PATHOPHYSIOLOGICAL ADAPTATION AND ROLE OF ASTROCYTES IN ALZHEIMER'S DISEASE: A LONGITUDINAL ANALYSIS IN A TRIPLE TRANSGENIC MOUSE MODEL.

A thesis submitted to the University of Manchester for the degree of PhD in Neuroscience in the Faculty of Life Science

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Pathophysiological Adaptation And Role Of Astrocytes In Alzheimer's Disease: A Longitudinal Analysis In A Triple Transgenic Mouse Model.
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Abstract

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease that deeply affects cognitive function and it is the primary and the most common cause of dementia. Histopathologically, AD is characterised by amyloid-β (Aβ) accumulation in the form of plaques and the presence of intracellular tangles of hyperphosphorilated tau protein. Loss of neurons and synaptic connectivity is also a main characteristic of AD. Different nmesic brain regions such as entorhinal cortex, prefrontal cortex, nucleus basalis of Meynert and hippocampus are affected in AD. Cholinergic neurotransmission is specially affected in AD, although other systems like glutamatergic are also involved.

Glial alterations in AD have been considerer secondary to neuronal changes. For decades, *astrogliosis* and *microglial activation* have been all the recognition they have received. However, nowadays, it is known that glia plays a central role in AD pathology and they are not longer neglected. Nevertheless, the current knowledge about their specific role in pathology is still limited.

In the present thesis we have studied both astroglial and microglial roles in the progression of AD. We have focused our work on the dentate gyrus (DG) and cornus ammonis 1 (CA1) areas of the hippocampus, which is one of the earliest and most affected brain regions in AD, as well as the major area involved in cognitive function. For that, we have used the triple transgenic mouse model of AD (3xTg-AD mouse) that mimics the human pathology with fidelity and it is considered one of the most relevant models of AD.

Our results show for the first time that astroglial atrophic and astrogliotic reaction in AD are concomitant processes. Interestingly, atrophy precedes Aβ plaque and tau tangle manifestation. Astrocytes in the DG showed reduced GFAP cytoskeleton (decrease of volume and surface; **Chapter 3)** and GS expression (decreased optical density; **Chapter 4**) at 12 months of age. At 18 months of age this alterations remained and expanded to the CA1 area. This reduction of cytoskeleton could reflect astroglial overall cell atrophy, and GS reduction would impact on the glutamate-glutamine cycle, therefore affecting glutamate metabolism and homeostasis. Both of these alterations could also have a direct effect on glutamatergic transmission and synaptic connectivity causing either: i) excitoxicity due to the reduced glutamate uptake of astrocytes from the synaptic cleft, ii) disruption of the neural connectivity due to the inefficient astroglial modulation of glutamatergic neurotransmission and iii) decreased availability of glutamate due to inefficient glutamate re-cycling, compromising, all of them, the cognitive function. Thus, all these dysfunctions could explain the early cognitive decline in AD that precedes the manifestation of pathological burden. In parallel, microglia results (**Chapter 5**) have shown proliferation of resting microglia that also precedes Aβ plaques appearance and pathological activation, suggesting that microglia responds to early pathological events. The specific interaction between astrocytes and microglia needs exploring. All in all, glial role in AD, although still mysterious, shows clear signs of active participation in the development of the pathology. Therefore, it is conceivable to think that future effective therapies will also target glial cells.

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Mendi magalekoentzat…

List of abbreviations

192 IgG Saporin	12 clone monoclonal antibody to the nerve growth factor receptor.
3xTg-AD	Triple transgenic mouse expressing APPSwedish, $PS1^{M146L}$, and Tau^{P301L}
4R/2N	Single transgenic mouse expressing human Tau R406W mutation
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
7TauTg	Single transgenic mouse model overexpressing tau protein without L neurofilament subunit
ACh	Acetylcholinergic
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AF64A	Ethylcholine mustard aziridinium ion
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3 hydroxy-5-methyl-4-isoxazole-propionic acid
APP	Amyloid precursor protein
$APP_{Swedish}$ 695.K670N/M671L	Double transgenic mouse expressing APP Swedish 695.K670N-M671L mutations
APP/Ld/2	Single transgenic mouse expressing APP London V642I mutation
APP_{695SWE}	Double transgenic mouse expressing Swedish double mutations K670N/M671L
APP_{751SL}	Single transgenic mouse expressing APP751SL mutation
APP_{751SL}/ $PS1_{M146L}$	Double transgenic mouse expressing APP751SL and PS1M146L mutations
APP_{Swe}	Double transgenic mouse expressing APP Swedish 695.K670N-M671L mutations
APP_{SWE}/$PS1_{dE9}$	Double transgenic mouse expressing APPswe (KM 593/594 NL) and PS1dE9 mutations
APPSwedish and $PS1^{M146L}$	Double transgenic mouse expressing APPswe (K595N, M596L) and PS1 (A246E) mutations
APPSwedish and V717F	Double transgenic mouse expressing APP Swedish (KM670/671NL) and V717F mutations
APP^{V717F}	Double transgenic mouse expressing Swedish and Indiana (D664A) mutations
ATP	Adenosine triphosphate
AxD	Alexander disease
Aβ	Amyloid β-peptide aggregates
BBB	Blood Brain Barrier
BM	Barnes maze
CA	Cornu ammonis
CC	Corpus callosum
ChAT	Choline acetyltransferase
CNS	Central nervous system
DG	Dentate gyrus
EAAC1	Glutamate transporters in neurons
EAAT	Excitatory amino acid transporter
EC	Entorhinal Cortex
ER	Endoplasmatic reticulum
ERM	Ezrin, radixin and moesin
FAD	Family Alzheimer's disease
FDA	Food and Drug Administration
FTD	Frontotemporal dementia
FTDP-17	Frontotemporal dementia with Parkinsonism linked to chromosome 17
G272V, P301L, R406W	Triple transgenic mouse expressing G272V, P301L and R406W mutations
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter in astrocytes
GLT	Glutamate transporter
GLT-1	Glutamate transporter 1
Grcl	Granular cell layer
GS	Glutamine synthetase
GS-IR	Glutamine synthetase immunoreactivity/-reactive
HAD	HIV-1 associated dementia
HIV	Human immunodeficiency virus
Htau	Mouse model with Endogenous tau knocked out
IA	Inhibitory avoidance
IF	Intermediate filament

IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
$InsP_3$	Inostitol triphosphate
IOD	Inversed optical density
K670M/N671L	Single transgenic Rat expressing APP Swedish K670N/M671L mutation
K670N/M671L and Indiana V717F	Double transgenic mouse expressing APP Swedish K670N/M671L and Indiana V717F mutations
K670N/M671L and V717F	Double transgenic mouse expressing APP Swedish K670N/M671L and V717F mutations
Lac	Stratum lacunosum moleculare
Lac mol	Lacunosum Moleculare
LTD	Long-term depression
LTP	Long-term potentiation
MMSE	Mini-Mental State Examination
Mol	Molecular cell layer
MRI	Magnetic resonance imaging
MWM	Morris water maze tasks
nbM	Nucleus basalis of Meynert
NFTs	Neurofibrillary tangles
NMDA	N- metyl- D-aspartate
N_V	Numerical density (cell number/ mm^3)
OD	Optical Density
Or	Stratum Oriens
P301L	Single transgenic mouse expressing tau P301L mutation
P301L TET-off	Single transgenic mouse model expressing P301L mutation regulated by tetracycline responsive promoters.
PB	Phosphate buffer
PcL	Pyramidal cell layer
PET	Positron emission tomography
PHF	Pared helical filaments
PS-1	Presenilin-1
PS-2	Presenilin-2
$PS1_{M146L}$	Single transgenic mouse expressing PS1M146L mutation
Rad	Stratum Radiatum
S Luc	Stratum Lucidum
SAD	Sporadic AD
SO	Stratum Oriens
SOD1	Superoxide dismutase
SR	Stratum Radiatum
Tau^{P301L} (4R,2-,3-)	Single transgenic mouse expressing tau P301L mutation
Tg1116	Single transgenic Rat expressing APP V717I mutation
Tg2576×JNPL3	Triple transgenic mouse expressing APP Swedish (KM670/671NL), V717F and tau P301L mutations
Tg2576×VLW	Triple transgenic mouse expressing APP Swedish (KM670/671NL), V717F and triple tau (G272V, P301L and R406W) mutations
Tg478	Single transgenic Rat expressing APP Swedish K670N/M671L mutation
Tg478/Tg1116	Double transgenic Rat expressing APP Swedish K670N/M671L and V717I mutations
Tg478/Tg1116/Tg11587	Triple transgenic Rat expressing APP Swedish K670N/M671L, V717I and PS-1 M146V mutations
TgAPPsw and PS1 M146L	Double transgenic mouse expressing APP Swedish K670N/M671L and PS1M146L mutations
TNF	tumour necrosis factor
TS	Trizma® base saline
V337M	Single transgenic mouse expressing human Tau V337M mutation
vim	Vimentin

Preface

I started my PhD training on January 2008 in the Laboratory of Prof. Rodríguez Arellano in The University of Manchester.

Since then I have been working on understanding the role of astrocytes and microglia in Alzheimer's disease (AD) in the triple transgenic (3xTg-AD) model of the disease. I have focused my investigation of age-related changes in hippocampal astrocytes (at light and electron microscopic level) as a critical region for mnesic function, which is also one of the initial brain regions affected in this devastating disease.

We firstly used quantitative light microscopic immunohistochemistry to study the changes in (i) GFAP positive astrocytes in the hippocampus, (ii) GFAP positive astrocytes in relation to Aβ and (iii) specific population alterations within the hippocampus. We have found a generalised decrease of astroglial GFAP cytoskeleton in the hippocampus that is concomitant with cytoskeleton hypertrophy on those astrocytes associated with Aβ deposits. Importantly, this cytoskeleton atrophy was not associated with cell loss as shown by cell density determination. These findings have been published initially in *Cell Death and Differentiation* and further expanded in a second manuscript in *Glia* (see bellow list of publications). Afterwards, we studied whether the observed astrocytic atrophy is associated with imbalanced glutamatergic neurotransmission. Indeed, we found a decrease in glutamine synthetase expressing astrocytes, which among its role to produce glutamine as a new substrate for neurons is also the responsible for the inactivation of extracellularly released glutamate. Interestingly enough, these results correlate with the GFAP atrophy, which has produced an additional paper that has been considered suitable for publication in *Molecular Neurodegeneration*. Regarding microglia we aim to understand what was the precise reaction in AD. For that we determined resting microglial and activated microglial population, and we found that microglia responses prior Aβ appearance and microglia activation, showing resting microglia proliferation. This finding has also been published in *Cell Death and Diseases.*

We hypothesised that astrocytes are profoundly affected during the progression of AD and that they could fail to support neurons causing neuronal contributing to transmitter imbalance and somehow to synaptic connectivity alterations in the hippocampus, therefore explaining the cognitive deficits manifestations of the disease. Interestingly, these alterations occur before Aβ plaque appearance and therefore could be involved in the early nmesic deficits of AD. Microglia, in turn, would contribute to the pathology by releasing pro-inflammatory molecules

Presently I am investigating the role of astrocytic neurotrophic factor (S-100β) to understand if cytoskeleton alterations are also reflected on total morphology and domain coverage. In addition, we will decipher whether exposure to enriched environment and/or voluntary wheel running reverses the astrocytic atrophy and astroglial decline in 3xTg-AD, as we have recently probed it to have an effect also in the altered neurogenic capabilities (Rodríguez et al, Curr Alzheimer Res, 2011). All these studies are of significant relevance for the comprehension of AD pathology and hopefully will be of relevant interest for the development of future effective therapies.

I addition to my direct PhD project, I have been actively involved in other ongoing studies in our laboratory including (i) the effect of environmental manipulation such as voluntary wheel running and enrichment on hippocampal neurogenesis in 3xTg-AD animals and (ii) the role of serotonergic transmission in 3xTg-AD mice. These active involvements in different projects have been or hopefully would be reflected into 5 authorship positions in peer-reviewed and highly respectable journals.

I have also been involved in teaching activities during my stay in The University of Manchester, including demonstration of science anatomical and neuroscience practicals as well as contributing to the conception, elaboration and fulfilment of final year projects for undergraduate students.

PUBLICATIONS IN PEER REVIEWED JOURNALS

-**Olabarria M**., Noristani H.N., Verkhratsky A., Rodríguez J.J. (2011). Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: Mechanism for deficient glutamatergic transmission? *Molecular Neurodegeneration.* **6**:55.

-Rodríguez J.J., Noristani H.N., **Olabarria M**., Fletcher J., Summerville T., Yeh C.Y., Verkhratsky A. (2011). Voluntary running and environmental enrichment restores impaired hippocampal neurogenesis in a triple transgenic mouse model of Alzheimer's disease. *Current Alzheimer's Research.* In press. [Epub ahead of print]

-Noristani H.N., Meadows RS., **Olabarria M.**, Verkhratsky A., Rodríguez J.J. (2011). Increased serotonin terminals and axons density in CA1 of the hippocampus in a triple transgenic mouse model of Alzheimer's disease: an ultrastructural study. *Cell Death and Disease.* In press.

-**Olabarria M**., Noristani H.N., Verkhratsky A., Rodríguez J.J. (2010). Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia*. **58**:831-838.

-Verkhratsky A., **Olabarria M**., Noristani H.N, Yeh C.Y., Rodríguez J.J. (2010). Astrocytes in Alzheimer's disease. *Neurotherapeutics*. **7**: 399-412.

-Noristani H.N., **Olabarria M**., Verkhratsky A., Rodríguez J.J. (2010). Serotonin fibre sprouting and increase in serotonin transporter immunoreactivity in the CA1 area of hippocampus in a triple transgenic mouse model of Alzheimer's disease. *Eur J Neurosci.* **32**: 71-79.

-Rodríguez J.J., Witton J., **Olabarria M**., Noristani H.N., Verkhratsky A. (2010). Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease. *Cell Death and Disease*. **1**:e1.

-Rodríguez J.J., **Olabarria M**., Chvatal A., Verkhratsky A. (2009). Astroglia in dementia and Alzheimer's disease. *Cell Death and Differentiation*.**16**, 378-385.

Note: Publications in red are part of this PhD research work

Chapter 1

General Introduction

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Abstract

Alzheimer's disease (AD) is an age-related progressive and irreversible neurodegenerative disease that deteriorates cognitive functions including memory, and it is the primary and most common cause of dementia in developed countries.

AD pathology is first manifested in the entorhinal cortex, spreading afterwards to the hippocampus and eventually to other areas like prefrontal cortex and nucleus basalis of Meynert. In this way, the hippocampus is one of the first and most affected brain regions in AD. In addition, the implication of the hippocampus in AD goes further, due to the fundamental role it plays in cognitive functions, including learning and memory.

Pathologically AD is characterised by β-amyloid and hyperphosphorilated tau accumulation, neuronal and synaptic loss. Over the last few years several hypotheses have been proposed to explain the pathogenesis of neurodegenerative diseases including AD. Synaptic loss and dysregulation of synaptic plasticity have been long recognised as early events in neurodegenerative diseases. This was due to the conception that neurons were the only implicated neural elements in plasticity and synaptic modulation. Thus, glial pathological changes have been regarded as secondary events that develop as a consequence of the neuronal changes, rather than an active contributor to the pathology. Today, we know that glial cells are essential for the adequate function of the central nervous system (CNS). Glial cells control brain homeostasis, modulate synaptic functions, protect the brain for external and internal threats and assure efficient axonal conduction by myelin ensheathment among others; hence guaranteeing a balanced and tuned brain performance. Therefore any glial alteration could have a dramatic impact on the overall system. Base on the current knowledge of glia, the central role that they play in neurodegeneration is undeniable. Indeed, astrogliosis and microglial activation are prominent features of AD. However, little is known about their specific role in the onset, progression and outcome of AD. Here, in the general introduction section of the thesis, we summarise the current knowledge regarding AD pathology, astroglial and microglial biology as well as their role in different disease with particular focus on AD, including some of our results.

1. INTRODUCTION

1.1. Alzheimer's disease

Alzheimer's disease (AD), was first observed in 1901, described in 1906 and published by the German neuropathologist Alois Alzheimer (Über eine eigenartige Erkrankung der Hirnrinde; (Alzheimer, 1907). The disease was initially observed in a 51 year old woman called Auguste D who presented with memory deficit, speaking and understanding difficulties as well as paranoia (Alzheimer, 1907). After the death of Auguste D (spring 1906), Alzheimer observed within her brain, what he described as "miliari foci" spread around the cortex and degenerating neurons with bundles of "fibrils" (Alzheimer, 1907); (Fig. 1), known today as senile plaques and neurofibrillary tangles, respectively. Alzheimer described meticulously the histopathology of "miliari foci" and "fibrils" in the evolution of the disease and also made the first observations about the changes in glia in relation to senile plaques (Alzheimer, 1907).

AD it is an age-related progressive and irreversible neurodegenerative disease that deteriorates cognitive functions including memory, and it is the primary and most common cause of dementia (Braak and Braak, 1991). AD is an incredibly common, devastating and always fatal disorder, which involves the degeneration of neurons and synapses in several brain regions including the hippocampus, entorhinal cortex, basal forebrain, amygdala, frontal cortex, nucleus basalis of Meynert (nbM) and inferior parietal cortex (Yankner, 1996). Cholinergic neurons of nbM bear a severe damage, leading to significant neuronal death (Whitehouse *et al.*, 1981). The dramatic rise in life expectancy, from roughly 49 years to more than 79 years in the first world within the last century, has resulted in a growing number of individuals achieving the age at which neurodegenerative disorders become common (Selkoe, 2001). There are two different forms of AD; presenil (familial) and senile or sporadic forms (Small and Cappai, 2006). Over 35 million people worldwide are affected by dementia (Cumming and Brodtmann, 2010) with AD being one of the most common causes of dementia in the elderly population (Braak *et al.*, 1999).

Epidemiologically, AD is classified into early-onset Family Alzheimer's disease (FAD) and late-onset sporadic AD, or SAD (Blennow *et al.*, 2006). Late-onset SAD accounts for the majority (95%) of AD cases that affect people above 65 years of age

(Kern and Behl, 2009). FAD is associated with mutations in three genes represented by amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2), which are inherited by an autosomal dominant mode (Shastry and Giblin, 1999). The FAD most commonly occurs in patients between 40 - 65 years of age and is characterised with a rapid progression (Shastry and Giblin, 1999). SAD, however, is not clearly associated with genetic factors. Nevertheless, the expression of APOE ε4 (one of the four genes responsible for the transport of cholesterol) has shown susceptibility to develop Aβ plaques (Fig. 1 and 2). Such increase in cholesterol levels increases Aβ aggregation and Aβ plaque formation (Martins *et al.*, 2006; Selkoe, 2001). Interestingly, drugs aiming to reduce the level cholesterol reduce the risk of developing AD (Martins *et al.*, 2006).

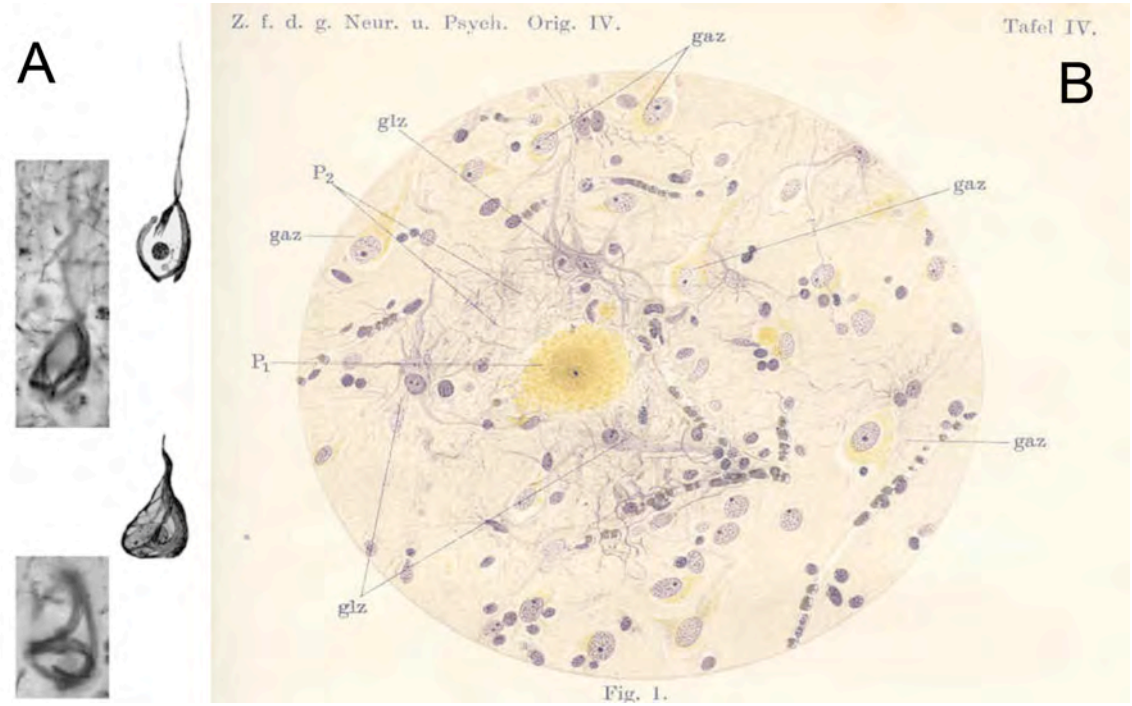


Figure 1. *Drawing of Alois Alzheimer illustrating (A)"fibrils" and (B) the glial reaction (astrogliosis and hypertrophy) in a pathological brain containing senile plaques. Key: gaz, neurone; glz, glial cell, P central part of the plaque; P_2, peripheral part of the plaque. From (Alzheimer, 1910).*

The major cognitive alterations associated with AD are memory deficits, language abnormalities and behavioural changes, which lead to the major neuropsychological features of AD (Harciarek and Jodzio, 2005). AD patients have trouble with memory of new events, most probably due to their inability to encode and store information for later recall (Knopman *et al.*, 2003). The loss of newly learned verbal information is one of the significant aspects of the memory disturbance in AD (Kaltreider *et al.*, 2000; Welsh *et al.*, 1992). AD patients also exhibit understanding difficulties and their verbal

communication is usually disturbed. In addition, impaired use of semantic representations has been also reported in patient with probable AD (Harciarek and Jodzio, 2005).

The most important behavioural symptoms of AD range from indifference, apathy, hallucinations and delusion, to agitation, aggression and unpredictable behaviour (Harciarek and Jodzio, 2005).

1.1.1. Pathological Hallmarks of AD

The two major histological hallmarks of AD patients are the presence of amyloid β-peptide aggregates (Aβ), first intracellular and after developing into senile plaques (Fig. 1 and 2A), and the presence of filamentous intracellular aggregates of the hyperphosphorylated tau protein (a microtubule-associated protein fundamental for neuronal cytoskeleton), which are the so-called neurofibrillary tangles (NFTs; Fig. 2C); (Yankner, 1996).

Senile plaques are focal extracellular deposits of fibrillar β-amyloid (also called neuritic plaques) that appear in brain parenchyma and in the wall of blood vessels (causing what is known as angiophilic angiopathy; Fig. 2B), and are associated with injured axons and dendrites, which are commonly found in great numbers in the limbic system (Dickson, 1997). Such plaques contain extracellular deposits of amyloid β-protein (Aβ) that occur principally in a filamentous form, i.e., as star-shaped loads of amyloid fibrils. The majority of the fibrillar Aβ found in the neuritic plaques is the variety ending at amino acid 42 ($A\beta_{42}$), the slightly longer and more hydrophobic form that is more prone to aggregation (Jarrett *et al.*, 1993). Extracellular pathological Aβ accumulation causes severe homeostasis disruption and result in neurotoxic damage to the surrounding tissue.

Neurofibrillary tangles are found in cell bodies, apical dendrites and axons as NFT, in distal dendrites as neuropil threads and in the abnormal neurites that are associated with some β-amyloid plaques (Gotz *et al.*, 2004a).

Another histopathological hallmark described by Alzheimer (Alzheimer, 1907) and later by Cajal, was the glia alteration (Fig. 1, 2A and 2B). Alzheimer observed in the

histological preparations of Auguste D, a clear hypertrophy of astrocytes (astrogliosis) surrounding the senile plaques, showing a large number of gliofilaments (Alzheimer, 1907) and microglial hypertrophy and activation (Alzheimer, 1907).

Despite the neuropsychological symptoms, post-mortem pathological-anatomical examination is fundamental to ultimately confirm the diagnosis of AD. In fact, to have a conclusive AD diagnosis the confirmation of the presence of neuritic plaques and neurofibrillary tangles is needed (Coll et al., 2003). Furthermore, the cerebral congophilic angiopathy, characterised by the deposition of the Aβ within cerebral vessels (Fig. 2B), may support AD diagnosis (Thal et al., 2008). Finally, macroscopic changes such us cerebral atrophy and dilatation of the ventricular system is also consistently observed in AD brain specimens (Chen et al., 2000).

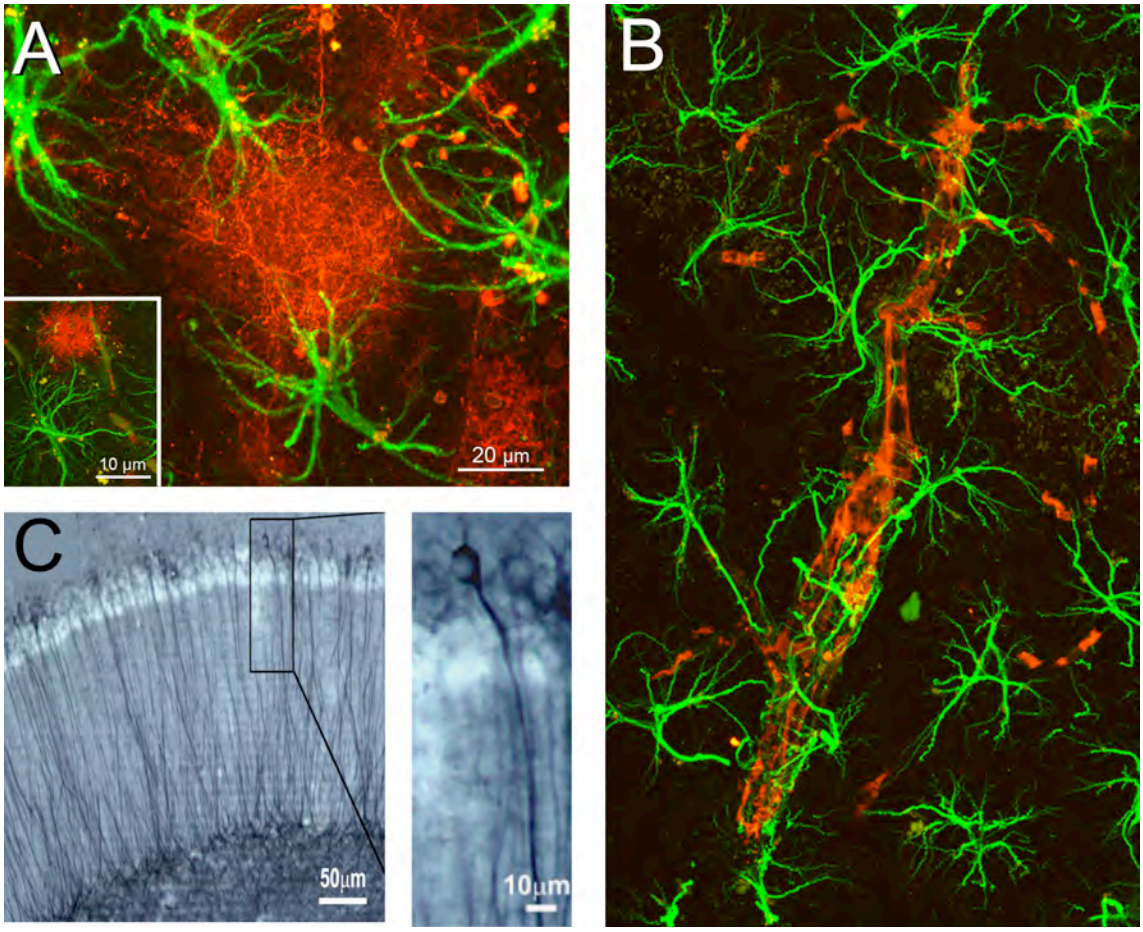


Figure 2. *Confocal photomicrographs of a triple transgenic AD mouse hippocampus showing: (A) Aβ plaque (red; senile plaque) surrounded by astrocytes (green; Rodriguez, Olabarria, et al, 2008). (B) Aβ loaded blood vessel (red; congophilic angiopathy) surrounded by astrocytes (green) (C) Phosphorylated tau protein within CA1 pyramidal cell of a mouse hippocampus (Olabarria et al., 2010; Rodriguez et al., 2008).*

1.1.2. Amyloid processing and accumulation in AD

The key of the formation of Aβ in AD pathology, resides in the abnormal processing of the amyloid protein precursor (APP). APP is a single transmembrane glycoprotein highly expressed in the brain (Gotz *et al.*, 2004b). Although the defined physiological function of APP is not clear, it is well recognised that it has an important role in neuronal differentiation by interacting with the Notch signalling pathway (Kimberly *et al.*, 2005). APP also acts as an epithelial growth factor (Schmitz *et al.*, 2002).

