of glutamate excitotoxicity in neurodegenerative diseases should be undertaken to make every therapeutic approach more effective (Walton and Dodd, 2007). Cerebrolysin is used within some European countries and Asia. Its mechanism of action is still not clear, but it reseambles endogenous growth factors, meaning that it has neurotrophic and supportive effects on neurons (Veinbergs et al., 2000). Studies and therapies exploring the use of cerebrolysin require further attention and comparative studies (Herrmann et al., 2011).

To conclude, Alzheimer's disease is a pathology that is the subject of large numbers of medical trials and much scientific guessing. Much effort has been made to develop and test theories of its causes and progression and to find new therapeutic targets; nevertheless, innovative approaches together with diligent work continue to be needed in order to achieve a cure.

2. AIMS AND HYPOTHESES:

The hypothesis behind this work is based on the assumption that astrocytes are actively involved in the progression of Alzheimer's disease, as well as in normal aging. In our opinion, the destructive cascade of events starts from an initial failure of astrocytes, which leads to reduced synaptic coverage and homeostatic imbalance, inluding inadequate nutrition to neurons. At the end, ongoing devastation in astrocytic functionality can result in total brain connectivity breakdown, as observed in AD patients, resulting in a complete deterioration of cognitive functions and memory. To closely investigate the astrocytic changes, we chose the medial prefrontal cortex (mPFC), an important region for managing complex cognitive functions as well as being a part of the connective limbic loop between other structures strongly affected in Alzheimer's disease, such as the hippocampus and the entorhinal cortex.

In our work we sought to prove that astrocytes can be a novel and promissing therapeutic target in AD.

The aims of this thesis are:

1. To characterize the changes in astroglial morphology in the mPFC at different ages in AD animals using 3D qualitative and quantitative neuroanatomical analysis;
2. To study the relation between β-amyloid and changes in GFAP-positive astrocytes in the mPFC;
3. To investigate the functional changes in astrocyte-dependent glutamate metabolism, crucial for proper neurotransmission within the brain, by analyzing the changes in glutamine synthetase (GS) and glutamate transporter – 1 (GLT-1);
4. To characterize the type of interrelation between the main brain structures (hippocampus, entorhinal cortex, medial prefrontal cortex) affected in Alzheimer's disease based on astrocytic alterations;
5. To study the alterations in astrocytic cytoarchitecture and the expression of specific markers (GFAP, S100β and GS) during non-pathological aging in the hippocampus and entorhinal cortex.

3. MATERIALS AND METHODS:

These studies were performed in accordance with the European Communities Council Directive of 24th of November 1986 (86/609/EEC) regarding the use of animals in research and were approved by the Ethical Committee of the Institute of Experimental Medicine of the Academy of Sciences of the Czech Republic, Prague, Czech Republic. All efforts were made to reduce the number of animals.

3.1. MICE:

Experiments were performed on male 3xTg-AD mice and their background-matching controls as described in detail in the **Introduction, part 1.2.6.b (Animal models)** and in scientific publications (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008; Rodriguez et al., 2009a). Briefly, the homozygous animals harbour mutant genes for $PS1_{M146V}$, APP_{Swe} and tau_{P301L}, which accounts for their ability to manifest the hallmarks of AD in an age-related and region-dependent manner. The generation of 3xTg-AD mice was based on the co-microinjection of two transgenes (APP_{Swe} and tau_{P301L}) into single-cell embryos from homozygous $PS1_{M146V}$ knockin mice (Fig.15). The animals were kept in the same-sex cages, in 12 hour light-dark cycles, with access to food and water *ad libitum*.

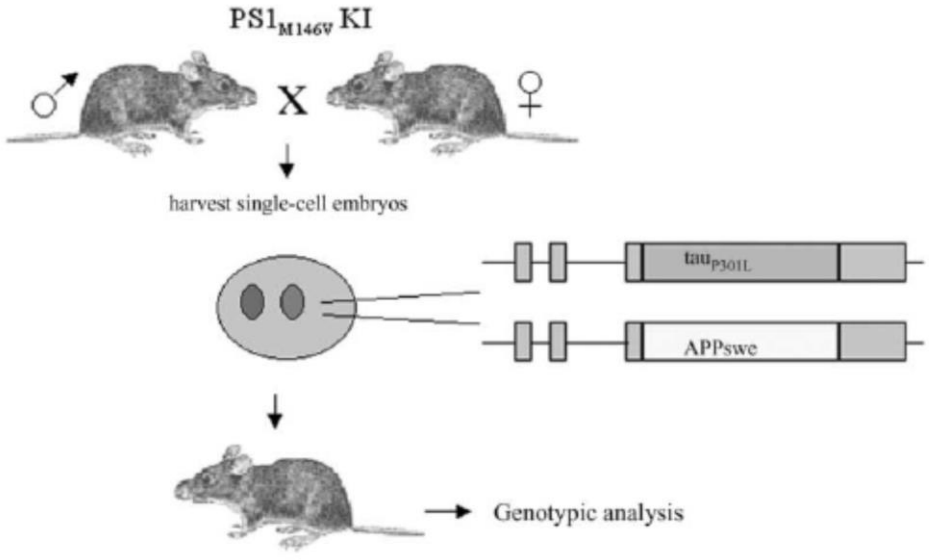

Fig.15 *The method used to develop 3xTg-AD mice (Oddo et al, 2003).*

3.2. IMMUNOHISTOCHEMICAL ANALYSIS:

3.2.1. Fixation and tissue processing:

3xTg-AD animals of different ages (1, 3, 6, 9, 12, 18 and 24 months; n=3-7 for all ages) and their equivalent Non-Tg controls were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ kg). The mice were perfused through the aortic arch with 3.75% acrolein (25 ml, Fluka Sigma-Aldrich, Germany) in a solution of 2% paraformaldehyde (Sigma, Germany) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75ml). The brains were then removed and cut into 4 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the mPFC. The brain sections were post-fixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Then, they were cut into 40–50 μm thick coronal sections using a vibrating microtome (MICROM HM 650 V, Thermo Scientific, USA). Free floating brain sections in 0.1 M PB, pH 7.4, were collected and stored in a cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels 1,98/1,54 mm anterior to Bregma were selected for immunochemistry, according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).

3.2.2. Antibodies:

To study the specific subtypes of astrocytes, as well as the interrelationships between them and/or Aβ, different antibodies were used. The tables give an overview of the primary and secondary antibodies used to fulfill the scientific goals of this thesis.

To assess possible nonspecific background labeling or cross-reactivity between antibodies derived from different host species, a series of control experiments was performed. The omission of the primary and secondary antibodies from the incubation solutions resulted in the total absence of target labeling.

Table 2. Primary antibodies used for immunohistochemistry

Antigen	Host	Type	Provider	Dilution	Reference
GFAP	Mouse	Monoclonal	Sigma-Aldrich (G3893)	1:5000	(Wilhelmsson et al., 2004)
GFAP	Rabbit	IgG fraction	Sigma-Aldrich (G9269)	1:5000	(Wilhelmsson et al., 2004)
GS	Mouse	Monoclonal	Millipore (MAB302)	1:500	(Wilhelmsson et al., 2004)
S100β	Rabbit	Polyclonal	DAKO (Z0311)	1:5000	(Hagen et al., 1986)
Aβ	Mouse	Monoclonal	Covance (SIG-39300)	1:2000	(Oddo et al., 2003b)

Table 3. Secondary antibodies used for immunohistochemistry

Antigen	Host	Type	Provider	Dilution	Reference
Mouse	Goat	FITC-conjugated IgG	Jackson Immunoresearch (115-096-146)	1:100	(Rodriguez et al., 2009a)
Rabbit	Goat	FITC-conjugated IgG	Jackson Immunoresearch (111-096-144)	1:100	(Olabarria et al., 2010)
Mouse	Goat	Alexa Fluor 594-conjugated IgG	Invitrogen (A11005)	1:200	(Olabarria et al., 2011)
Mouse	Goat	TRITC-conjugated IgG (Rhodamine)	Jackson Immunoresearch (115-026-075)	1:200	(Olabarria et al., 2010)
Mouse	**Horse**	**Biotinylated IgG**	**Vector Laboratories (BA-1400)**	**1:200**	**(Rodriguez et al., 2008)**

3.2.3. Immunohistochemistry:

3.2.3.a. Peroxidase:

To minimise methodological variability, sections through the mPFC containing both hemispheres of all animals were processed at the same time under precisely the same experimental conditions. For this procedure, the vibratome sections were first incubated for 30 min in 30% methanol in 0.1M PB and 3% hydrogen peroxide (Sigma-Aldrich, Germany). Sections were rinsed with 0.1M PB for 5 mins and placed in 1% sodium borohydride (Sigma-Aldrich, Germany) for 30 minutes. Subsequently, the sections were washed with PB profusely before rinsing in 0.1M TS for 10 minutes. Brain sections were then incubated with 0.5% albumin bovine serum (BSA, Sigma-Aldrich, Germany) in 0.1M TS and 0.25% Triton X-100 (Sigma-Aldrich, Germany) for 30 minutes. For single labeling, sections were incubated for 48 hours at room temperature with the primary antibody. The sections were rinsed in 0.1M TS for 30 minutes and incubated in the appriopriate dilution of the biotinylated secondary antibody for 1 hour at room temperature. Subsequently, the sections were rinsed in 0.1M TS for 30 minutes, followed by incubation for 30 minutes in avidin-biotin peroxidase complex (Vetor Laboratories Ltd, UK). The peroxidase reaction product was visualized by incubation in a solution containing 0.022 % of 3,3'diaminobenzidine (DAB, Sigma-Aldrich, Germany) and 0.003 % H_2O_2 for 1.5 minutes, as described previously (Rodriguez et al., 2008; Rodriguez et al., 2009a; Olabarria et al., 2011). The reaction was stopped by rinsing the tissue in 0.1M TS for 5 minutes followed by 0.1M PB for 15 minutes. Brain sections were permanently mounted onto gelatinized slides and then dehydrated in ascending concentrations of ethanol (50, 70, 80, 90, 95 and 100 %) followed by xylene. Finally, they were permanently coverslipped with entellan (Merck, Czech Republic).

3.2.3.b. Fluorescence:

For the detection and determination of GFAP-positive cells and their relation to Aβ aggregates, as well as in case of GFAP-GS colocalization, we used both single and double indirect immunofluorescence labeling. The initial stages in preparation for antigen detection were the same as in the case of peroxidase staining. After incubation in 30% methanol in 0.1M PB and 3% of hydrogen peroxide, the sections were placed in 1% sodium borohydride (Sigma-Aldrich, Germany) for 30 min, washed profusely with PB

and rinsed in 0.1M TS for 10 min. Then, the brain sections were incubated with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Germany) in 0.1M TS and 0.25% Triton X-100 (Sigma-Aldrich, Germany) for 30 min. For single labeling, the sections were incubated for 48 h at room temperature in the appriopriate primary antibody. The sections were rinsed in 0.1M TS for 30 min and incubated in a dilution of fluorochrome-conjugated secondary antibody for 1 h at RT, then rinsed in 0.1M TS for 30 min.
For double labeling, the sections were incubated for 48 h at room temperature in a primary antibody cocktail. Each antigen was detected in a sequential manner on the same sections by incubation with their correspondent fluorochrome-conjugated secondary antibody.

Finally, the sections were rinsed with 0.1M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).

3.2.4. Cell count in the ventral medial prefrontal cortex:

We determined the numerical density (Nv, # cells/mm^3) of GFAP-positive and GS-positive astrocytes in the mPFC in at least three representative non-consecutive sections, analyzing an area of 600,000 μm^2 in coronal sections of 40 μm thickness, thus representing a total volume of 24,000,000 μm^3 per section. Confocal images were used for this purpose in the case of GFAP-positive astrocytes, while light microscopy images enabled the counting of GS-positive glia. Both subtypes of glial cells were intensively labeled, which made them easy to identify with an equal chance of being counted by a single observer to reduce counting bias to a minimum.

3.2.5. Morphological analysis of the astrocyte cytoskeleton:

GFAP-positive astrocytes (n=35 per animal, with a minimum of 5 per layer in case of mPFC region) were imaged using confocal scanning microscopy (Leica TCS SP), recording layers every 0.2μm. Morphological analysis was carried out by Cell Analyst (Chvatal et al., 2007), the software developed in the Department of Neurobiology, Institute of Experimental Medicine, Prague, Czech Republic. In general, the software allows the performance of 2 types of cell visualization: (1) 3D image reconstruction by **rendering the cell surface** as well as (2) 2D reconstruction done by **volume rendering**,

meaning that recordings from all the layers are superimposed (Chvatal et al., 2007) (Fig.16A). At the basis of the technique is sectioning of the image along the vertical axis into a stack of 2D parallel images, with known xyz size. Subsequently, several techniques are utilized, such as filtering the images (including the removal of background noise), thresholding and the final morphometric calculations. Digital filters applied for the purpose of our study were: average 3x3, convolution, gauss 5x5, despeckle and simple objects removal. Thresholding (in a 0-255 greyscale) was used to determine which part of the image will be taken into consideration as a part of the cell. The image area where pixels had a value above the threshold was ignored. The cell volume was calculated by the Cavalieri method, while the cell surface area was established using the iso-contouring or iso-intensity contouring method (Kubinova et al., 1999; Duerstock et al., 2003). In the case of 3D surfaces of the cells, those were constructed using the pixel value of the threshold – "iso-values" (Fig.16B) (Chvatal et al., 2007).

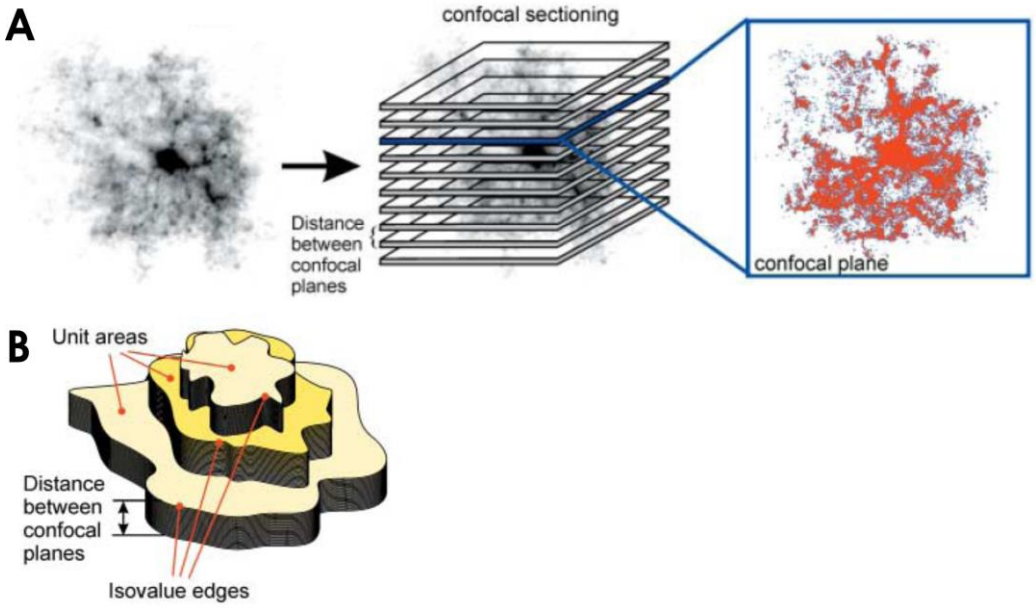

Fig.16 *(A) Presentation of GFAP-IR astrocyte confocal sectioning. All cell layers recordings (shown as a stack of images in the middle drawing) were superimposed and underwent the procedure of digital filtering. (B) Draft showing unit areas bounded by isovalue edges and the distance between sections used in the morphometric calculations (Chvatal et al, 2007).*

3.2.6. Measurements of the domain and cell body surface area of GS-immunoreactive cells:

We used ImageJ software to measure the domain and cell body surface area in representative cells from each age group (N=15 in both controls and 3xTG-AD animals).

For this purpose, higher magnification micrographs (objective 40x) obtained with light microscopy (Zeiss Observer D1) were analyzed.

3.2.7. Colocalization of GS-IR and GFAP-IR astrocytes:

To determine the colocalization of GS-IR and GFAP-IR astrocytes, representative higher magnification stacks of images throughout the mPFC region of control and 3xTg-AD animals (N = 4 in both cases) were taken with a confocal microscope (Zeiss LSM 5 DUO) at 0.2 μm z-step. Both GS-IR and GFAP-IR cells were imaged at the same time and then counted in an approximate area of 40,500 μm^2 in a section of 40μm thickness.

3.3. WESTERN BLOT ANALYSIS:

3.3.1. Tissue processing:

Transgenic and control animals of different ages (1, 6, 9 and 12 months; n = 5-7) were euthanized by cervical dislocation. Brain tissue samples containing the mPFC were collected immediately and lysed with 100 μl of STEN lysis buffer [50 mM Tris (pH 7.6), 150mM NaCl, 2 mM EDTA and 1% Triton-X (Sigma-Aldrich, Germany)] with protease inhibitors (Complete mini, Protease Inhibitor Cocktail Tablets, Roche, Czech Republic) on ice for 30 min. When ready to use, the lysates were centrifuged (13.000 rpm, 5 min) and the supernatants tranferred to new eppendorf tubes.

3.3.2. SDS polyacrylamide gel electrophoresis (PAGE) and transfer of proteins onto a nitrocellulose membrane:

The protein concentrations from brain tissue lysates were determined using the Bradford method (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). Samples containing 20 μg of protein and 1X Laemmli buffer (Laemmli, 1970) were boiled at 95^{o}C for 4-5 min. Samples were loaded together with 5μl of protein marker (prestained Protein Ladder, Page Ruler, Fermentas) and run on 12% sodium dodecyl sulfate-polyacrylamide gels [30% acrylamide: bisacrylamide (37,5:1), 1,5 M Tris (pH 8,8), 10% Ammoniumpersulfate (APS), 10% SDS and 0,1% Tetramethylethylene-diamine (TEMED)]. The gels were submerged into running buffer 1X (Tris Base 25 mM; Glycine 119 mM; SDS 1%) and run initially at 100 V until the samples passed the stacking gel

and then at 150 V until the mercaptoethanol dye reached the bottom of the gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane in an electrical field in order to immobilize them in a specially designed chamber (BioRad). Prior to transfer, the gel and nitrocellulose membrane were dunked in 1X transfer buffer (Tris Base 25 mM; Glycine 119 mM; Methanol 20%; pH 7,6) and run at 400 mA (constant) for 120 min. The polyacrylamide gel was placed onto the nitrocellulose membrane (Sigma) and sandwiched in a blotting cassette between a sponge and Whatman filter paper. All parts were clinched together after ensuring that no air bubbles had been formed between the gel and the membrane.

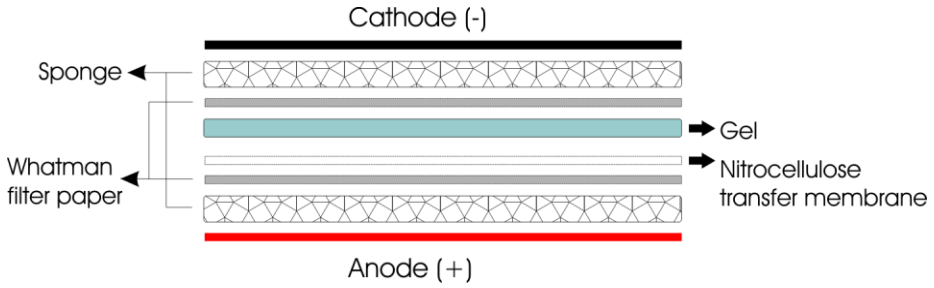

Fig.17 *The arrangement of the parts of the transfer cassette.*

After electroblotting the membranes were rinsed in water and stained briefly with Ponceau S Red dye in 0.1% acetic acid to verify the protein transfer as well as the equal loading of the samples.

To prevent non-specific binding of the primary and secondary antibodies, membrane blocking was performed using a blocking solution consisting of 5% non-fat dry milk soluted in Tris Buffer Saline Tween20 (TBST) buffer (Tris Base 10 mM; NaCl 100 mM; Tween-20 0.1%; pH 7.6). Blots were incubated for 1 h at RT with agitation.

3.3.3. Antibodies:

The primary and secondary antibodies used for WB are summarized in the tables below.

Table 4. Primary antibodies used for Western blot

Antigen	Host	Type	Provider	Dilution	Reference
GS	Mouse	Monoclonal	Millipore (MAB302)	1:20000	(Sen et al., 2011)
GLT-1 (EAAT2)	Rabbit	Polyclonal	Cell Signaling (3838)	1:1000	(Amara and Fontana, 2002)
***β-actin**	**Mouse**	**Monoclonal**	**Sigma-Aldrich (A2228)**	**1:20000**	**(Gimona et al., 1994)**

* antibody used as a loading control

Table 5. Secondary antibodies used for Western blot

Antigen	Host	Type	Provider	Dilution	Reference
Rabbit	Goat	Peroxidase-conjugated IgG	Jackson Immunoresearch (111-035-003)	1:20000	(Kulijewicz-Nawrot et al., 2013)
Mouse	**Goat**	**Peroxidase-conjugated IgG**	**Jackson Immunoresearch (115-035-003)**	**1:15000**	**(Kulijewicz-Nawrot et al., 2013)**

3.3.4. Protein detection and band analysis:

The primary antibodies were diluted in the same blocking buffer (5% non-fat dry milk/TBST) and the membranes were incubated for 1h (in the case of anti-GS and anti-β-actin antibodies) or 2h (in the case of anti-GLT-1 antibody) at RT. Following the incubations, the membranes were washed three times in TBST at RT with agitation for 15 min to remove residual primary antibodies. The blots were then probed with HRP-

conjugated secondary antibodies and incubated for 1h with agitation at RT. Finally, the membranes were washed three times in TBST.

Visualization of the secondary antibodies was achieved with enhanced chemiluminescence substrate (ECL). The membrane was covered with the substrate solution 1:1 (Sol1: 250 mM luminol in DMSO and 90 mM p-coumaric acid in DMSO, Sigma-Aldrich, Germany; Sol2: 1M Tris pH 8,5 and H_2O_2, Sigma-Aldrich, Germany) and incubated for 5 min in the dark at RT and afterwards exposed to XBM x-ray film (Retina, Fotochemische Werke GmbH, Berlin, Germany). After scanning the images, ImageJ free software was used to quantify the intensity of the bands. The ratio of GS or GLT-1 to β-actin, used as loading control, was first assessed. In order to perform comparisons across different Western blots, an internal control was always included on each blot as the same reference point, regarding the GS or GLT-1/β-actin ratio.

3.4. Statistical analysis:

Unpaired t-tests alone (in the GS AD experiments, as well as in the aging study) or combined with one-way ANOVA (in the GFAP experiments) were used to examine differences in the number, surface area, volume as well as the relative ratios of GS or GLT-1 to β-actin in labeled cells from 3xTg-AD and Non-Tg animals. Additionally, a linear regression test was used to analyze the relation between age and the relative ratios of GS to β-actin in 3xTg-AD and Non-Tg animals. All data are expressed as mean ± SEM. Statistical analysis was done using GraphPad Prism (GraphPad Software) and significance accepted at $p \leq 0.05$.

4. RESULTS:

4.1. ASTROCYTIC CYTOSKELETAL ATROPHY IN THE MEDIAL PREFRONTAL CORTEX OF A TRIPLE TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE.

Kulijewicz-Nawrot M, Verkhratsky A, Chvátal A, Syková E, Rodríguez JJ.

J Anat. 2012 Sep;221(3):252-62. doi: 10.1111/j.1469-7580.2012.01536.x. Epub 2012 Jun 27.

We analyzed the astrocytic cytoskeletal changes within the mPFC of a triple transgenic mouse model of AD (3xTg-AD) by measuring the surface area and volume of glial fibrillary acidic protein (GFAP)-positive profiles in relation to the build-up and presence of amyloid-β (Aβ) and compared the results to those found in non-transgenic control animals at different ages. 3xTg-AD animals showed clear astroglial cytoskeletal atrophy, which appeared at an early age (3 months; 33% and 47% decrease in GFAP-positive surface area and volume, respectively) and remained throughout the disease progression at 9, 12 and 18 months of age (29% and 36%; 37% and 35%; 43% and 37%, respectively). This atrophy was independent of Aβ accumulation, since only a few GFAP-positive cells were localized around Aβ aggregates, which suggests no direct relationship with Aβ toxicity.

4.1.1. General astrocytic appearance and cell count:

In both Non-Tg and 3xTg-AD mice, GFAP-immunolabeled astrocytes showed typical characteristics of protoplasmic astrocytes with numerous, elongated and extended processes arising from the astrocyte somata in a star-shaped radial pattern (Fig. 18A-D). These GFAP-immunoreactive (GFAP-IR) processes differed in thickness and length, from clearly visible solid branches to thin, subtle and elaborated ones, which corresponded to proximal and distal processes, respectively. GFAP-IR astrocytes were much more abundant in layer I and appeared in lower numbers through layers II to VI. However, independently of the gradient of GFAP-positive astrocytes, the Nv (number of cell/mm^3) of GFAP-IR astrocytes was constant and equal at different ages in both Non-Tg and 3xTg-AD animals, with no significant differences between them (Fig. 19).

4.1.2. Early and sustained astroglial cytoskeletal atrophy in 3xTg-AD mice:

GFAP-positive astrocytes in the 3xTG-AD mice showed a significant reduction in their cytoskeletal surface area and volume as early as 3 months of age; both parameters decreased by 33% and 47%, respectively, when compared to Non-Tg controls (2526.58 ± 103.44 μm^2 vs. 3770.23 ± 166.88 μm^2, p=0.0007; 507.78 ± 22.28 μm^3 vs. 950.13 ± 86.99 μm^3, p=0.0026). This cytoskeletal atrophy remained at 9 months (a decrease of 29% in surface area, 2580.81 ± 288.53 μm^2 vs. 3633.10 ± 244.97 μm^2, p=0.0458; and a decrease of 36% in volume, 536.51 ± 73.30 μm^3 vs. 834.16 ± 88.32 μm^3, p=0.0475), 12 months (a decrease of 37% in surface area, 2279.37 ± 340.39 μm^2 vs. 3633.35 ± 121.88 μm^2, p=0.0096; and a decrease of 35% in volume, 507.91 ± 74.82 μm^3 vs. 787.37 ± 54.76 μm^3, p=0.0236) and 18 months, when the surface area was reduced by 43% (2210.96 ± 143.36 μm^2 vs. 3857.58 ± 391.03 μm^2, p=0.0075) and the volume by 37% (526.55 ± 58.08 μm^3 vs. 841.94 ± 91.41 μm^3, p=0.0269) (Fig. 18E-F).

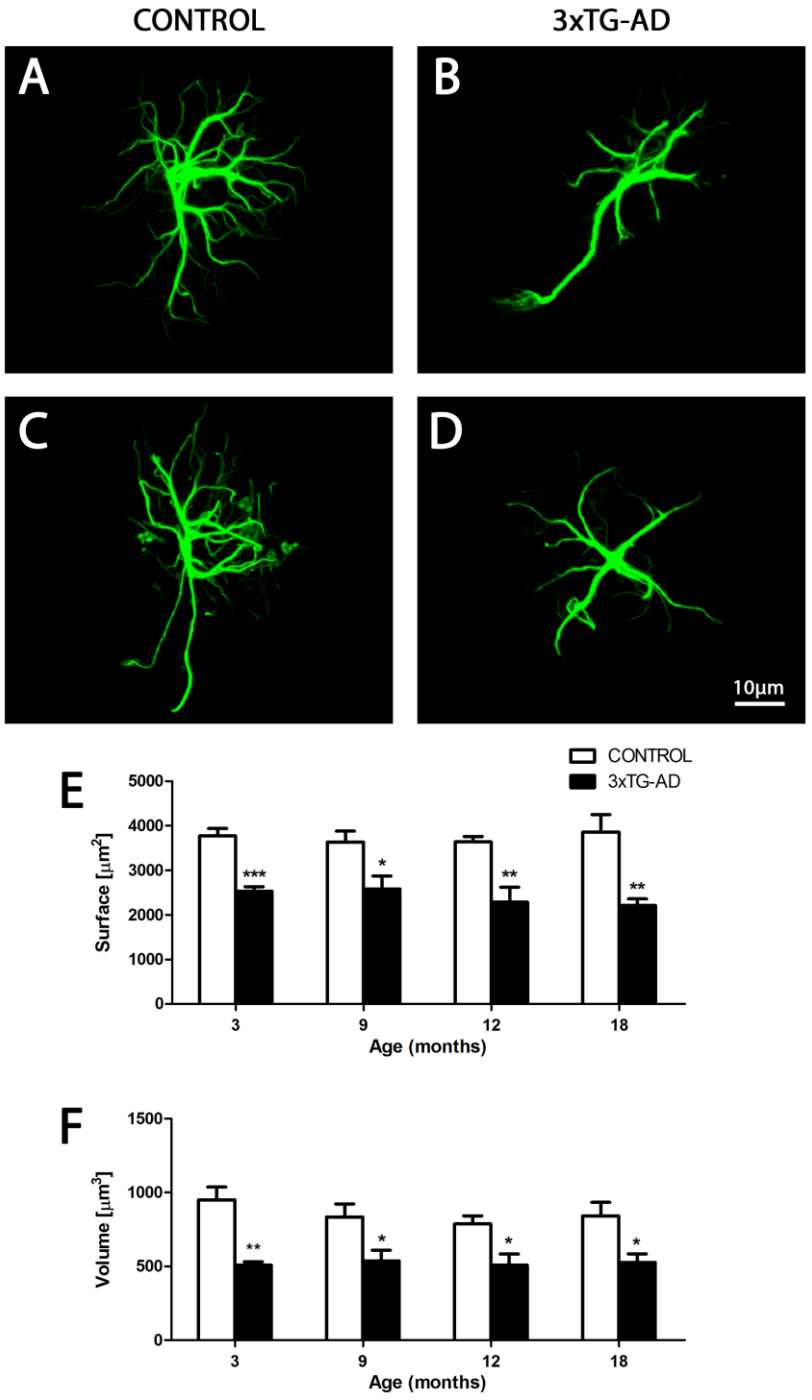

Fig.18 *Confocal images showing the classical morphology of GFAP-positive astrocytes in control Non-Tg animals and astrocytic atrophy in the 3xTg-AD animals at 3 months (A and B, respectively) and 18 months (C and D, respectively) in the mPFC. Bar graphs showing the decreases in the GFAP-positive surface area and volume throughout the whole extent of the mPFC (E-F) in 3xTg-AD mice when compared with control animals. Bars represent mean ± SEM.*

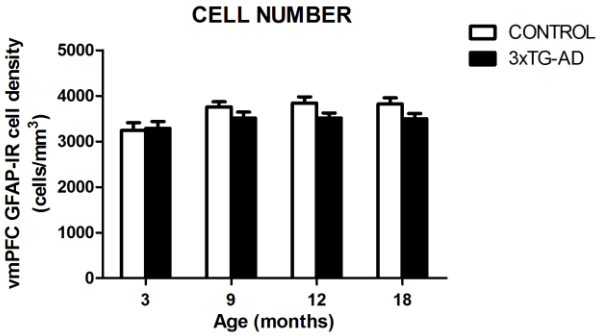

Fig.19 *Bar graphs showing the numerical density (number cells/mm3) of GFAP-IR cells in 3xTg-AD mice and Non-Tg controls. Bars represent mean ± SEM.*

4.1.3. Layer-specific astrocytic atrophy:

Our results show that astrocytic atrophy is not only a generalized phenomenon, but also layer-specific. The affected layers at both young (3 months) and old ages (18 months) were the superficial layers 1 and 2 together with the deep layers 4 and 5. However, layer 3 only appeared to be affected at advanced ages, starting at 12 months of age (Fig. 20C and 20H).

4.1.3.a. Superficial layers 1-3:

In general, astrocytes from the superficial layers in 3xTg-AD animals were characterised by shorter, less numerous and rather horizontally oriented processes. At the age of 3 months astrocytes in superficial layer 1 were reduced in surface area by 37% (2017.35 ± 293.54 μm^2 vs. 3219.53 ± 346.18 μm^2, p=0.0381) and in volume by 48% (438.24 ± 55.06 μm^3 vs. 844.05 ± 81.32 μm^3, p=0.0061) when compared to those in Non-Tg control animals. At very late ages this atrophy, which was absent at middle and advanced ages (9 to 12 months), re-appeared, and the astrocytic cytoskeletal surface area underwent a reduction of 31% (1972.83 ± 111.31 μm^2 vs. 2843.57 ± 166.21 μm^2, p=0.0048) along with a 32% decrease in volume (410.70 ± 39.60 μm^3 vs. 603.36 ± 35.91 μm^3, p=0.0113) (Fig. 20A and 20F).

Astrocytic atrophy in layer 2 at 3 months was manifested by a 50% reduction in surface area (1900.20 ± 355.75 μm^2 vs. 3787.24 ± 595.47 μm^2, p=0.0346) and a 52% reduction

in volume (390.97 ± 46.66 μm^3 vs. 813.33 ± 52.94 μm^3, p=0.001). Similarly to layer 1 at 18 months, equivalent decreases were observed: 56% and 53% in surface area and volume, respectively (1715.77 ± 92.83 μm^2 vs. 3945.10 ± 507.82 μm^2, p=0.005; and 374.96 ± 51.09 μm^2 vs. 791.10 ± 66.44 μm^2, p=0.0025) (Fig. 20B and 20G).

The surface area and volume of astrocytes in layer 3 were affected only at advanced and late ages, the changes being significant at 12 months (a decrease of 37%, 3004.46 ± 527.54 μm^2 vs. 4763.42 ± 363.08 μm^2, p=0.0334; and a decrease of 32%, 663.33 ± 113.59 μm^3 vs. 981.10 ± 55.21 μm^3, p=0.0455, respectively) and at 18 months of age (a decline in surface area of 41%, 2521.97 ± 237.91 μm^2 vs. 4244.60 ± 661.12 μm^2, p=0.0497; and a decline in volume of 40%, 540.11 ± 65.38 μm^3 vs. 906.31 ± 129.89 μm^3, p=0.0454) (Fig. 20C and 20H).