The abnormal processing of APP is carried out by APP secretases, which are α-, β- and γ-secretases in neurons (Fig. 3). The APP cleavage takes place within the amyloid sequences, on the N-terminal and C-terminal ends of the amyloid sequence respectively (Nunan and Small, 2000). APP processing via α-secretase is the main processing pathway, where Aβ sequence is cleaved on the C-terminal side of residue 16, generating an 83-residue portion (Esch *et al.*, 1990). As α-secretase destroys the Aβ sequence, it is believed that α-secretase pathway prevents neurotoxic amyloid formation (Nunan and Small, 2000). Some studies have shown that stimulation of muscarinic receptors can increase APP processing via α-secretase (Muller *et al.*, 1997; Nitsch *et al.*, 1992).

There is, however, an alternative proteolytic cleavage by β-secretase that results in the secretion of the slightly shortened sAPP-β molecule and the retention of a 99-residue C-terminal portion. The C99 fragment can also undergo cleavage by γ-secretase to release the neurotoxic Aβ peptides (Selkoe, 2001); (see also Fig. 3 and 4). The precise location of cleavage by -secretase is essential for the development of AD (Nunan and Small, 2000) and production of Aβ. The cleavage of APP by γ-secretase is narrowly related to AD pathology (Scheuner *et al.*, 1996; Small and McLean, 1999). Although the γ-secretase has not been recognised, there are accumulating evidences supporting that presenilins (PSs) are these secretases. It has been proved that PS knockout mice inhibit all γ-secretase activity (Herreman *et al.*, 2000). Thus, due to the role of α- and β- secretases, the use of specific inhibitors for AD therapy gathers

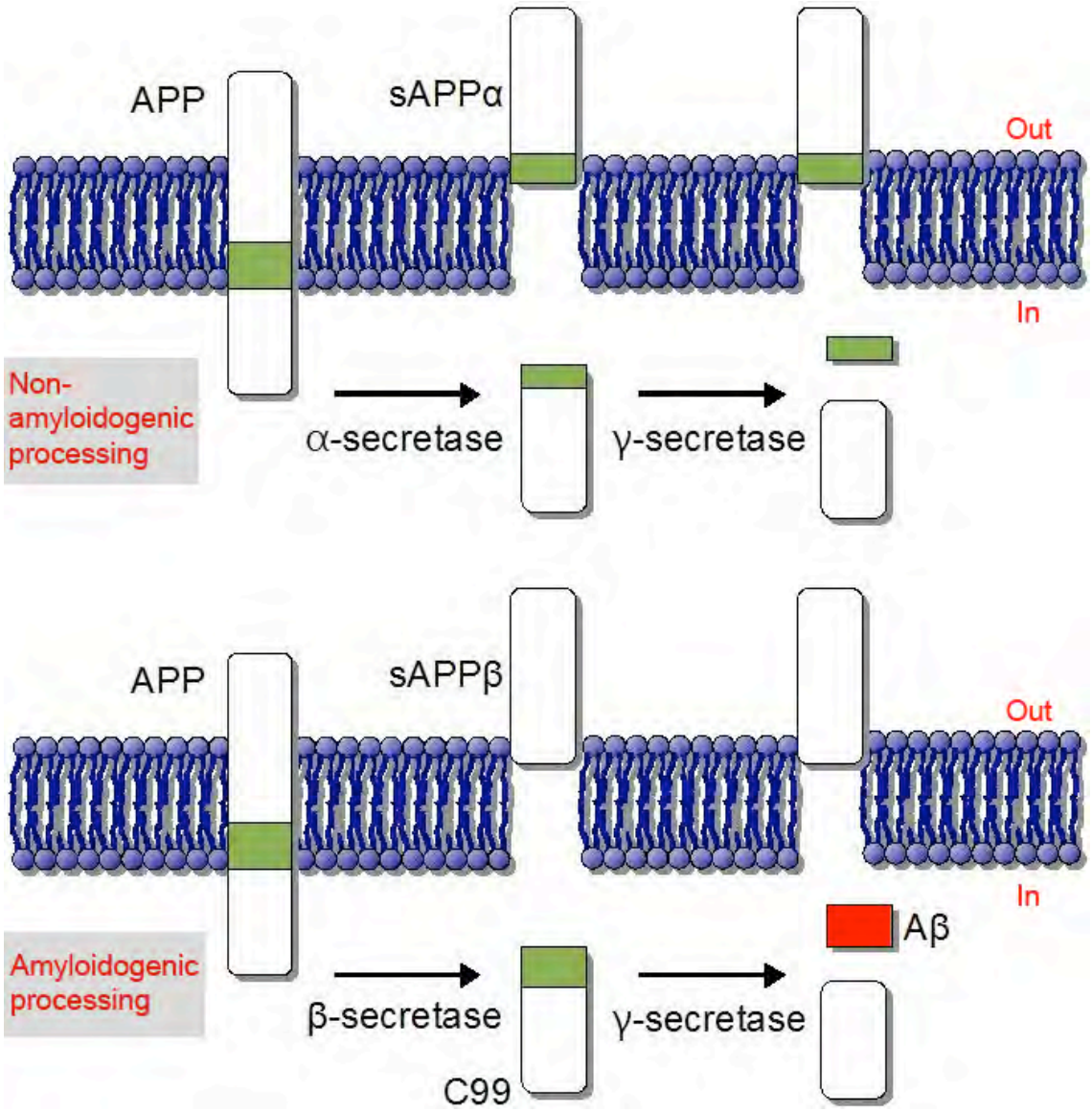


Figure 3. *Scheme of the two possible APP cleavages. (Generous gift from Miss Yeh)*

strength (Nunan and Small, 2000; Small and McLean, 1999). However, γ-secretase may be an important enzyme for the processing of others proteins such us Notch, which is involved in the regulation of neuronal differentiation, gametogenesis, myogenesis. Therefore, these inhibitors may have undesirable side effects, altering Notch signalling pathway (Fischer *et al.*, 2005; Nunan and Small, 2000).

1.1.3. The Amyloid cascade Hypothesis of AD

The abnormal production and accumulation in AD of Aβ, which precedes the tangle formation has led to the development of "amyloid cascade hypothesis". This suggests a synergistic effect of Aβ on tau pathology (Delacourte and Buee, 2000; Gotz *et al.*, 2004a; Thal *et al.*, 2002). Mutations in APP, PS1 and PS2 genes alter the metabolism of

APP processing (Fig. 4), leading to either increased levels of total Aβ or a selective augmentation of the longer more amyloidogenic $A\beta_{42}$ species (Scheuner *et al.*, 1996). This genetic evidence has provided the strongest support for the amyloid cascade hypothesis, which predicts that Aβ is the trigger for all cases of AD. In contrast, mutations in the tau gene do not lead to AD (Gotz *et al.*, 2004b).

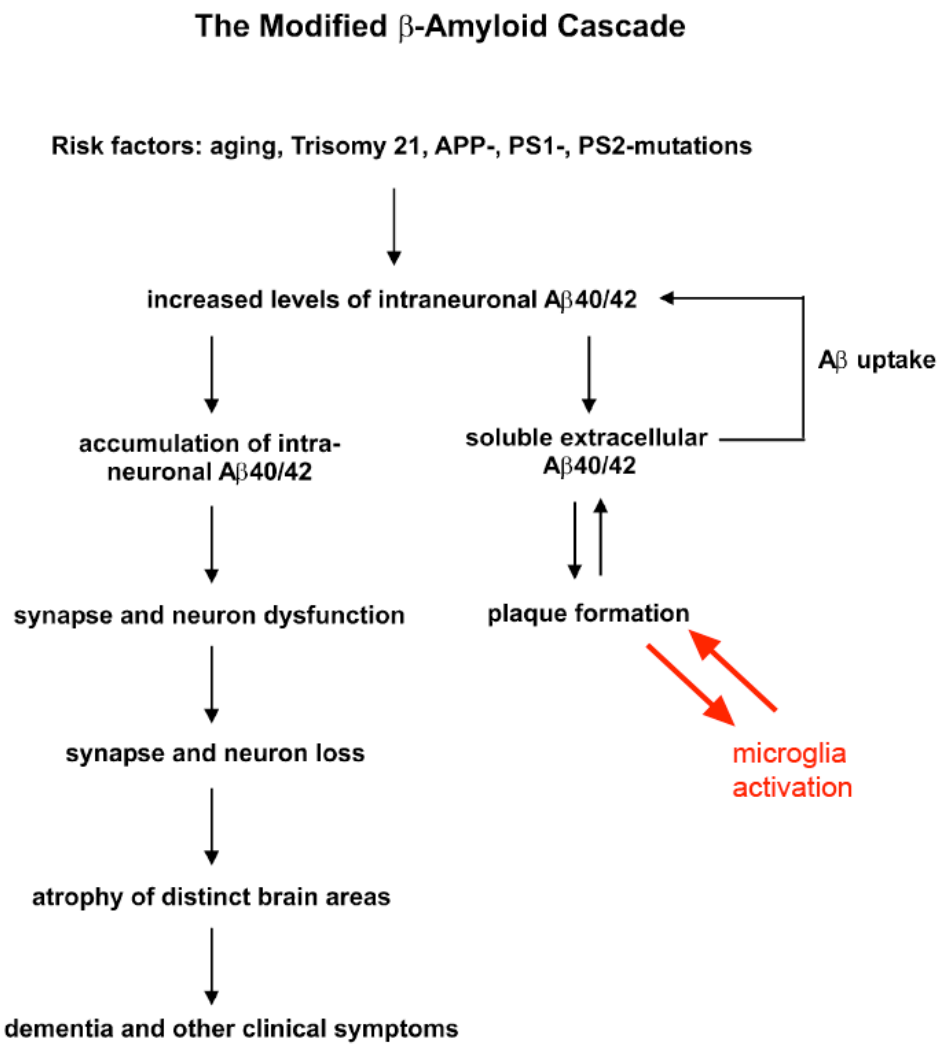


Figure 4. *Schematic diagram displaying pathogenic progression in AD. Multiple factors including ageing, Down syndrome and mutant APP, PS-1 or PS-2, contribute to the increase of intraneuronal Aβ42 level, leading to synaptic and neuronal dysfunction as well as glial alteration and degeneration in specific brain areas involved in cognitive functions. In parallel, increased secretion and deposition of Aβ42 leads to extracellular plaque formation and glial activation. Adapted from (Wirths et al., 2004).*

In addition, it is known that exposure of cells to Aβ increases the phosphorylation of the microtubule-stabilising protein tau, suggesting that the two lesions do not occur independent of each other (Seyb *et al.*, 2008). Hyperphosphorylation of tau results in dissociation of the protein from microtubules leading to a loss of microtubule stability, and ultimately cell death (Braak *et al.*, 1999). Different studies have shown that the Aβ treatment of primary neuron cell culture triggers an increase of intracellular calcium and

arise of calpain due to the augment of intracellular calcium protein in AD brains has also been described. The activation of calpain enzyme, which is a calcium-dependent non-lysosomal cysteine proteases, due to the exposure of Aβ may be an important signalling pathway linking the extracellular Aβ accumulation to intracellular formation of hyperphosphorylated tau tangles (Seyb *et al.*, 2008); (Fig. 2C).

The progression of AD occurs with initial neurodegenerative events appearing in the trans-entorhinal cortex, which spread afterwards to the entorhinal cortex and hippocampus. Subsequently the spreading wave of neurodegeneration swallows the rest of the temporal, frontal, and parietal lobes (Thompson *et al.*, 2003; Thompson *et al.*, 2007). At the more advanced stages of the AD, the grey matter undergoes severe damage manifested by a profound loss of neurones and synaptic contacts.

Controversially, the spatio-temporal appearance of Aβ plaques and neurofibrillary tangles do not coincide; Aβ pathology begins in the neocortex and spread towards the forebrain (Thal *et al.*, 2002) whilst tangles are first shown in the hippocampal formation, spreading afterwards towards the neocortex (Gotz *et al.*, 2004a).

1.1.4. Tau protein alteration in AD

Neurofibrillary tangles (NFTs) are found in neurons and are formed by paired helical filaments (PHF); (Braak *et al.*, 1999); (see also Fig. 2C). The main protein component of PHF is tau, which is a microtubule-associated protein (Goedert *et al.*, 1988; Gotz *et al.*, 2004a; Novak *et al.*, 1991). In physiological conditions, the functions of tau include microtubule assembly and stabilisation, signal transduction, cytoskeleton organisation and intracellular vesicle transport. During the progression of AD, tau undergoes abnormally hyperphosphorylation, suffering a conformational change which results in the dissociation of tau from microtubules and hence the tangle formation (Gotz *et al.*, 2004a). These events consequently, lead to the degeneration of neuronal axons and dendrites, by destabilisation of the cytoskeleton and protein transport mechanisms. As a consequence, cholinergic neurotransmission is directly affected (Niewiadomska *et al.*, 2006) and gives rise to the cognitive impairments and finally to neurodegeneration of the affected cells (Braak *et al.*, 1999); (see also Fig. 4).

Conclusive evidence correlates the presence and localisation of NFTs with the degree of cognitive impairment and memory disturbances in AD (Braak and Braak, 1991). Further studies focused on tau dysfunctions showed truncation and phosphorylation as two major post-translational modifications responsible for toxic increase of function as a primary cause of tauopathies including Alzheimer's disease (Alonso *et al.*, 1996; Novak *et al.*, 1991). In addition, the other major type of dementia (frontotemporal dementia; FTD) also shows tau gene mutations (Hutton *et al.*, 1998).

1.1.2. Neurochemical Alterations in AD

1.1.2.1. Cholinergic alteration in AD

A turning point in AD research came in the early 80's when degeneration of cholinergic neurons throughout specific nuclei of the brain was discovered (Bartus *et al.*, 1982). These evidences came as result of different pharmacological studies using specific cholinergic drugs such as, atropine and scopolamine. These antimuscarinic drugs induced a learning deficit, which was restored by the acetylcholinesterase enzyme inhibitors (AChE); (Deutsch *et al.*, 1971). Today it is considered that the decrease of the activity of choline acetyltransferase (ChAT; which is the responsible enzyme of the synthesis of acetylcholine) in the cortex and the hippocampal regions is the most consistent neurochemical change in AD pathogenesis. In addition, also appears a reduced presence of cholinergic projections (Fodale *et al.*, 2006). These alterations give rise to difficulty in maintaining attention, and provoke profound cognitive impairment, such as loss of memory and learning ability (Fodale *et al.*, 2006).

Based on the cholinergic deficiency hypothesis, the initial pharmacological therapies for AD focused on the enhancement of the cholinergic system. Physiostigmine was one of the earliest and showed to increase acetylcholine in the synaptic cleft by inhibiting AChE (Geerts and Grossberg, 2006). Since then, multiple AChE inhibitors have been developed that are widely used in AD treatment. However, they seemed to be efficient merely at the early stages of the disease and lose efficacy over-time (Oddo and LaFerla, 2006). Although AChE inhibitors remain as the drugs of first choice, other cholinergic drugs have been also designed such as muscarinic and nicotinic agonists (Caccamo *et al.*, 2006). However, the involved of other neurotransmission systems has been started

to be considered in AD such as glutamatergic, serotonergic systems; hence research has been expanded to these systems to try to uncover the obscurities of AD pathology.

1.1.2.2. Glutamatergic alteration in AD

Learning and memory are closely related to the expression of cholinergic receptors, synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD); (Fodale *et al.*, 2006). Cholinergic system is intimately linked, as well as the above mentioned plastic processes, to glutamatergic system forming the so-called "glutamatergic/aspartategic-acetylcholinergic circuit", which is the morphofunctional base of learning, memory and cognition (Jacob *et al.*, 2007). Memory is in general believed to be stored as strength changes of the synapses, and although its precise mechanism remains mysterious, glutamatergic system play an important role in memory function (Collingridge *et al.*, 2004). In addition, glutamatergic function in memory is related with the synaptic plasticity including LTP especially in the hippocampus. A high mobility of glutamatergic receptors have been reported between intracellular site and the plasma membrane, where glutamate receptors are required for the induction and gamma-aminobutyric acid (GABA) receptor for modulation of this plasticity (Collingridge *et al.*, 2004). In fact, the new approach to pharmacological treatment of AD is focused on the glutamatergic system. This new pharmacological strategy, aims to modulate glutamatergic neurotransmission by the drug called memantine.

Memantine (1-amino-3,5-dimethyl-adamantance) is a low-affinity, non-competitive N- methyl- D-aspartate (NMDA) receptor antagonist, which block NMDA open channel competing with Mg^{2+} to bind in the channel pore (Chen *et al.*, 1992). It was initially approved by the Food and Drug Administration (FDA) in 2003 to treat moderate to severe AD in USA and it is used commonly worldwide nowadays in the treatment of AD. Indeed, clinical trials have shown that memantine improves cognitive functions compared to placebo in patients with probable AD diagnosis (Kirby *et al.*, 2006; Reisberg *et al.*, 2003). Aβ injections in mice under memantine treatment showed less memory impairment and neuronal degeneration suggesting a neuroprotective effect (Miguel-Hidalgo *et al.*, 2002). Some authors have hypothesised that the decrease of glutamate NMDA receptor functionality might contribute to neurodegeneration, therefore opposing to the beneficial effects of the memantine treatment. Opposing this hypothesis, several studies have shown that glutamate up-take blockers lead to neuronal

death, while suggesting that glutamate receptors antagonist could prevent neuronal death (McBean and Roberts, 1985; Rothstein *et al.*, 1993). In addition, there are more evidences in favour of the use of memantine than opposed to them (Walton and Dodd, 2007).

1.1.2.3. Serotonergic alteration in AD

Patients with AD-type pathology also show a decrease in central and peripheral serotonergic (5-HT) neurotransmission as revealed by reduced concentration of 5-HT in cerebrospinal fluid (Tohgi *et al.*, 1992; Tohgi *et al.*, 1995), and platelets (Mimica *et al.*, 2008; Muck-Seler *et al.*, 2009). Positron emission tomography (PET) and magnetic resonance imaging (MRI) studies further corroborated AD-related decrease of 5-HT neurotransmission in the CNS (Hasselbalch *et al.*, 2008; Kepe *et al.*, 2006; Meltzer *et al.*, 1998). Post-mortem analysis of AD brains confirmed decrease in extracellular levels of 5-HT, of its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and decrease in expression of 5-HT receptors in various brain regions including the neocortex and the hippocampus (Chen *et al.*, 1996; Garcia-Alloza *et al.*, 2005; Lai *et al.*, 2002; Lorke *et al.*, 2006; Nazarali and Reynolds, 1992; Palmer *et al.*, 1987; Truchot *et al.*, 2008). Impairment of 5-HT neurotransmission in AD is consistent with loss of 5-HT neurones from raphe nuclei with associated loss of cortical 5-HT projections (Chen *et al.*, 2000a; Yamamoto and Hirano, 1985).

Regarding the clinical symptoms, AD-related deficits in 5-HT neurotransmission are associated with accelerated cognitive decline determined by Mini-Mental State Examination (MMSE); (Lai *et al.*, 2002) as well as with behavioural symptoms including depression, aggressive behaviour and psychosis (Garcia-Alloza *et al.*, 2005). Altered 5-HT neurotransmission correlates with the severity of dementia and therefore can be considered responsible for the cognitive and non-cognitive abnormalities associated with AD (Garcia-Alloza *et al.*, 2005; Lai *et al.*, 2002).

Reduced 5-HT neurotransmission in the CNS correlates with deficient cognitive function in AD patients (Newhouse *et al.*, 2002; Porter *et al.*, 2000). **In this line of evidence, recent data form our lab has shown an increase of serotonergic fibres and terminals in the hippocampus of the 3xTg-AD mouse model, suggesting an imbalanced serotonergic transmission in AD (Noristani *et al.*, 2011; Noristani *et al.*,**

2010). In fact, treatment with selective inhibitors of serotonin reuptake increases CSF concentration of 5-HT and improves cognitive function and memory in patients with dementia of AD type (Marksteiner *et al.*, 2003; Mossello *et al.*, 2008; Mowla *et al.*, 2007; Tohgi *et al.*, 1995). Drugs acting at specific 5-HT receptors have also been suggested as therapeutic agent to enhance cognitive function in AD (Terry *et al.*, 2008).

1.1.2.4. Cannabinoids in AD

Although not much is known about the cannabinoid system in pathology, the role that cannabinoids have seems to be more involved in protection rather than failure. Indeed, cannabinoid agonists like anandamide and noladin have shown to be neuroprotective against Aβ damage *in vitro*, probably mediated by CB1 receptor and MAPK-mediated mechanism (Milton, 2002). Anandamida protect neurons from inflammatory response (Fernandez-Ruiz *et al.*, 2007) and cannabinoid receptor CB2 was localised in reactive microglia in pathological condition while other study showed that CB2 activation inhibits Aβ -induced microglia activation as shown by the prevention of morphological changes and the reduction of the production of TNF-α and cytokines (Ramirez *et al.*, 2005). However, these results are in contrast to the *in vivo* studies. Cognitive impairment and microglial activation were prevented in rats after Aβ i.c.v. injection (Ramirez *et al.*, 2005). All these studies suggest that cannabinoids can ameliorate pathological response and be instrumental in the protection of the CNS in neurodegeneration (Campillo and Paez, 2009).

1.2. Anatomy of AD regions

AD clinical symptoms are indisputably related to the brain regions that are affected in the disease. In this sense memory related structures such as the septo-hippocampal complex, entorhinal cortex, amygdala, the nucleus basalis of Meynert, and in particular the hippocampus are deeply affected (Braak and Braak, 1991). The work of this thesis is specifically focused on the hippocampus due to the central role it plays in the disease, being one of the key regions regarding the long-term declarative memory establishment in mammals (Lavenex and Amaral, 2000; Nadel, 1995) working as a storeroom for higher-order cortices (Squire and Alvarez, 1995) and one of the first and most vulnerable regions affected in AD.

1.2.1. Entorhinal cortex

The entorhinal cortex (EC) is located at the rostral end of temporal lobe and is deeply involved in mnesic processes by establishing the cortico-hippocampal circuits (Yeh *et al.*, 2011). The EC is divided into superficial (I-III) and deep layers (IV-VI) and show differential anatomical and functional organisation (Suzuki and Amaral, 1994; Witter and Amaral, 2004). The superficial layers, receive most of the of intracortical information and are the major output source, in particular layer II, to the hippocampus, whereas the deep layers are mainly responsible for the projections to cortical regions (Suzuki and Amaral, 1994; Witter and Amaral, 2004; Yeh *et al.*, 2011). More specifically, neurons of layer II project to the middle and outer molecular layer of the dentate gyrus (DG) and send collaterals to the hippocampal CA2 and CA3 fields (Suzuki and Amaral, 1994; Tamamaki and Nojyo, 1993; Witter *et al.*, 1989); (see also Fig. 6A). Layer III projects mainly to the CA1 and subiculum, which in turn projects to the layer V of the EC (Naber *et al.*, 2001). Functionally, the EC is involved in the process of working memory (Fransen, 2005; McGaughy *et al.*, 2005; Ranganath and D'Esposito, 2001), is also essential for memory consolidation (Remondes and Schuman, 2004) and spatial navigation memory (Fyhn *et al.*, 2004; Yeh *et al.*, 2011).

1.2.2. Prefrontal cortex

Also known as PFC, is located at the anterior part the frontal lobe and it is widely connected to different brain regions such as hypothalamus and hippocampus (Forbes and Grafman, 2010). Importantly, only forms reciprocate connections with basal ganglia and cortical motor areas. Functionally, it is essential for setting and achievement of long-term goals. The PFC is divided into dorsolateral (DLPFC), ventrolateral (VLPFC), dorsomedial (DMPFC), ventromedial (VMPFC) and orbifrontal (OFC) regions (Forbes and Grafman, 2010). Medial and orbitofrontal regions are involved in integrating emotional and viscerally arousing information. They receive projection from amigdala, hippocampus and most of other limbic structures, and also reply information to the DLPFC. This one in turn, is believed to be involved in the execution of movement and planed behaviour as well as integration of sensory information (Barbey *et al.*, 2009). It is bidirectionally connected to basal ganglia, premotor cortex, the cingulate cortex and indirect input from substancia nigra cerebellum via mediodorsal and ventrolateral thalamic nuclei (Fuster, 1997).

1.2.3. Nucleus basalis of Meynert

The nucleus basalis of Meynert (nbM) is deeply affected in AD as was described in 1982 by Whitehouse (Whitehouse *et al.*, 1982). NbM is included in the cholinergic system and bears a dramatic neuronal loss. Most of this cholinergic neurons project to the hippocampus. The nbM also provide efferents to the amigdala, neocrotex and the rest of cortical areas (Schliebs and Arendt, 2006).

1.2.4. The Hippocampus

The hippocampus term emerged from its similarity with the seahorse (Fig. 5). From very early times, the hippocampus has drawn the attention of scientist perhaps for the well-defined structure of the hippocampus. Its so well stratified architecture and its role in memory have made this region extremely attractive to uncover many of the enigmas of the complex brain wiring and memory function.