A slight difference was noticeable between astrocytes in the different superficial layers. Astrocytes from layer 2 in comparison to those from layer 1 had slightly more expanded processes, but less numerous than the more bushy astrocytes from the deeper superficial layer 3. At 3 months of age in 3xTg-AD animals, there were some significant internal differences between the GFAP-positive profiles of layer 3 and those of other layers, despite the fact that this layer was unchanged compared to the same layer in the control group. Layer 3 astrocytes appeared to be larger compared to those in layer 1 (53.08% larger in surface area, 4299.96 ± 644.34 μm^2 vs. 2017.35 ± 293.54 μm^2, p=0.0181; and 47.42% larger in volume, 833.54 ± 93.17 μm^3 vs. 438.24 ± 55.06 μm^3, p=0.0107) and also those in layer 2 (55.81% in surface area, 4299.96 ± 644.34 μm^2 vs. 1900.2 ± 355.75 μm^2, p=0.0178; 53.1% in volume, 833.54 ± 93.17 μm^3 vs. 390.97 ± 46.66 μm^3, p=0.0054); this phenomenon was observed only at early ages and exclusively in 3xTg-AD animals, not in control mice.

4.1.3.b. Deep layers 4 and 5:

Astrocytes in the deep layers were star-like displayed a more typical protoplasmic anatomy (Fig. 21) but with rather thin, numerous, but limited in length processes, sent in all directions. The reduction in astrocytic cytoskeletal branching in layers 4 and 5 was

significant not only in the early and late stages of AD, but also at mid-advanced ages (3, 12 and 18 months of age).

The surface area and volume of astrocytes in layer 4 were decreased at 3 months by 42% (3024.84 ± 329.86 μm^2 vs. 5210.35 ± 697.60 μm^2, p=0.0299) and by 53% (597.18 ± 45.41 μm^3 vs. 1269.32 ± 232.94 μm^3, p=0.0299), respectively. At 12 months the surface area was decreased by 49% (2944.96 ± 539.49 μm^2 vs. 5810.24 ± 383.12 μm^2, p=0.0049) and the volume by 48% (609.86 ± 87.45 μm^3 vs. 1183.26 ± 165.95 μm^3, p=0.0223). In the oldest group of animals at 18 months of age, the atrophy of mPFC astrocytes in layer 4 remained evident, showing a decrease in both surface area and volume (50% in surface area, 2910.83 ± 556.42 μm^2 vs. 5832.46 ± 613.04 μm^2, p=0.0124; and 47% in volume; 654.01 ± 126.84 μm^3 vs. 1235.76 ± 200.46 μm^3, p=0.0496) (Fig. 20D and 20I).

The reduction in astrocytic cytoskeletal surface area and volume in layer 5 at 3 months of age was manifested to the same extent by a 51% decrease in surface area (1805.06 ± 584.22 μm^2 vs. 3702.41 ± 291.62 μm^2, p=0.0271) and a 70% decrease in volume (356.60 ± 30.53 μm^3 vs. 1200.08 ± 241.64 μm^3, p=0.0134). At 12 and 18 months of age, these reductions persisted even though the differences were less pronounced: 36% and 41% in surface area (2509.24 ± 484.45 μm^2 vs. 3907.91 ± 131.85 μm^2, p=0.0318; and 2053.31 ± 205.48 μm^2 vs. 3460.72 ± 359.09 μm^2, p=0.0145, respectively) and 36% and 37% in volume (596.90 ± 118.90 μm^3 vs. 930.23 ± 46.78 μm^3, p=0.0402; and 528.75 ± 53.96 μm^3 vs. 837.37 ± 101.99 μm^3, p=0.0368) at 12 and 18 months, respectively (Fig. 20E and 20J).

As observed in superficial layers 1 and 2, astrocytes in layer 5 were significantly smaller than those in layer 3. Indeed, layer 5 astrocytes were smaller by 41.98% in surface area (1805.06 ± 584.22 μm^2 vs. 4299.96 ± 644.34 μm^2 vs., p=0.0285) and by 43.02% in volume (833.54 ± 93.17 μm^3 vs. 356.6 ± 30.53 μm^3 p=0.0028), while no clear differences were evident compared to astrocytes from layer 4.

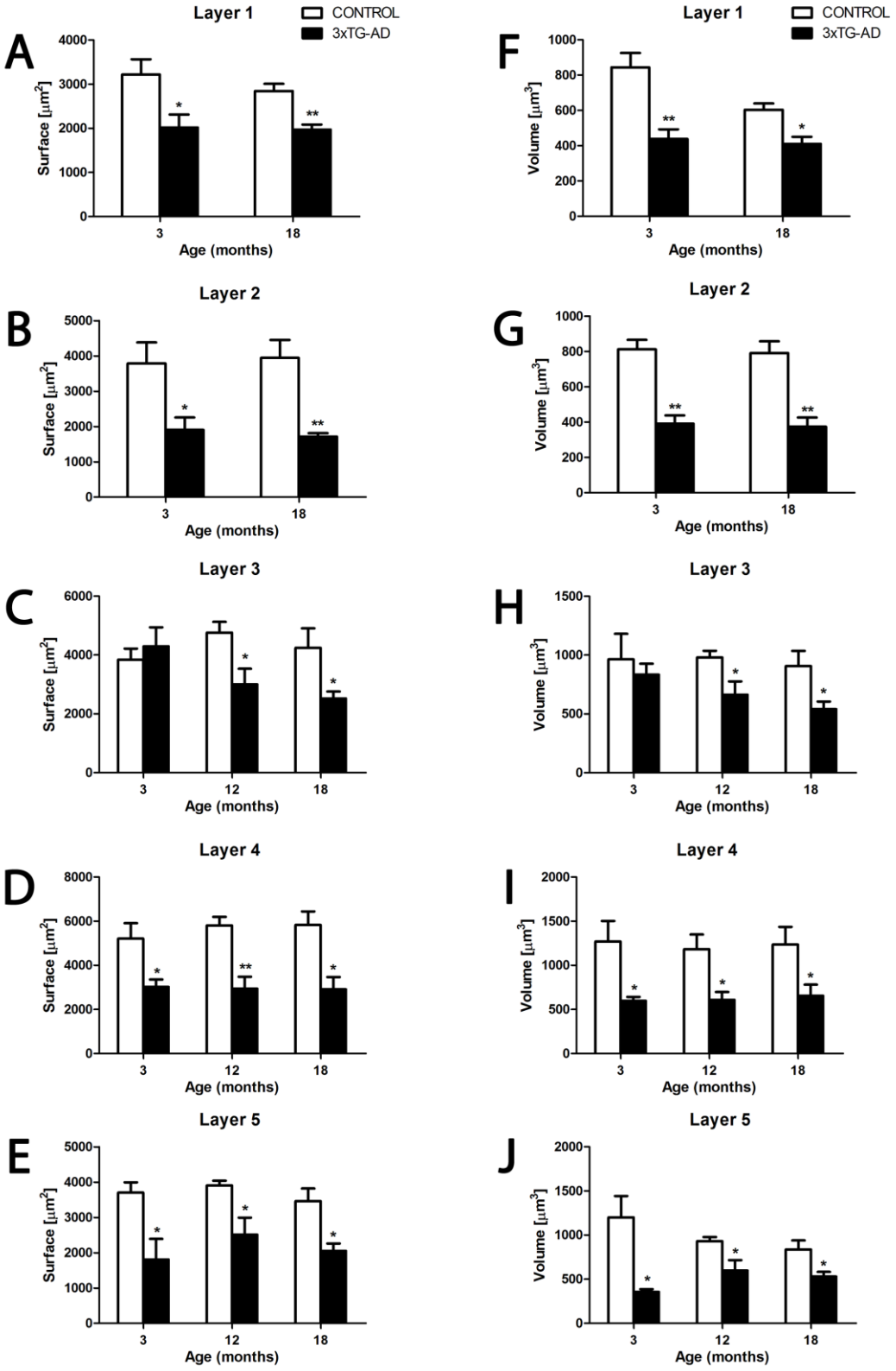

Fig.20 *Bar graphs illustrating the decreased GFAP-positive surface area (A-E) and volume (F-J) in the different cortical layers of the mPFC. Bars represent mean ± SEM.*

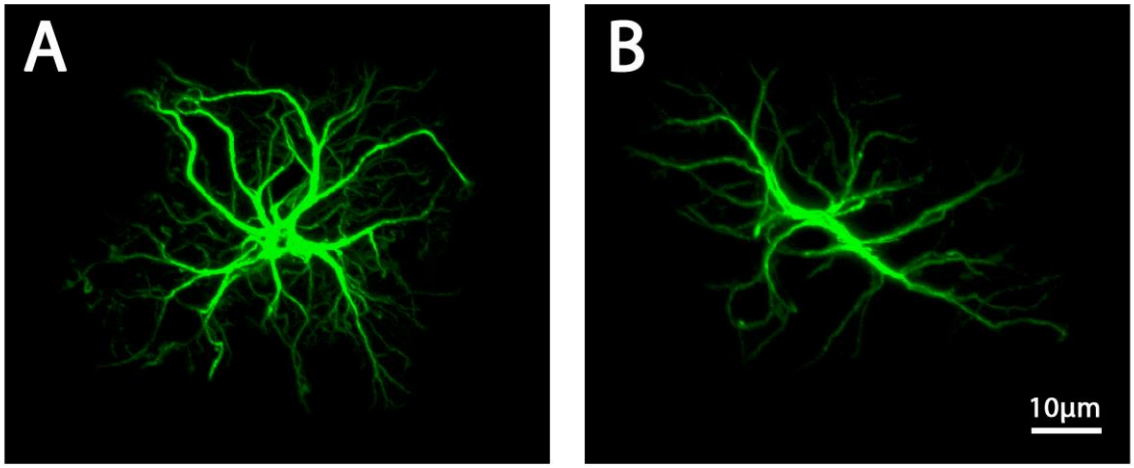

Fig.21 *Confocal images showing a typical protoplasmic astrocyte (A) compared to a less classical cortical astrocyte (B) from the deep layers of 3xTg-AD mice***.**

4.1.4. Intracellular Aβ accumulation but rare astrocytic association with neuropil Aβ aggregates:

Up to 18 months of age, no plaques were formed in the mPFC of 3xTg-AD animals. However, vascular Aβ accumulation and a vast intracellular accumulation of amyloid β were observed (Fig. 22C and inset, 22B), with a minimal tendency to progress to extracellular plaque deposition, except for aggregates (Fig. 22A-B). The intracellular accumulation of Aβ started to appear around 6 months of age and became more robust with disease progression. Neurones filled with Aβ were located throughout the cortex, with a tendency towards a slightly more prominent presence in the deeper layers (4-5). These neuropathological signs were not generally associated with the presence of neighbouring astrocytes positive for GFAP (Fig. 22A-B) and, if so, only with astrocytes displaying evident atrophic characteristics (Fig. 22A and asterisk).

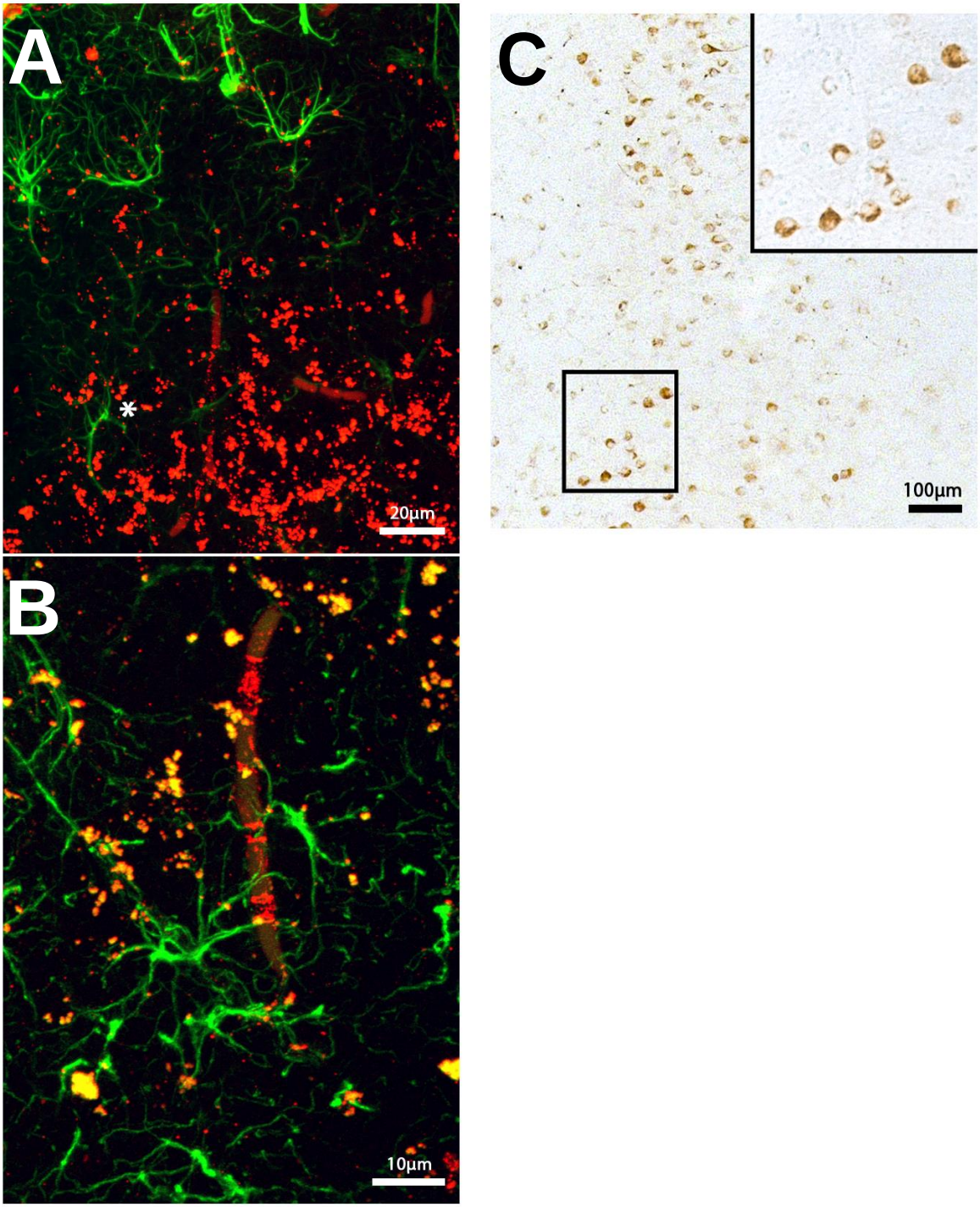

Fig.22 *Confocal images illustrating Aβ aggregates in the mPFC, but few GFAP-positive neighbouring astrocytes (A and asterisk), and an Aβ loaded blood vessel surrounded by some reactive astrocytes in an 18-month-old 3xTg-AD animal (B); Green – GFAP, red – Aβ. Brightfield micrograph showing the intracellular accumulation of Aβ in the mPFC of an 18-month-old transgenic animal (C).*

4.2. ASTROCYTES AND GLUTAMATE HOMEOSTASIS IN ALZHEIMER'S DISEASE: A DECREASE IN GLUTAMINE SYNTHETASE BUT NOT IN GLUTAMATE TRANSPORTER-1 IN THE PREFRONTAL CORTEX.

Kulijewicz-Nawrot M, Syková E, Chvátal A, Verkhratsky A, Rodríguez JJ.

ASN Neuro 2013 5(4):art:e00123.doi:10.1042/AN20130017. Epub 2013 Sep 23.

We analyzed the expression of glutamine synthetase (GS) and glutamate transporter-1 (GLT-1) in astrocytes in the mPFC during the progression of AD in a triple-transgenic mouse model (3xTg-AD). GS is an astrocyte-specific enzyme, responsible for the intracellular conversion of glutamate to glutamine, whereas the removal of glutamate from the extracellular space is accomplished mainly by astroglia-specific GLT-1. We found a significant decrease in the numerical density (Nv, cells/mm^3) of GS-positive astrocytes from early to middle ages (1-9 months) (at the age of 1 month by 17%, 6 months by 27% and at 9 months by 27% when compared to control animals) in parallel with a reduced expression of GS (determined by Western blots), which started at the age of 6 months and was sustained up to 12 months of age. We did not, however, find any changes in the expression of GLT-1, which implies an intact glutamate uptake mechanism. Our results indicate that the decrease of GS expression may underlie a gradual decline in the vital astrocyte-dependent glutamate-glutamine conversion pathway, which in turn may compromise glutamate homoeostasis, leading towards failures in synaptic connectivity with deficient cognition and memory.

4.2.1. General characterization of GS-positive astrocytes in control and transgenic animals and their relation to GFAP:

Cortical GS-immunoreactive (GS-IR) astrocytes were uniformly distributed in both the superficial and deep layers of the mPFC. GS-IR astrocytes showed clear labeling of their primary and distal processes, thus faithfully delineating the astrocytic domains (Fig. 23A and C). GS-IR astrocytes showed typical characteristics of protoplasmic astrocytes with multiple elaborated processes emanating from the cell somata (Fig. 23A). In control animals the astrocytic cell bodies were well defined and almost perfectly round in shape. In 3xTg-AD animals, the GS-IR cell bodies were also clearly outlined, but slightly less

spherical and markedly smaller. In 3xTg-AD animals, GS-positive astrocytes also had less branching in both their main and secondary processes (Fig. 23B and D). The processes were directed in a random fashion, with thin extensions oriented in various directions.

As revealed by co-staining, there was a different tendency to co-express GFAP and GS in mPFC astrocytes in control and 3xTg-AD mice. In general, in controls and transgenic animals three subpopulations of astrocytes were identified: GS-IR, GFAP-IR and a population expressing both proteins (GS/GFAP-IR) (Table 6). The majority of astroglial cells in the region of interest in control animals were GS-IR 72.90%, while the population of GFAP-IR astrocytes constituted 9.03%. In 3xTg-AD mice 82.50% of astrocytes were GS-IR, while 12.5% were GFAP-IR. The most interesting difference was observed in the case of astrocytes co-expressing GS and GFAP (GS/GFAP-IR). In 3xTg-AD animals just 5% expressed both proteins simultaneously (Fig. 23F), while up to 18.07% of astrocytes in control animals did so (Fig. 23E).

Table 6. Different distribution of astrocytic subpopulations in the mPFC. Numbers represent changes in numerical density (cells/mm^3) in Non-Tg and 3xTg-AD animals (± SEM).

	NON-TG	**3xTG-AD**
GS-IR	17,423 ± 388	15,265 ± 918
GFAP-IR	2,158 ± 308	2,312 ± 388
GS/GFAP-IR	4,317 ± 752	925 ± 178
Total mean Nv	23,899 ± 1,108	18,503 ± 1,153

GS-IR – glutamine synthetase immunoreactive cells; GFAP-IR – glial fibrillary acidic protein immunoreactive cells; GS/GFAP – glutamine synthetase and glial fibrillary acidic protein immunoreactive cells; Nv- numerical density (cells/mm3)

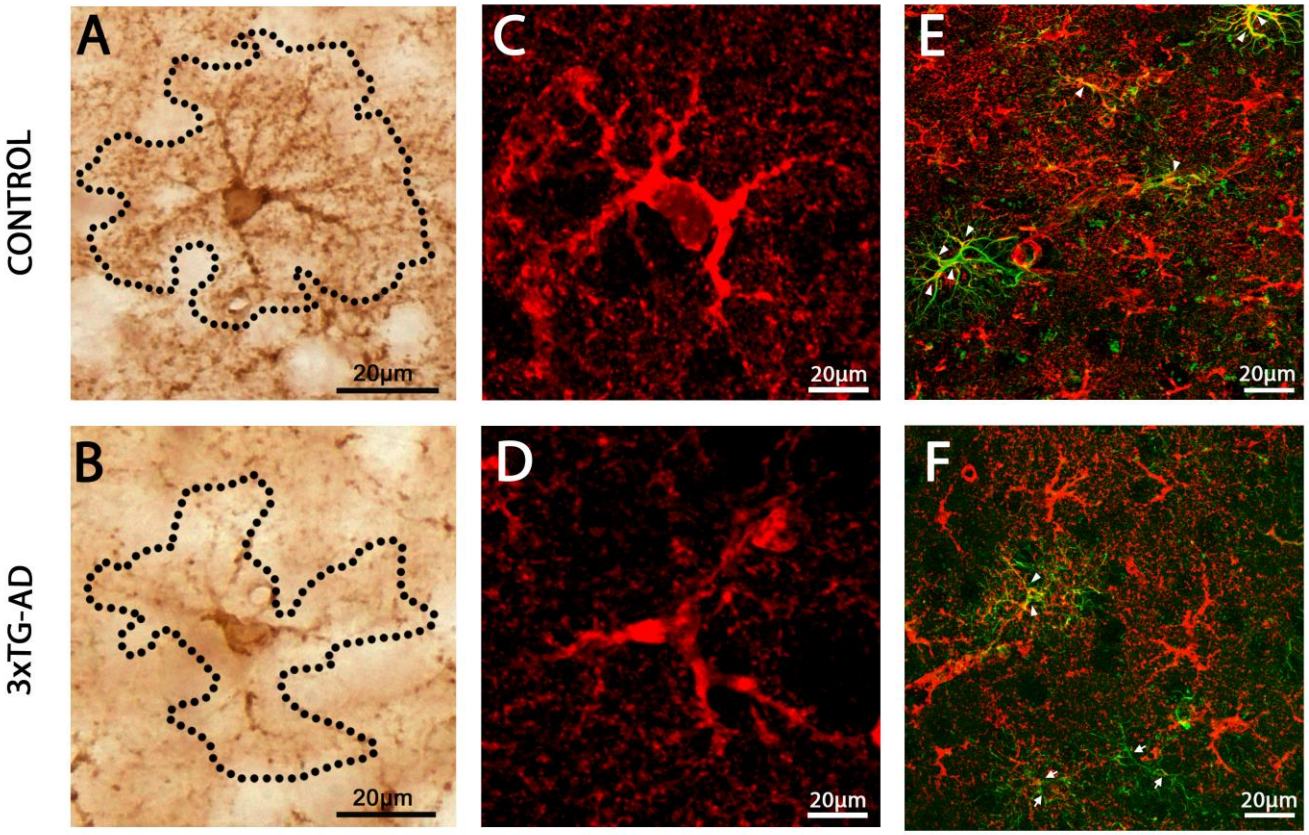

Fig.23 The astrocytic phenotype of GS-IR astrocytes and their colocalization with GFAP are altered in AD mice. *Light and confocal images of GS-IR astrocytes in the mPFC of Non-Tg control animals (A and C) and 3xTg-AD mice (B and D), illustrating the difference in astrocytic morphology and appearance. (E and F) Confocal images demonstrating astrocytic GFAP and GS co-expression, which is much reduced in 3xTg-AD animals. The majority of GFAP-IR astrocytes from control mice do coexpress GS (E; arrowheads). In contrast, even if some 3xTg-AD GFAP-IR astroglia show co-expression with GS (F; arrowheads), many GFAP-IR astrocytes fail to express both proteins at the same time (F; arrows).*

4.2.2. Reduced GS-IR Nv (cell number/mm^3) astrocytes in early and middle stages of Alzheimer's disease:

From 1 month of age, a significant reduction appeared in the Nv of GS-IR cells in 3xTg-AD mice in the mPFC, when compared to control animals (17.38%; p=0.0337). This reduction was sustained and further progressed through more advanced ages, being 27.21% at 6 months (p=0.0079) and 27.52% at 9 months (p=0.0252) (Fig. 24 and Table 7).

The decrease in GS-IR cell numbers in 3xTg-AD animals was paralleled by a decrease in the surface area of the cell bodies, which reached significant levels at the age of 1 and 6

months (by 40.69%, $p<0.0001$ and by 39.24%, $p<0.0001$, respectively). The decrease in the size of the GS-IR astroglial profiles was associated with a shrinkage of the astrocytic domain, parallelled with less branchy processes as compared with controls (at 1 month by 46.71%, $p<0.0001$ and at 6 months by 41.28% $p<0.0001$).

Table 7. Reduced Nv of GS-IR astrocytes in the mPFC during early and middle stages of Alzheimer's disease. Numbers represent changes in numerical density (cells/mm^3) in the Non-Tg and 3xTg-AD animals at different ages (± SEM).

Age (months)	*NON-TG*	3xTG-AD
1	10,706 ± 609	8,845 ± 299
6	9,250 ± 384	6,732 ± 517
9	9,317 ± 827	6,753 ± 254

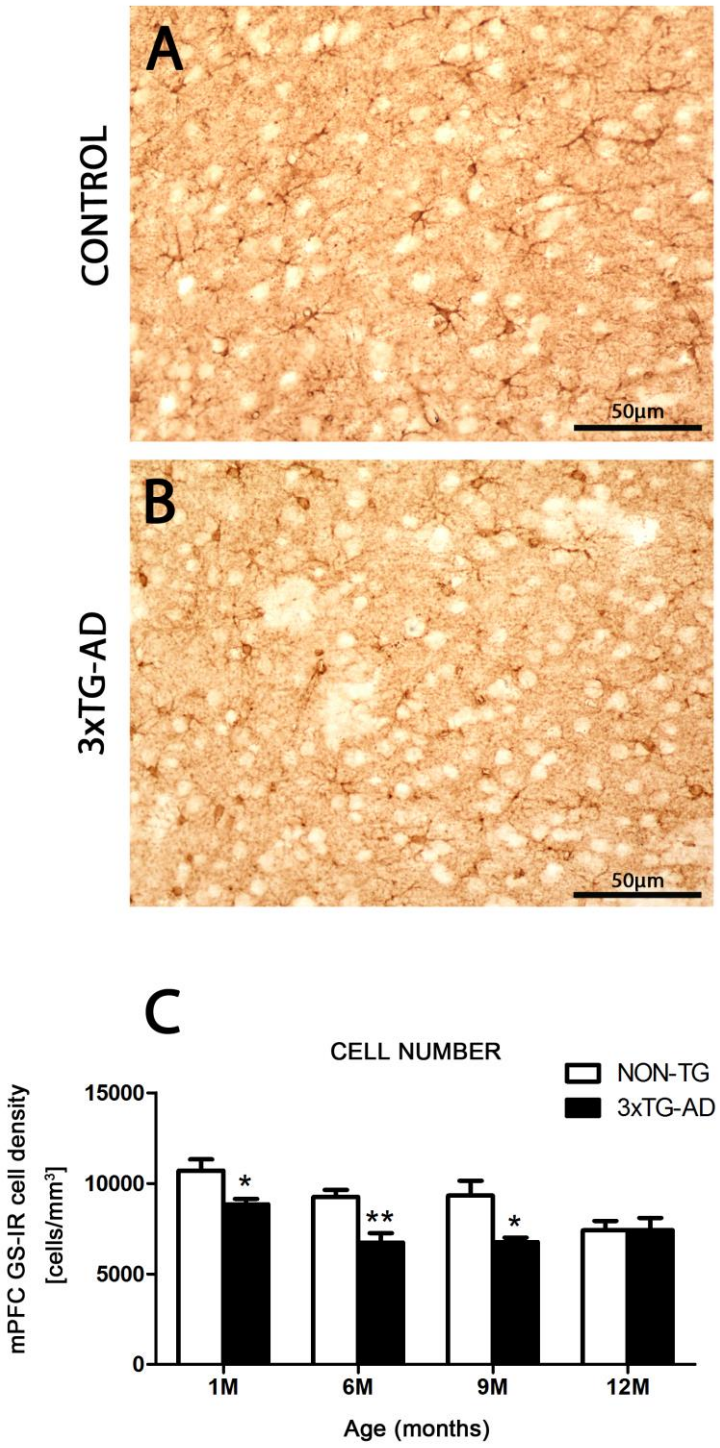

Fig.24 Differential distribution and number of GS-IR astrocytes between control and transgenic mice. *Light micrographs showing the distribution of GS-IR astrocytes within the mPFC in Non-Tg control (A) and 3xTg-AD (B) animals. (C) Bar graphs showing the numerical density (number cells/mm^3) of GS-IR cells in the mPFC of 3xTg-AD and Non-Tg controls. Bars represent mean ± SEM.*

4.2.3. GS expression decrease from early to middle AD stages:

As determined by Western blots, GS expression was significantly decreased in 3xTg-AD animals compared with controls at both middle and advanced stages of AD (Fig. 25A). A significant decrease was observed at 6 months of age (0.561 ± 0.191 vs. 1.534 ± 0.291; p=0.021), at 9 months of age (0.507 ± 0.116 vs. 1.843 ± 0.509; p=0.0444) and at 12 months of age (1.076 ± 0.260 vs. 2.857 ± 0.807; p=0.0364) (Fig. 25B). After performing linear regression analysis, no statistically significant positive correlation between the age value and relative levels of GS/beta-actin was found neither in Non-Tg nor in 3xTG-AD animals (r^2=0.8347, p=0.0864 and r^2=0.0166, p=0.8713, respectively) (Fig. 27).

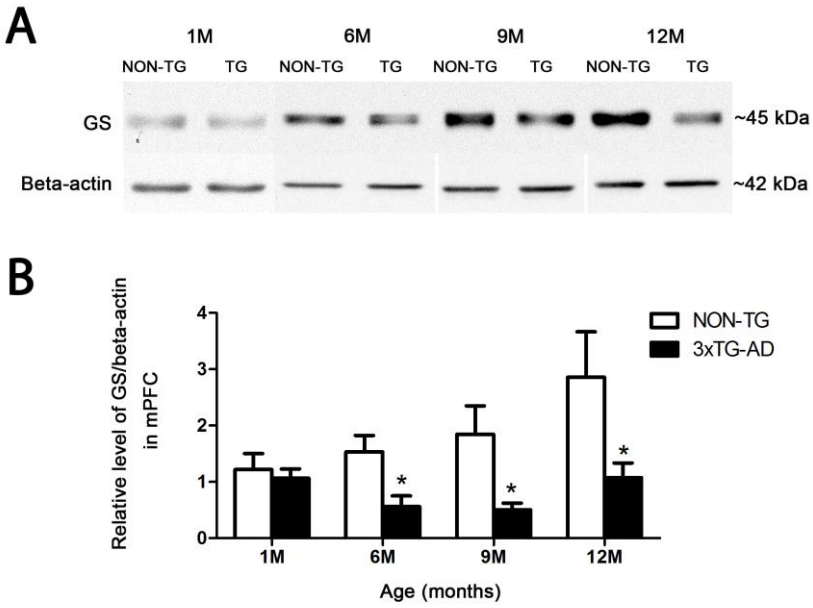

Fig.25 *Bar graphs and representative Western blots showing the relative levels of GS (A-B) in the mPFC of 3xTg-AD mice and Non-Tg controls. Bars represent mean ± SEM.*

4.2.4. GLT-1 remains stable during the progression of AD:

Astrocytic GLT-1 expression in 3xTg-AD mice showed no significant difference at all ages when compared to control animals (Fig. 26C). Nevertheless, at any given point there was a slight reduction of GLT-1 levels, concomitant with the significantly reduced GS expression (Fig. 26D).

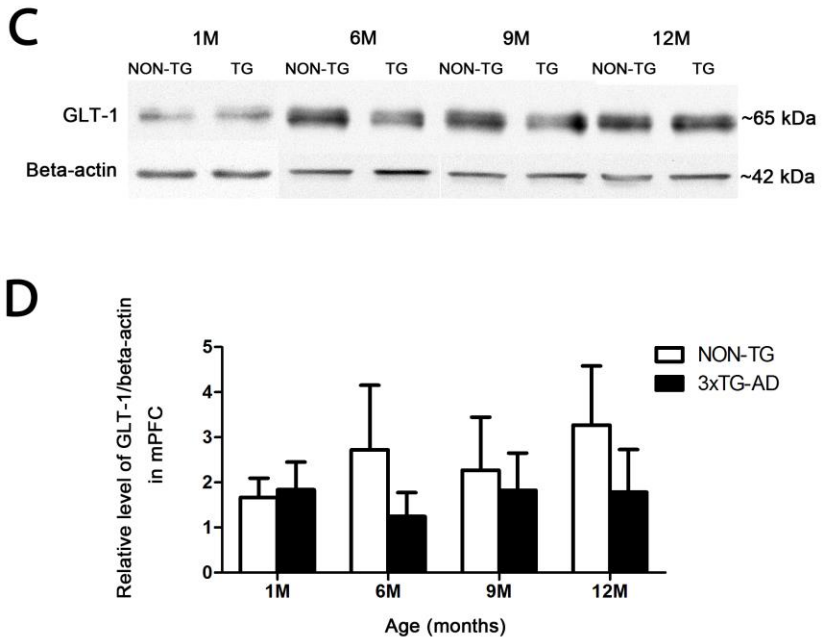

Fig.26 *Bar graphs and representative Western blots showing the relative levels of GLT-1 (C-D) in the mPFC of 3xTg-AD mice and Non-Tg controls. Bars represent mean ± SEM.*

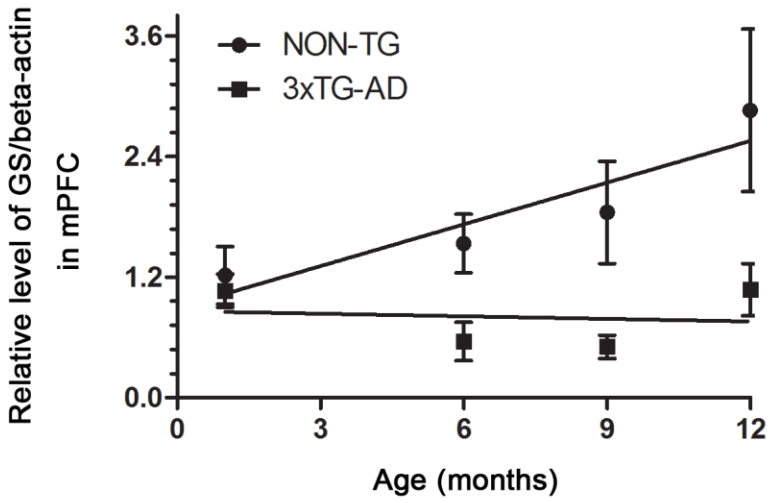

Fig.27 *Lack of positive correlation between the age and relative levels of GS. Linear regression analysis showing no statistically significant relation between the age and relative levels of GS/beta-actin in both Non-Tg and 3xTg-AD animals. Bars represent mean ± SEM.*

4.3. COMPLEX AND REGION-SPECIFIC CHANGES IN ASTROGLIAL MARKERS IN THE AGING BRAIN

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The morphological aging of astrocytes in the entorhinal cortex (EC), dentate gyrus (DG) and cornu ammonis 1 (CA1) regions of the hippocampi of male SV129/C57BL6 mice of different ages (3, 9, 18 and 24 months). Astroglial profiles were visualized by immunohistochemistry using glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and S100β staining; these profiles were imaged using confocal or light microscopy for subsequent morphometric analysis. GFAP-positive profiles in the DG and the CA1 of the hippocampus showed age-dependent hypertrophy, as indicated by an increase in surface area, volume and somata volume at 24 months of age compared with 3-month-old mice. In EC the changes were just opposite: the surface area, volume and cell body volume of astroglial cells at 24 months of age were decreased significantly compared with the 3-month group. The GS-positive astrocytes displayed smaller cellular surface areas at 24 months compared with 3-month-old animals in both areas of the hippocampus, whereas the GS-positive profiles remained unchanged in the EC of old mice. The analysis of S100β-immunoreactive profiles revealed a substantial increase in the EC, a more moderate increase in the DG and no changes in the CA1 area. Based on the obtained data from 3 astroglial markers, we conclude that astrocytes undergo a complex age-dependent remodeling in a brain region-specific manner.