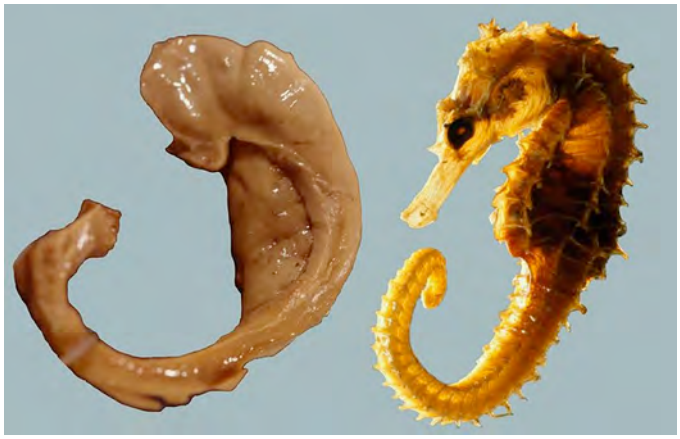


Figure 5. *Illustrative photograph showing the appearance similarities of a human hippocampus and fornix with a seahorse.*
(From László Seress' 1980)

The hippocampus is located in the medial temporal lobe of the forebrain and is divided into four main subfields, CA1, CA2 and CA3 (CA: *cornu ammonis*); (Lorente de Nó, 1933; Rapp and Gallagher, 1996; Witter and Amaral, 2004) and an adjacent subfield named the dentate gyrus (DG; see Fig. 6). The CA subfields' main layer, pyramidal cell layer, is formed by the pyramidal cells and also other types of neurons like basket, axo-axonic, bistratified and radial trilaminar cells (Witter and Amaral, 2004); (Fig. 6A). Pyramidal neurons extend apical and basal dendritic trees to the over- and underlying layers called the stratum radiatum and the stratum oriens respectively (Fig. 6B). In addition a number of interneurons, horizontal trilaminar and O-LM cells as well as basket and bistratified cells, populate these two strata (Witter and Amaral, 2004). Other layer, the stratum lacunosum moleculare, occupies the most superficial

portion of the CA areas, right below the stratum radiatum, which receives massive thalamic innervation mainly from the nucleus reuniens (Herkenham, 1978; Witter and Amaral, 2004; Wouterlood *et al.*, 1990). The apical dendritic tree of the pyramidal neurons also reach in this strata (Amaral *et al.*, 2007).

DG also has a highly organised trilaminar structure that consists of the granular and the molecular cell layers and the hilus; Lorente de Nó originally named the latter as CA4, Fig. 6A). The granular cell layer is densely packed with somata of different types of neurons. Dentate granule cells, unlike pyramidal neurons in CA areas, only extend the dendritic tree apically to the molecular layer while axons are basally projected through the hilus reaching the CA3; known as mossy fibres after Ramón y Cajal described them in detail (Amaral *et al.*, 2007); (see also Fig. 6C). Other type of neuron in the granule cell layer is the dentate pyramidal basket cell. These inhibitory interneurons are located in the sub-granular cell layer and extend their dendritic tree apically to the molecular layer and basally to the hilus. The basket plexus of these neurons are GABAergic and form inhibitory contacts with the proximal dendrites and cell bodies of granule cells, being one single dentate pyramidal cell capable of modulating 10,000 granule cells (Amaral *et al.*, 2007; Seress and Pokorny, 1981; Witter and Amaral, 2004). In addition, the DG has the unique distinctiveness of the adult neurogenesis, which takes place in the subgranular layer, integrating newly generated neurons in the hippocampal network (Treves *et al.*, 2008).

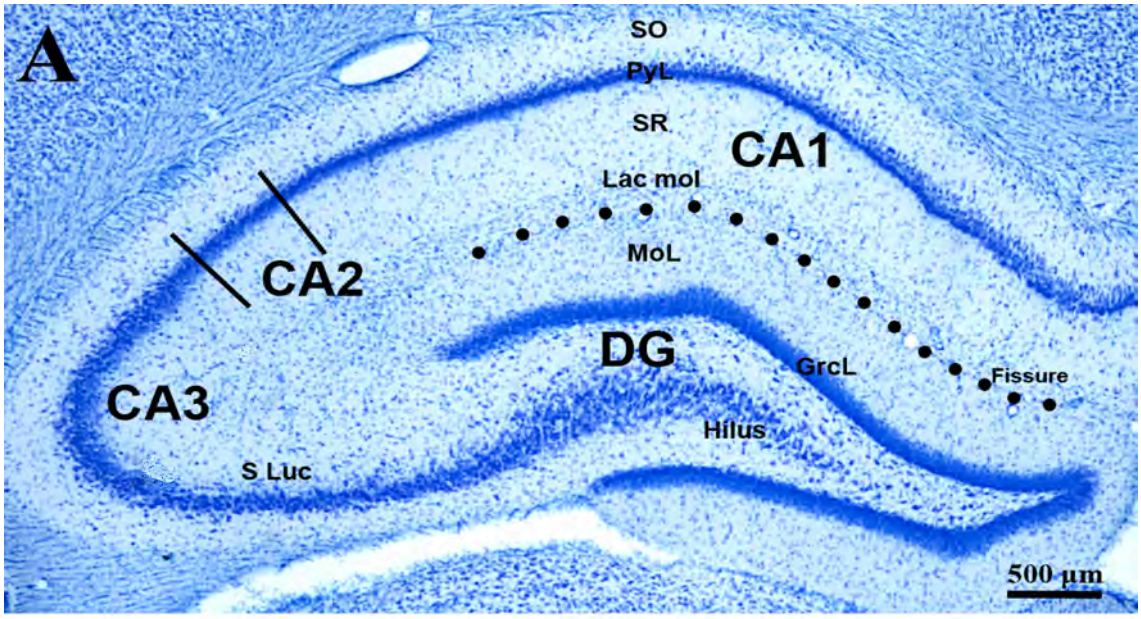
Very interestingly, all hippocampal areas, together with other parahippocampal regions (entorhinal cortex and subiculum), are interconnected describing the so-called tri-synaptic circuit (Fig. 6C). In this way, fibers coming from the layer II of the entorhinal cortex (Scharfman, 2007) project to the dendritic trees of the medial molecular layer of the DG neurons (Ohm, 2007), via the perforant path, which in turn, send projections to the stratum lucidum reaching the neurons located in the CA3 and making strong and sparse synapses near the pyramidal cell somata (Treves *et al.*, 2008). Finally, the CA1 region receives input from the CA3 and sends afferent fibers towards the subiculum (Rapp and Gallagher, 1996). This network is characterised by long-term potentiation capabilities, which set the base for cognitive process (Collingridge and Lester, 1989).

In this thesis we have focused our work in the DG and CA1 areas of the hippocampus. The anatomical organisation of the hippocampus makes the DG, and in particular the molecular layer, very important as it receives direct input from the entorhinal cortex, the first brain region affected in AD, and initiates the tri-synaptic circuit potentially influencing the hippocampal function. One could say that the molecular layer is the main entrance to the hippocampus. In addition, many asymmetric, excitatory, contacts take place in the molecular region, having a special relevance for LTP, LTD and in summary for cognition and memory. In addition, excitatory/glutamatergic neurotranmission occupies a central role within the excitotoxic hypothesis of AD pathology (Choi, 1992). CA1 in turn, is involved in associative memory and also shows the earliest pathological burden within the hippocampus displaying aberrant Aβ load before other hippocampal areas.

Therefore, considering the anatomical organisation, the functional involvement in cognition and the affection during the progression of AD, the importance of the investigation of DG and CA1 areas is unquestionable.

Hippocampal architecture is ruled by neuronal distribution and although neurons are unanswerably essential in hippocampal function, glia cells also have a vital role in it. The main glial cells in the hippocampus are astroglia and microglia, and their distribution in the hippocampus is truly extensive, covering virtually every corner of the hippocampal parenchyma (Olabarria et al., 2010; Rodriguez et al., 2009); (see also Fig. 2A and 2B). The only exceptions are the main neuronal layers; pyramidal and granular cell layers, where the dense arrangement of neuronal cell bodies hardly allows space for other cells. However, glial processes are commonly located in these layers (Olabarria *et al.*, 2010, 2011). Glial interactions with neurons, synapses and extracellular space are decisive in the CNS and will the main focus of this thesis.

Figure 6 (→). (A) *Toluidine blue stained rat hippocampus showing the distribution of different layers.* ***Key:*** *DG: Denate Gyrus; Grcl: Granular cell layer; Mol: Molecular cell layer; in: Inner molecular layer; med: medial molecular layer; out: outer molecular layer; S Luc: Stratum Lucidum; Lac mol: Lacunosum Moleculare; SR: Stratum Radiatum; SO: Stratum Oriens (adapted from Rodríguez- unpublished photograph)* ***(B)*** *Scheme of the hippocampus illustrating the main types of neurons of the principal layers: Granular cell neurons populate the Grcl extending the dendritic tree to the MoL and projecting mossy fibres the pyramidal neurons of the CA3. Both pyramidal neurons in the CA3 and CA1 display apical and basal dendritic trees (Adapted form Rolls, 2009).* ***(C)*** *Drawing of the neural circuitry of the young rabbit hippocampus showing the main subfields and the try-synapse loop (Adapted from Santiago Ramón y Cajal. 1911).*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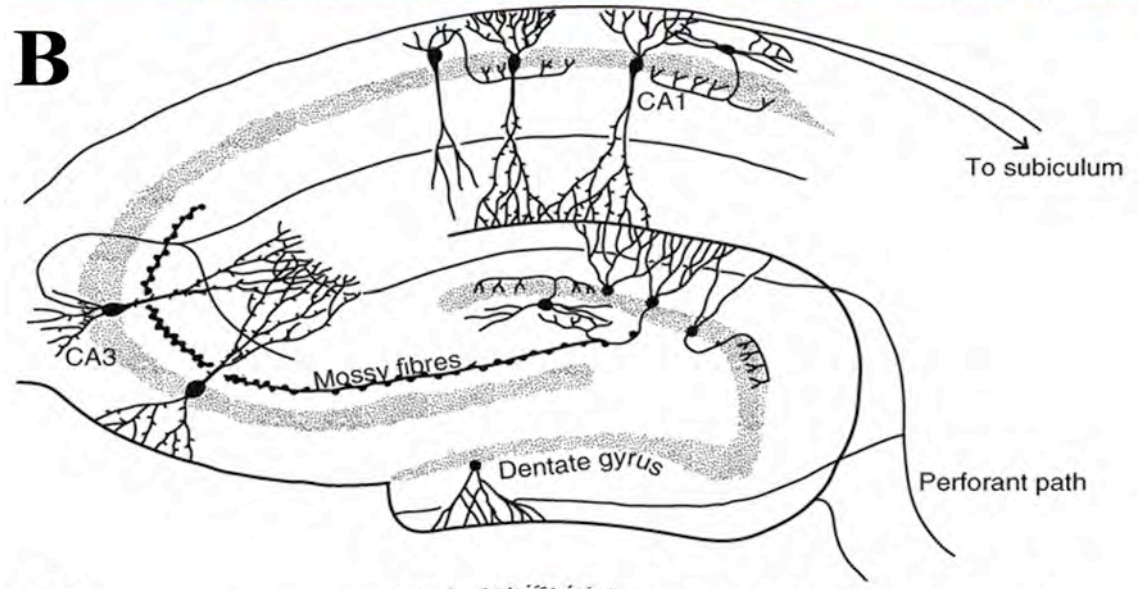
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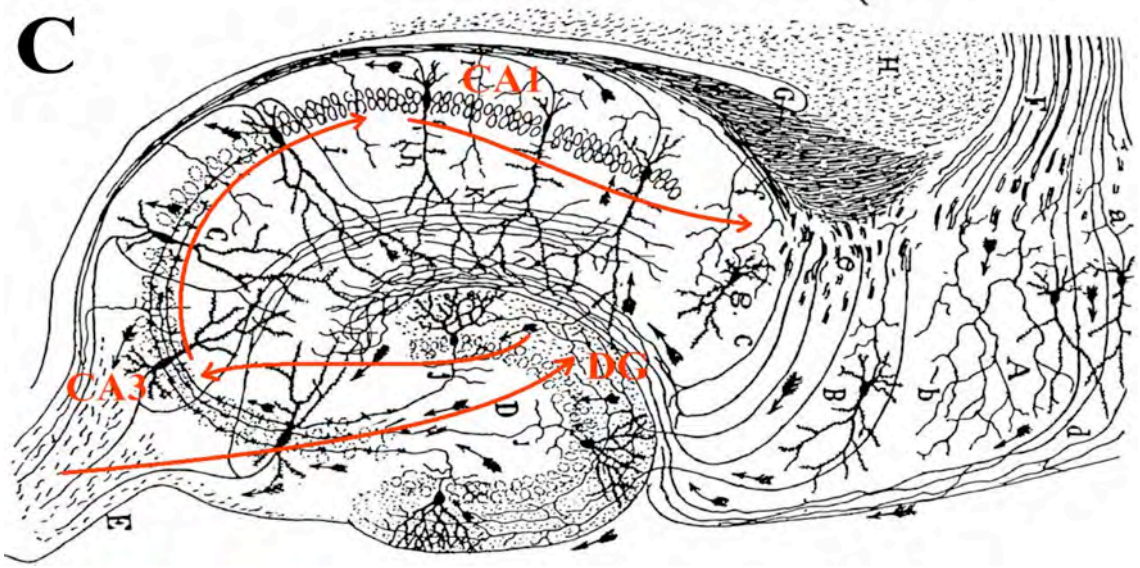
C
CA1
CA3
DG

2. ANIMAL MODEL OF AD

Different strategies have been used throughout biomedical research in order to find relevant animal models to approach the understanding of the disease and offer potential solutions for its delay and hopefully cure. However, no animal suffers AD (Toledano and Alvarez, 2004). AD researchers have been using distinct animal models, which mimic one or more biochemical, histo-pathological and behavioural abnormalities associated with AD (Cassel *et al.*, 2008; Gotz *et al.*, 2004b). In this direction, a wide range of experimental animal models has been developed for better deciphering disease progression. The first animal models of AD were normal aged or senile animals (Bertoni-Freddari *et al.*, 1986; Biegon *et al.*, 1986), usually monkeys (Sani *et al.*, 2003), and rats that exhibited cholinergic involution and deficits in synaptic plasticity associated with amyloid deposition. The use of these models carries the need to consider the parallel involution of other neurotransmission systems regulating cortical functions (Arranz *et al.*, 1996).

2.1. Lesion animal models of AD

Since the cholinergic theory explained the senile memory dysfunction (Bartus *et al.*, 1982) and the AD-related degeneration of cholinergic basal forebrain neurons (Davies and Maloney, 1976; Whitehouse *et al.*, 1981), several AD animal models with deficit in the cholinergic system have been created (Toledano and Alvarez, 2004); (see also Table 1). Within AD models, cholinergic neuron lesions or dysfunctions induced animal models have been the most commonly used ones, especially models with lesions in the nucleus basalis magnocellularis (nbm) of rodents, which is the equivalent of nbM in man (Toledano and Alvarez, 2004). Lesion models, offer the possibility to study the differences of the structure, function and behaviour of the cholinergic systems in young and aged animals (Pepeu and Marconcini Pepeu, 1994; Wellman and Pelleymounter, 1999). Nevertheless, it is very important to take into account the exact type of lesion produced and its evolution, if meaningful results are to be obtained (Toledano and Alvarez, 2004).

Subsequently, basal forebrain cholinergic lesion models were developed using different methods (Toledano and Alvarez, 2004). Some models were created by non-

selective cholinergic methods using toxins which act on different glutamatergic receptors such us NMDA, ibotenic acid (potent AMPA receptor agonist), quisqualic acid, α -amino-3 hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and NMDA acids. Neuronal death or dysfunction is carried out by changes in the Ca^{2+} intake. Other non-specific cholinergic neurotoxins have been also used including quinolic acid (neuroexcitatory tryptophan metabolite) (Boegman *et al.*, 1985) and other vinca alkaloid substances (Di Patre *et al.*, 1989a; Di Patre *et al.*, 1989b). The other big group of basal forebrain cholinergic lesion models are those produced using what is called cholinotoxins. These models have been considered the closest to AD as was accepted the non-cholinergic neuron preservation (Toledano and Alvarez, 2004). AF64A cholinotoxin, which binds to the high affinity choline uptake system, has different effects depending on the dose and region of the brain where is administrated. When high doses are used (more than 2 nmol), the entire cholinergic cells are affected, while when low doses are administrated, only cholinergic neurons with long projections are damaged, specially those in the septum, nmb and the diagonal band of Broca (Wiley, 1992). In addition, the induced lesion varies throughout the different regions of the forebrain, to the extent that cholinergic neuronal death, short-term cholinergic dysfunctions and cholinergic recovery can all be performed in different models of this kind. The need for a more selective toxin led to the development of immunotoxins such us 192IgG-saporin, which binds selectively and irreversibly to low affinity nerve growth factor receptor and as consequence interrupts protein synthesis in cholinergic neurons (Wiley, 1992) leading to the impairment of the cholinergic neurotransmission.

Interestingly, other studies have revealed, for example, that many of the learning and memory impairments traditionally attributed to the cholinergic corticoparietal system are not due to degeneration of the cholinergic neurons of the nucleus basalis of Meynert (nbM), but instead may be due to damage of more rostral elements of the cholinergic basal forebrain system (Muir, 1997). Other lesion models have been also developed including ethanol intake in the drinking water. This method is based on the evidences of neuropsychological studies that show the similar cognitive impairment takes place in early AD and Korsakoff syndrome (Moss *et al.*, 1986), with loss of cholinergic neurons in nucleus basalis (Arendt *et al.*, 1983). In addition, the administration of β -amyloid directly into nbm has been used to produce AD models, which results in dysfunctions in the basalcortical cholinergic neurons and alterations in cortical acetylcholine release (Casamenti *et al.*, 1998; Giovannini *et al.*, 2002). It is important to note that lesioned

animals do not exhibit pathological hallmarks (plaques and tangles) associated with AD that is critical for understating the relation between AD-related pathology and cognitive dysfunction (Gotz *et al.*, 2004a; Gotz *et al.*, 2004b).

2.2. Single and Double Transgenic animal models of AD

In the search of the better understanding of the role of amyloid plaques and neurofibrillary tangles in AD, experimental transgenic animal models have been developed, which replicate some neuropathological features of AD (Gotz *et al.*, 2004b); (see also Table 1). Firstly, simple transgenic models were created harbouring single mutations. The first APP transgenic animal with AD-like neuropathology was developed in 1995 by Games et al. This model was called PDAPP and showed many pathological features of AD including extracellular amyloid deposits, neuritic dystrophy and astrocytosis (Games *et al.*, 1995). Memory impairments were reported but there was no correlation between this impairment and amyloid deposition degree (Gotz *et al.*, 2004b). Hsiao et al (1996) developed the transgenic APPswe mutation (Tg2576 mice) using prion protein vector (Hsiao *et al.*, 1996). This mutation consists of a double base-pair substitution at codon 670 and 671 and results in abundant Aβ plaque depositions (Hsiao *et al.*, 1996). Memory and learning impairment in Tg2576 transgenic mice is patent at 9 months of age (Hsiao *et al.*, 1996). Following the discovery of Swedish mutation in AD, Novartis researchers developed the Swedish double APP mutation (APP23) mouse models with a 7-fold increased expression of APP (Sturchler-Pierrat *et al.*, 1997). In contrast to PDAPP and Tg2576 models, APP23 revealed a 14% loss of hippocampal CA1 pyramidal neurons compared with controls (Gotz *et al.*, 2004b). Subsequent studies led to development of other APP transgenic mice with amyloid pathology including APP751SL and APPV717F (Blanchard *et al.*, 2003; Dodart *et al.*, 2000); (see also Table 1).

The discovery of Presenilin-1 (PS-1) and presenilin-2 (PS-2) as most common mutations identified in familial cases of AD, triggered the development of multiple transgenic mouse models displaying these mutations (Gotz *et al.*, 2004a; Gotz *et al.*, 2004b). In addition, to decipher the role of genetic risk factors in APP processing and memory, double transgenic have been created. Double PS-1 X APP transgenic mice display accelerated deposition of amyloid plaques and memory deficits without tangle formation (Holcomb *et al.*, 1998; Richards *et al.*, 2003). Borchelt and colleagues

crossbred PS-1 transgenic mice (expressing mutant proteins M146L, A246E or PS1DE9) with APP transgenic mice expressing Swedish mutation (Borchelt *et al.*, 1997). Similarly, PSAPP double transgenic model was generated by cross breeding PS-1 transgenic mice (expressing mutant protein M146L or M146V) with Tg2576 transgenic mice (Holcomb *et al.*, 1998). Other double transgenic model (PS2APP mice, generated by crossing PS2 (N141I) and APPSwe, also display accelerated amyloid deposition concomitant with age-dependent spatial learning and memory deficits although no tangle formation was evident (Richards *et al.*, 2003). APP double transgenic mice (Tg-CRND8; generated by combined insertion human APP695 with Swedish and Indiana mutations in the cosSHaPrP vector) also display early and aggressive amyloid deposition associated with learning impairment that becomes evident at 3 months of age (Chishti *et al.*, 2001).

Mutation in Tau proteins has been linked with neurodegeneration associated with frontotemporal lobe dementia (Gotz *et al.*, 2004a; Philipson *et al.*, 2010). Transgenic mouse models also have been developed harbouring tau related mutations. The first model was created in 1995 (Gotz *et al.*, 1995) when pathogenic mutations in tau had not yet been identified (Gotz *et al.*, 2004a; Gotz *et al.*, 2004b). This model does not show neurofibrillary tangle (NFT) pathology, nevertheless, somatodendritic localisation and hyperphosphorylation of tau is observed. Three years after the development of the first tau transgenic mouse model, the identification of the pathogenic mutations of tau in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) led to development of different transgenic mice models exhibiting not only NFT formation in neurons (Gotz *et al.*, 2001b; Gotz *et al.*, 2001c; Lewis *et al.*, 2000; Tatebayashi *et al.*, 2002) but also reactive glia cells (Gotz *et al.*, 2001a; Gotz *et al.*, 2001b; Higuchi *et al.*, 2002). The Tau301L mutation was one of the first FTDP-17 mutations that had been identified in human patients (Hutton *et al.*, 1998) and is the most common mutation linked with frontotemporal dementia (Lewis *et al.*, 2000). Transgenic mice with Tau301L mutation display NFT in brain and spinal cord; more specifically in the motor neurons of the spinal cord, hence reducing their population by half in spinal cord (Lewis *et al.*, 2000).

Most of the transgenic animal models mentioned so far neglects to express both histopathological features of AD (namely Aβ and NFT); (Gotz *et al.*, 2004a; Gotz *et al.*, 2004b). In addition, selective and drastic neuronal loss has not been achieved in any tau

transgenic models, not taking into account the loss of motor neurons in spinal cord as this is not an AD-related feature (Gotz *et al.*, 2004a; Gotz *et al.*, 2004b). In order to improve the AD models, triple-transgenic AD model was developed.

Table 1. *Neuropathology in the main AD animal models. Modified from (Rodriguez and Verkhratsky, 2011)*

Lesion and transgenic mouse, rat and primate models	Neuropathology	Reference
Ageing	Cholinergic involution and amyloid deposition	(Sani *et al.*, 2003) (Fischer *et al.*, 1992) (Michalek *et al.*, 1989)
Electrolytic lesion	Neuronal death	(Lescaudron and Stein, 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)
Specific toxins (AF64A, 192Ig-G saporin)	Cholinergic neuronal death	(Arendt, 1994) (Waite *et al.*, 1995) (Chrobak *et al.*, 1988) (Hanin, 1996)
β-Amyloid	Cholinergic dysfunction	(Wiley, 1992; Wiley *et al.*, 1991) (Giovannini *et al.*, 2002) (Pavia *et al.*, 2000)
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)
$PS1_{M146L}$	Diffused plaques	(Blanchard *et al.*, 2003)
PS-1/APP_{Swe}	Plaques	(Borchelt *et al.*, 1997)
PSAPP (PS-1/Tg2576)	Plaques	(Holcomb *et al.*, 1998)
Tg-CRND8 (APPSwedish 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)
APPSwedish and $PS1^{M146L}$	Plaques	(Janus *et al.*, 2000)
K670N/M671L and V717F	Plaques	(Janus *et al.*, 2000)
TgAPPsw and PS1 M146L	Plaques	(Takeuchi *et al.*, 2000)
ALZ7	Tangles	(Gotz *et al.*, 1995)
Tau^{P301L} (4R,2-,3-)	Tangles	(Lewis *et al.*, 2000)
P301L	Tangles	(Gotz *et al.*, 2001a)
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 knocked out	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 and Janus, 2007)
Tg2576×JNPL3 (APP_{SWE})	Plaques and Tangles	(Lewis *et al.*, 2001)
Tg2576 and VLW	Plaques and Tangles	(Ribe *et al.*, 2005)
3xTg-AD	**Plaques and Tangles**	**(Oddo *et al.*, 2003b)**
Tg478	None	(Flood *et al.*, 2009)
Tg1116	None	(Flood *et al.*, 2009)

2.3. Triple transgenic mice model of AD (3xTg-AD)

As mentioned previously, in an attempt to better represent the major neuropathological features of AD, the triple transgenic (3xTg-AD) model was generated (by Salvatore Oddo and Frank la Ferla in 2003); (Oddo *et al.*, 2003a; Oddo *et al.*, 2003b). The 3xTg-AD transgenic mice harbour mutant genes for amyloid precursor protein (APPSwe), PS1M146V and tau Tau301L displaying temporal and region specific Aβ and tau pathology that closely resembles that seen in the human AD brain (Oddo *et al.*, 2003b). The 3xTg-AD model was generated by microinjections of two independent transgenes encoding human APPSwe and human TauP301L into a single-cell embryo from PS1M146V knockin mice (PS1-KI); (Oddo *et al.*, 2003b). This technique has the advantage of minimising genetic interactions (Gotz *et al.*, 2004b) and facilitates the establishment and maintenance of the mouse colony as well as reduces the independent grouping of the mutations the next generations (Oddo *et al.*, 2003a; Oddo *et al.*, 2003b). The 3xTg-AD mouse model is considered as one of the most relevant animal models in which to study AD that gives the opportunity to understand interactions between Aβ and NFT and their effect in synaptic functions (see Table 1).

The 3xTg-AD mice develop an age-dependent and progressive neuropathology that includes plaque and tangle pathology. Aβ deposits initiate in the cortex and progress to the hippocampus with aging, whereas tau pathology is first apparent in the hippocampus and then progresses to the cortex (Oddo *et al.*, 2003a). Despite equivalent over-expression of human APP and tau, Aβ pathology precedes typical indications of tau pathology such as conformational or hyperphosphorylation changes in the tau protein (Oddo *et al.*, 2003a); these results are consistent with the amyloid cascade hypothesis, which predicts that Aβ deposition is the earliest pathological trigger of AD. In addition, the 3xTg-AD transgenic mice also display selective loss of nicotinic receptors (Oddo *et al.*, 2005), which is consistent with post mortem studies of AD brain (Burghaus *et al.*, 2000; Wevers *et al.*, 2000). Furthermore, we have also recently reported reduced adult neurogenesis in 3xTg-AD mice neurogenesis (Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2009a; Rodriguez *et al.*, 2011), which may also contribute to deficits in learning and memory functions observed in this model.