4.3.1. General description of astroglial profiles identified with different markers:

GFAP-positive astrocytes showed a characteristic stellate shape and multiple branched morphology (Fig. 28A-L), although there were clear differences between the hippocampus and the EC. Hippocampal astrocytes emanated several primary processes with numerous secondary processes, all of them extending radially (Fig. 28A-H). Astrocytic GFAP profiles in the EC showed fewer branches with a very low number of secondary processes (Fig. 28I-L). Similarly, the distribution of astrocytes differed between the two brain regions. Astrocytes in the hippocampus uniformly covered the

whole parenchyma with the pyramidal and granular cell layers being the only exception, where fewer astrocytes were present. In the EC, the presence of GFAP-positive astrocytes was less prominent (Olabarria et al., 2010; Yeh et al., 2011).

Cells immunoreactive for S100β (100β-IR) showed a typical astrocytic stellate shape in both the hippocampus and the EC with round somata and multiple branched processes (Fig. 30); proximal processes extended outward from the somata in a radial manner, whereas the distal processes branched randomly. Both in the hippocampus and the EC, the S100β-IR cells were widely and evenly distributed, with the exception of the granular and pyramidal cell layers of the DG and CA1 subregions of the hippocampal formation, where fewer S100β-IR were present.

GS-positive astroglial profiles displayed small round cell bodies with primary branches and a few secondary processes extending randomly and radially (Fig. 32). In the hippocampal subfields, the DG and CA1, the distribution of GS-positive astrocytes was similar to that of GFAP- and S100β-positive astrocytes, being widely present throughout the regions despite fewer GS-positive astrocytes being found in the pyramidal and granular cell layers, as we previously described (Olabarria et al., 2011). In the EC, GS-positive astrocytes were evenly and extensively distributed throughout the entire region with only partial and/or minimal colocalization of GS-IR with GFAP labeling. Of note, most EC astrocytes were GFAP negative and comprised three populations: only 10.3% were GS/GFAP-IR and 11.3% were single positive GFAP-IR cells, whereas 78.1% of all astrocytes in the EC were GS-IR single positive cells (Yeh et al., 2013).

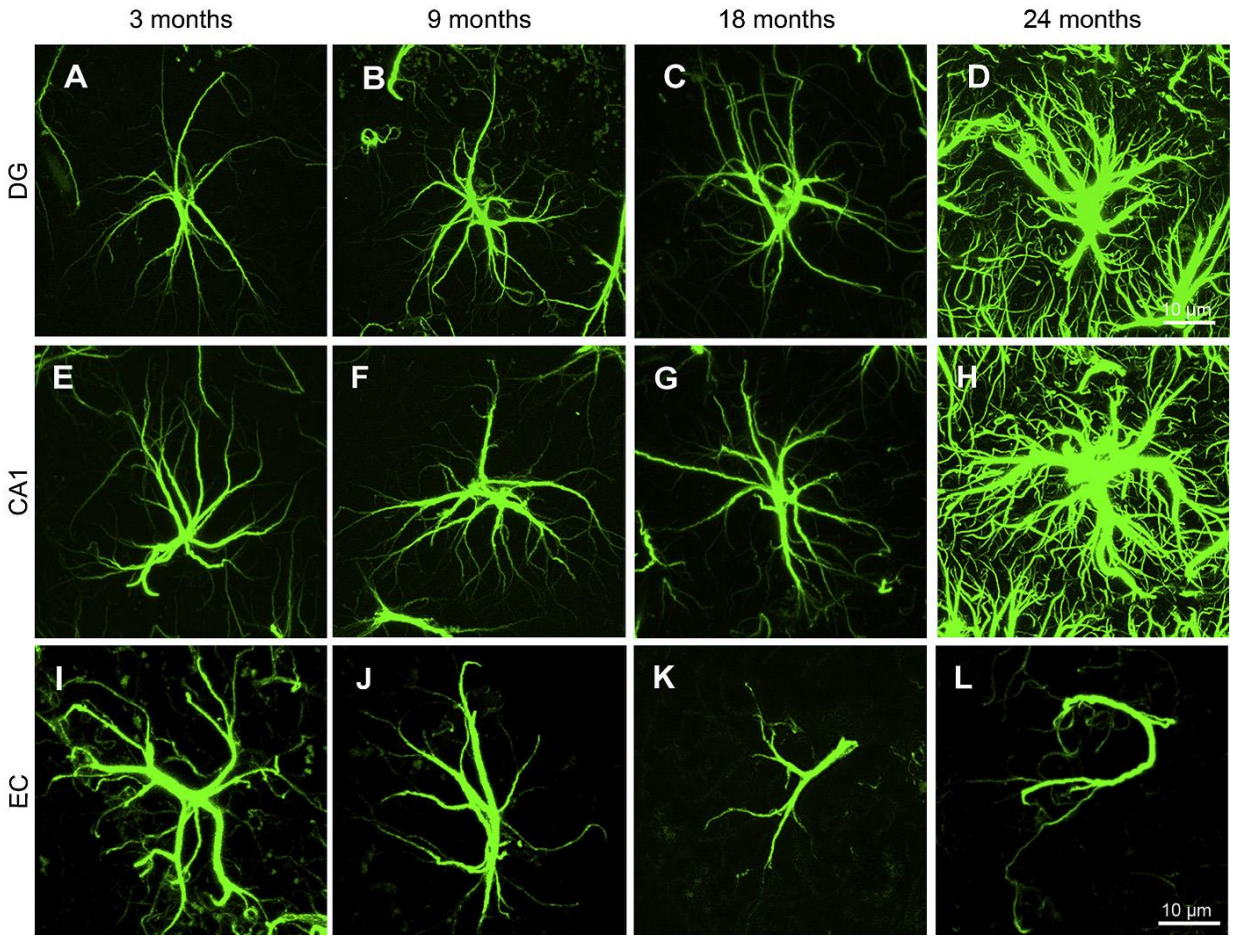

Fig.28 *Representative confocal 3-dimensional reconstructed images showing glial fibrillary acidic protein-immunoreactive astrocytes in the dentate gyrus (DG), cornu ammonis 1 (CA1) and entorhinal cortex (EC) of animals at 3 months (A, E and I), 9 months (B, F and J), 18 months (C, G and K) and 24 months of age (D, H and L), respectively.*

4.3.2. GFAP-IR astrocytes in the DG show a progressive age-related increase in their profile parameters:

GFAP-positive astroglial profiles in the DG were similar in terms of their measured parameters at 3 and 9 months of age (Figs. 28A and B and 29A-C). At 18 months of age, we detected a significant increase of 78.56% in the surface area of GFAP-IR cells (1793.84 ± 138.53 vs. 1004.64 ± 174.88 μm^2, p=0.0228), 95.89% in volume (665.95 ± 64.18 vs. 339.96 ± 70.83 μm^3, p=0.0225) and 128.14% in somata volume (274.03 ± 25.90 vs. 120.11 ± 28.19 μm^3, p=0.0107) compared with 3-month-old mice (Figs. 28A and C and 29A-C). The increase was also evident compared with 9 months of age, the surface area being increased by 83.41% (1793.84 ± 138.53 vs. 978.03 ± 165.58 μm^2,

p=0.0178), the volume by 104.99% (665.95 ± 64.18 vs. 324.87 ± 68.97 μm^3, p=0.0184) and the cell body volume by 115.25% (274.03 ± 25.90 vs. 127.31 ± 31.32 μm^3, p=0.0222) (Figs. 28B and C and 29A-C). At 24 months of age, a marked hypertrophy of GFAP-IR cells developed as evidenced by a marked increase of their surface area of 390.70% and 404.05% (4929.75 ± 1353.56 vs. 1004.64 ± 174.88 μm^2, p=0.007; 4929.75 ± 1353.56 vs. 978.03 ± 165.58 μm^2, p=0.0034), volume of 404.43% and 427.87% (1714.89 ± 492.87 vs. 339.96 ± 70.83 μm^3, p=0.0088; 1714.89 ± 492.87 vs. 324. 87 ± 98.97 μm^3, p=0.0044) and somata volume of 291.16% and 269.06% (469.85 ± 130.96 vs. 120.11 ± 28.19 μm^3, p=0.0127; 469.85 ± 130.96 vs. 127.31 ± 31.32 μm^3, p=0.0092) compared to 3- and 9-month-old animals, respectively (Figs. 28A-C and 29A-C). Compared with a later age, 18 months, there was also a clear but statistically insignificant increase in surface area of 174.81%, volume by 157.51% and somata volume of 71.46% (Figs. 28C-D and 29A-C).

4.3.3. GFAP-IR astrocytes in the CA1 show a progressive age-related increase in their profile parameters:

An increase in astroglial GFAP profiles in the CA1 region was detected throughout the aging process. At 9 months of age, there was already an increase of 206.27% in the surface area of GFAP-positive cells (1278.63 ± 236.02 vs. 417.48 ± 57.48 μm^2, p=0.0098), 286.56% in volume (428.65 ± 98.27 vs. 110.89 ± 18.49 μm^3, p=0.0141) and 266.96% in cell body volume (165.54 ± 40.22 vs. 45.11 ± 5.46 μm^3, p=0.0174) compared with 3 months of age (Figs. 28F and 29D-F). At 18 months of age, the surface area, the volume and the somata volume increased by 75.75% (2247.19 ± 120.76 vs. 1278.63 ± 236.02 μm^2, p=0.0217), 113.32% (914.41 ± 58.88 vs. 428.65 ± 98.27 μm^3, p=0.0133) and 108.29% (344.81 ± 7.26 vs. 165.54 ± 40.22 μm^3, p=0.0118), respectively, compared with 9 months of age (Figs. 28F-G and 29D-F). The difference between 18 and 3 months was more prominent, with the surface area of GFAP-IR astrocytes being increased by 438.27% (2247.19 ± 120.76 vs. 417.48 ± 57.48 μm^2, p<0.0001), the volume by 724.62% (914.41 ± 58.88 vs. 110.89 ± 18.50 μm^3, p<0.0001) and the somata volume by 664.34% (344.81 ± 7.2 vs. 45.11 ± 5.46 μm^3, p<0.0001) (Figs.28E and G and 29D-F). The most significant change was detected at 24 months of age with an increase of the surface area

of 1203.91% (5443.59 ± 1641.09 vs. 417.48 ± 57.48 μm^2, p=0.0338), the volume of 2209.98% (2561.52 ± 843.14 vs. 110.89 ± 18.50 μm^3, p=0.0407) and the cell body volume of 1580.31% (758.03 ± 234.81 vs. 45.11 ± 5.46 μm^3, p=0.0349) (Figs. 28E and H and 29D-F). Compared to mice at 9 and 18 months of age, increases in the surface area of GFAP-IR astroglia, their volume and their somata volume were also apparent but not statistically significant (Figs. 28F-H and 29D-F).

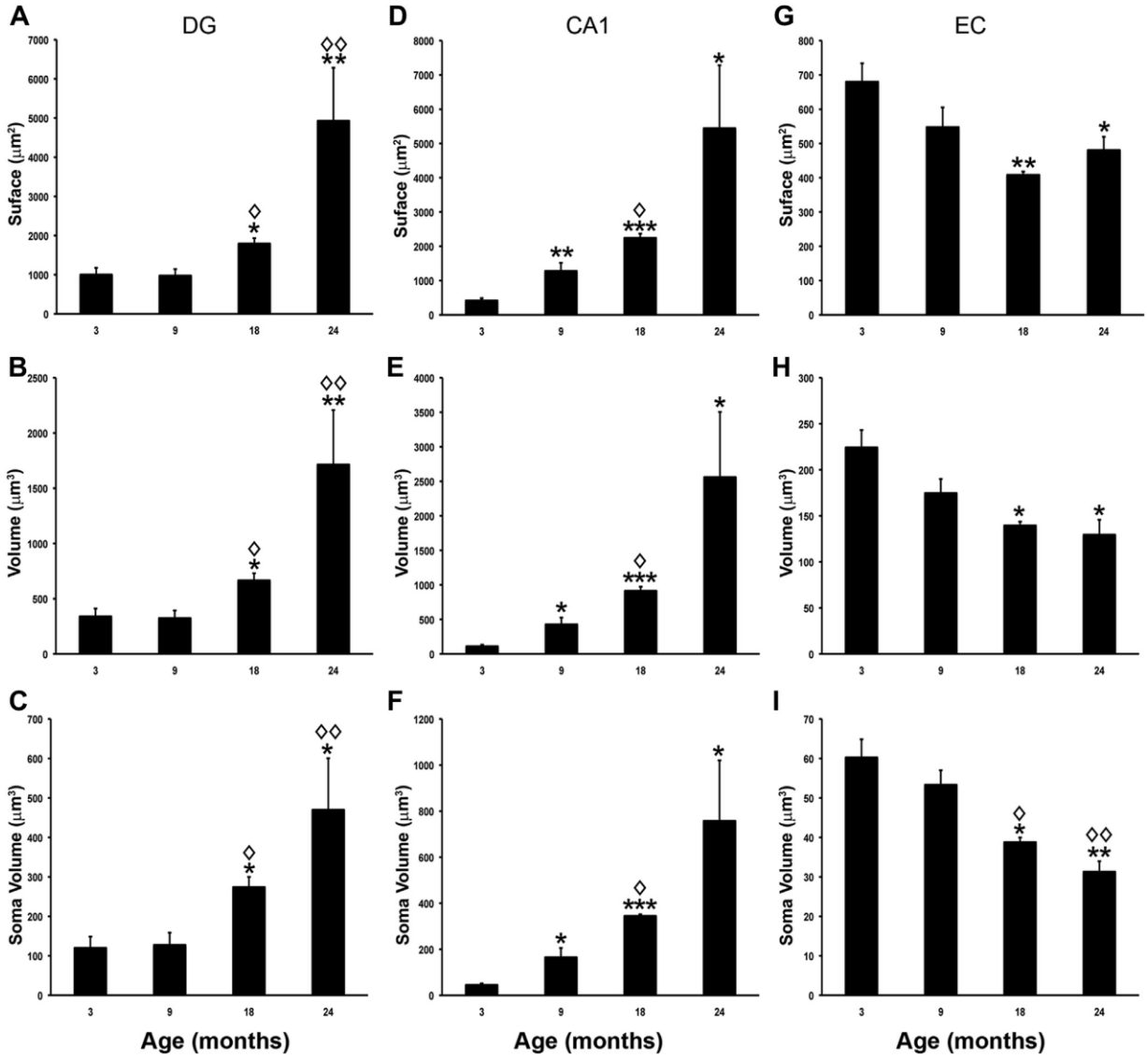

Fig.29 *Bar graphs showing regional comparisons of the surface area, volume and somata volume of glial fibrillary acidic protein-positive cells in the dentate gyrus (DG) (A-C), cornu ammonis 1 (CA1) (D-F) and entorhinal cortex (EC) (G-I) across ages. Bars represent mean ± standard error of the mean (*p≤0.05, **p≤0.01, ***p≤0.0001 compared with 3 months of age; ◊ p≤0.05, ◊◊ p≤0.01 compared with 9 months of age; in DG, n-6, 7, 3 and 4 for 3, 9, 18 and 24 months, respectively; CA1, n-4, 3, 3 and 4 for 3, 9, 18 and 24 months, respectively; in EC, n-4, 5, 3 and 3 for 3, 9, 18 and 24 months, respectively).*

4.3.4. Astrocytes in the EC show a progressive age-dependent decrease in their GFAP-IR parameters:

In contrast with the hippocampus, astrocytes in the EC showed a slight decrease in GFAP profiles parameters already at middle age (9 months) (Figs. 28J and 29G-I). The entorhinal astrocytes in aged animals (18 and 24 months) displayed an atrophy as revealed by a reduction in their surface area, volume and somatic volume (Figs. 28K-L and 29G-I). At 18 months of age, the GFAP-IR astrocytes showed a significant decline in their surface area of 40.28% (406.3 ± 9.30 vs. 680.2 ± 53.70 μm^2, p=0.0076), in volume of 37.76% (139.6 ± 4.06 vs. 224.32 ± 18.80 μm^3, p=0.0131) and in body volume of 55.24% (38.81 ± 1.17 vs. 60.25 ± 4.60 μm^3, p=0.0117) compared with the EC of young animals (3 months) (Figs. 28I and K and 29G-I). At the age of 24 months, GFAP-IR astrocytes in the EC show a further reduction in their surface area of 40.68% (403.6 ± 9.31 vs. 680.2 ± 53.70 μm^2, p=0.0121), the volume of 42.22% (129.6 ± 12.15 vs. 224.32 ± 18.80 μm^3, p=0.0149) and the cell body volume of 47.99% (31.33 ± 2.63 vs. 60.25 ± 4.60 μm^3, p=0.0044) compared with mice at 3 months (Figs. 28I and L and 29G-I). Meanwhile, mice of 9 months compared with older groups (18 and 24 months) also showed a significant reduction in their total volume of 27.24% (53.34 ± 3.67 vs. 38.81 ± 1.17 μm^3, p=0.0265) and of 41.26% (53.34 ± 3.67 vs. 31.33 ± 2.63 μm^3, p=0.0058), respectively (Figs. 28J-L and 29G-I).

4.3.5. Age-dependent changes in S100β-IR astrocytic profile parameters: an increase in the DG with no changes in the CA1:

Compared with the 3-month-old mice, the surface area and the volume of S100β-positive cells in the DG of 24-month-old mice increased by 167.17% (595.5 ± 45.08 vs. 1591 ± 293.6 μm^2; p=0.0285) and by 196.96% (147.9 ± 16.12 vs. 439.2 ± 90.23 μm^3; p=0.0336), respectively (Figs. 30A and B and 31A and B). In contrast, the S100β-IR astrocytes in the CA1 region of the hippocampal formation did not show any significant difference in either surface area (751.8 ± 78.63 vs. 638.4 ± 99.67 μm^2, 15.10%; p=0.4) or cell volume (205.7 ± 19.98 vs. 190.0 ± 32.98 μm^3, 7.63%; p=0.7054) when comparing 24-month-old animals with the young 3-month-old mice (Figs. 30C and D and 31C and D).

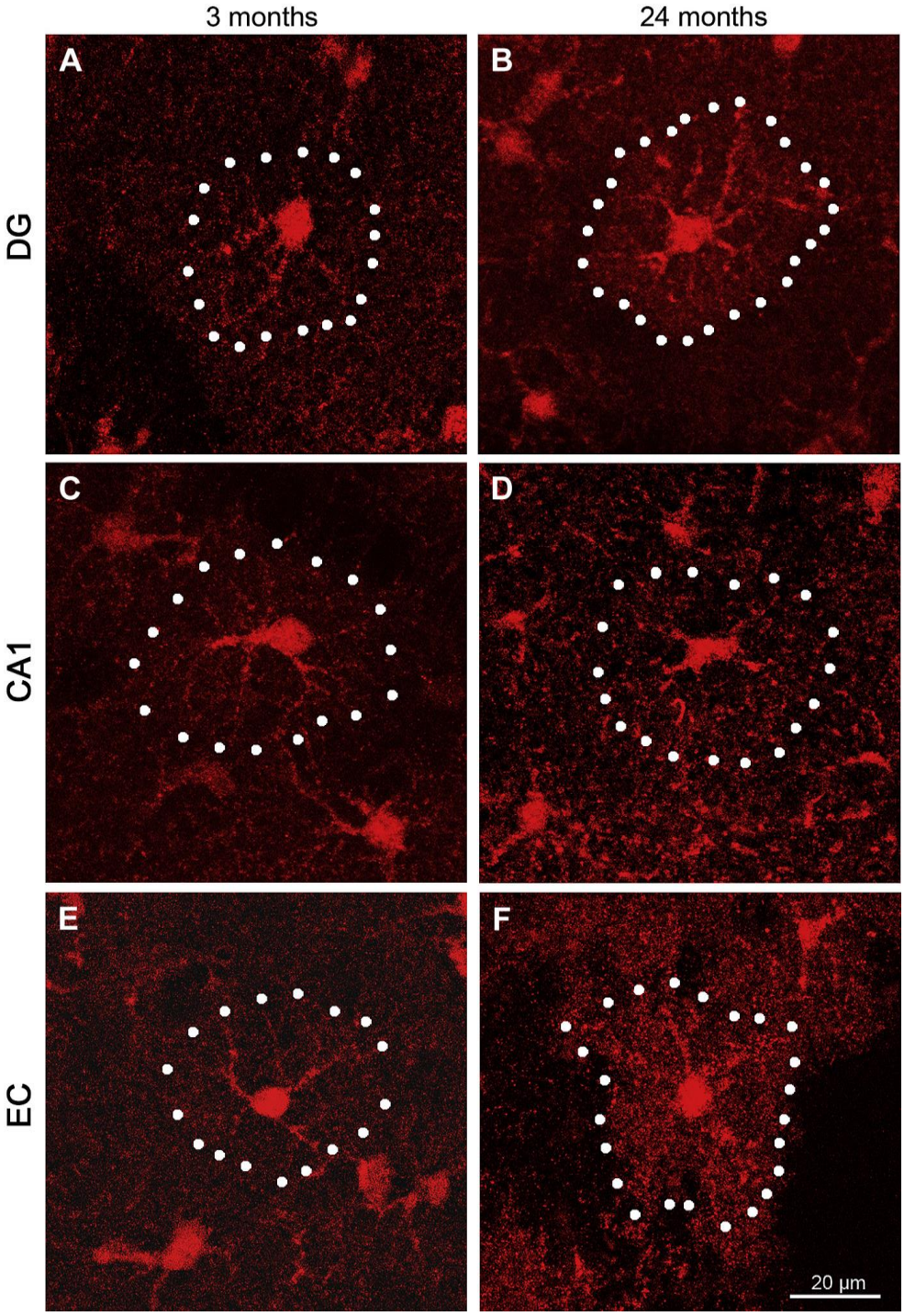

Fig.30 *Representative confocal 3-dimensional reconstructed images showing S100β-immunoreactive astrocytes in the dentate gyrus (DG), cornu ammonis 1 (CA1) and entorhinal cortex (EC) of animals at 3 months (A, C and E) and 24 months of age (B, D and F), respectively.*

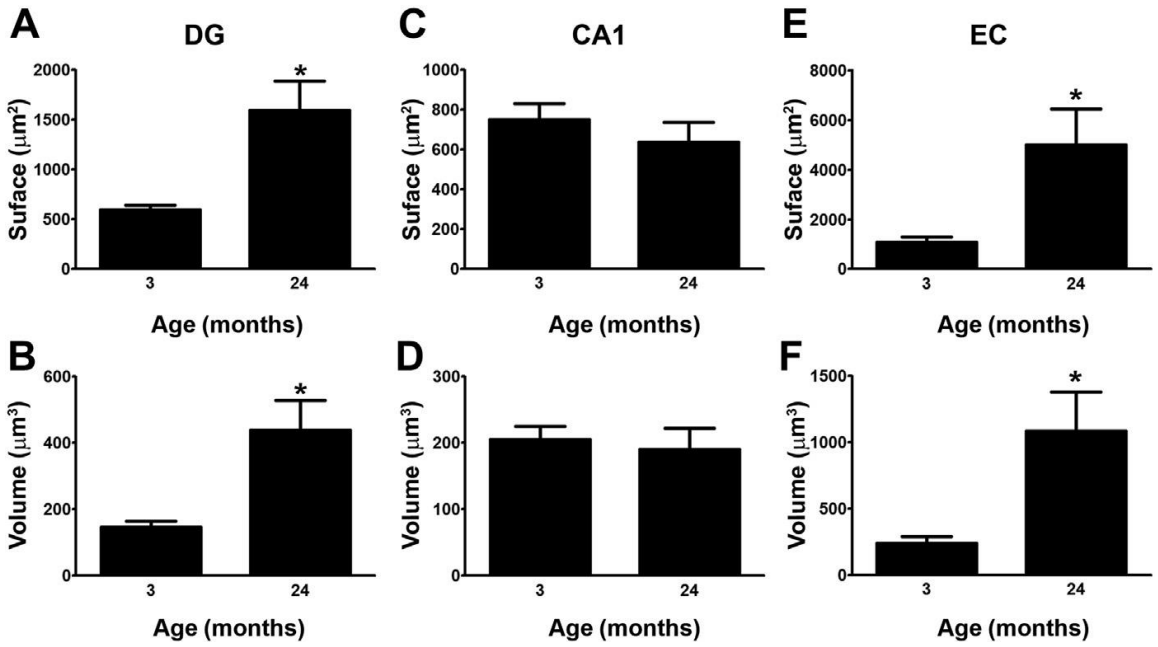

Fig.31 *Bar graphs showing the regional comparisons of the surface area and volume of S100β-immunoreactive cells in the dentate gyrus (DG) (A and B), cornu ammonis 1 (CA1) (C and D) and entorhinal cortex (EC) (E and F) at 3 and 24 months of age. Bars represent mean ± standard error of the mean (*p≤0.05 compared with 3 months of age; in DG, n-3 and 4 for 3 and 24 months, respectively; in CA1, n-3 for both 3 and 24 months; in EC, n-4 and 3 for 3 and 24 months, respectively).*

4.3.6. Age-dependent increase in S100β-IR astrocytic profile parameters in the EC:

A significant increase in the parameters of S100β-IR astroglia was detected in the EC. The surface area of the entorhinal S100β-positive cells at 24 month was increased by 377.51% (1072 ± 214.9 vs. 5001 ± 1438 μm^2; p=0.0241) compared with young 3-month-old mice (Figs. 30E and F and 31E). Likewise, there was a significant increase in the volume of S100β-positive cells (of 354.93%; 238.5 ± 54.34 vs. 1085 ± 297.8 μm^3; p=0.0217; Fig. 31F).

4.3.7. Age-dependent decrease in astrocytic GS-IR profile parameters in the DG and CA1:

The surface area of the GS-positive cells in the DG was decreased significantly at 24 months compared with 3 months (by 6.64%; 852.19 ± 18.21 vs. 916.97 ± 18.23 μm^2; p=0.0471; Figs. 32A and B and 33A). Similarly, in the CA1 a similar decline in GS-

positive cells suface area was detected at 24 months compared with 3 months (of 6.2%; 988.73 ± 19.26 vs. 1053.70 ± 6.25 μm^2; p=0.0235; Figs. 32C and D and 33B).

4.3.8. Aging does not affect GS-IR profiles in the EC:

In contrast to the hippocampal regions, entorhinal astrocytes displayed unchanged parameters of their GS-IR profiles, as evidenced by the similar values of the surface area of GS-positive cells at 24 months compared with 3 months (806.27 ± 14.28 vs. 851.52 ± 22.90 μm^2; Figs. 32E and F and 33C).

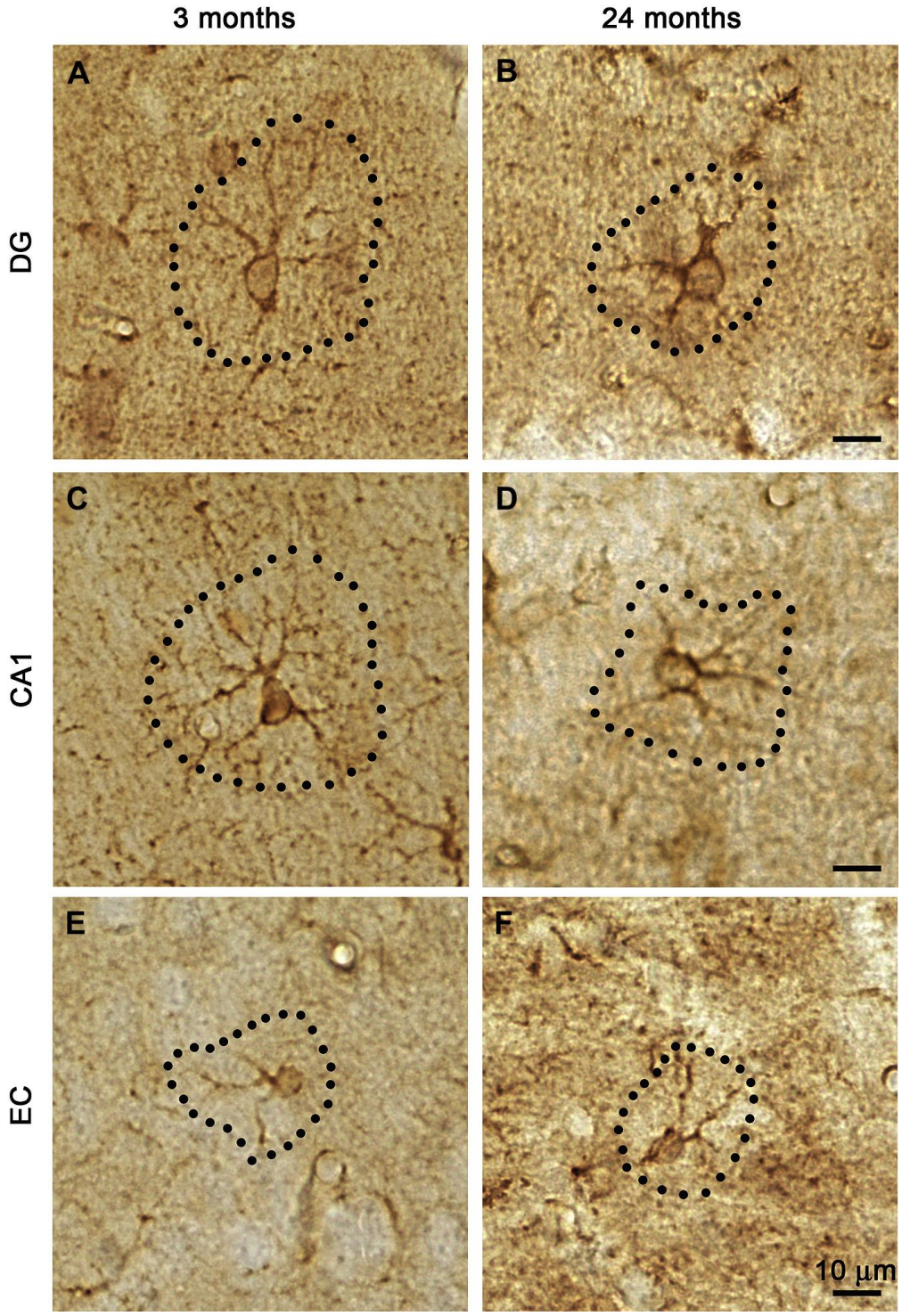

Fig.32 *Light micrographs showing the morphology and surface area of glutamine synthetase-positive astrocytes in the dentate gyrus (DG), cornu ammonis 1 (CA1) and entorhinal cortex (EC) of 3-month-old mice (A, C and E, respectively) and 24-month-old mice (B, D and E, respectively).*

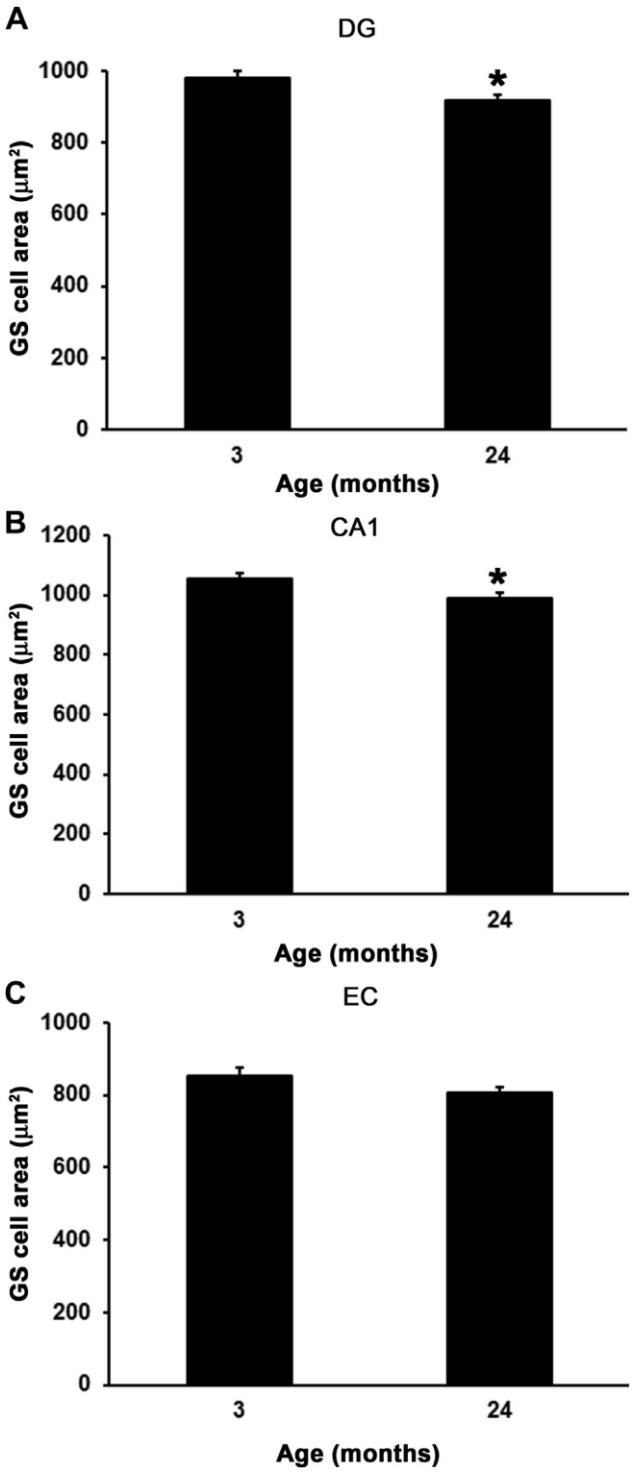

Fig.33 *Bar graphs showing regional comparison of the surface area of the glutamine synthetase-positive cells in the dentate gyrus (DG) (A), cornu ammonis 1 (CA1) (B) and the entorhinal cortex (EC) (C) at 3 and 24 months of age (*p≤0.05 compared with 3 months of age ; in DG, n-4 for both 3 and 24 months, in CA1, n-4 and 5 for 3 and 24 months, respectively; in EC, n-4 for both 3 and 24 months).*

5. DISCUSSION:

Alzheimer's disease (AD) is characterized by progressive brain dysfunction, strongly affecting neurons and synapses and leading to the complete breakdown of cognitive functions. Over the time, patient stops being aware of his/her ongoing degeneration and is left without anything that identifies him or her as a resolute and mindful human being. That is why there is such a strong impetus to find a successful treatment and ideal therapeutic targets.