Astrogliosis is a prominent feature of AD neuropathology (Beach and McGeer, 1988; Beach *et al.*, 1989), which was recognised by Alois Alzheimer early during the

description of the disease (Alzheimer, 1910); (see also Fig. 1). Double labelling in brain sections from 3xTg-AD mice using antibodies against Aβ and the astrocytes-specific marker GFAP revealed reactive astrocytes adjacent to extracellular Aβ deposits (Oddo *et al.*, 2003a; Oddo *et al.*, 2003b).

Behavioural tests have shown that 2 months old 3xTg-AD mice, which do not show the typical histopathology yet (Oddo *et al.*, 2003b), display no cognitive impairment (Billings *et al.*, 2005). This, therefore, confirms that 3xTg-AD mice are not born with the cognitive impairment. Cognitive deficits in the 3xTg-AD model show good correlation with the accumulation of intraneuronal Aβ (Carroll *et al.*, 2007; McKee *et al.*, 2008). At 6 months of age 3xTg-AD mice display the earliest sign of cognitive impairment in the form of delayed learning compared to control mice (Billings *et al.*, 2005). The 3xTg-AD mice need longer time to learn the Morris water maze tasks (MWM), which suggests that 6 months old 3xTg-AD mice have learning dysfunctions related to an incapacity to retain the learnt information from day to day (Billings *et al.*, 2005). This cognitive impairment increases as the disease progressed (Billings *et al.*, 2005). Other behavioural studies using inhibitory avoidance (IA) and Barnes maze (BM) tests also revealed significant impairment in spatial memory, long-term memory and contextual learning in 3xTg-AD mice by 6 months of age (Billings *et al.*, 2005; Caccamo *et al.*, 2006; Clinton *et al.*, 2007; McKee *et al.*, 2008). Impairment in objective memory is evident in 3xTg-AD mice at 9 months of age (Clinton *et al.*, 2007). Longitudinal studies using MWM revealed age-related progressive decline in learning and spatial memory from 6 to 18 months of age in 3xTg-AD mice correlating with increase in Aβ and tau neuropathology (Billings *et al.*, 2007; Blurton-Jones *et al.*, 2009; Clinton *et al.*, 2007; Gimenez-Llort *et al.*, 2007; Halagappa *et al.*, 2007; Martinez-Coria *et al.*, 2010).

3. GLIAL CELLS

In 1858, Rudolf Virchow introduced glia for the first time in a lecture entitled *"Rückenmark und Gehirn"* (Spinal cord and the brain); (Kettenmann and Verkhratsky, 2008). Here, glial cells, now also known as neuroglia, were presented as passive neural elements that were holding neurons together. This concept of "cells filling the space not occupied by neurons" (Weigert, 1895) further remained in time. In fact, the name neuroglia comes from "nervenkitt" or nerve-cement (Kettenmann and Verkhratsky, 2008; Virchow, 1856) clearing up any doubt about their historical concept. However, this original connective role of glial cells, although rudimentary, was not completely wrong. In fact, for instance, glial cells present in grey matter define the architecture of the tissue by their non-overlapping domains, covering all neural elements within the same (Bushong *et al.*, 2002) and execute most of their functions that nowadays we know they perform.

Glial cells outnumber neurons (up the 90% in human cortex) and are present in both white and grey matter (Rodriguez et al., 2009); (see also Fig. 2A and 2B). They represent the non-excitable neural cell population and perform a wide variety of functions ranging from structural to homeostatic and from developmental to immunological, highlighting their essential role in the nervous system (Verkhratsky and Butt, 2007).

Glial cells are classified in two main groups that are macroglia and microglia (Lin and Bergles, 2004). Macroglia is the most heterogeneous of these two cell groups and includes astrocytes, oligodendrocytes, NG2 glia cells and other minor glia cells populations like ependimocytes, radial glia and Schwann cells, whilst microglia (on its own) constitutes the immune system of the CNS (Verkhratsky and Butt, 2007). The work of this thesis is focused on astrocytes and microglia. However, it is worth mentioning the unique nature of oligodendrocytes.

Oligodendrocytes (*oligo,* few; *dendro,* process and *cyte,* cell: cell with few processes), are present almost exclusively in white matter (Butt, 2006; Fields, 2008; Sherman and Brophy, 2005; Sherman *et al.*, 2005) and enwrap long segments of axons with a multilayer sheath of extended cell membrane: the myelin sheath. Phonotypical,

adult oligodendrocytes are heterogeneous and classified into four types (I-IV) depending on diameter of axons they enwrap (Butt and Berry, 2000). They myelinate several axons simultaneously, define Nodes of Ranvier and enable saltatory impulse propagation in the CNS (Nave, 2010). These properties make oligodendroglia essential in the CNS and their failure will inevitably lead to severe diseases like multiple sclerosis, psychiatric disorders (Matute, 2010; Rodriguez *et al.*, 2011) and different types of leukodystrophies like Alexander disease (Tian *et al.*, 2010; Towfighi *et al.*, 1983). A high percentage of AD patients exhibit evidence of white matter degeneration with severe loss of oligodendrocytes by apoptosis (Brown *et al.*, 2000). In addition, *in vitro* evidences show that Aβ can inflict damage (Xu *et al.*, 2001) and increase the vulnerability to glutamate toxicity in oligodendrocytes (Pak *et al.*, 2003). This is further supported by an *in vivo* experiment in which amyloid $\beta_{1\text{-}42}$ was injection in white matter causing axon disruption and myelin damage as well as oligodendrocytes loss (Jantaratnotai *et al.*, 2003; Rodriguez *et al.*, 2011). Although it is not fully understood, there are evidence suggesting that amyloidosis and Ca^{2+} dyshomeostasis might be the underlying mechanisms (Mattson and Chan, 2003; Matute, 2010). Studies on AD mutant mice (Preselin-1 knock-in) have show to be more prone to glutamate excitotoxicity and when exposed to cuprizone, demyelinising agent, show exacerbated myelin damage and impoverished cognitive capabilities (Pak *et al.*, 2003). Recent findings on the 3xTg-AD have shown myelination alterations precede amyloid and tau pathology (Desai *et al.*, 2009; Rodriguez *et al.*, 2011). This suggests myelin and oligodendroglia alterations might be involved in the development of the disease form early stages (Burns *et al.*, 2005).

3.1. Astroglia

Astrocytes were first observed by Otto Deiters in 1865 and named by Michael von Lenhossek in 1893 (Lenhossek, 1893). Astrocytes, also known as astroglia, have been classically defined by their star-like morphology and expression of glial fibrils, and they were originally considered as part of the connective tissue (Wang and Bordey, 2008); (see also Fig. 2A and 2B). However, nowadays we have understood the diverse nature of astrocytes and we are able to classify them based on their anatomical, antigenic and physiological properties. In fact, astroglia is a very heterogeneous group of glia cells under which there are included many different types of cells (Kimelberg, 2004). They are, among others, retinal Müller glia (Newman and Reichenbach, 1996), cerebellar Bergmann glia (Chan-Palay *et al.*, 1974) and tanycytes, being protoplasmic and fibrous astrocytes the main two types which are present in grey (Andriezen, 1893) and white matter (Kölliker, 1889) respectively (Kettenmann and Verkhratsky, 2008). The fibrous astrocytes exhibit a straight morphology with few branches, overlapping with adjacent astrocytes (Butt *et al.*, 1994) and have homogeneous distribution through the white matter (Oberheim *et al.*, 2006). Based on their simple morphology and their little distinction between primates and non-primates mammals, it could be stated that their functions are limited to structural and metabolic, fitting them fairly well into the classical definition of astrocytes as supportive cells (Oberheim *et al.*, 2006).

Protoplasmic astrocytes, in turn, form the main and most important astroglial population in grey matter (Rodriguez *et al.*, 2009). They are believed to be involved in the evolution of high cognitive tasks of humans since the glia-neuron index is higher in primates than in non-primate mammals (Slezak *et al.*, 2006). Protoplasmic astrocytes define the architecture of the grey matter and in some cases constitute up to 90% of cells i.e. in the human cortex. In general, in all mammals protoplasmic astrocytes display a complex branched morphology, showing a spongiform appearance –challenging the historical concept of star shaped cells– (Bushong *et al.*, 2002) with numerous secondary thin processes (Oberheim *et al.*, 2006). Protoplasmic astrocytes are organised in non-overlapping domains (Bushong *et al.*, 2002) contacting blood vessels, neighbouring astrocytes, neurons and synapses (Wilhelmsson *et al.*, 2006). Astrocytic processes may be classified as lamellopodia and filopodia depending on their morphology. Filopodia are thin membrane elongations that monitories the parenchyma by the relatively rapid contractive and withdrawal movements, whilst lamellopodia are flat, tongue-shaped

processes that can cover neuronal surfaces like soma, synapses and/or blood vessels (Verkhratsky and Butt, 2007).

3.1.1. Astroglia Cytoskeleton and Architecture

Astroglial cytoskeleton is one of the most important aspects of astrocytes. Glial fibrillary acidic protein (GFAP) is the main component of astroglial cytoskeleton and is one the pillars of this thesis (Fig. 2A, 2B and 7A).

GFAP was first isolated form multiple sclerosis brains and it was introduced to the scientific community in 1969 by Dr. Eric Shooter (Eng *et al.*, 1970). GFAP is the main intermediate filament (IF) of mature astroglial cytoskelton. Other IF like vimentin, nestin and synemin also form astroglial cytoskeleton (Middeldorp and Hol, 2011). GFAP cytoskeleton defines the so called characteristic astroglial morphology; star-like cells. Primary GFAP process extend radially bifurcating and branching several times leading to secondary and tertiary fine profiles (Olabarria *et al.*, 2010, 2011; Rodriguez *et al.*, 2009); (see also Fig. 7).

Classically, GFAP has been an astroglial marker and in many cases astroglial identification relied on GFAP immunodetection. This was due to the assumption that all astrocytes express GFAP in a specific manner. However, now we know that not all astrocytes express GFAP and that not all the GFAP is expressed by astrocytes (Wang and Bordey, 2008). For instance, virtually all hippocampal astrocytes express GFAP, whilst GFAP expression in cortex is reduced to few cells (Kimelberg, 2004; Yeh *et al.*, 2011). In addition, in the mature CNS, GFAP immunoreactivity is detected in both protoplasmic and fibrous astrocytes, radial glia in the cerebellum (Bergmann glia) and subependymal astrocytes adjacent to the ventricles. At the surface of the brain, GFAP expression is found mainly in astrocytes, which form the outer limiting membrane (Eng *et al.*, 2000). There is also a degree of GFAP expression in immature oligodendrocytes, Schwann cells myenteric plexus cells, Kupffer cells of the liver, epiglotic cartilage and several neoplasic tumours (Eng *et al.*, 2000). Indeed, medical centres throughout the world have used routinely GFAP immunodetection for tumour diagnosis.

However, interpretation of GFAP immunodetection may be not straightforward. Tissue fixation has high influence in the preservation of GFAP antigenity (Eng *et al.*,

2000). It is also possible that there is an increase of immunostaining of GFAP without an increase of GFAP content. For instance, increase of GFAP had been reported based on the increase of GFAP immunostaining without an actual overexpression of GFAP in a multiple sclerosis model (Smith *et al.*, 1983). Later on, using electron microscopy it was proved that such increase of GFAP immunodetection was due to the disruption of the blood-brain barrier and its consequent oedema. In oedema astrocytes absorb the infiltrating water to minimise the damage (see astroglia and Blood Brain Barrier [BBB]). This excess of intracellular fluid allowed the tight bundles of glial filaments to dissociate, exposing more antigenic sites to GFAP antibodies (Eng *et al.*, 1989).

In addition, in the past monoclonal antibodies against GFAP had been generated that were specific and other that bound GFAP and vimentin (Eng, 1985). Nowadays, apart from more refined GFAP antibodies we also count with detection antisera for different GFAP isoforms. This allows us to differentiate between $GFAP^{+1}$, GFAP delta and GFAP-α offering new astroglial horizons to explore (Middeldorp and Hol, 2011).

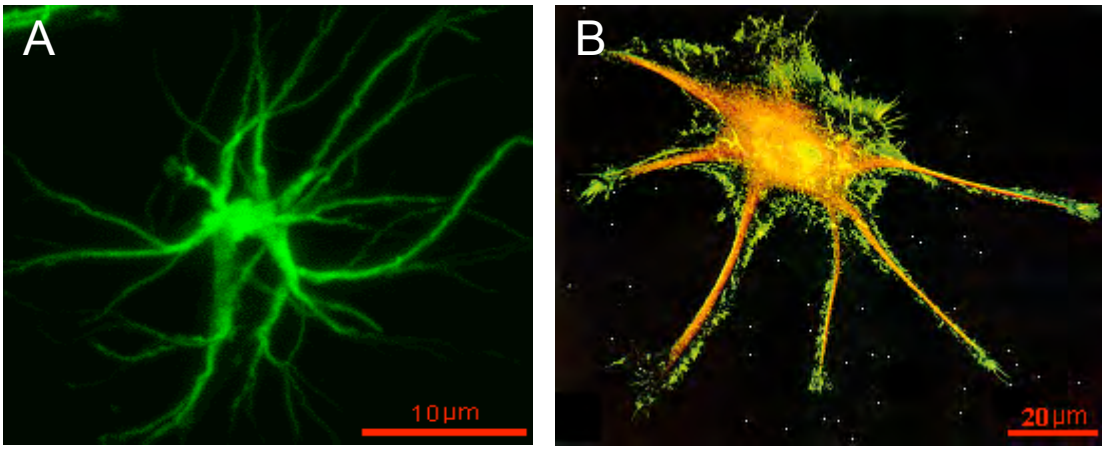

Figure 7. (A) *Confocal photomicrograph showing GFAP structure in a hippocampal astrocytes.* ***(B)*** *Immunofluorescence of an astrocyte indicating the main structured of GFAP (red) and ezrin forming thinner filaments (green), (B) from (Derouiche and Frotscher, 2001)..*

Functionally, GFAP has been regarded as structural protein for many years. Nevertheless, now we are able to associate GFAP to the majority of the astroglial functions. The generation of GFAP knockout mouse ($GFAP^{-/-}$) by deletion and/or disruption of the GFAP gene in embryonic stem cells (Gomi *et al.*, 1995; Liedtke *et al.*, 1996; McCall *et al.*, 1996; Pekny *et al.*, 1995) significantly contributed to the understanding of the specific role of GFAP in normal and pathological condition. Very

surprisingly, GFAP$^{-/-}$ mice, although were absolutely devoid of GFAP as shown by Northern and Western blotting analysis and by immunohistochemistry, their appearance was indistinguishable from the wild types. The first conclusion was that GFAP had no role. More detail studies expanded these findings and found that GFAP$^{-/-}$ astrocytes in these mice still had vimentin expression, therefore IFs, and that astrogliosis was inducible. In order to explore this unexpected finding, a double knockout was generated lacking both GFAP and vimentin (GFAP$^{-/-}$Vim$^{-/-}$). This time, GFAP$^{-/-}$Vim$^{-/-}$ mice were entirely deficient in IFs both in normal and pathological condition as shown *in vivo* and *in vitro* (Eliasson *et al.,* 1999). Thanks to the GFAP$^{-/-}$Vim$^{-/-}$ mice, other essential roles of astrocytes have been evidenced. A motility study of primary cultured astrocytes from GFAP$^{-/-}$, Vim$^{-/-}$ and GFAP$^{-/-}$Vim$^{-/-}$ showed that fast-moving subpopulation of astrocytes were affected in GFAP$^{-/-}$ and Vim$^{-/-}$ astrocytes, and more severely in GFAP$^{-/-}$Vim$^{-/-}$ astrocytes, evidencing the importance of GFAP and IFs in astroglial motility (Lepekhin *et al.*, 2001). Moreover, AxD astrocytes that show GFAP overexpression, showed greater migration rate (Yoshida *et al.*, 2007).

It is known that astrocytes are essential in overall neurotransmission and in particular in glutamate neurotransmission (see bellow section 3.1.2.3). Indeed, there are growing evidences showing that GFAP plays an important role in glutamate homeostasis. Surprisingly, GFAP has shown to have not only effect on astroglial glutamate transporters but also in neuronal glutamate transporters. Hippocampal GFAP$^{-/-}$ preparations showed less glutamate uptake and decreased glutamate transporters in astrocytes (GLAST) and in neurons (EAAC1); (Sullivan *et al.*, 2007). Other study in cortical astrocytes from GFAP null mice evidenced deficient GLT-1 transporter trafficking to cell membrane (Hughes *et al.*, 2004). GFAP has also been proofed to be essential for GLAST anchoring in the cell membrane (Sullivan *et al.*, 2007). Glutamine synthetase expression is also affected by GFAP. Suppressed GFAP expression resulted in glutamine synthetase increase. However, opposing results rose when increase of GFAP was correlated with a decrease in glutamine levels and glutamate-glutamine conversion rate (Lieth *et al.*, 1998; Pekny *et al.*, 1999).

Astrocytes are fundamental for the BBB, so is GFAP. First generated GFAP$^{-/-}$ mice showed no apparent physical handicap but retinal and spinal cord bleeding. Further research showed that very few GFAP filaments were covering the BBB, causing the weakening of the BBB and thus the bleeding (Liedtke *et al.*, 1996).

It is worth clarifying that all these GFAP-dependant functions have been studied in GFAP null mice. It is therefore important to remind that as not all astrocytes expressed GFAP, those astrocytes that in normal condition do not express GFAP may have other mechanisms to perform these functions.

GFAP over-expression is characteristic of astrogliosis; a term which refers to the astroglia reaction to many pathological insult, be it mechanical, environmental, genetic or metabolic. Indeed, increase of GFAP mRNA had been suggested as a sensitive and early marker for neurotoxicity (Eng *et al.*, 2000). Astrogliosis involves modifications in morphology and gene expression, which aims to limit the insult and protect the non-affected parenchyma. Pekny and colleagues demonstrated that astrocytes do not necessarily need to express GFAP or vimentin to show astrogliotic phenotype. This was evidenced by the fact that wild type, $GFAP^{-/-}$ and $Vim^{-/-}$ mice showed very similar astrogliotic phenotype while no astrogliotic reaction was detected in mice lacking both GFAP and vimentin (Pekny *et al.*, 1999). Nevertheless, in other mice the sole absence of GFAP was enough not to cause astroglial reaction (Nawashiro *et al.*, 1998). In addition these mice show increased susceptibility for cervical spinal cord injury produced by sudden acceleration of the head, and also were very sensitive to cerebral ischemic injury (Nawashiro *et al.*, 2000; Tanaka *et al.*, 2002). Similarly, $GFAP^{-/-}$ mice showed to be more sensitive to kainic acid-induced neurotoxicity (Otani *et al.*, 2006). All in all, theses evidences seem to suggest that GFAP is required as a protective factor (Middeldorp and Hol, 2011). Nevertheless, these data does not show whether GFAP plays a protective role in astrogliosis or not.

It is generally accepted that there is a gradual increase of GFAP in astrocytes throughout the adult lifespan of mice, rat and humans (Eng *et al.*, 1998; Nichols *et al.*, 1993).

Astrocytic gliosis is a prominent neuropathologic change in Alzheimer's disease (AD). Numerous reports have shown reactive astrocytes in AD brains, most frequently in association with neuritic plaques (Beach and McGeer, 1988; Duffy *et al.*, 1980).

Other non-IF structural proteins complete astroglial cytoskeleton. These are ezrin, radixin and moesin (ERM) and are the responsible for the motility of the finest

astroglial profiles (Derouiche and Frotscher, 2001). Within the CNS, these proteins are specifically located in astrocytes, preferentially in the fine processes where no GFAP filaments are found (Fig 7). These very thin processes ensheath synapses and are able to perform rapid structural changes (Derouiche and Frotscher, 2001; Landgrebe *et al.*, 2000; Oliet *et al.*, 2001).

3.1.2. Functions of astroglia

Control of the extracellular composition is mainly astroglial responsibility (Verkhratsky *et al.*, 2011). Extracelullar ion, metabolite and active molecules are under tight homeostatic control. In particular, the extracellular concentration of K^+ is essential. Neuronal activity causes K^+ efflux that is accumulated in the extracellular space. Excess of K^+ would alter the excitability of neurons due to the K^+ depolarisation (Kofuji and Newman, 2004). Astrocytes liberate the extracellular environment by uptaking the K^+ and buffering it throughout the astroglial syncytium (see bellow). In addition as we will explain later, astrocytes control glutamate extracellular levels (Kofuji and Newman, 2004; Verkhratsky *et al.*, 2011).

Astrocytes are entirely integrated in the neural machinery and are intimately associated with all the neural components. These interactions define astroglial functions and set the basis for the functional brain unit; the neuro-glial-vascular unit (Rodriguez *et al.*, 2009; Takano *et al.*, 2009; Wilcock *et al.*, 2009); (see also Fig. 8 and 9). Astrocytes within their anatomical domain contact blood vessels, neighbouring astrocytes, neurons and synapses (Olabarria *et al.*, 2010; Rodriguez *et al.*, 2009; Wilcock *et al.*, 2009; Wilhelmsson *et al.*, 2006). In addition astrocytes play an active role in neurotransmission, gliotransmission and information processing.

3.1.2.1. Neuro-glial-vascular unit

Astroglia and blood vessels

Astrocytic processes envelop the cerebral microvessels and form, together with endothelial cells, the blood-brain barrier (Fig. 9). This physical barrier is the most fundamental of all brain defences limiting the entrance of solutes and other damaging elements hence protecting the integrity of the CNS, (see Fig. 2B and Fig. 8).

Endothelial cells, and in special the tight junctions between them, are the key component of the blood-brain barrier (Abbott *et al.*, 2006). Due to the tight junctions all

molecules present in the blood torrent are forced to take a transcellular route across endothelial cells, instead of moving paracellularly around endothelial cells like in the peripheral capillaries (Wolburg and Lippoldt, 2002). This particularity of CNS microvessels allow mainly lipophilic and small molecules to enter in the CNS since this physicochemical properties of the molecules limit the crossing through the endothelial phospholipidic bilayer, limiting significantly the access to a great number of molecules (Abbott and Romero, 1996).

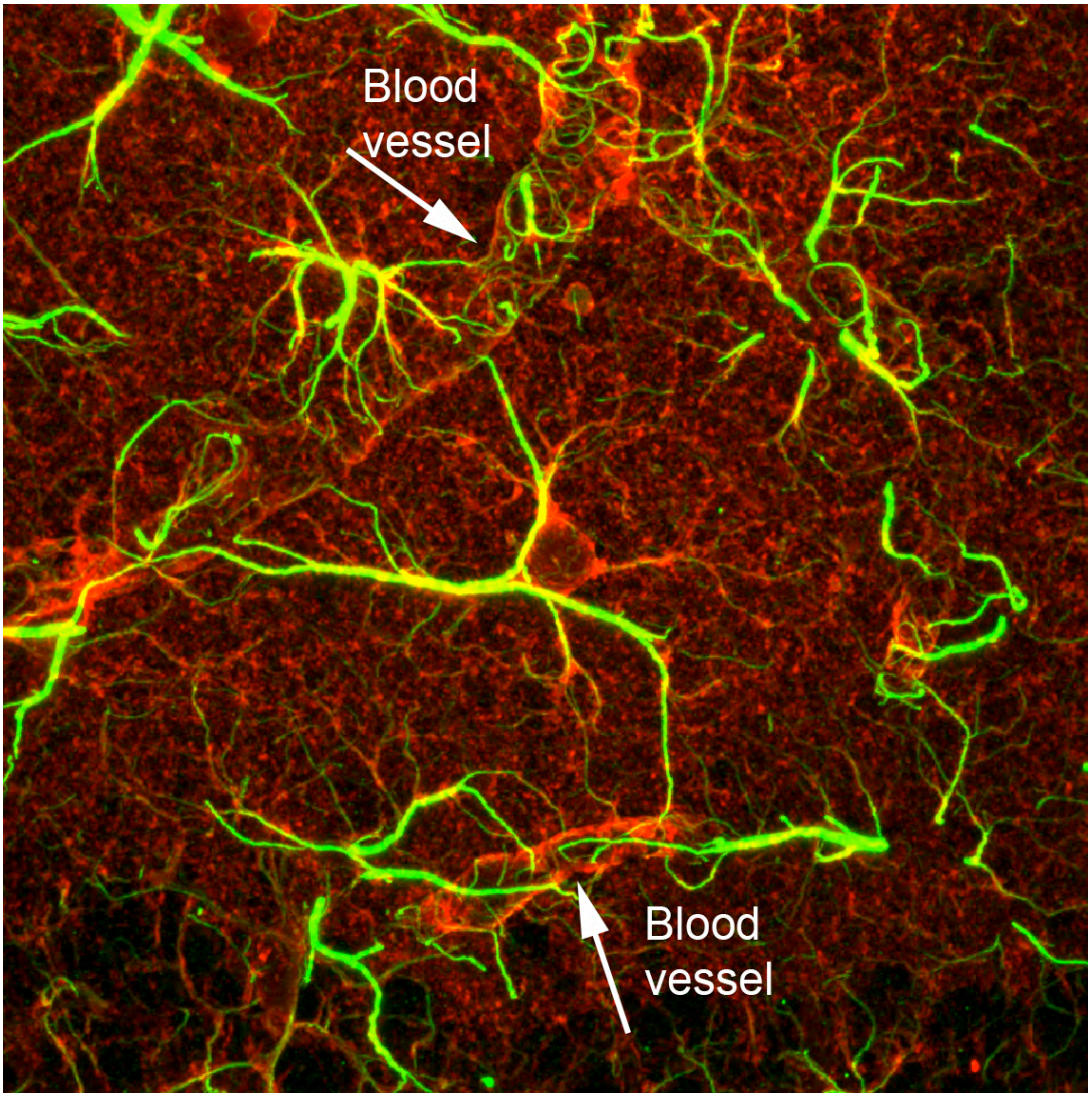

Figure 8. *Hippocampal protoplasmic astrocyte expressing GFAP (green) and GS (red) making contacts with various blood vessels. Processes of different astrocytes complete enwrap blood vessels. GFAP outlines the cytoskeleton, whilst GS fills up the cytosol showing a spongiform appearance. Interestingly, just by using astroglial specific markers is possible to outline clearly blood vessels' profiles.*

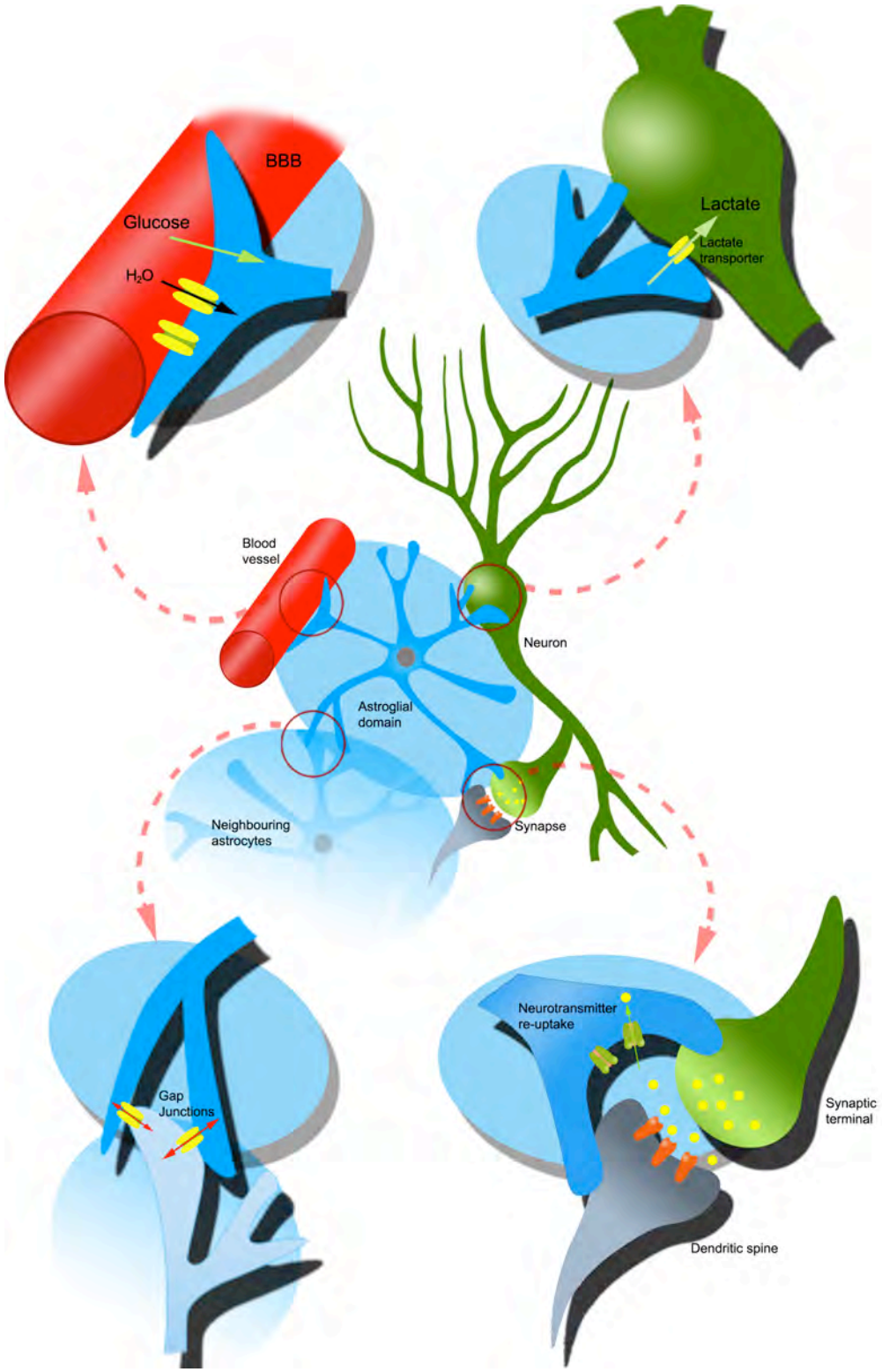

Figure 9. *Schematic* diagram *representing the neuro-glial-vascular unit. Astrocytes are organised in non-overlapping domains, in which they contact blood vessels, neighbouring astrocytes, neurons and synapses (centre). Astroglia processes are involved in blood-brain barrier formation, control blood flow, absorb glucose and water (top left). Astrocytes also convert glucose to lactate, which is the main energy source of neurons. Lactate is transported to neurons by specific transporters (top right). Adjacent astrocytes are interconnected by gap junctions forming the astroglial syncytium, allowing* Ca^{2+} *wave propagation (bottom left). Astroglial processes also contact synapses and modules synaptic activity (bottom right).*

Very importantly, the presence of astrocytes is essential to induce the formation of endothelial tight junctions. This was evidenced by studies that showed that cultured endothelial cells did not develop BBB phenotype (tight junctions) unless they were in the presence of astrocytes or astrocytic condition media (Arthur et al., 1987; Bauer and Bauer, 2000). Therefore endothelial cells depend on astrocytes to form tight junctions and form an efficient BBB (Fig. 8).