Synaptic loss and synaptic impairment, broadly understood, are assumed to correlate most strongly with the cognitive deficits that can be detected long before the clinical diagnosis of AD (Coleman et al., 2004). A significant loss of synapses within the prefrontal cortex was demonstrated to correlate with dementia in Alzheimer's patients (Masliah et al., 1993). These features are also strictly connected with frontotemporal dementia and normal aging (Lipton et al., 2001; Uylings and de Brabander, 2002). Synaptic deterioration may be the result of an altered cycle of neurotransmitter conversion, from synthesis to reuptake, and vesicle trafficking (Yao and Coleman, 1998; Yao et al., 2003).

In this thesis, based on our results, we propose that morphological and functional changes in astrocytes, indispensable modulators of neuronal and synaptic activity, might negatively influence synaptic performance and affect the delicate homeostasis within the brain, contributing to the cognitive and mnesic decline observed in AD patients.

5.1. MORPHOLOGICAL CHANGES IN ASTROCYTES AND THEIR RELATION TO Aβ PRESENCE IN THE 3xTG-AD MODEL:

First, we focused on a detailed analysis of GFAP-positive astrocytes (their morphology and cell density) at different ages to identify the type of change astrocytes undergo in 3xTg-AD animals, the most advanced model of AD available. We found that the overall density of GFAP-positive astrocytes does not vary significantly between Non-Tg and 3xTg-AD animals with age. Also, no evident age-dependent cell loss was observed. These results correlate with previous findings in the hippocampus (HC) and entorhinal cortex (EC) in the same animal model, where no signs of astrocytic death were found

(Olabarria et al., 2010; Yeh et al., 2011). Those observations are in line with those from other neurological diseases that involve neuroglia, such as schizophrenia, where no significant changes in glial density were detected (Selemon et al., 1995, 1998). This could suggest that astrocytes are constantly involved constantly in some morphofunctional changes due to progressive biochemical and molecular alterations that are long-lasting and connected with neuronal defects. To answer this question we measured the surface area and volume of GFAP-positive astroglia. We have identified a general atrophy of GFAP-positive astrocytes, which was already significant at early pre-symptomatic and pre-pathological ages (3 months) and was sustained during disease progression in all age groups up to 18 months. Interestingly, the decrease showed a clear layer-specific pattern: layers 1-2 were strongly affected and similar changes were found in the deep layers 4 and 5. Layer 3 was only affected from middle age onwards (Kulijewicz-Nawrot et al., 2012).

The prefrontal cortex is one of the brain regions most sensitive to the detrimental effects of aging (Liu et al., 1996; West, 1996; West, 2000; Lamar and Resnick, 2004). Age-related degeneration in the prefrontal cortex is more significant than in other brain areas (Raz et al., 1997). Extensive PFC atrophy has been observed in Alzheimer's disease patients (Salat et al., 1999, 2001). In this regard, Burgmans and colleagues in their recent study (Burgmans et al., 2009) suggested that PFC atrophy is specifically associated with dementia and can be used as a predictor/biomarker of Alzheimer's disease. In general, the observed cortical decrease is assumed to be the result of cell shrinkage, rather than a reduction in cell numbers (Kemper, 1994; Uylings and de Brabander, 2002). This cell diminishing was described mainly in neurons; nevertheless, it is in agreement with our data which broaden the concept of atrophic cortical astroglia. Changes in the appearance of astrocytes from transgenic animals were manifested mainly in thinner and shorter astrocytic processes, with a tendency to be more horizontally oriented (in the superficial layers) or with a preserved star-like protoplasmic look (in the deep layers). Such layer-specific GFAP reductions and alterations also appear in other neurological disorders, such as schizophrenia and depression, for which the mPFC (and particularly layer 5) is highly pathologically relevant (Kulijewicz-Nawrot et al., 2012). Alterations in the mPFC are even more important for frontotemporal dementia (Miguel-Hidalgo et al., 2000;

Martin et al., 2001; Rajkowska et al., 2002). One can conclude that regarding the important role of astrocytes in maintaning brain homeostasis, the observed mPFC astroglial atrophy during the progression of AD can result in reduced synaptic coverage, decreased metabolic support to neurons and synapses, and neurotransmitter imbalance, all leading to alterations in the flow and processing of information within the brain.

When thinking about the arrangement of afferents and efferents of the prelimbic cortex (PL), one of the subdivisions of the mPFC, this brain region is responsible for cognitive functions. It is directly involved in the integration and management of sensory and mnemonic information, intellectual functions and actions, all of which are strongly affected in AD. The second subdivision of interest, the infralimbic cortex (IL), represents a visceromotor center and appears to be crucial when it comes to reward-related behavior and initiating the state of the mood (Ongur and Price, 2000; Hoover and Vertes, 2007). Although they perform different functions, the IL and PL are generally treated as a single region of the ventral medial prefrontal cortex, and this thesis treats them in the same way (Vertes, 2004). An intriguing conception is that concerning the very complex nature and organization of the PFC, it is extremely hard to fully characterize it, with radical opinions even holding that this region does not display any systematic organization at all (Miller, 2000; O'Reilly, 2010). What is more, the development of the prefrontal cortex is not completed until the third decade of life or later, while the myelination of axons from the prefrontal and associated areas extends until the end of the fifth decade (Bartzokis, 2004). All of these facts make the PFC an appealing region to study.

The mPFC receives a wide range of afferent projections from such structures as the hippocampus (layers 1-3,5-6), the entorhinal (layers 1-2) as well as the perirhinal (layers 1-3) cortices and the medial basal forebrain (layers 1,3,5-6) (Jay and Witter, 1991; Delatour and Witter, 2002; Vertes, 2004; Hoover and Vertes, 2007). Also, the midline thalamus nucleus reuniens (RE), the major source of thalamic afferents to the hippocampus, densely innervates the IL and PL, terminating mainly within layers 1 and 5/6 (Wouterlood et al., 1990; Bokor et al., 2002; Vertes, 2006). Even though there are no direct return projections from the mPFC to the hippocampus, the hippocampal terminals form mainly asymmetric synapses on prefrontal pyramidal neurons and mPFC fibers shape the same contacts on the dendritic shafts of those RE cells, which in return project

to the hippocampal formation (Hurley et al., 1991; Carr and Sesack, 1996; Vertes, 2004). Thus, the RE is ideally positioned to strongly influence the activity of the hippocampus and the mPFC as an integral part of the limbic loop between these structures (Di Prisco and Vertes, 2006; Vertes et al., 2007). Also, considering the salient function of the hippocampus/prefrontal cortex connections in the pathophysiology of major depression and schizophrenia, specific notable alterations could appear due to an altered connectivity of these areas (Szeszko et al., 2003; Goldapple et al., 2004; Jay et al., 2004), associated with a potential homeostatic deficiency due to the observed generalized astrocytic atrophy. In line with the rewiring hypothesis, Dumitriu and colleagues (Dumitriu et al., 2010) found a selective spine loss in aged monkeys in the neurons of layer III in the homologous PFC, suggesting a decreased ability to reconnect prefrontal cortical circuits with advancing age. Layer III, as an origin and "end station" for cortico-cortical connections, is highly significant for the formation of memory by association and is assumed to be very sensitive to neurodegenerative changes (Fuster, 2008; Kulijewicz-Nawrot et al., 2012). Considering all its complexity and its role in connection management, every change in the cells involved, in our case astrocytes, can have a dramatic outcome for the brain functionality.

Our second interesting finding was that the astrocytic atrophy, determined by changes in GFAP-positive astrocytes' surface area and volume, was not directly connected with the toxic effect of Aβ. In contrast to other brain regions affected in Alzheimer's disease, such as the hippocampus and entorhinal cortex (Olabarria et al., 2010; Yeh et al., 2011), no plaque formation was observed in the mPFC until advanced ages. Nevertheless, amyloid β aggregates were clearly visible and robust, especially in the deep layers. Even in the close vicinity of Aβ aggregates astrocytes were not broadly present; however, some were visible and had atrophic features. This observation makes astrocytic atrophy and Aβ's harmful effects two crucial but independent features of AD pathology in the mPFC. The lack of plaques, but the presence of Aβ aggregates in the mPFC could result in different synaptic performance and alterations, compared to, for example, the hippocampus, where plaques have been noticed (Olabarria et al., 2010). Still, it cannot be forgotten that there is controversy concerning the relationship between the presence of plaques and the devastation of cognitive functions – no clear correlation has been found so far (Ferreira

and Klein, 2011). Nevertheless, taking into consideration the differences in cytoarchitecture between the PFC and hippocampus, such as the cortex is not so stratified and is less strictly organized into precisely defined layers and fiber pathways, slightly different disruptive effects of Aβ alone can be observed on the molecular or physiological level (Amaral and Witter, 1989; Uylings and van Eden, 1990). Despite these differences, generally the presence of Aβ is connected with altered cognitive performance (Roder et al., 2003; Koffie et al., 2011).

It is important to emphasis that our results, which point to very early changes in astrocytic GFAP profiles, could signal ongoing pathological processes far before the appearance of the classic and well known histological hallmarks of AD. Similar data were obtained from analyzing the entorhinal cortex and the hippocampus of 3xTg-AD mice (Olabarria et al., 2010; Yeh et al., 2011). Changes within the EC also started from very early ages (1 month), before the classic signs of the disease appeared, and were maintained till advanced ages (12 months); in the hippocampus atrophy was observed from the age of 12 months in the case of the DG and from 18 months of age in the case of the CA1 region. The differences between the brain structures and the onset of astrocytic atrophy could have their roots in dissimilarities in the detailed cytoarchitecture and the propagation pattern of the disease. Normally, we assume that AD initiates within the EC, reaches the HC and spreads to other cortical regions, including the mPFC, which directly or indirectly projects back to the mentioned places. In this case the specific limbic loop, described in the previous section, would be of major importance for therapeutic intervention, as the core of memory and cognition lies right there. In line with our results, Bossers and colleagues in their recent work (Bossers et al., 2010) describe the upregulation of genes within the PFC connected with synaptic activity and plasticity, preceding the appearance of any neuropathological alteration associated with AD and signaling some kind of compensatory mechanism of brain activity to maintain a normal cognitive level. It also strengthens our hypothesis that the observed early and sustained astrocytic atrophy, both general and layer-specific, as well as the spatial propagation of the pathology or the interrelation between the affected structures can initiate a sustained influence on connective processes and interactions within certain circuits within the CNS. Astroglial atrophy may represent the ongoing pathological remodeling within the brain. It

can impact upon the number and functional status of synapses, leading to decreased connectivity together with the neurotransmitter imbalance that underlies the progression of Alzheimer's disease.

5.2. HOMEOSTATIC CHANGES WITHIN 3xTG-AD ASTROCYTES AND THEIR INFLUENCE ON ASTROCYTIC FUNCTIONALITY:

After analyzing the GFAP profiles of astrocytes and finding the atrophy described above, questions about further astrocytic changes were raised. As a first step in revealing homeostatic alterations, we decided to focus on glutamine synthetase (GS), an enzyme expressed by astrocytes and crucial for the conversion of glutamate to non toxic glutamine (Martinez-Hernandez et al., 1977; Westergaard et al., 1995). Glutamate is the main excitatory neurotransmitter in the CNS, being involved in the vast majority of brain functions, including cognition, memory and learning (Fonnum, 1984; Headley and Grillner, 1990; Danbolt, 2001). In this regard, the glutamate-glutamine metabolic cycle is of the highest importance for rescuing neurons from the toxic effects of excess glutamate presence (Broer and Brookes, 2001). We found a significant and sustained decrease in astrocytic GS-IR and GS expression, which appear already at very early ages (1 month of age in the case of GS-positive cells number per mm^3 and 6 months of age in case of GS expression analyzed by WB). The decrease in GS expression was also present in 12-month-old animals, when robust intracellular Aβ, with a tendency to form extracellular deposits, is clearly visible. The decrease at that age in 3xTg-AD animals was revealed by Western blot analysis, but not by the GS-IR cell number (unchanged at 12 months). The lack of a significant difference is due to the simultaneous decrease in the number of GS-IR astrocytes in control animals at 12 months (Kulijewicz-Nawrot et al., 2012). Our results underline the substantial metabolic disruptions in the glutamate-glutamine cycle starting early during disease progression in the mPFC, which contrasts with findings in the hippocampus, where a decline in GS-IR astrocytes was observed at advanced ages, around 12-18 months of age (Olabarria et al., 2011). The pattern of above findings resemble the tendency observed after analyzing GFAP-positive cells profiles, where hippocampal astrocytes located far from amyloid plaques were atrophic also at advanced ages, while in mPFC changes were observed far before appearance of any Aβ form (Olabarria et al., 2010; Kulijewicz-Nawrot et al., 2012).

Also, when analyzing the astrocytes in the mPFC, we found three different subpopulations of astroglia: single GS-IR, single GFAP-IR and GS/GFAP-IR. Similar observations were made in the hippocampus and entorhinal cortex (Olabarria et al., 2011; Yeh et al., 2013). In line with our results, Robinson in his study on the human cortex described complementary populations of astrocytes, GFAP-IR and GS-IR, with quite heterogeneous regional distribution (Robinson, 2000). The novelty in our case is the presence of a third, GS/GFAP-IR population of astrocytes, which furher supports the idea of strong heterogeneity among astrocytes, both morphological and functional (Kimelberg, 2004; Matyash and Kettenmann, 2010; Verkhratsky, 2010; Theis and Giaume, 2012). As an example, this heterogeneity is manifested by the different functional behavior of astrocytes in the case of injury, by an increase in the co-expression of GFAP and GS or the de novo expression of one of them (Humphrey et al., 1997; Walz and Lang, 1998; Kulijewicz-Nawrot et al., 2013).

Previously, we hypothesized that the atrophy of astrocytes is involved in AD pathology and may be responsible for a decrease in metabolic support for neurons as well as an altered synaptic environment, including neurotransmitter inactivation and homeostasis (Heneka et al., 2010; Verkhratsky et al., 2010; Rodriguez and Verkhratsky, 2011a).

As was already described, glutamate is a key neuromodulator of the CNS and its metabolism strongly depends on astrocytes, specifically on the enzyme GS (Fonnum, 1984; Anderson and Swanson, 2000; Walton and Dodd, 2007). Two different types of changes in enzyme expression have been detected in a variety of brain disorders. The first type is an increase in GS concentration, which was reported in such pathologies as vascular dementia and ALS (Tumani et al., 1999). The second type is a reduction in the activity of GS, found in hepatic encephalopathy, spinocerebellar atrophy and schizophrenia (Lavoie et al., 1987; Smith et al., 1991; Kish et al., 1994; Le Prince et al., 1995; Burbaeva et al., 2003; Volterra and Meldolesi, 2005; Steffek et al., 2008). Taking into consideration the great importance of astrocytic GS in the glutamate-glutamine cycle, each of the mentioned type of change in enzyme expression can influence the accumulation of glutamate and the ability of astrocytes to take up this neurotransmitter. Also, the reduction observed in GS levels in Alzheimer's disease patients is not

topographically associated with aggregated Aβ, suggesting no direct restrictive connection to the expression of GS (Robinson, 2001). However, significantly decreased expression of GS (measured by optical density; OD) was found at the age of 18 months in the hippocampus of 3xTg-AD animals, when the Aβ plaques are robust (Olabarria et al., 2011). As already metioned, we demonstrated significant alterations in mPFC GS-IR astrocytes already at the age of 1 month and decreased GS expression manifested in 6-month-old 3xTg-AD mice, suggesting ongoing metabolic pathology regarding astrocytes. In line with our results, several research groups have reported lowered concentrations of GS in patients with AD, showing similar reductions in GS and glutamate levels in the initial stages of AD (Smith et al., 1985; Csernansky et al., 1996; Jimenez-Jimenez et al., 1998; Robinson, 2001). Additionally, magnetic resonance spectroscopy at 0.5T in an in vivo study confirmed a reduction in the levels of glutamate and glutamine in AD patients (Antuono et al., 2001) This could suggest the hypothesis that dysfunction of the glutamate-glutamine cycle is a quite early event in the progression of this terminal neurogedenerative disease and make such dysfunction an issue of significant concern for treatment approches.

A meaningful fact is that alterations in glutamatergic transmission in the PFC can be a direct source of depression (Miguel-Hidalgo et al., 2010). Based on current research data, a strong bound is known to exist between depression and dementia, which points towards the innovative hypothesis that depression does not appear as a result of AD, but rather exists as an independent risk factor for AD (Ownby et al., 2006; Caraci et al., 2010). Microarray and Western blot analyses have revealed that the expression of GS is significantly lower in depressed patients, further corroborating the fundamental importance of astrocytes to the depression pathology (Choudary et al., 2005; Miguel-Hidalgo et al., 2010).

According to our previous study, a crucial feature of the mPFC is the atrophy of GFAP-positive astrocytes, observed before the typical histopathological onset of the disease (Kulijewicz-Nawrot et al., 2012). This atrophy is further corroborated by a reduced GS - IR profiles. Similarly, in studies of the prefrontal cortex of patients with schizophrenia and major depressive disorder (MDD), reductions in GS and GFAP expression have been reported (Miguel-Hidalgo et al., 2002; Steffek et al., 2008).

Alterations in the expression of GFAP should be taken into serious consideration due to its essential role in the proper guidance and expression of astrocytic and neuronal glutamate transporters as well as solid process formation, resulting in accurate functional interaction with neuronal cells (Weinstein et al., 1991; Oliet et al., 2001; Hughes et al., 2004; Sullivan et al., 2007). Astrocytic glutamate transporters (GLT-1 and GLAST) are "tools" to protect the inner environment from excitotoxicity by clearing the excitatory neurotransmitter from the extracellular space (Maragakis et al., 2004). GLT-1 is responsible for 95% of glutamate uptake in the cortex and hippocampus (Lehre et al., 1995; Danbolt, 2001). The downregulation of GLAST/EAAT1 and GLT-1/EAAT2 was found in patients suffering from major depressive disorder (MDD) (Miguel-Hidalgo et al., 2010). We did not find any change in GLT-1 expression in the mPFC, which can suggest the absence of any alterations in the mechanism of glutamate inactivation. In contrast, Li and his group (Li et al., 1997) described a significant decrease in GLT-1 transporter in the frontal cortex of AD patients. Still, there is much controversy surrounding the question of whether the downregulation of these transporters is prominent in Alzheimer's disease. Beckstrom and colleagues in their elegant study of AD patients aged from 69 to 94 (Beckstrom et al., 1999) claim individual differences in the levels of glutamate transporters, thus rejecting a straightforward correlation between reduced glutamate transporter expression and AD (Li et al., 1997). We found GLT-1 expression in the mPFC to be generally unchanged, which can suggest the preservation of glutamate uptake or possible differences in transporter expression between subjects. Our results are in line with Beckstrom's findings highlighting the variability in transporter expression and suggesting a common insight into cognitive function decline during severe brain diseases associated with astrocyte alterations and malfunction (Kulijewicz-Nawrot et al., 2013).

We believe that structural changes in astrocytes affect synaptic performance. Perisynaptic glial processes provide a structure that shields the individual synapse from interference from synapses in the vicinity and other extrasynaptic signaling acts. By ionotropic receptors, importantly glutamatergic and purinergic ones, astrocytes are able to generate local increases in $[Na^+]i$ and $[Ca^{2+}]i$ in perisynaptic processes, which enable astrocytes to

control and support synaptic transmission in various ways, e.g. by modulating the activity of Na^+/K^+ ATPase (pump) (Verkhratsky et al., 2010; Nedergaard and Verkhratsky, 2012).

Also, as was shown in studies similar to ours, a loss of GS was detected in the perisynaptic regions of the neuropil and in astrocytic endfeet – the locations of glutamate transporters (Schmitt et al., 1997; Robinson, 2001). As GS is highly sensitive to oxidation, among the treatment possibilities could be antioxidant therapy, although there is still a lack of common agreement on the subject (Schor, 1988; Vina et al., 2011; Teixeira et al., 2013). Also, the area of stem cells research provides hope. An example could be a therapy based on MSCs, which release a vast repertoir of growth factors, including GDNF, VEGF and FGF-2 (Maltman et al., 2011). Those growth factors are known to be indispensible for astrocytes to modulate neuronal activity, and their level was shown to decrease during the aging process (Bernal and Peterson, 2011). Theoretically, it is then possible to use MSCs to stimulate the activity of affected astrocytes to return to their baseline levels, which could be beneficial for all of their homeostatic functions and protective roles.

5.3. HETEROGENOUS REGIONAL DISTRIBUTION OF ASTROCYTES WITHIN THE BRAIN DURING PHYSIOLOGICAL AGING:

Aging is a multiplex biological event, involving numerous genetic and environmental factors (Wu et al., 2005). Regarding its physiological background in the brain, it is generally believed that aging takes place due to the progressive loss of the neuronal homeostatic reservoir, including delicate changes in synaptic connectivity, not because of substantial neuronal loss (Turlejski and Djavadian, 2002; Toescu and Verkhratsky, 2007). Far less is known when it comes to astrocytic changes, though. Nevertheless, changes in the morphology and functionality of these main glial cells can cause a serious decrease in neuroprotective capability, triggering detrimental processes in senescent neurons (Pertusa et al., 2007). Well documented are differences in astrocytic density during aging, showing both an increase or decrease (Nishimura et al., 1995; Cotrina and Nedergaard, 2002; Wu et al., 2005; Mansour et al., 2008). When it comes to changes in morphology specifically, the data are still scarce (Matyash and Kettenmann, 2010; Rodriguez et al., 2013). Astrocytes constitute a very heterogenous population of cells, characterized by

different appearances and properties (Matyash and Kettenmann, 2010). To fill an important gap in our knowledge of senescent astrocytes, we performed a systematic analysis of the morphological changes of astrocytes in the aging hippocampus (HIP) and entorhinal cortex (EC), using such specific markers as GFAP, S100β and GS.

Considering changes in GFAP expression, the majority of studies have shown that the aging process corresponds closely with an increased level of astrocytic GFAP, in the same manner as in reactive astrogliosis (Nichols, 1999; Cotrina and Nedergaard, 2002). We report two opposite reactions, depending on brain region. In the hippocampus GFAP-positive cells showed age-dependent hypertrophy, indicated by a significant increase in their surface area, volume and somata volume at 24 months, while entorhinal cortex astrocytes showed marked atrophy at the same age point (Rodriguez et al., 2013). Astrocytic astrogliosis and the connected hypertrophy are assumed to be a defensive reaction, probably a response to the inflammatory and oxidative state of the aging brain (Cotrina and Nedergaard, 2002). Undergoing a process of complicated morphofunctional modifications (see chapter 1.1.4.a), astrocytes are driven to find the source of damage, to assist the neuronal survival and to re-establish connectivity within neuronal networks (Sofroniew, 2009; Verkhratsky et al., 2012).

Inflammation, which possibly underlies the significant increase in hippocampal GFAP, is considered to cause alterations in calcium signaling, the basic astrocytic modulatory mechanism of neuronal function (Squier and Bigelow, 2000). However, astrocytic atrophy and degeneration can be of high pathological importance, especially by reducing the support for neurons and very probably having destructive effects on neuronal connectivity, as was already broadly described in the previous chapters of the discussion. In line with our data, longitudinal MRI studies have shown a reduced volume of the entorhinal cortex associated with advanced age, as well as atrophy of the perforant path and diminished synaptic connectivity between the EC and hippocampus (Du et al., 2006; Scheff et al., 2006; Stranahan and Mattson, 2010). As reported previously, the EC is a very sensitive and vulnerable region when it comes to AD, being the first affected by this deletorious disease. Importantly, the atrophic changes of EC astrocytes are also observed in the 3xTg-AD model, even in the presence of Aβ deposits, which provides further

support for the hypothesis of their defective defensive and supportive potential (Yeh et al., 2011; Rodriguez et al., 2013).

After analyzing the age-dependent changes in the GS we found s significant decrease in the parameters of GS-positive astrocytic profiles in the hippocampus, while no changes were observed in the case of the EC (Rodriguez et al., 2013). The coexistence of GFAP upregulation and GS downregulation was already shown during in vitro studies and points out its functional implications: inhibition of GABAergic and glutamatergic transmission (Weir and Thomas, 1984; Verkhratsky and Kirchhoff, 2007; Ortinski et al., 2010). The age-dependent changes and the relation between GFAP and GS were region-specific due to the fact that the majority of astrocytes within the hippocampus expressed both proteins of interest, while in the entorhinal cortex 3 subtypes of astrocytes were detected (GS-IR; GFAP-IR and GFAP/GS-IR) (Olabarria et al., 2011; Rodriguez et al., 2013; Yeh et al., 2013).

The expression pattern in the case of S100β was rather opposite to that of GS. The profile parameters of S100β-immunoreactive cells were highly (around 4-fold) increased in the EC, moderately increased in the dentate gyrus (DG) and remained unchanged in the CA1 hippocampal region at 24 months (Rodriguez et al., 2013). Astrocytic-specific S100β is a calcium binding protein, involved in various interactions between astrocytes and neurons (see chapter 1.1.2.b) (Kubista et al., 1999). Its elevated level was reported in many neurodegenerative diseases, as well as in healthy adults with impaired cognitive functions (Yadavalli et al., 2008; Steiner et al., 2011). An excess of S100β can be directly or indirectly neurotoxic (via secondary release by astrocytes and microglia cells of cytokines and NO) (Van Eldik and Wainwright, 2003). Nevertheless, the precise functional consequences of the reported changes remain enigmatic and the subject of more detailed study.

Based on the morphological analysis of 3 astroglial markers, we conclude that astrocytes undergo a complex age-dependent remodeling in a brain-specific manner (Rodriguez et al., 2013).

6. CONCLUSIONS:

The main findings and functional implications of the present thesis are:

1. We found an early and sustained general reduction in the surface area and volume of the GFAP astrocytic cytoskeleton. This atrophy is layer-specific, which suggests a direct link with the limbic loop established between the thalamic nucleus reuniens, hippocampus, entorhinal cortex and mPFC, therefore accounting for the memory disturbances observed in AD,
2. The presence of Aβ aggregates surrounded by only a few GFAP-positive astrocytes implies that the astrocytic generalized atrophy does not have a direct relationship with β-amyloid toxicity,
3. The decrease in GS-expressing astrocytes demonstrates a potential glutamatergic homeostatic failure, especially in the astrocyte-dependent glutamate-glutamine conversion pathway, accompanied by changes in the astroglial GFAP phenotype throughout the progression of AD. The fact that we did not find any significant changes in the expression of GLT-1 could mean an intact glutamate inactivation mechanism; nevertheless, there was a tendency towards a decline, which could indicate some changes in the efficacy of glutamate uptake via this specific astrocytic glutamate transporter,
4. The astrocytic atrophy together with reported homeostatic changes found in 3xTg-AD mice could account for the severity and progression of the neuropathological changes as well as synaptic breakdown resulting in disconnection problems and therefore the associated cognitive and mnesic alterations observed in AD patients,
5. During physiological aging, astrocytes undergo morphofunctional changes that are region-specific, as revealed by the differential and opposite expression of specific astrocytic markers (GFAP, GS and S100β) between the hippocampus (CA1 and DG regions) and the entorhinal cortex.

All of above findings demonstrate that mPFC astroglia are involved in the evolution and progression of Alzheimer's disease, having the potential to be considered as therapeutical targets. Additionally, astrocytes were shown to be deeply involved in the non-pathological aging process in an active and diversified way, depending on the brain region. Following their changes further could finally lead to the discovery of successful remodeling strategies for affected networks in the senescent brain.

7. SUMMARY:

In this thesis we reported astrocytic atrophy characterized by a reduction in the surface area and volume of GFAP-positive glial cells in the prefrontal cortex of 3xTg-AD mice – an important morphological alteration starting far before any well known histopathological hallmark of AD. This change is present in parallel with homeostatic failure suggested by the decreased expression of GS. Those alterations can have drastic effects on brain connectivity and the biochemistry of the main neurotransmitters within the brain, such as glutamate and GABA. GFAP is implicated in a variety of processes, such as cell migration and proliferation, neurite outgrowth, astrocytic glutamate transporter expression (GLAST and GLT-1) and synaptic plasticity, so that every change can shift the astrocytes' role from physiology to pathology. In the case of affected GFAP-IR astrocytes, the withdrawal of processes from neurons and synapses can lead to a severe transmission crush, due to the uncontrolled spillover of the neurotransmitter from the synaptic cleft, inadequate metabolic support and the lack of a physiological barrier between the affected synapse and other synapses in its close vicinity. This will directly disturb the reciprocal connections between the affected brain regions, inluding the important structures for memory and emotions, such as the entorhinal cortex, the hippocampus and the PFC.

Through the glutamate transporters (GLT-1, GLAST) and specific enzymes (GS), astrocytes are able to successfully protect the brain from excess of glutamate. After cytoskeletal astrocytic atrophy (independent of Aβ accumulation), the observed GS deficiency can result in a shortage of glutamine for neurons due to the distorted glutamate-glutamine cycle and subsequently in an insufficient synaptic effect.

The mechanisms and regulations of the aging brain are complicated and still not fully understood. Different markers can show opposite changes depending on brain region, as was proven by studying GFAP, GS and S100β expression in senescent astrocytes. Further study of astroglial aging in needed to reach the next step in helping the brain to remain less affected in the face of advancing age. The common agreement underlines the fundamental importance of maintaining harmony between the elimination and compensatory remodeling of neuronal nets. This reshaping is highly dependent on homeostatic stability, support and defence.

The basis for proper brain function lies in well functioning networks, in which neurons and glia are evenly involved. The results of studies presented in this thesis underline the crucial role of astrocytes in maintaining metabolic stability within the synaptic and neuronal environment, which makes them a promising therapeutic target in the prevention as well as the treatment of AD.

8. SOUHRN:

V této práci jsme popsali důležité morfologické změny astrocytů způsobené Alzheimerovou chorobou (AD), které předcházejí typickým histopatologickým změnám v mozkové tkáni postižené AD. U 3xTg-AD myšího modelu jsme v prefrontální kůře objevili atrofované GFAP-pozitivních astrocyty, jejichž povrch i objem byly významně menší. Současný pokles exprese glutamin-syntentázy (GS) u astrocytů postižených AD signalizoval porušení mechanismů glutamátové homeostázy. Takové změny pak mohou negativně ovlivňovat činnost nervových spojů a funkční stabilitu hlavních mozkových neurotransmiterů (glutamátu a GABA). Je známo, že gliální acidický fibrilární protein (GFAP) se účastní řady cytokinetických dějů (migrace a proliferace), má zásadní vliv na růst neuritů, podílí se na tvorbě transportních proteinů pro glutamát a aspartát v gliových buňkách (GLAST a GLT-1) a na vzniku nových synapsí. Tudíž i sebemenší změny v morfologii astrocytů mohou vést k patologii mozkové funkce. V případě poškození GFAP-IR astrocytů může dojít k progresivní poruše nervového přenosu způsobené nekontrolovatelným vyléváním neurotransmiteru v okolí synaptické štěrbiny, metabolickou nerovnováhou a ztrátou fyziologické bariéry okolních synapsí. To má za následek oboustranné porušení nervových spojů mezi postiženými oblastmi mozku zodpovědnými za paměť a emoce (entorhinální kůra, prefrontální kůra a hippokampus).

Astrocyty zprostředkovávají ochranu mozku před nadměrným uvolňováním glutamátu pomocí glutamátových transportérů (GLT-1 a GLAST) a specifických enzymů (glutamin syntetázy, GS). Pokles hladiny GS pozorovaný v populaci atrofovaných astrocytů (nezávisle na hromadění Aβ) by mohl vysvětlovat sníženou dodávku glutaminu do neuronů; syntéza a metabolismus glutaminu závisí na GS a jeho nedostatek může vést k poruše glutamátové homeostázy a k narušení synaptického přenosu signálu.

Mechanismy a regulační děje probíhající ve stárnoucím mozku nejsou ještě zcela známy. Sledováním exprese různých biochemických ukazatelů (GFAP, GS a S100β) ve stárnoucích astrocytech jsme zjistili rozdílné změny v závislosti na testovaných oblastech mozku. Právě nové poznatky ohledně stárnutí glií by nám mohly pomoci zjistit, jak zmírnit účinek stárnutí na mozkové funkce. Schopnost mozku udržovat rovnováhu mezi odstraňováním a kompenzační přestavbou nervových spojů je klíčová a vyžaduje homeostatickou vyváženost.

Správná činnost mozku se odvíjí od dobře fungující, vysoce organizované sítě nervových okruhů, do kterých jsou rovnoměrně zapojeny jak neurony, tak glie. Výsledky této studie podtrhují ústřední roli astrocytů v udržování stability metabolických dějů mezi neurony a synapsemi. To je činí slibným terapeutickým cílem v prevenci i léčbě AD.

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10. PUBLICATIONS:

1) **Magdalena Kulijewicz-Nawrot**, Alexei Verkhratsky, Alexander Chvátal, Eva Syková, José J. Rodríguez; „Astrocytic cytoskeleton atrophy in the medial prefrontal cortex during the progression of Alzheimer's disease". J Anat. 2012 Sep;221(3):252-262. doi: 10.1111/j.1469-7580.2012.01536.x. Epub 2012 Jun 27. **IF 2.357**

2) **Magdalena Kulijewicz-Nawrot**, Eva Syková, Alexander Chvátal, Alexei Verkhratsky, José J. Rodríguez; „Astrocytes and glutamate homeostasis in Alzheimer's disease: a decrease in glutamine synthetase but not in glutamate transporter-1 in the prefrontal cortex". ASN Neuro. 2013 doi:10.1042/AN20130017. Epub 2013 Sep 23. **IF 3.638**

3) José J. Rodríguez, Chia-Yu Yeh, Slavica Terzieva, Markel Olabarria, **Magdalena Kulijewicz-Nawrot**, Alexei Verkhratsky; „Complex and region-specific changes in astroglial markers in the aging brain". Neurobiol Aging. 2013 Aug 19. pii: S0197-4580(13)00289-3. doi: 10.1016/j.neurobiolaging.2013.07.002. **IF 6.166**

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