Astrocytes also integrate neural activity with the blood flow. Synaptic firing increases astroglial intracellular Ca^{2+} which travels to perivascular endfeet where vasoactive molecules are released to modulate vasodilatation to cope with the metabolic demands (Iadecola and Nedergaard, 2007). In this way, astrocytes transport nutrients from the blood torrent to active neurons. The main energy source of neurons is lactate and it is metabolised from glucose via the glucose-lactate shuttle (Verkhratsky *et al.*, 2010). This metabolic step takes place in astrocytes, neurons being dependent on astrocytes for survival and function (Bittar *et al.*, 1996; Magistretti, 2009; Tsacopoulos and Magistretti, 1996).

Astroglial syncytium

Astrocytes are integrated in a continuous superstructure formed by the contacts of fine processes with neighbouring astrocytes through gap junctions (Benarroch, 2005), providing the route for intercellular communications integrating astrocytes in a functional syncytium (Rodriguez *et al.*, 2009). Gap junctions are formed by connexons and are located intercellularly between distal astroglial processes of adjacent astrocytes. Connexons form relatively big pores being permeable for molecules with molecular weight of approximately 1KD. Interestingly, astroglial syncytia is anatomically organised in disconnected nuclei, responding to a functional mapping (Giaume *et al.*, 2007; Giaume and McCarthy, 1996).

Glial cells are the non-excitable neural cells and are unable to produce propagating action potential (Verkhratsky *et al.*, 2010). However, gap junctions provide a route for intercellular communications allowing long-range glial signalling. For this purpose, astrocytes employ Ca^{2+} as signalling substrate. Ca^{2+} is an intracellular second messenger that modulates a wide variety of cellular reactions. Ca^{2+} is ubiquitously present in all types of cells and is stored in the endoplasmatic reticulum (ER), which plays an important central role in Ca^{2+} signalling (Berridge and Irvine, 1989; Kostyuk and

Verkhratsky, 1994). Ca^{2+} accumulation and release from the ER is decisive in the modulation of Ca^{2+} signalling. For that stimulation of Ca^{2+} channels, ryanodine and inostitol triphosphate ($InsP_3$) receptors, in the ER will cause Ca^{2+} release to the cytosol increasing intracellular Ca^{2+} concentration (Bezprozvanny, 2005; Hamilton, 2005). Both receptors are sensitive to intracellular Ca^{2+} while $InsP_3$ is also sensitive to $InsP_3$ second messenger. Intracellular Ca^{2+} increase is capable of propagating through the astroglial syncytium as a Ca^{2+} wave. In addition, $InsP_3$ can also diffuse through gap junctions activating Ca^{2+} channels are contributing to the Ca^{2+} wave propagation (Hansson and Ronnback, 2003).

Astroglial synaptic modulation

Synapses are also contacted by astrocytic endfeet and their coverage by astrocytic processes varies depending on the brain region (Wang and Bordey, 2008). Around 60% of the synapses in the hippocampus are ensheathed by astrocytes (Ventura and Harris, 1999) while a much higher degree of astrocytic synaptic coverage (approximately 85%) is displayed is the cerebellum (Xu-Friedman *et al.*, 2001). Each protoplasmic astrocyte can ingrate within its anatomical domain approximately 2 million synapses in humans while 20.000 to 120.000 in rodents (Verkhratsky *et al.*, 2010).

Astrocytic endfeet together with the presynaptic and postsynaptic terminals form what we know as tripartite synapse (Araque *et al.*, 1999; Hansson and Ronnback, 2003). Astrocytes monitor very closely synaptic function and an active member of neurotransmission. Synaptic activity can cause glutamate release to the extrasynaptic space and activates glutamate receptors on astrocytes, which causes an increase of intracellular Ca^{2+}. This leads to the release of glutamate from astrocytes, which may serve as a feedback signal to neurons (Araque *et al.*, 1998). In addition, astrocytes involvement in synapses goes further. Astrocytes can have different effects on synapse, by producing an MGlur-dependant depression or an NMDA-receptor-dependant increase in neurotransmitter release (Araque *et al.*, 1998, 1999).

Other important aspect about the presence of astroglial processes surrounding synapses is that they re-capture the released neurotransmitters. In some cases, astrocytes are the main cells that uptake majority of the released neurotransmitters. This can serve as refinement of the neurotransmission by reducing the noise, as re-cycling metabolic

way and as protection system to avoid over-activation of the synaptic receptors (Danbolt, 2001; Olabarria *et al.*, 2011).

3.1.2.2 Astroglia as a communication element

We already have explained some aspects of astroglial communication capabilities like Ca^{2+} signalling and modulation of neurons in synapses. However, astroglial participation in communication goes beyond. Astrocytes express the same diversity of neurotransmitters as neurons. First evidences came in 1984 when *in vitro* experiments showed that glutamate and GABA induced astroglial and oligodendroglial responses (Bowman and Kimelberg, 1984; Kettenmann *et al.*, 1984). Further experiments have shown that astroglial neurotransmitter distribution is conditioned by the environment to which astrocytes are exposed to (Verkhratsky *et al.*, 2010). For instance, processes of astrocytes contacting serotonergic synapses in hippocampus, which receives dense serotonergic input from the raphe nuclei, are endowed with serotonergic receptor (Peddie *et al.*, 2008; Vertes *et al.*, 1999), whilst those ones in contact with Purkinje neurons in cerebellum deploy adrenergic and histaminergic receptors (Kirischuk *et al.*, 1996). Nevertheless, most astrocytes express purinoreceptor and glutamate receptor (Verkhratsky *et al.*, 2010). Astrocytes also have a number of other communication molecules known as gliotransmitters. Glutamate, ATP, D-serine and GABA are referred as gliotrasmitter and are released with astroglial excitation and Ca^{2+} waves, targeting both neurons and other glial cells (Angulo *et al.*, 2008; Bezzi *et al.*, 1998).

All these evidences expand the role that astrocytes have in neurotransmission, showing the existence of neuronal-glial bidirectional communication in a way that astrocytes openly “listen” and “talk” to synapses (Araque *et al.*, 1999). Indeed, astroglial involvement in information processing begins to be considered.

Following the nature of the thesis and considering the special relevance of glutamatergic transmission in hippocampus and astroglial involvement on its modulation, we will focus on it.

3.1.2.3. Glutamate-Glutamine Cycle

Majority of glutamatergic synapses are enwrapped by astrocytes (60%); (Ventura and Harris, 1999). This leads to the central role that astrocytes play in the glutamine-glutamate cycle, making neurons and glutamate homeostasis entirely dependent on astrocytes (Robinson, 2001; Rothstein *et al.*, 1996).

Glutamate is as essential for normal brain functioning, as it is potentially lethal. Glutamate is the major mediator of excitatory signals as well as of nervous system plasticity (Danbolt, 2001). It is essential for long-term potentiation (Collingridge and Lester, 1989), which in turn is an essential feature of hippocampus and cognition. However, glutamate is toxic at high concentration and can lead to cell dead (Choi, 1992). Several mechanisms have been proposed to link extracellular high concentration of glutamate and cell death (Choi, 1992; Danbolt, 2001). Today, the most accepted one is that the excess of glutamate over-stimulates postsynaptic receptors leading to a Ca^{2+} influx to the cell via NMDA receptors. Mitochondria buffer the excess of cytosolic Ca^{2+} into the organelle compartment that consequently leads to the loss of mitochondrial membrane integrity. Mitochondrial membrane disruption will lead to energy supply failure, causing rapid necrotic cell death (Young *et al.*, 2010). In parallel, such energy failure might lead to massive glutamate release because glutamate membrane gradient is no longer maintained, spreading glutamate-derived excitotoxic damage.

Brain is very rich in glutamate and majority of it is located intracellularly. The highest concentration is in nervous terminals causing very elevated concentration gradient of glutamate across the synaptic plasma membranes (Danbolt, 2001). Maintenance of this gradient has a high energy cost, thus any energy shortage will cause glutamate massive release to the extracellular space. In addition, glutamate is in constant turnover being released to the extracellular space and re-uptaken continuously (Danbolt, 2001). Therefore, the fine and adequate coordination of all contributors in glutamate turnover is decisive for both correct glutamatergic neurotransmission and avoidance of glutamate-mediated excitotoxic events.

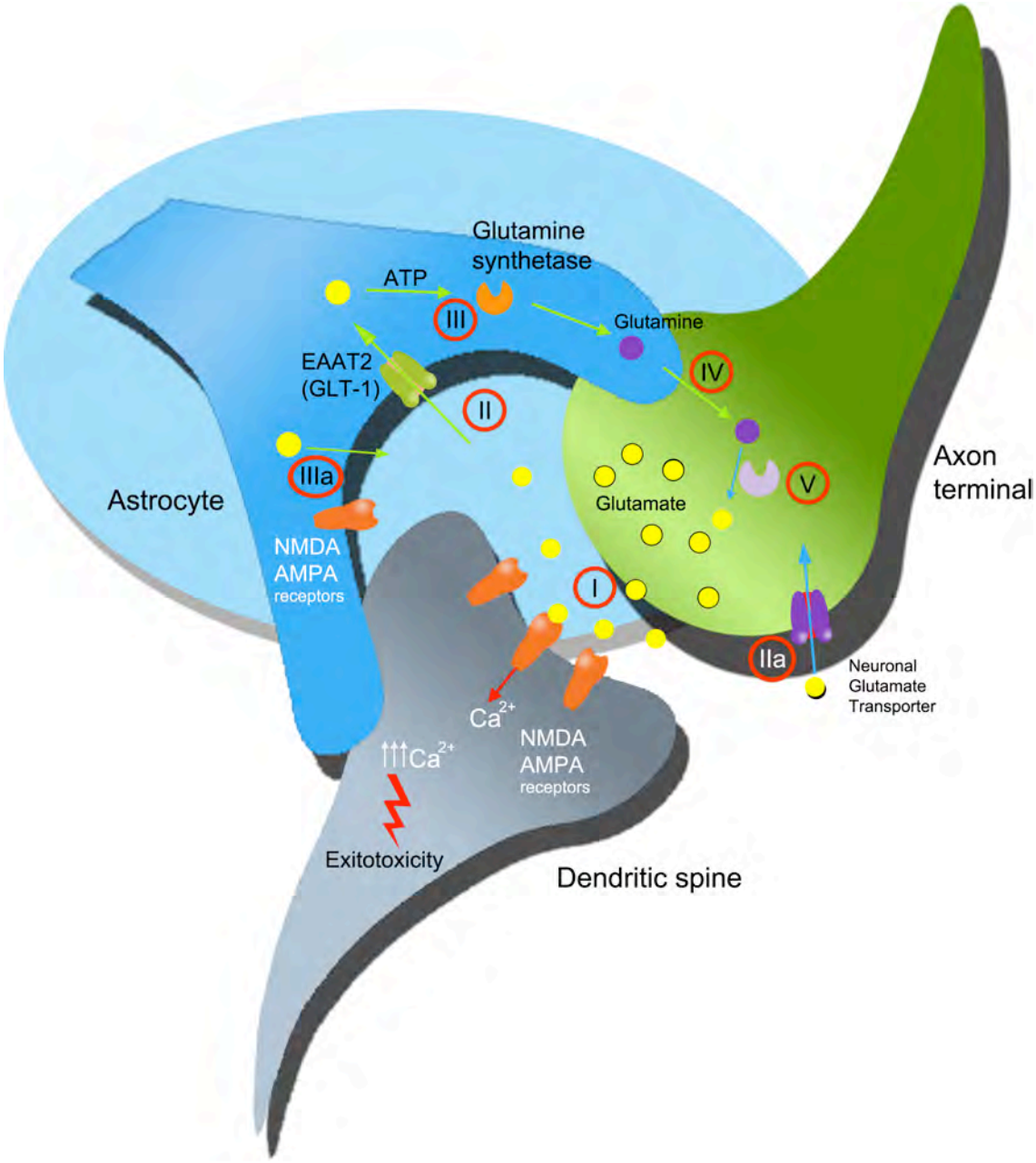

Figure 10. *Diagram of the glutamate-glutamine cycle.* ***(I)*** *Stored glutamate in vesicles is released from the synaptic terminal to the synaptic cleft were it binds to glutamate receptors, causing* Ca^{2+} *influx into the dendritic spine. An excess of glutamate or prolonged glutamate exposure to glutamate receptors would lead to an elevated influx of* Ca^{2+} *to the neuron causing excitotoxicity.* ***(II)*** *Glutamate re-uptake from the extracellular space is mainly done by perysynaptic astroglial process (80%) and it is carried out by the glutamate transporter EAAT2 (GLT-1 in rodents).* ***(IIa)*** *Neuronal contribution to glutamate up-take is minor (20%).* ***(III)*** *Astrocytes inactivate glutamate by the ATP-dependent transformation into glutamine by Glutamine Synthetase enzyme.* ***(IIIa)*** *In parallel, astrocytes also participate in neurotransmission by releasing glutamate.* ***(IV)*** *Glutamine is transported to neurons,* ***(V)*** *where it is transformed again into glutamate by glutaminase (in light purple). Finally glutamate is stored in synaptic vesicles until next action potential triggers their release.*

Glutamate acts on synaptic receptors located in the surface of plasma membrane. Extracellular levels of glutamate must be kept low in order to reduce synaptic noise (signal-to-noise ratio) and to avoid toxicity. In contrast, intracellular glutamate is generally innocuous. Glutamate dose not count with a synaptic enzymatic system as cholinergic system to inactivate extracellular glutamate. Therefore the only way to reduce extracellular glutamate level is by cellular uptake (Logan and Snyder, 1972).

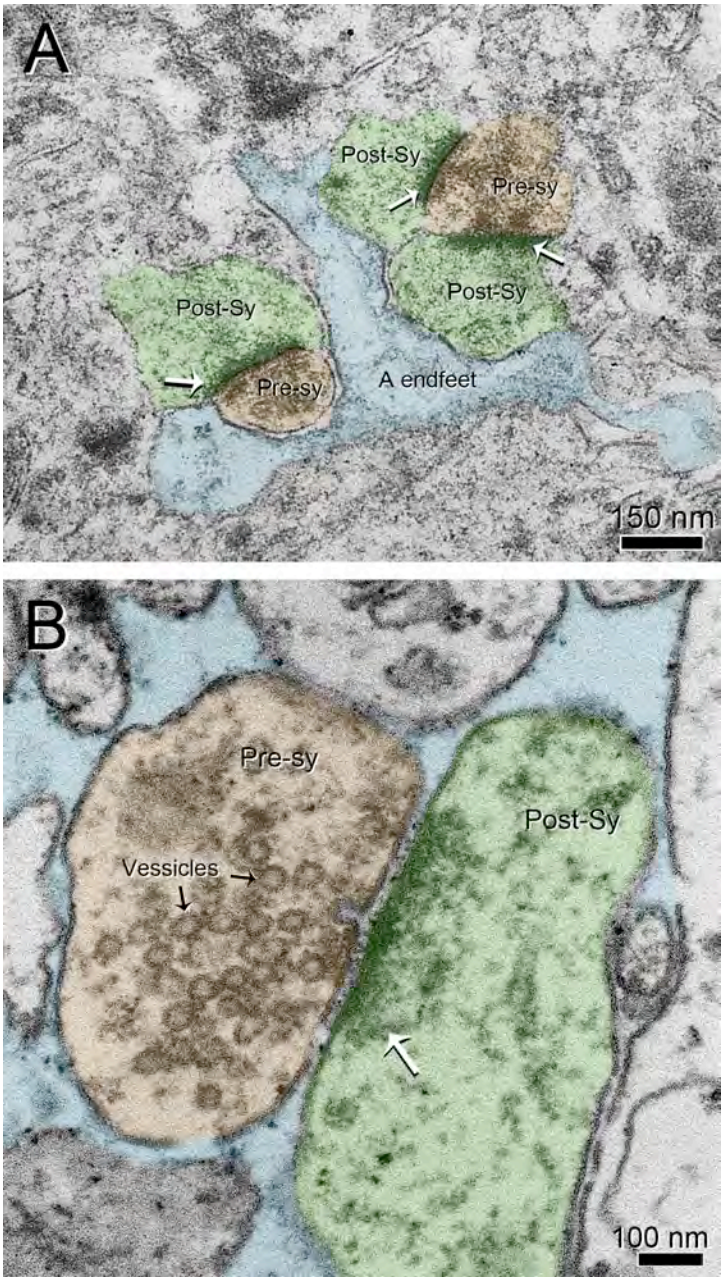

Figure 11.
Electromicrographs illustrating astroglial and synaptic spatial association. ***(A)*** *Astroglial endfeet (blue) surrounds several glutamatergic synapses in the hippocampus, contacting both pre- and post-synaptic terminals (Orange and green respectively).* ***(B)*** *Excitatory synapse of the hippocampus with the characteristic vesicular presence in the pre-synaptic terminal. One of the vesicles is fusioned with the plasma membrane liberating the vessicular content. Key: Pre-sy, pre-synaptic terminal; post-sy, post-synaptic terminal. White arrow: post-stnaptic density (Personal library).*

In nerve terminals glutamate is released by exocytosis to the synaptic cleft, where after binding with glutamatergic receptors is re-uptaken mainly by glutamate transporters located principally in astrocytes (GLT-1; 80%) and, in some degree, in neurons (GLAST and EAAC1; 20%); (Kirischuk *et al.*, 2007; Verkhratsky and

Kirchhoff, 2007; Verkhratsky *et al.*, 2010). Neurons transport glutamate into synaptic vesicles by glutamate vesicular transporters. In astrocytes glutamate is transformed to glutamine, which is no longer active/excitotoxic, in an ATP-dependent process by astroglia-specific glutamine synthetase. Glutamine is subsequently transported to neurons where is converted into glutamate by glutamate dehydrogenase (McKenna *et al.*, 2000) and stored in synaptic vesicles.

Beyond glutamate and glutamine bidirectional transformations, glutamate-glutamine cycle has in addition other parallel metabolic fates. Glutamate can be transformed, after several metabolic steps (glutamate, α-ketoglutarate, maleate, pyruvate), into lactate (McKenna *et al.*, 1996a; McKenna *et al.*, 1996b) that serves as energy substrate for neurons. In addition, neurons do not exclusively rely on astrocytic glutamine to synthetase glutamate. Neurons are capable of producing *de novo* glutamate independently from astroglial supply of glutamine. However, this process might need astroglial support by providing amino groups to complete the synthesis of glutamate.

3.1.3. Astroglia in brain pathology

Astrocytes are involved in virtually every type of brain pathology (Chvatal *et al.*, 2008); (see also Table 2). As astrocytes participate in such an extent variety of functions (Rodriguez *et al.*, 2009), their performance in pathological conditions will determine the survival of the nervous tissue as well as the degree of the neurological damage (Chvatal *et al.*, 2008).

Moreover, pathological insults will rise to a specific astrocytic reaction known as reactive astrogliosis, which aims to protect the brain parenchyma (Chvatal *et al.*, 2008; Nishiyama *et al.*, 2005), reconstruct the blood–brain barrier (Rodriguez *et al.*, 2009) and promote the remodelling of the neural circuitry (Pekny and Nilsson, 2005). Within astrogliosis we can distinguish two different astrocytic reactions. Astrocytes close to the damage experiment a severe hypertrophy and proliferation that eventually will remodel the affected area forming the glia scar. While astrocytes distant to the injury undergo multiple biochemical changes and they will not alter the normal architecture of CNS tissue. Additionally, they permit growth of neurites and synaptogenesis, as a result facilitating the remodelling of neuronal networks (Rodriguez *et al.*, 2009).

However, astrocytes can play a dual role in brain pathology, providing defence as well as damage; the latter contributing to damage exacerbation and propagation (Chvatal *et al.*, 2008). Certain neurological pathologies, such us ischemia, result in a massive release of glutamate, which induces depolarisation of further neurons, thus causing excitotoxicity, which makes up the central role in brain damage (Nicotera *et al.*, 2007). Astrocytes are the main defence against glutamate excitotoxicity, being responsible for the majority of the glutamate re-uptake (Kirischuk *et al.*, 2007) in excitatory synaptic transmission.

If excitotoxicity causing pathological condition is prolonged, glutamate cannot be any longer contained in astrocytes and eventually, as a consequence of depolarisation, will be immensely released exacerbating the damage.

Massive release of glutamate also has been described in human glioma (Sontheimer, 2003). Deficient glutamate decrease have been also reported in other pathologies like epilepsy (Wong *et al.*, 2003), Wenickie's encephalopathy (Hazell, 2009), Human

herpesvirus-6 HIV associated dementia (Fotheringham *et al.*, 2007), amyotrophic lateral sclerosis (Rothstein *et al.*, 1995), Alexander disease (Tian *et al.*, 2010) and Alzheimer's disease (Parsons *et al.*, 1998). Contrarily, schizophrenic patient have decreased glutamatergic neurotransmission as a result of the overexpression of EAAT2 by astrocytes in the prefrontal cortex (Matute *et al.*, 2005).

Table 2. *Summary of astrocytic dysfunction in different brain pathologies.*

Disorder	Astrocytic dysfunction	Reference
Ageing	GFAP up-regulation, Astrogliosis, No cell proliferation, No branching	(Unger, 1998) (Eng *et al.*, 1998)
Ischemia	Massive glutamate release	(Nicotera *et al.*, 2007)
Epilepsy	Reduced glutamate uptake (↓GLAST, ↓GLT-1)	(Wong *et al.*, 2003)
Wernickie's encephalopathy	Decrease of EAAT1, EAAT2, GFAP and GS	(Hazell *et al.*, 2001) (Hazell *et al.*, 2010)
Rasmussen's enthephalitis	Astrocytic loss (apoptosis)	(Bauer *et al.*, 2007)
Alexander's disease	Gliosis, GLT-1 decrease, CD44 expression in hippocampus	(Tian *et al.*, 2010)
Human herpesvirus-6	Impaired glutamate uptake	(Fotheringham *et al.*, 2007)
Human glioma	Massive glutamate release	(Sontheimer, 2003)
Schizophrenia	Increased GLT-1 mediated glutamate uptake	(Matute *et al.*, 2005)
Amyotrophic Lateral Sclerosis	Reduced glutamate uptake (↓EAAT2) Increased GFAP and S100β immunoreactivity	(Rothstein *et al.*, 1995) (Migheli *et al.*, 1999)
Multiple sclerosis	β_2-adregenergic receptor loss	(De Keyser *et al.*, 2010)
Parkinson's disease	Provokes oxidative stress in dopaminergic neurons	(Blum *et al.*, 2009)
Huntington's disease	Reduced glutamate uptake (↓GLT-1) and deficient trophic neuronal support	(Hsiao and Chern, 2010)
HIV associated dementia	Reduced glutamate uptake	(Xing *et al.*, 2009)
FTD	Apoptosis and dystrophy	(Broe *et al.*, 2004)
Alzheimer's disease	GFAP overexpression: Protein (tissue) Protein (CSF) mRNA	(Beach *et al.*, 1989) (Noppe *et al.*, 1986) (Le Prince *et al.*, 1993) (Kato *et al.*, 1998)
	↓GLT-1 expression	(Simpson *et al.*, 2008)
	↓GS expression in cortex	(Robinson, 2000) (Robinson, 2001)
	Hypertrophy around Aβ plaques	(Olabarria *et al.*, 2010)
	Atrophy in Aβ free parenchyma	(Olabarria *et al.*, 2010)
	↓GS expression in hippocampus	(Olabarria *et al.*, 2011)

Key: Aβ: Amyloid-β; CD44= cell-surface glycoprotein 44; CSF= cerebrospinal fluid; FTD: Frontotemporal dementia; GLAST: Glutamate aspartate transporter; GLT-1: Glutamate transporter 1; GFAP: Glial fibrillary acidic protein; GS: Glutamine synthetase; EAAT=Excitatory amino acid transporter; HIV: Human immunodeficiency virus. Note: Details in red respond to the need of covering the update literature in astroglia in AD, based on the present thesis results.

Astrocytic involvement in neuropathological diseases goes beyond glutamate homeostatic imbalance. Astrocytes can be involved in other neurotransmission systems like in dopanimergic neurotransmission in which they are believed to cause oxidative stress to dopaminergic neurons (Blum *et al.*, 2009), adrenergic system in which there is a reduction of β2-adregenergic receptor in astrocytes, and can also undergo apoptotic like has been described in Rasmussen's enthephalitis (Bauer *et al.*, 2007).

The question whether astrocytes are the originating cause of these pathologies or simply affected counterpart is yet to be addressed. However, an increasing body of evidence suggests that astrocytes are active contributors to neuropathology. Indeed, astroglial degeneration has been described in different pathologies (Rodriguez *et al.*, 2009; Verkhratsky *et al.*, 2010); (see also Table 2).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a motor neurodegenerative disease that was first described by Charcot in 1869 that affects the spinal cord, brain stem and motor cortex (Barbeito *et al.*, 2004). Mutations in the Cu/Zn superoxide dismutase (SOD1) gene are the responsible in the familial ALS, which represent around the 20% of cases (Rosen et al, 1993).

Astrocytes have been described to become reactive and downregulate EAAT2 in both patients and animal models of ALS (Howland *et al.*, 2002; Rothstein *et al.*, 1995). Similar astrogliotic reaction was described at late stage of ALS pathology (McGeer and McGeer, 2002). Contrarily, other evidences show prominent astroglial degeneration in human ($SOD1^{G93A}$) transgenic mouse. Importantly, astroglial degeneration preceded neuronal damage and clinical symptoms (Rossi *et al.*, 2008). It was also shown showed that ALS astrocytes were more sensitive to glutamate damage (Rossi *et al.*, 2008). In addition, normal lifespan of experimental ALS models was achieved by the enhancement of antioxidative protection using transcription factors such as Nrf2 (Sarlette *et al.*, 2008; Vargas *et al.*, 2008). Similarly, selective silencing of SOD1 mutation in astrocytes slowed the progression of the pathology.

Huntington's disease

Huntington's disease is an autosomal dominant neurodegenerative disease that was first described by Huntington in 1872. It is characterised by movement disorder, dementia and it is cause of death (Hsiao and Chern, 2010). A defect in Huntingtin gene causes aggregation of a variety of proteins leading to neuronal dead of GABAergic neurons in the striatum (Oliveira, 2010). Astrocytes are also affected and have shown decreased level of GLT-1 level in HD mice and provide deficient trophic support to neurons making them more vulnerable to excitotoxicity (Chou *et al.*, 2008; Lievens *et al.*, 2001), and an *in vitro* study proved that such reduction was caused by mHtt mutation in astrocytes (Shin *et al.*, 2005). Moreover, mice models with only Htt

mutation in astrocytes exhibit characteristic symptoms of the disease, not only showing the involvement of astrocytes in the pathology, but also the sufficiency to provoke the dysfunction (Bradford *et al.*, 2010).

Parkinson's disease

Degeneration of the dopaninergic neurons of the substantia nigra causes Parkinson's disease that is characterised by akinesia, rigidity, tremor at rest and postural abnormalities (Parkinson, 1817). Astrogliosis have been described at late stages of the disease. In addition, it has been observed that astrocytes can contribute to microglial recruitment and activation by releasing pro-inflammatory factors. Contrarily, astrocytes also may release growth factors and diminish microglial reaction. Beyond the consideration of whether these findings are contradictory or not, the role that astrocytes have in Parkinson's disease is still poorly understood (McGeer and McGeer, 2008; Mena and Garcia de Yebenes, 2008). It is known that substantia nigra has less presence of astrocytes than other brain region and it is conceivable that this fact might make this brain region more vulnerable if astroglial degeneration occurs. However, this hypothesis needs experimental confirmation (Verkhratsky *et al.*, 2010).

Alexander disease

Alexander disease (AxD) is leukodystrophy characterised by demyelination and dysmyelination, megathephaly, phycomotor retardation, seizures and accumulation of Rosenthal fibres in astrocytes (composed among others by GFAP), being very often fatal at early age (Alexander, 1949; Head *et al.*, 1993; Li *et al.*, 2002). AxD can appear in infants, children or adults, and the earlier the onset is, the more severe the disease is, progressing faster and causing death earlier. Some adults have been asymptomatic throughout their life and only the presence of Rosenthal fibres in the postmortem examination evidenced the pathology (Li *et al.*, 2002).

A mutation in the gene encoding for GFAP is the etiogenic factor in AxD, which usually takes place in the domain crucial for polymerisation of GFAP into filaments (Hsiao *et al.*, 2005). To the best of our knowledge, AxD is the only astroglial primary disorder and this fact makes it of extreme importance to understand astroglial role in neurodegeneration.

The mutation varies from some patients to others; being R239 amino acid changes the most common and severe ones (Hsiao *et al.*, 2005). As a consequence of the mutation, GFAP protein polymerisation is affected and it is prone to accumulate together with ubiquitin, hsp27 and αB-crystallin, suggesting a defective protein degradation pathway. As a consequence, astrocytes undergo different pathological changes including GFAP accumulation, gliosis, GLT-1 and CD44 expression, leading eventually to the collapse of the system (Tian *et al.*, 2010).

3.1.4. Astrocytes and dementia

Within all the different brain pathologies, probably dementia is the most devastating one, since it seizes the human identity leaving behind the defendless being (Rodriguez *et al.*, 2009). Dementia, defined as the progressive decline in cognitive function beyond what might be expected from normal aging, characterises the elderly society of developed countries and can be caused by a wide variety of reason, ranging from traumatic injuries (mechanical, chemical or as a result of irradiation) to the intrinsic neurodegenerative processes associated with genetic factors, predispositions and other yet unknown reasons (Rodriguez *et al.*, 2009). The role of astrocytes in dementia relies relatively unexplored even though, nowadays some generalisation can be stated as we know astrocytic pathology is irremediable linked to the progression of dementia (Verkhratsky *et al.*, 2010). Both astrogliosis and astroglial dystrophy are patent in different types of dementia; moreover in some cases both astroglial pathological responses may happen concomitantly depending on the disease form and/or stage of the pathology (Rodriguez *et al.*, 2009).

Frontotemporal dementia

Under this type of dementia are included all non-AD dementia and also known as FTD. Astroglial reaction in FTD is associated with a very early and profound apoptotic death and dystrophy of astrocytes (Broe *et al.*, 2004; Rodriguez *et al.*, 2009). Astroglial progressive loss directly correlates with the severity of dementia. Controversially, other study using tissue from 21 frontotemporal dementia brains, found marked astrogliosis in the frontal and temporal cortices at the very early stages of the disease, with aberrant increase of astroglial density (Kersaitis *et al.*, 2004).

Wernickie's encephalopathy

This thiamine deficient pathology is characterised by an aberrant loss of neurons due to an excitotoxic process affecting the thalamus and the mammillary bodies, and in a lower extent the cortex (Hazell *et al.*, 2001; Hazell *et al.*, 2010). Although, the exact mechanism involved in such process is unknown, several mechanisms are suggested including different metabolic elements of the glutamatergic neurotransmission (Hazell *et al.*, 1993; Hazell *et al.*, 1998). Astrocytic essential role of glutamate clearing has been shown to be affected as has been shown in a thiamine-deficient animal model, in which there is a region specific decrease of GLT-1 and GLAST in the medial thalamus (Hazell

et al., 2001) and decreased level of GFAP in frontal cortex (Hazell *et al.*, 2010). Other studies have shown that changes in astrocytic morphology are one of the earliest pathological evidences. Astrocytic therapeutical potential has been already tested by the application of N-acetylcysteinum, improving glutamate homeostasis capability and promoting neuronal survival (Hazell *et al.*, 2010).

Other dementias

In other dementias, such as thalamic dementia, astroglial alterations play a pathological central role, characterised by highly localised proliferation of perivascular and peryneuronal astroglial processes, which is believed to be a primary pathological change (Rodriguez *et al.*, 2009). Indeed, astrocytes might produce dementia even without severe neuronal loss (Potts and Leech, 2005). In HIV-1 associated dementia (HAD), similarly, both hippocampal and entorhinal astrocytes showed significant hypertrophy and increase in GFAP expression (Vanzani *et al.*, 2006). Furthermore, there are evidences that suggest that astrocytes with HIV-1 infected, together with reactive microglia, may be the responsible for the neuronal damage, through the release of inflammatory and death factors (Deshpande *et al.*, 2005; Nardacci *et al.*, 2005). At the same time, however, HAD also leads to a prominent apoptotic death of astrocytes especially in the subjects with rapidly progressing cognitive deficits (Thompson *et al.*, 2001).

3.1.5. Astroglia in Alzheimer's disease

In AD, astrocytes, together with microglia, are the most participating glial cells. Astrocytes, like in other brain pathologies, are directly involved in AD. Indeed, the so characteristic AD plaques are formed by Aβ aggregates, degenerating neurites, activated microglia and reactive astroglial processes (Chvatal *et al.*, 2008; Kato *et al.*, 1998; Wisniewski and Wegiel, 1991), thus astrocytes can be considered an inseparable components from AD. Astroglial activation was already observed by Alois Alzheimer and Santiago Ramón y Cajal (Garcia-Marin *et al.*, 2007). Although the nature of the cause that triggers this activation in AD remains unclear, studies have shown both aggregated, β-Amyloid protein and the intact cores of amyloid plaques isolated from human AD brain tissue can stimulate activation of astrocytes *in vitro* (DeWitt *et al.,* 1998). Increasing body of evidence suggest that astrocytic function in AD is complex and variable depending on the brain area and/or disease's stage (Rodriguez *et al.*, 2009; Verkhratsky *et al.*, 2010). In addition, a juxtapose reaction of astrocytes has been suggested, in which astrocytes can undergo both astrogliosis and dystrophy depending on the proximity to the neuritic plaques (Olabarria *et al.*, 2010; Rodriguez *et al.*, 2009). Furthermore, activated astrocytes can release trophic factors that will have a dose dependant protective or detrimental effect in the brain parenchyma (Rothermundt *et al.*, 2003). All in all, even though some specific data is beginning to generate related to astroglial role in AD, we still ignore the detailed involvement of astrocytes to the onset, progression and outcome of the AD pathology.

Astrocytes are believed to clear Aβ accumulation (Verkhratsky *et al.*, 2010). Cortical astrocytes in AD brains have shown intracellular Aβ accumulation (Nagele *et al.*, 2003). Although the source this Aβ could be either astroglial internal production or external source, the former seems very unlikely since the expression APP in astrocytes is very low (Nagele *et al.*, 2004). Indeed, experimental evidences show astrocytes in the vicinities of senile plaques express the Aβ-degrading neprilysin enzyme (Apelt *et al.*, 2003), supporting the Aβ internalising role that astrocytes can have in AD. However, Nagele and colleagues further evidenced that astrocytes, due to an excess of Aβ accumulation, could contribute to senile plaque burden after liberating the pathological Aβ build up (Nagele *et al.*, 2003; Nagele *et al.*, 2004). Interesting observations came from an *in situ* study, in which astrocytes were plated on APP transgenic mice brain sections (Wyss-Coray *et al.*, 2003). Application of exogenous astrocytes from wild type

control mice showed to clear Aβ deposits, though endogenous astrocytes from APP transgenic mice showed to be inefficient at this task, suggesting the crucial role of astrocytes in AD (Wyss-Coray *et al.*, 2003).

Brain capillaries are importantly affected in AD due to the accumulation of Aβ in the walls of the blood vessels (Fig. 2B and 9). This has an obvious effect on brain microcirculation and could affect water electrolytes and glucose transport from blood torrent to the CNS (Verkhratsky *et al.*, 2010).

Metabolic alterations are one of the first detected pathological alteration in AD patients (Verkhratsky *et al.*, 2010). AD early stages are associated with reduced use of glucose (Mosconi *et al.*, 2008). However experimental data shows controversy on this matter. Some authors claim Aβ decreases astroglial use of glucose (Parpura-Gill *et al.*, 1997; Schubert *et al.*, 2009) while others report the opposite (Allaman *et al.*, 2010). Neurones co-cultured with Aβ pre-treated astrocytes showed significant reduction on neuronal survival in comparison to co-cultures with naive astrocytes (Allaman *et al.*, 2010). This pattern controversy regarding the effect of Aβ in astroglial glucose metabolism might respond to differences in the studied AD stage as was claimed by Allaman and colleagues (Allaman *et al.*, 2010; Verkhratsky *et al.*, 2010).

3.1.5.1 Astroglial Architecture and Cytoskeleton (GFAP)

Astrogliosis is a prominent pathological hallmark of AD (Fig. 1, 2A and 2B). Indeed, the degree of AD severity has shown to correlate with the increase of astrogliosis (Muramori *et al.*, 1998) and increase of GFAP expression (Noppe *et al.*, 1986). The degree of reactive astrogliosis has been reported to increase concomitantly to increasing progression of Braak stage of AD (Simpson *et al.*, 2008).

Astroglial activation is classically reported based on GFAP over expression and cytoskeleton hypertrophy being GFAP one the most studied aspects of astrocytes in multiple pathologies including AD. Examination of AD brains revealed that astrogliosis displays a specific laminar pattern in cortical layers II, III and V. In addition, both grey and white matter interface also showed prominent GFAP immunoreaction (Beach *et al.*, 1989).

Astrogliosis has been found most frequently in association with neuritic plaques (Beach and McGeer, 1988; Duffy *et al.*, 1980; Eng *et al.*, 2000). Astrogliosis has been proofed to be intimately associated with amyloid plaques or diffuse deposits of amyloid and surround them with dense layers of processes (Sofroniew and Vinters, 2010), probably limiting the neurotoxic potential of neurotic plaques (Fig. 2A). An anatomical study in AD brains revealed that astroglial processes intensely surround Aβ plaques and some astroglial processes penetrating to the core of the senile plaque (Kato *et al.*, 1998). An *in vitro* experiment of astroglial pure culture showed that Aβ causes Ca^{2+}-depented GFAP upregulation 12 hours after Aβ treatment. This was verified by BAPTA (Ca^{2+} chelating agent) or thapsigargin (inhibitor of calcium entrance in the ER) post-treatment that showed reduced GFAP expression compared to controls (Chow *et al.*, 2010). AD transgenic mice expressing APP and PS1 mutations, showed severe astrogliosis associated with neuronal loss. Interestingly, both pathological events correlated with intraneuronal Aβ accumulation and no with extracellular Aβ plaque burden (Casas *et al.*, 2004).

Neuritic plaque density correlates with the increase of GFAP mRNA levels (Le Prince *et al.*, 1993; Pike *et al.*, 1995). Contrarily, other study found that astrogliosis was associated with dense plaques of Aβ and not with diffuse Aβ (Simpson *et al.*, 2008). Controversial results from AD mice models showed GFAP positive astrocytes density appeared to be increased in APPSw and APPSwDI mice models when compared to wild type control, while APPSwDI/NOS2-/- mouse model, which shows more severe AD-like pathology, showed decrease in GFAP positive astrocytes density (Wilcock *et al.*, 2009). In addition, astrogliosis also has been found not only in association with Aβ accumulation but also with neurofibrillary tangles (Beach *et al.*, 1989; Ingelsson *et al.*, 2004; Porchet *et al.*, 2003). Other study of GFAP in relation to tau tangles showed a borderline tendency towards GFAP immunoreactivity increase associated with local tangle burden (Simpson *et al.*, 2008).

GFAP also has been studied in relation to cognitive function, showing inverse correlation (Kashon *et al.*, 2004). Other detail study investigated cognitive variations in relations to GFAP independently to AD pathology. They concluded that although there was a wide variety of GFAP expression level along the studied population segment, GFAP elevation might be an early marker for neurodegenerative processes (Middeldorp and Hol, 2011; Wharton *et al.*, 2009).

3.1.5.2. Glutamate-glutamine cycle and AD

Glutamatergic neurotransmission imbalance is believed to be the underlying mechanism of a number of neurodegenerative diseases. Glutamate-glutamine cycle is essential for the housekeeping of glutamatergic neurotransmission and different elements of this cycle are the responsible for the perturbation of the glutamate homeostatic balance.

Significant reduction of glutamate re-uptake has been reported in temporal, parietal and cortices as well as in the hippocampi of AD (Cross *et al.*, 1987; Greenamyre *et al.*, 1985). AD frontal cortex showed reduction of EAAT2 (glia specific glutamate transporter); (Li *et al.*, 1997). EAAT1 and EAAT2 have been reported to decline with increasing progression of Braak stages in AD, which may raise the vulnerability of local neurons to excitotoxicity (Simpson *et al.*, 2010). However, others have found no change regarding EAAT2 immunoreactivity in AD brains (Beckstrom *et al.*, 1999). Antibody epitope, post-mortem interval and tissue treatment was attributed to these discrepancies (Walton and Dodd, 2007). Altered glutamate transporter localisation has been observed in AD brains. Pyramidal neurons in AD cortex showed strong expression of EAAT1, colocalising with hyperphophorilated tau (Scott *et al.*, 2002). Neuronal expression of EAAT2 has been also reported in AD brains (Thal *et al.*, 2002).

Co-cultures of neuron-astroglia are spared from excitotoxic damage by pretreatment with Aβ through the activation of glutamate transporters (Baba *et al.*, 2003). Non-toxic doses of Aβ up-regulate EAAT2 protein expression in astrocytic cultures (Rodriguez-Kern *et al.*, 2003). The same has been shown in cultures of rat cortical astrocytes for GLAST (Abe and Misawa, 2003). The protein precursor of Aβ, APP, induces neuroprotection via elicitation of EAAT1 and EAAT2 (Masliah, 1997, 1998; Mattson *et al.*, 1999).

Increasing age correlates with GS activity decline (Takahashi *et al.*, 2002) and in AD this decline is even more profound (Le Prince *et al.*, 1995; Smith *et al.*, 1991). Robinson (1991) found general reduction of GS in AD brain cortices, probably due to a loss of astroglial distal processes (Robinson and Robinson, 1991). Later on it was described that astroglial GS reduction in cortex could be compensated by neuronal GS expression, as was evidenced by the presence of GS positive neurons in AD brain

cortices (Robinson, 2000, 2001). On the contrary, increased levels of GS immunoreactivity was found in the prefrontal cortex of AD brains (Burbaeva *et al.*, 2005). Regarding Aβ, decreased GS expression was found in the vicinities neuritic plaques (Robinson, 2000). In addition, other study showed that Aβ can cause reduction of GS activity (Castegna *et al.*, 2002; Robinson, 2000). Another aspect of GS worth mentioning is that it is sensitive to oxidation and, according to several authors; this could affect GS immunodetection (Butterfield *et al.*, 1997; Hensley *et al.*, 1995). It was postulated that a built-up of glutamate due to the lack, decrease or inefficient GS activity could cause reversal glutamate transport, contributing to the excitotoxic damage (Nicholls and Attwell, 1990). Moreover, GS reduction could affect glutamate cycling and therefore affect cognitive functions.

3.2. Microglia

Microglial cells are the immunocompetent neural cells and form the first defence line in the CNS (van Rossum and Hanisch, 2004). They were introduced by Del Rio Hortega in 1919, (Del Rio-Hortega, 1919); (see also Fig. 12) describing in detail microglial particularities like the mesodermal developmental origin, the ramified morphology, the activation migration and proliferation in pathology (Kettenmann *et al.*, 2011). Nowadays, these precise observations of Del Rio Hortega are still considered perfectly valid (Kettenmann *et al.*, 2011), despite the technical limitations of the beginning of the XX century.

Microglial cells are the most distinct type of neuroglial cells because, unlike the rest of the neuroglial cells, have myelomonocytic origin (Ransohoff and Perry, 2009) and invade the CNS at embryonic and postnatal stages (Pocock and Kettenmann, 2007). Functionally, microglia cells can be found in three different conditions which are resting microglia, activated microglia and phagocytic microglia depending on the pathophysiological state of the CNS (Verkhratsky and Butt, 2007); (see also Fig. 12).

Resting microglia is the resident of the healthy CNS and display a ramified morphology composed by fine processes centralised in a small soma in the grey matter (Kreutzberg, 1996), whilst white matter resting microglia extends processes in parallel to myelinated axons (Verkhratsky and Butt, 2007). Resting microglia use their processes to scan (survey) the brain parenchyma in their domain (Nimmerjahn *et al.*, 2005) searching for "off" neural signals to remain inactive. This surveillance state although it is not reactive, it is highly active and "resting microglia" cannot longer be considered a passive state (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005).

Pathological "on" neural signals rapidly provoke microglial activation (Biber *et al.*, 2007). Microglia is characterised by this rapid reaction that involves morphological and functional profound and complex changes that are embraced under the term "microglial activation" (Kettenmann *et al.*, 2011; Kreutzberg, 1996). Microglia activation is a complex and multistep process that is conditioned by the triggering pathological events. For instance, pathological activation due to a bacterial invasion will differ from the activation caused by capillary damage. In addition, microglial activation does not follow

an "all-or-none" pattern, being possible to find different degrees of activation (Kettenmann *et al.*, 2011).

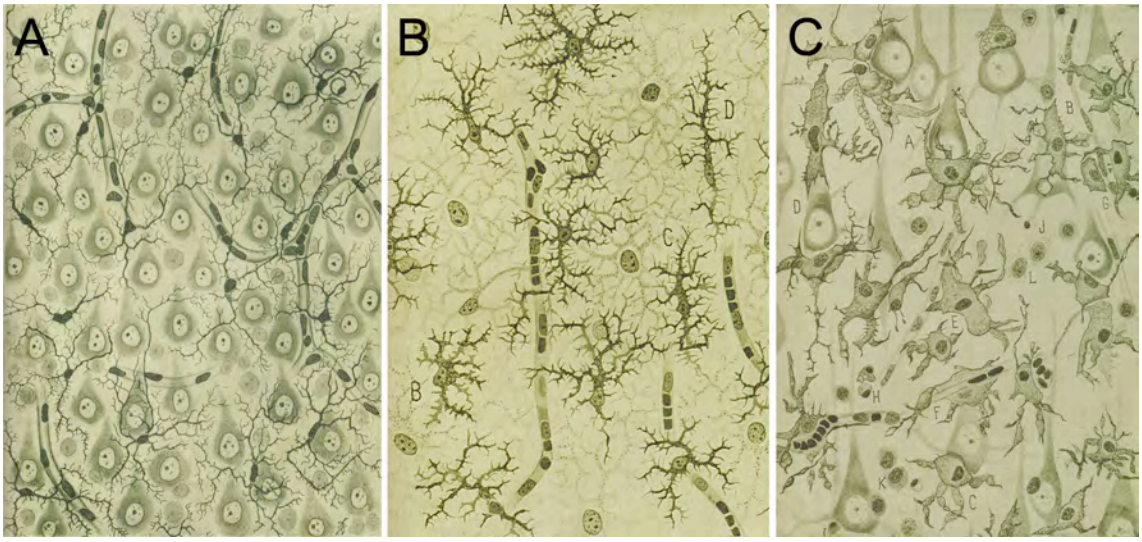

Figure 12. *Drawings of Pío del Río-Hortega showing the morphological differences between resting (A) activated (B) and (C) amoeboid macrophagic microglial cells in the rodent brain.*

However, generally, microglia retracts fine processes adopting an amoeboid appearance and become motile allowing cell migration to the lesion area guided by chemotactic gradients (Jinno *et al.*, 2007; Kettenmann *et al.*, 2011). Together with morphological changes, activated microglia express and secrete a wide variety of cytokines and pro-inflammatory chemokines such as tumour necrosis factor (TNF) α, interferon γ and interleukin 1β. Microglia can also upregulate oxidant molecules such as NO and O_2 that can protect against pathogenic organisms (Philips and Robberecht, 2011). Microglial pro-inflammatory reaction promotes tissue repair. Further secretion of anti-inflammatory molecules like insulin-like growth factor 1 (IGF1), interleukin 4 and interleukin 10, resolve pro-inflammatory reactions limiting inflammation and also contribute to tissue restore. Microglial intervention as pro-inflammatory or anti-inflammatory agent and its proportion depends on the surrounding microenvironment and the pathological events (Philips and Robberecht, 2011). Microglial activation can further evolve and become macrophage-like cell. This ovoid-shaped activated microglia clear hazards by phagocyting derbies.

Once the insult has been resolved, activated microglia can return to resting state. "Post-activated" microglia, due to the immune experience, will harbour long-lasting modifications that will determine future pathological activations, contributing to the

CNS immune system "memory" and ensuring more efficient future responses (Kettenmann *et al.*, 2011).

3.2.1. Microglia in cerebral ischemia

Experimental focal cerebral ischemia have shown to provoke rapid (within minutes) recruitment and activation of microglial cells, which in turn produce an overabundance of pro-inflammatory molecules such as IL-1β and TNF-α amplifying the damage (Rothwell *et al.*, 1997). Controversially, microglial initial reaction may also attempt to protect the parenchyma from the ischemic injury (Jin *et al.*, 2010; Raivich *et al.*, 1999). Microglial activation is followed by infiltration of peripheral immune cells like neutrophils, T cells, and monocytes/macrophages, although with a delay of hours to days.

One of the major difficulties in understanding the time correlation of the inflammation after ischemic injury is that both microglia and blood-derived macrophages that are phenotypically very similar (Jin *et al.*, 2010). In this way, mice models with GFP bone marrow have contributed to the comprehension of inflammation in cerebral ischemia. Middle cerebral artery occlusion in this model showed that resident microglia rapidly reacts to ischemic injury and that blood-derived cells invade the CNS days after, never outnumbering resident microglia (Kokovay *et al.*, 2006; Tanaka *et al.*, 2003).

3.2.2. Microglia in AD

Microglia, being the CNS immune residents, are inevitably involved in all types of brain and spinal cord pathologies (Giaume *et al.*, 2007) including AD.

Aβ plaques are commonly surrounded by activated microglia (Heneka and O'Banion, 2007) and activation of microglia is believed to appear in parallel to the formation of Aβ, being part of late stage of AD (Heneka *et al.*, 2010; McGeer and McGeer, 1999). However, many aspects of microglial role remain controversial and whether they are beneficial or detrimental is unclear. For instance, some studies suggest that microglia may clear the Aβ accumulation (Bolmont *et al.*, 2008; Frautschy *et al.*, 1998) while others have shown that activated microglia can release pro-inflammatory

factors (Heneka and O'Banion, 2007) and also contribute to plaque formation (Stalder *et al.*, 1999).

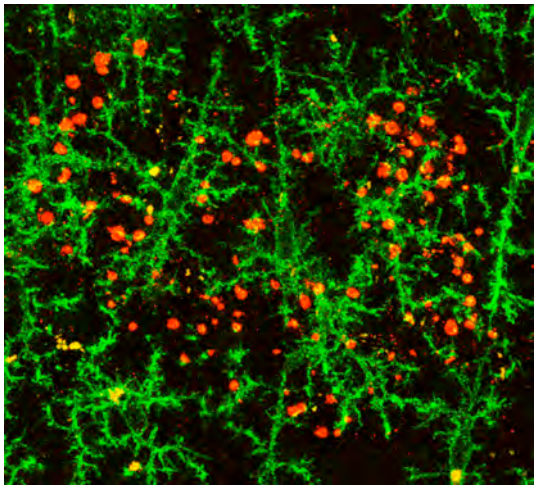

Figure 13. Confocal florescence micrograph showing activated microglia is association with Aβ accumulation in the CA1 of the hippocampus of the 3xTg-AD mouse model of AD. (Rodriguez *et al.*, 2010).

Amyloid peptides, although probably not exclusively, trigger microglia activation in AD (Barger and Harmon, 1997; Schubert *et al.*, 2000). However it remains unidentified whether soluble amyloid or formed plaques are the responsible for this reaction (Heneka *et al.*, 2010) since there are contentious studies reporting opposite activation patterns (Heneka *et al.*, 2005; Meyer-Luehmann *et al.*, 2008). In B6C3-YFP transgenic mice microglial activation was reported only after Aβ plaque formation (Meyer-Luehmann *et al.*, 2008) while opposite reaction was observed in APP V717I transgenic mice that showed microglial activation considerably early that senile plaque formation (Heneka *et al.*, 2005).

Microglial role in AD in discussed in detail in Chapter 6, where is shown an early proliferation of resting microglia and a subsequent activation of microglia.

3.2.3. Pathological interaction between astroglia and microglia

Pathological interactions between astroglia and microglia are as intriguing as unknown. Both astroglia and microglia represent the most solid defence of the nervous system and the well being of the CNS relies completely on their correct and proportional pathological response. During inflammatory response, the most common pathological event, this glial cell coupling is evidenced. As we mentioned before, astrocytes are integrated in a continuous superstructure allowing long-range signalling thanks the gap junctions that are composed by connexons. Astrocytes involved in

inflammation modify their Cx43 expression (Rouach *et al.*, 2002). Culture astrocytes exposed to interleukin-1β downregulates the expression of both protein and mRNA of Cx43 (John *et al.*, 1999). Similar results have been observed after treatment with TNF-α and Aβ (Hinkerohe *et al.*, 2005). Therefore, microglia, and even Aβ, can modulate astroglial main communication route, disconnecting injury-related astrocytes from the rest and thus, restricting the damage. Interestingly, astrocytes are also capable of modulating microglia reaction. Indeed, it is believed that gliotransmitter like ATP, glutamate, D-serine and TNF-α regulate many functional aspects of microglial function (Hung *et al.*, 2009). In the case of ATP, is known that it acts like microglia activating signal. In addition, ATP released from astrocytes will stimulate microglial migration to the injured site (Davalos *et al.*, 2005). Astrocytes co-cultured with microglial cells suppress microglial expression of interlukin-10 receptor (Ledeboer *et al.*, 2002). Therefore, astrocytes can also have strong influence on microglia clearing task. Microglial capacity to phagocyte Aβ is suppressed in microglia astroglia co-culture, whilst pure microglia culture clears Aβ efficiently (DeWitt *et al.*, 1998).

Scope and outline of the thesis

Alzheimer's disease (AD) is an age-related, irreversible and progressive neurodegenerative pathology that forms the most common type of dementia in the elderly population. AD is characterised pathologically, by accumulation of intra/extracellular amyloid beta (Aβ) plaque and intracellular hyperphosphorelated tau proteins accompanied by concomitant synaptic and neuronal loss (Selkoe, 2001, 2002). AD affects specific brain regions including cortex and hippocampus that are intimately involved in learning and memory. Clinically AD is characterised by deterioration of cognitive function including learning and memory. Although classically AD pathology has been linked with loss of neurones more resent studies emphasise involvement of glial cells including astocytes and microglia. Astrocytes are the main homeostatic cellular elements of the central nervous system (CNS), which define its structure and provide for numerous aspects of CNS function. Microglial cells are the resident immune cells in the CNS and make up the most susceptible sensors in brain pathology.

The main research question of this thesis involves investigating alterations in astrocytes and microglial cells in Alzheimer's disease. We use triple transgenic mouse model (3xTg-AD), which resembles closely the development of AD pathology in humans. Specifically, our research questions include: (i) How does astrocytes and microglia cells are altered in AD, (ii) What is the exact alterations in astrocytic cytoskeletal protein during the progression of AD using the 3xTg-AD animal model of the disease, (iii) How does alterations in astrocytic cytoskeleton affect their normal function in relation to glutamatergic homeostasis, and (iv) what is the alterations in microglial cell populations throughout progression of AD.

In the first chapter, we comprehensively review the current literature in relation to altered astrocytes and their involvement in multiple neuropathology with special focus on AD. We also review the role of microglial cells in normal and pathophysiology. In addition, we also briefly review the available literature relating to development of different animal models of AD including 3xTg-AD.

Chapter 2
Materials and methods

In **chapter 2**, we describe the general methodological approach and techniques used to obtain the results described in my PhD thesis. We explain the method used for generation of 3xTg-AD animals, fixation and tissue processing, antibodies used, immunohistochemistry-peroxidase, immunohistochemistry-fluorescence, morphological analysis of astrocyte cytoskeleton, GFAP-positive cell count in hippocampus, optical density (OD) measurement , numerical density and statistical analysis.

Chapter 3

Astrocytes are homeostatic cells of the CNS and are invariably involved in multiple brain neuropathology (Giaume et al., 2007; Nedergaard and Dirnagl, 2005; Seifert et al., 2006). Several studies reported both aggregated form of Aβ protein and the intact cores of Aβ plaques isolated from human AD brain tissue, as well as Aβ fragments can stimulate astrogliosis, which is also observed in human AD brains (Nagele et al., 2004).

Our primary study in chapter 3 involved investigating the alterations in (i) astroglia morphology throughout the age-dependent progression of AD in triple transgenic mouse model of Alzheimer's disease (3xTg-AD) that mimics the progression of the human disease; (ii) the specific morphological alteration of astrocytes in relation to amyloid plaques and (iii) the numerical density of astrocytes in the hippocampus. Using quantitative light microscopy immunohistochemistry we found found reduction of surface and volume of GFAP profiles in the hippocampus. Interestingly, while astroglial atrophy appears as a generalised process, astrocytes surrounding plaques are clearly hypertrophic as revealed by increased surface and volume of GFAP profiles. We suggest differential effects of AD on astroglial populations depending on their association with plaques accounting for the progressive disruption of neural networks connectivity and neurotransmitters imbalance which underlie mnesic and cognitive impairments observed in AD.

Part of the results of this paper were published prior the conception of this paper in a preliminary way in the factual review (Rodríguez J.J., Olabarria M., Chvatal A., Verkhratsky A. (2009). Astroglia in dementia and Alzheimer's disease. Cell Death and Differentiation.16, 378-385.) As a factual review, combines an exaustive review of the current literature with our mentioned preliminary data. Due to this fact and the fact that

the data included here is expanded in Olabarria et al, 2010 (Chapter 3), we have not included it as an independent chapter. However, we consider it is relevant for the purpose of the thesis and it has been included in *apendix 2*.

Olabarria M., Noristani HN., Verkhratsky A., Rodríguez JJ. (2010). Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia*. 58:831-838.

My personal contribution to above manuscript includes carrying out the experiment, data analysis, preparation of the figures and contributing to the writing of the manuscript.

Chapter 4

Astrocytes are essential for CNS glutamatergic transmission. They are the main elements in glutamatergic homeostasis by maintaining glutamate synthesis, uptake and turnover via glutamate-glutamine shuttle. Glutamine synthetase (GS) is specifically expressed in astrocytes, which forms glutamine by an ATP-dependent amination of glutamate.

In chapter 3 we have described that; in 3xTg-AD mouse model of AD; there is a generalised atrophy of astrocytes occurring at the middle stages of the disease (9-12 months of age). Formation of the senile plaques triggers secondary astrogliosis in astrocytes associated with Aβ depositions, and the later stages of the pathology are characterised by concomitant astroglial atrophy and astrogliosis that in any case is not associated with astrocytic density alterations (12-18 months of age). In chapter 4 we extend our previous findings by analysing the changes in hipplcampal GS-expressing astrocyte in 3xTg-AD animals aged between 9 and 18 months. We found a significant reduction in mumerical density Nv (number of cell/mm3) of GS immunoreactive (GS-IR) astrocytes. In addition, reduced Nv of GS-IR astrocytes is paralleled by a decrease in overall GS expression (determined by its optical density). Furthermore, reduced GS-IR Nv is more evident adjacent of Aβ deposits comparred to areas free of Aβ. Reduced GS containing astrocytes and GS-immunoreactivity suggests AD-related deficiency in glutamate homeostatic system, which at the advanced and late stages of the disease may affect the efficacy of glutamatergic transmission in the diseased brain and contribute to the cognitive deficiency.

Olabarria M., Noristani H.N., Verkhratsky A., Rodríguez J.J. (2011). *Molecular Neurodegeneration.* **6**:55. Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: Mechanism for deficient glutamatergic transmission? **6**:55.

My personal contribution to above manuscript includes carrying out the experiment, analysis of the data, and preparation of the figures as well as contributing to the writing of the manuscript.

Chapter 5

Microglia are the resident immune cells in the CNS and make up the most susceptible sensors during brain pathology. In the healthy CNS, microglial processes are constantly scanning their adjacent microenvironment and their territorial domains. Microglia can also migrate into all regions of the CNS acquiring specific ramified morphological phenotype termed "resting microglia". Any CNS insults stimulate a complex and multi-stage process where microglia undergo morphological and functional changes termed "activated microglia".

Some studies have shown that activated microglia contribute to neurodegeneration in AD, either via the chronic secretion of pro-inflammatory peptides (Zilka et al. 2006) or by being involved in the formation of Aβ plaques (Stalder et al. 1999). However, other reports have suggested a neuroprotective role of microglia in AD through, for example, Aβ clearance (Hickman et al. 2008; Bard et al. 2000). At any rate the concentration of activated microglia in the vicinity of Aβ plaques is routinely observed, (Frautschy et al. 1998; Jimenez et al. 2008; Simard et al. 2006; Wegiel et al. 2003) although the changes in the resting microglia population in AD remain unknown.

In chapter 5 we quantify the density of resting and activated microglia in the CA1 subfield of the hippocampus in 3xTg-AD mouse model of AD. We found a significant increase in the area density of resting microglia in 3-Tg-AD animals both at plaque-free stage (9 months) and after the development of Aβ plaques (12 and 18 months). We also found a significant increase in activated microglia at 12 and 18 months of age compared with non-transgenic (non-Tg) controls. Interestingly, the increase in the density of resting microglia precedes both plaque formation and activation of microglia by extracellular Aβ accumulation. We suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting microglia

increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

-Rodríguez JJ., Witton J., **Olabarria M**., Noristani HN., Verkhratsky A. (2010). "Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease. *Cell Death and Disease*. 1:e1

My personal contribution to above manuscript includes participating in the carrying out of the experiment, preparation of figures and contributing to the writing of the manuscript.

Chapter 6
General discussion/conclusion

Finally in chapter 6, we describe an overview of the main findings from proceeding chapters and discuss the obtained results in relation to future research. To conclude, astrocytes and microglia cells play an important role in AD neuropathology. The 3xTg-AD animal model of the disease display generalised astrocytic cytoskeleton atrophy in the hippocampus comcomitant with astrogliosis specifically observed in astrocyte located adjacent to Aβ palques. Astrocytic cytoskeleton atrophy is also associated with astrocytic dysfunction including expression of glutamine synthease. Such decrease in expression of glutamnine synthase is more evident in astrocytes located in the close vicinoty of Aβ palque depositions. With respect to microglia, the 3xTg-AD animals exhibit an increase in resting microglia proceeding the multiplication of activated microglial populations and accumulation of Aβ palques in the hippocampus. The next step would be to decipher the effect of astrocytic cytoskeleton atrophy on synaptic coverage throughout progression of AD. In additon, investigating the effect of environmental manupulation including voluntary wheel running and environmental enrichment not only on astocytic cytoskeleton atrophy/dysfunction, but also on microglial population in relation to underlying AD-related neuropathology. These studies are essential in uncovering the prcise role of glial cells in AD and the development of cell-specific therapy for better treatment of the disease neuropathology.

Chapter 2

Materials and methods

Chapter 2 - Material and Methods

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice

In Chapter 3, Chapter 4, Chapter 5

Experiments were performed on male 3xTg-AD mice and their background-matching controls. The procedures for generating 3xTg-AD mice have been described previously (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008; Rodriguez et al., 2009a; Rodriguez et al., 2009b). In brief, human APP cDNA harbouring the Swedish mutation (KM670/671NL) and human four repeat Tau, harbouring the P301L mutations were co-microinjected into single-cell embryos of homozygous PS1M146V knockin mice. The background of the PS1 knockin mice is a hybrid 129/C57BL6. The non-Tg control mice used were also from the same strain and genetic background as the PS1 knockin mice, but they harbour the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12h light-dark cycles with free access to food and water.

Fixation and tissue processing

In Chapter 3, Chapter 4, Chapter 5

Animals of different age groups (3, 6, 9, 12 and 18 months; n = 3 - 7) were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75 % acrolien (25 ml, TAAB, UK) in a solution of 2 % paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2 % paraformaldehyde (75 ml). Brains were then removed and cut into 4 - 5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodriguez et al., 2008). The brain sections were post-fixed in 2 % paraformaldehyde for 24 hours and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40 – 50 μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25 % sucrose and

3.5 % glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels -1.58 mm/-2.46 mm (hippocampus) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).

Antibodies

In Chapter 3, Chapter 4, Chapter 5

Different antibodies were used to study specific groups of glial cells and their relation with Amyloid- β plaques. Specific details are given in the Material and Methods section of each chapter. Bellow a table summarising all primary antibodies used in this thesis:

Table 1. *Summary of primary antibodies used and their respective sources.*

Antigen	Host	Type	Source & Cat Ref	Dilution	Reference
Aβ	Mouse	Monoclonal (6E10)	Covance; SIG-39300	1:2.000	(Oddo *et al.*, 2003a)
Aβ	Mouse	Monoclonal (6E10) Alexa Fluor® 488 Labelled	Covance; SIG-39347	1:2.000	(Oddo *et al.*, 2003a) (Olabarria *et al.*, 2011)
GFAP	Mouse	Monoclonal	Sigma-Aldrich, UK; G3893	1:30.000	(Wilhelmsson *et al.*, 2004)
GFAP	Rabbit	IgG Fraction	Sigma-Aldrich, UK; G9269	1:30.000	(Wilhelmsson *et al.*, 2004)
GS	Mouse	Monoclonal	Millipore; MAB302	1:500	(Wilhelmsson *et al.*, 2004)
MAC-1 (CD11b)	Rat	Polyclonal	Serotec, UK; MCA802G	1:1000	(Rodriguez *et al.*, 2010)
Tomato lectin*	-	Biotinylated	Sigma-Aldrich, UK; L0651	1:500	(Rodriguez *et al.*, 2010)

(*) Lectin is a glycan recognising protein isolated from *Lycopersicon esculentum* (tomato), not an antibody.

Immunohistochemistry – Peroxidase

In Chapter 4, Chapter 5

To minimise methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using 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, UK). Sections were rinsed with 0.1 M PB for 5 minutes and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. Subsequently the sections were washed with PB profusely before rinsing in 0.1 M TS for 10 minutes. Brain sections were then incubated with 0.5% albumin bovine serum (BSA, Sigma, Dorset, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, Dorset, UK,) for 30 minutes. Sections were incubated for 48 hours at room temperature with primary antibody. After sections were rinsed in 0.1 M TS for 30 minutes and incubated in 1:200 dilution of biotinylated secondary antibody against the host antigen for 1 hour at room temperature (see Table 2). Sections were rinsed in 0.1 M TS for 30 minutes, followed by incubation for 30 minutes in avidin-biotin peroxidase complex (Vector Laboratories Ltd, Peterborough, UK). The peroxidase reaction product was visualised by incubation in a solution containing 0.022 % of 3,3'diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003 % H2O2 for number of minutes (check specific times in detailed M&M of chapters) as described previously (Rodriguez et al., 2008; Rodriguez et al., 2009a). The reaction was stopped by rinsing the tissue in 0.1 M TS for 6 minutes followed by 0.1 M PB for 15 minutes. Brain sections were permanently mounted onto gelatinised slides. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100 %) followed by xylene; and then permanently coverslipped.

Table 2. *Summary of secondary antibodies used for peroxidase immunohistochemistry and their respective sources.*

Antigen	Host	Type	Source	Dilution	Reference
Mouse	Horse	Biotinylated IgG	Vector laboratories, UK; BA-001	1:200	(Rodriguez *et al.*, 2008)
Rat	Donkey	Biotinylated IgG	Jackson Immunoresearch 712-065-153	1:200	(Rodriguez *et al.*, 2010)

Immunohistochemistry – Fluorescence

In Chapter 3, Chapter 4, Chapter 5

Following the initial incubation in 30% methanol in 0.1M PB and 3% hydrogen peroxide (Sigma, UK), for 30 min; for single antigen detection, the sections were washed with PB profusely before rinsing in 0.1 M TS for 10 min. Brain sections were then incubated with 0.5% albumin bovine serum BSA (Sigma, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, UK) for 30 min. For the single labelling, sections were incubated for 48 h at room temperature in primary antibody. The sections were rinsed in

0.1 M TS for 30 min and incubated in 1:200 dilution of fluorochrome-conjugated secondary antibody (for 1 h at room temperature and finally rinsed in 0.1M TS for 30 min.

For dual immunofluorescence labelling, the sections were incubated for 48 h at room temperature in both primary antibody cocktail simultaneously. Subsequently, each antigen was detected in a sequential manner on the same sections by incubation with their correspondent fluorochrome-conjugated secondary antibody, sequentially.

For triple immunofluorescence labelling, the sections were incubated for 48 h at room temperature in primary antibody cocktail simultaneously. Subsequently, each antigen was detected in a sequential manner on the same sections by incubation with their correspondent fluorochrome-conjugated secondary antibody, sequentially. Then, the sections were washed in 0.1M TS for 30 min and incubated in 0.5% BSA in 0.1 M TS and 0.25% Triton X-100 for 30 minutes. Subsequently, sections were incubated in the third fluorochrome-conjugated secondary antibody for 20 hours at room temperature.

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

Table 3. *Summary of secondary antibodies used for fluorescence immunohistochemistry and their respective sources.*

Antigen	Host	Type	Source	Dilution	Reference
Mouse	Goat	FITC-conjugated IgG	Jackson Immunoresearch; 115-096-146	1:200	(Rodriguez *et al.*, 2009b)
Rabbit	Goat	FITC-conjugated IgG	Invitrogen, Paisley, UK; 111-096-144	1:200	Olabarria et al, 2010
Rat	Goat	FITC-conjugated IgG	Invitrogen, UK; MR6001	1:200	(Rodriguez *et al.*, 2010)
Mouse	Goat	TRITC-conjugated IgG (Rhodamine)	Invitrogen, Paisley, UK; 115-026-075	1:200	(Olabarria *et al.*, 2010)
Mouse	Goat	Alexa Fluor® 594	Invitrogen, Paisley, UK; A11005	1:200	(Olabarria *et al.*, 2011)
Rabbit	Goat	Alexa Fluor® 633	Invitrogen, UK, A21072	1:200	(Olabarria *et al.*, 2011)

Morphological analysis of GFAP positive astrocytes cytoskeleton

In Chapter 3

GFAP positive astrocytes were imaged using confocal scanning microscopy (Leica SP5 upright), recording layers at every 0.2 μm. Parallel confocal planes were superimposed and morphological analysis was carried out by Cell analyst (Chvatal et al., 2007a; Chvatal et al., 2007b) using digital filters (average 3x3, convolution, gauss 5x5, despeckle, simple objects removal) to determine the surface (S) and the volume (V) of the cytoskeleton of astrocytes both in the vicinity of and distant to β-amyloid plaques, as well as regardless to β-amyloid accumulations in CA1 subfield of the hippocampus. When analysing astroglial morphology in relation to Aβ plaques we considered all cells with somata within 50 mm from the plaque border as plaque-associated, and cell with somata positioned more distantly as cells not associated with plaques. Additionally, first data produced with this method also showed cytoskeleton complexity (Rodriguez et al., 2009b). This magnitude corresponds to the volume and surface ratio (see formula bellow). However, due to difficulties of the audience in the comprehension of this valuable magnitude, it was not longer used to express the variations of cytoskeleton structure.

$$C = {}^{2}\sqrt{S} / {}^{3}\sqrt{V}$$

C=Complexity; S=Surface; V=Volume

Cell count in hippocampus

In Chapter 3, Chapter 4, Chapter 5

We determined the total number of specific groups of glial cells in the DG and CA1 subfields in 4-5 representative non-consecutive sections throughout the dorsal hippocampus (analysed in an area of 152.000 μm^2 for DG and 235.000 μm^2 approximately for CA1 in coronal sections of 40 mm thickness). Confocal stack images were used to this propose in the case of fluorescent labelling, while single snapshot images were used for peroxidase labelling. Specific groups of glial cells were intensely labelled which made them easy to identify with equal chance of being counted. The number of specific groups of glial cells was determined blindly by a single observer; therefore, counting bias was kept to a minimum.

Optical Density (OD) Measurement

In Chapter 4

Using computer-assisted imaging analysis (Image J 1.32j, NIH, USA), we analysed the expression and density of GS labelling at 9, 12 and 18 months of age in both 3xTg-AD and non-Tg mice by measuring their optical density (OD) as described previously (Cordero et al., 2005). Briefly, to exclude any experimental errors and/or bias, all images were taken at constant light intensity. Optical filters were used to ensure the specificity of the signal recorded by the camera. The staining was observed throughout the thickness of the section (40 μm) using light microscopy (Nikon Eclipse 80i). No differences were observed in GS immunoreactivity throughout the thickness of the section between 3xTg-AD and non-Tg control animals; hence the changes in OD were used as measure of increased GS expression. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with readout of 250 corresponding to the area with very low GS expression and 0 corresponding to the densest area of labelling. The calibration density was kept constant for measuring all section to avoid experimental variances. Sections background OD was determined from the corpus callosum (CC) that was considered as blank since GS labelling in the CC is virtually absent. GS density of the entire DG MoL and CA1 (with the exception of the pyramidal cell layer) was measured independently and a single measurement was obtained from every sub-region in each hemisphere. To analyse the change in GS density against constant control, the 255 was divided by control region (CC) and the obtained factor was multiplied by the region of interest in every given section. Inverse optical density was obtained by subtracting from the obtained background level (set at 255). Measurement of mean density were taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each slice. The results are shown as inverse GS density (IOD/pixel).

Electron microscopy

In Chapter 1

Following the DAB treatment (Immunohistochemistry), brain sections were rinsed and flattened in 0.1 M PB and post-fixed in 2 % osmium tetroxide in 0.1 M PB for 1 hour as described previously (Peddie *et al.*, 2008a, b). The sections were then washed in 0.1 M PB for 10 minutes followed by sequential dehydration through replacement with graded ethanol series (30, 50, 70 and 95 %, 5 minutes each). The sections were further

dehydrated using 100 % ethanol (10 minutes), propylene oxide (2 x 15 minutes) and propylene oxide:EPON (1:1, Agar scientific Ltd, Stanstead, UK) overnight at room temperature rotating. The propylene oxide:EPON was replaced with 100 % EPON and rotated for further 2 hours. The sections were then flat embedded between sheets of Aclar fluo halocarbon film (Agar scientific Ltd, Stanstead, UK); (Peddie *et al.*, 2008a, b). Polymerisation was carried out by incubating at 60 °C overnight. Following polymerisation, the region of interest (CA1 subfield of the hippocampus) was selected and microdissected from the flat embedded tissue and mounted on the tip of EPON blocks (Peddie *et al.*, 2008a, b). Diamond knife was used to cut series of ultrathin sections of these regions at a thickness of approximately 70 nm. Series of sections were collected on degreased copper mesh grids (200 mesh) and counterstained using uranyl acetate and lead citrate (Reynolds, 1963) prior to examining under Philips FEI Tecnai 12 BioTwin electron microscope.

Statistical analysis

In Chapter 3, Chapter 4, Chapter 5

All data were expressed as mean ± SEM. Statistical analysis was done using GraphPad Prism (GraphPad Software) and Minitab (Minitab 15 Software), significance being accepted at $p \leq 0.05$. In Chapter 3, one way and two way analysis of variance (ANOVA) were used to determine the changes in surface and volume of GFAP profiles within and between each experimental group (3xTg-AD or non-Tg), followed where appropriate by Bonferroni post-hoc test. Paired or unpaired t-test were used to examine differences in the number, surface, and volume of GFAP labeled cells between the 3xTg-AD and non-Tg animals, as well as was to analyse astrocytic cytoskeleton differences in relationship with Aβ senile plaques. In Chapter 4, one-way and two-way analysis of variance (ANOVA) was used to determine the changes in N_v and OD of GS within and between each experimental group (3xTg-AD or non-Tg), followed by Bonferroni post-hoc test. Unpaired t-tests were used to examine differences between 3xTg-AD and non-Tg animals at different time points and differences between away and around conditions in 3xTg-AD. In chapter 5, analysis of variance (one-way and two-way ANOVA) was used to examine differences in the mean number of Mac-1- IR and TL-IR cells in the 3xTg-AD and non-Tg animals during ageing, followed by a Bonferroni's post-hoc test where appropriate. Whenever two groups were compared, an unpaired t-test was applied.

Chapter 3

Concomitant Astroglial Atrophy and Astrogliosis in a Triple Transgenic Animal Model of Alzheimer's Disease

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Abstract

Astrocytes are fundamental for brain homeostasis and are at the fulcrum of neurological diseases including Alzheimer's disease (AD). Here, we monitored changes in astroglia morphology throughout the age-dependent progresión of AD. We used an immunohistochemical approach that allows us to determine the domain of glial cytoskeleton, by measuring the surface, volume, and the relationship between astrocytes and neuritic plaques. We investigated astroglia in the hippocampus of a triple transgenic mouse model of AD (3xTg-AD) that mimics the progression of the human disease. The numerical density of astrocytes is affected neither by AD nor by age. Indeed, from early age we found lesser astrocytic GFAP surface and volume (6 months; 43.84 and 52.76%, respectively), persisting at 12 (40.73 and 45.39%) and 18 months (64.80 and 71.95%) in the dentate gyrus (DG) of 3xTg-AD, whereas in CA1 it appears at 18 months (29.42 and 32.74%). This cytoskeleton atrophy is accompanied by a significant lower glial somata volumen in DG at 12 and 18 months (40.46 and 75.55%, respectively), whereas in CA1 it is significant at 18 months (42.81%) when compared with controls. However, while astroglial atrophy appears as a generalised process, astrocytes surrounding plaques are clearly hypertrophic as revealed by increased surface (48.06%; 66.66%), and volume (57.10%; 71.06%) of GFAP profiles in DG and CA1, respectively, at 18 months. We suggest differential effects of AD on astroglial populations depending on their association with plaques accounting for the progressive disruption of neural networks connectivity and neurotransmitters imbalance which underlie mnesic and cognitive impairments observed in AD.

Introduction

Astrocytes are the main homeostatic cellular elements of the central nervous system (CNS), which define its structure and provide for numerous aspects of CNS function (Nedergaard et al., 2003; Oberheim et al., 2009; Oberheim et al., 2006; Verkhratsky, 2006, 2009; Wang and Bordey, 2008). Astrocytes generate the blood–brain barrier, control extracellular ion homeostasis and neurotransmitter balance, and maintain synaptic connectivity, strength and plasticity (Abbott et al., 2006; Bundgaard and Abbott, 2008; Halassa et al., 2007; Haydon and Carmignoto, 2006; Perea et al., 2009; Volterra and Meldolesi, 2005).

Neurological diseases result from disruption of a fine equilibrium among neural cell damage, neuroprotection and regeneration. Astrocytes, being homeostatic cells in the CNS are invariably involved in every kind of neuropathology (Giaume et al., 2007; Nedergaard and Dirnagl, 2005; Seifert et al., 2006). Astroglia forms the first line of brain defense through its wide-ranged homeostatic activity. Astrocyte regulates the volume and composition of extracellular space (Kofuji and Newman, 2004), contain extracellular levels of glutamate (Danbolt, 2001; Kirischuk et al., 2007), thereby limiting the intrinsic excitotoxicity of the latter, regulate fluid movements, and provide the main antioxidant system (Dringen, 2000; Simard and Nedergaard, 2004). However, there is an intrinsic dichotomy in the function of astroglia, which can contribute to neuronal damage through failure or reversal of various homeostatic molecular cascades (Giaume et al., 2007; Nedergaard and Dirnagl, 2005). Pathological insults to the CNS trigger a specific astrocytic reaction known as reactive astrogliosis, which aims at protecting the brain parenchyma, isolating the damaged area, reconstructing the blood–brain barrier, and promoting the remodeling of the neural circuitry through isomorphic gliosis (Wilhelmsson et al., 2006).

Alzheimer disease (AD), (Alzheimer, 1907; Kraepelin, 1919) is one of the most common form of dementia (Braak et al., 1999), clinically manifested by cognitive deficits such as memory and learning impairment, and histopathologically by substantial neuronal and synaptic loss associated with extracellular β-amyloid (Aβ) accumulation in the form of plaques and appearance of intraneuronal hyperphosphorilated tau protein fibrillary tangles (Selkoe, 2001). The first and most

affected areas in AD are those directly responsible for cognitive tasks; these areas include entorhinal cortex, hippocampus, basal forebrain, and nucleus basalis of Meynert (Yankner, 1996). The pathological potential of glia in dementia was originally suggested by Alois Alzheimer in 1910 (Alzheimer, 1910). Several studies have shown that both aggregated Aβ protein and the intact cores of Aβ plaques isolated from human AD brain tissue, as well as Aβ fragments can stimulate astrogliosis, which is also observed in human AD brains (Nagele *et al.*, 2004). Experiments in vitro, have demonstrated that Aβ can trigger astroglial Ca^{2+} signaling, mitochondrial depolarisation, and oxidative stress (Abramov *et al.*, 2003, 2004); the Aβ was also reported to trigger apoptosis in cultured embryonic astrocytes (Assis-Nascimento *et al.*, 2007) and in human AD brains (Kobayashi *et al.*, 2004). Reactive astrocytes were postulated to accumulate large amounts of Aβ (Nagele *et al.*, 2003; Nagele *et al.*, 2004) forming astroglial Aβ deposits, although this ability has not been conclusively confirmed. Here, we report the results of detailed analysis of astroglial morphology in the hippocampus of transgenic AD animal model of different ages.

Materials And Methods

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice

Experiments were performed on male 3xTg-AD mice and their background-matching controls as described in detail previously (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008; Rodriguez et al., 2009a).

Fixation and Tissue Processing

3xTg-AD animals of different age groups (3, 6, 9, 12, and 18 months; n=5, 6, 6, 7, 7, 3; respectively) and their equivalent (3, 6, 9, 12, and 18 months; n=5, 6, 7, 7, 4, 3; respectively) non-Tg controls were anesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75% acrolien (25 mL, TAAB, UK) in a solution of 2% paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75 mL). Brains were then removed and cut into 4–5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodriguez et al., 2008). The brain sections were postfixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40–50 µm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels –1.58 mm/–2.46 mm (hippocampus) posterior to Bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).

Antibodies

A monoclonal mouse antiserum generated against GFAP from pig spinal cord (anti-GFAP; Sigma-Aldrich, UK; #G3893) was used for the determination of glial cytoskeleton properties and change and rabbit anti-GFAP IgG fraction of antiserum (Sigma-Aldrich, UK; #G9269) for the analysis of glial cytoskeleton in relationship with

neuritic plaques. For the identification of beta amyloid (Aβ) plaques, we used a monoclonal mouse antiserum that reacts with abnormally processed isoforms, as well as precursor forms of Aβ, recognising an epitope within amino acids 3–8 (EFRHDS; anti-Aβ 6E10 [SIG-39320]; Signet Laboratories, Dedham, MA). The immunolabeling pattern we obtained with this antibody is equivalent to that obtained previously in different brain regions (Oddo et al., 2003a). The specificity of the antibodies has been reported previously using immunohistochemistry and western blot (Eng et al., 2000; Halliday et al., 1996; Rodriguez et al., 2008). To assess for nonspecific background labeling or cross-reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and secondary antibodies from the incubation Solutions resulted in a total absence of target labeling (data not shown).

Immunohistochemistry

For the detection and determination of GFAP cells and its relationship with Aβ senile plaques we used both, single, and dual indirect immunofluorescence labeling. To minimise methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using precisely the same experimental conditions. However, since the same animals have been used for both experiments we could only use sequential sections that did not allow an exact matching in anatomical levels for both single and dual labeling. Nevertheless, this was minimised by collecting sections that ranged between 40 and 100 μm apart therefore guaranteeing the maximum possible anatomical proximity. For this procedure, the vibratome sections were first incubated in 1% sodium borohydride (Aldrich, UK) for 30 min. The sections were then washed with PB profusely before rinsing in 0.1 M TS for 10 min. Brain sections were then incubated with 0.5% albumin bovine serum BSA (Sigma, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, UK) for 30 min. For the single labeling, sections were incubated for 48 h at room temperature in primary antibody (mouse anti-GFAP, 1:30,000; SIGMA, Saint Louise, MI). The sections were rinsed in 0.1 M TS for 30 min and incubated in 1:200 dilution of fluorescein (FITC)-conjugated goat antimouse IgG (Jackson Immunoresearch, Baltimore Pike, PA) for 1 h at room temperature and finally rinsed in 0.1M TS for 30 min.

For dual labeling, the sections were incubated for 48 h at room temperature in

primary antibody cocktail containing: (1) mouse anti-beta amyloid monoclonal antibody (Aβ; 1:2,000; Covance, Emeryville, CA) and (2) rabbit anti-GFAP (1:30,000; SIGMA, Saint Louise, MI) simultaneμously. Subsequently, Aβ and GFAP were detected in a sequential manner on the same sections by incubation with Rhodamine (TRITC)-conjugated goat anti-mouse and (FITC)-conjugated goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).

Morphological Analysis of Astrocyte Cytoskeleton

Astrocytes (n=30–35, in both single and dual labeling experiments) were imaged using confocal scanning microscopy (Leica SP5 upright), recording layers at every 0.2 μm. Parallel confocal planes were superimposed and morphological analysis was carried out by Cell Analist Programe (Chvatal et al., 2007) using digital filters (average 3 x 3, convolution, gauss 5 x 5, despeckle, simple objects removal) to determine the surface (S) and the volumen (V) of the GFAP-stained cytoskeleton of astrocytes. When analysing astroglial morphology in relation to Aβ plaques, we considered all cells with somata within 50 μm from the plaque border as plaque-associated, and cell with somata positioned more distantly as cells not associated with plaques.

GFAP-Positive Cell Count in Hippocampus

To determine whether the changes in hippocampal GFAP cytoskeleton surface and volume are linked with changes in the number of astrocytes expressing GFAP, we determined the total number of GFAP-positive astrocytes in the dentate gyrus (DG) and CA1 subfields in five representative nonconsecutive sections throughout the dorsal hippocampus (analysed in an area of 152,000 μm^2 for DG and 235,000 μm^2 for CA1 in coronal sections of 40 μm thickness). The specific analysed areas were the molecular layer in the DG and stratum radiatum and stratum lacunosum-moleculare in the CA1. Confocal stack images were used to this propose. GFAP-positive astrocytes were intensely labeled against dark background that made them easy to identify with equal chance of being counted. The number of GFAP-positive astrocytes was determined blindly by a single observer; therefore, counting bias was kept to a minimum.

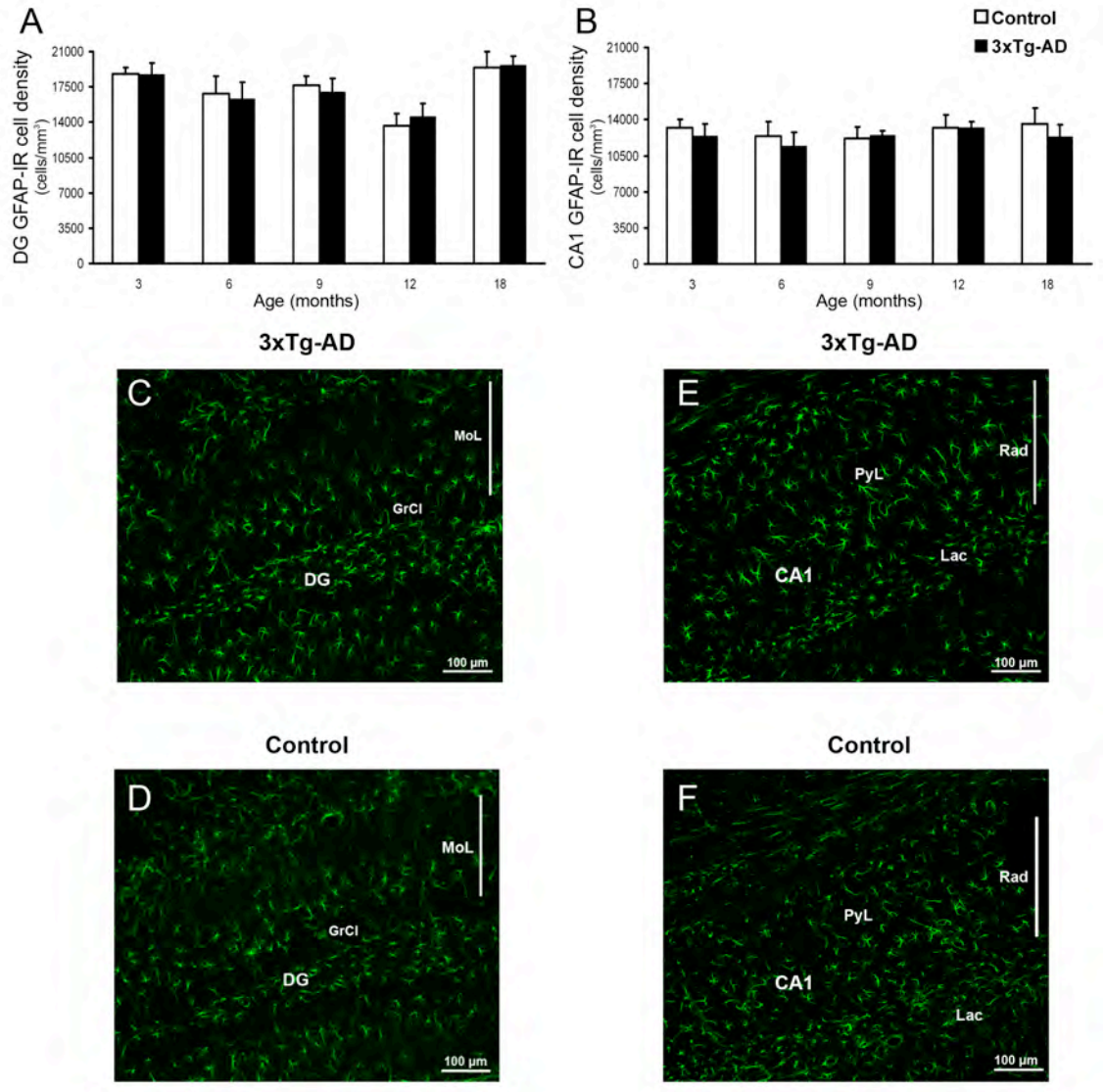
Statistical Analysis

Data were expressed as mean ± SE. One-way and two-way analysis of variance (ANOVA) were used to determine the changes in surface and volume of GFAP profiles within and between each experimental group (3xTg-AD or non-Tg), followed by Bonferroni post-hoc test. Paired or unpaired t-test were used to examine differences in the number, surface, and volume of GFAP labeled cells between the 3xTg-AD and non-Tg animals, as well as was to analyse astrocytic cytoskeleton differences in relationship with Aβ senile plaques.

Results

Ageing and AD Conditions Are Not Associated with Changes in Numbers of GFAP-Positive astrocytes

We analysed the astroglial morphology in the hippocampus, where the majority (~80%) of astrocytes express GFAP (Kimelberg, 2004). Numerous GFAP immunoreactive cells (GFAP-IR), showing stellate shape and multiple branched processes that are typical of astrocytes (Figs. 1–3), were widely distributed throughout the hippocampus in both non-Tg and 3xTg-AD mice; some of them having the typical morphology of perivascular astrocytes (Fig. 3C). When we calculated the density of astrocytes within the DG and CA1 of the 3xTg-AD mice and compared it with the non-Tg, we found no changes in the density of GFAP-IR astrocytes at all ages (Fig. 1).



Astroglial Cytoskeletal Modifications in the 3xTg-AD

From 6 months of age, astrocytes in the 3xTg-AD mice showed a clear tendency towards a lower GFAP surface shown by a difference of 43.84% (781.11 ± 87.44 μm^2 vs. 1390.89 ± 340.01 μm^2; n.s) and volume by 52.76% (254.61 ± 36.62 μm^3 vs. 559.08 ± 152.39 μm^3; n.s), when compared with their non-Tg controls in the DG (Fig. 2A,B, respectively) but not in the CA1 subfield where similar changes only appears at later age (18 months; Figs. 2C and 3D). However, these changes in the DG become significant only at 12 and 18 months of age in 3xTg-AD mice when compared with controls. Astrocytes in DG show less GFAP surface 40.73% (575.04 ± 89.65 μm^2 vs. 970.33 ± 54.84 μm^2, $P < 0.0001$) and 64.79% (631.25 ± 78.17 μm^2 vs. 1793.84 ± 138.53 μm^2, $P < 0.0019$), respectively (Fig. 2A). Likewise, there was a significant lower GFAP volume at 12 months (45.39%; 165.18 ± 32.32 μm^3 vs. 302.48 ± 21.72 μm^3, $P < 0.0162$) and at 18 months of age (71.98%; 186.76 ± 22.19 μm^3 vs. 665.95 ± 64.18 μm^3, $P < 0.0103$; Fig. 2B). These changes in GFAP surface and volume were accompanied by a reduction in glial branching, indicative of the process of atrophy (Fig. 2F, H). One way ANOVA showed that age had not an effect on GFAP surface and volume in DG of nor non-Tg or 3xTg-AD condition ($p > 0.05$ in both cases), whilst two way ANOVA determined that genotype significantly affects GFAP surface and volume ($F_{3,36}$=17.25; $P < 0.001$ and $F_{3,36}$=15.69; $P < 0.001$ respectively). In the CA1 subfield, ageing affected both non-Tg and 3xTg-AD groups as shown by an increase of GFAP surface by 113.33% and 82.50% ($P < 0.0296$ and $P < 0.004$) and of GFAP volume by 142.53% and 122.85% ($P < 0.0412$ and $P < 0.0099$) respectively. However, 3xTg-AD manisfested a significant lower level of GFAP surface and volume at 18 months (Fig. 2C,D). The difference in surface was 29.42% (1586.00 ± 106.97 μm^2 vs. 2247.19 ± 120.76 μm^2, $P < 0.0095$) whereas the volume difference was 32.74% (615.02 ± 66.92 μm^3 vs. 914.44 ± 58.88 μm^3, $P < 0.0237$; Fig. 2C,D).

Figure 1. (←) *Bar graphs showing the numerical density (# cells/mm^3) of GFAP-IR cells number in the DG (A) and CA1 (B) of 3xTg-AD mice compared to control animals. Bars represent mean ± SEM. Confocal micrographs illustrating the expression and number of GFAP-IR cells in the DG (C) and CA1 (E) of 3xTg-AD mice compared to non-Tg animals (D and F, respectively).* ***DG****: dentate gyrus,* ***GrCL****: granule cell layer,* ***Mol****: molecular layer,* ***PyL****: pyramidal layer,* ***Rad****: stratum radiatum,* ***Lac****: stratum lacunosum moleculare.*

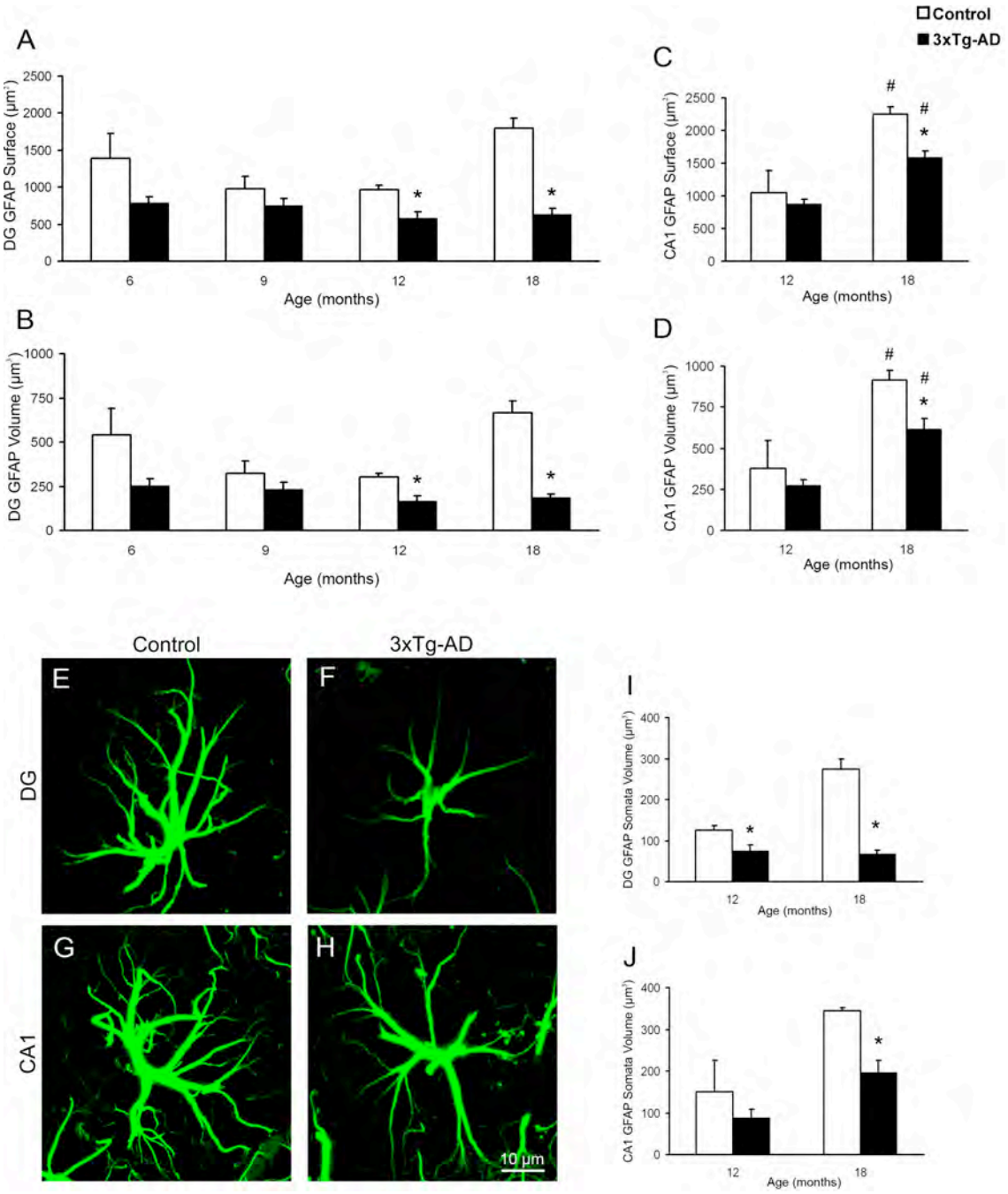


Figure 2. *Bar graphs showing GFAP surface, volume, and body volume differences in both the DG (A, B, I) and the CA1 (C, D, J) of the hippocampus of the 3xTg-AD mice when compared to control animals. Bars represent mean ± SEM. ∗ =p<0.05 when compared to age match control. # = p<0.05 when compared to genotype match 12 months of age group. G-J: Confocal micrographs illustrating the astrocytic atrophy in 3xTg-AD mice in the DG (F) and CA1 (H) compared to control animals (E and G, respectively) revealed by GFAP labeling.*

Changes in Astroglial Somata Volume in the 3xTg-AD

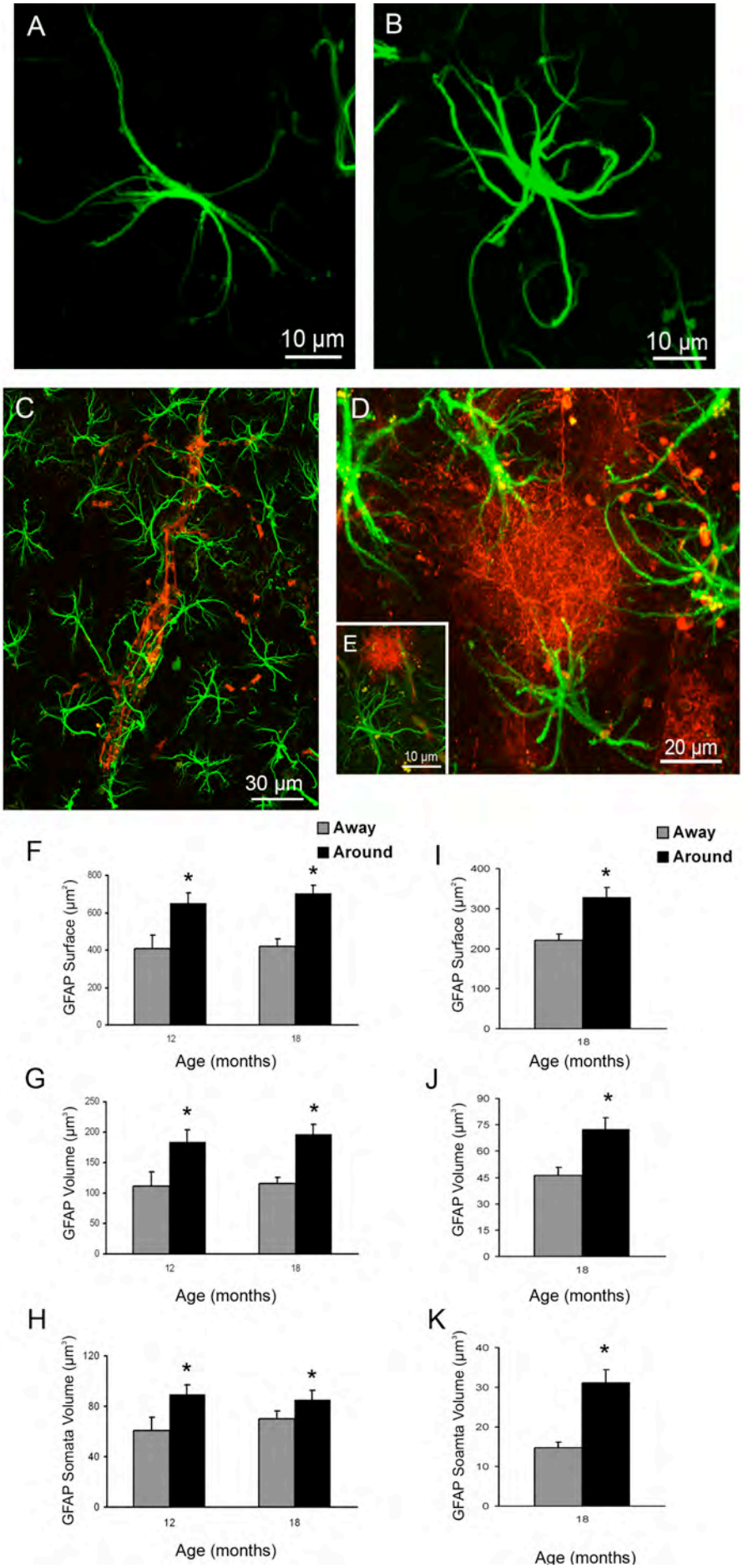
Hippocampal astrocytic somata were normally separated between each other determining their different cellular domains. The astroglial processes were extending outward from the somata in multiple directions; generally, proximal primary processes had a tendency to extend radially whereas distal and secondary order extended in random fashion (Figs. 2E–H and 3A, B).

From our results, it is evident that the astroglial somata was rarely placed in the center of the astrocytic extensions and domain (Figs. 2E–H and 3A, B). As happened with the overall GFAP profiles, the glial somatic volumes also showed significant lower level, but without any evidente change in their shape (Fig. 2F, H). This difference (of 40.46%, 106.53 ± 16.14 μm^3 vs. 178.93 ± 57.83 μm^3, n.s) appeared already at 6 months of age in the DG, but was significant only at 12 and 18 months; when the volumes of somata difference compared to non-Tg was of 40.46%, (74.14 ± 14.97 μm^3 vs. 124.53 ± 11.72 μm^3, $P < 0.0476$) and of 75.55% (66.97 ± 8.88 μm^3 vs. 274.03 ± 25.90 μm^3, $P < 0.0016$), respectively (Fig. 2I). In CA1, this effect was evident at 18 months of age (difference of 42.81%, 197.19 ± 28.87 μm^3 vs. 264.18 ± 7.26 μm^3, $P < 0.0081$; Fig. 2J).

Differential Changes in Morphology of Astrocytes Depending on Their Relation to Aβ Plaques

The accumulation of Aβ plaques in the hippocampus of 3xTg-AD animals starts at 12 months, with a clear prevalence in the CA1 compared with the DG. These plaques are surrounded by astrocytes, which display typical astrogliotic characteristics such as hypertrophy and increased branching (Fig. 3B–E). Some of these reactive astrocytes are also associated with blood vessels highly loaded with Aβ (Fig. 3C).

Reactive astrocytes surrounding Aβ plaques in CA1 had significantly larger GFAP surface when compared with astrocytes situated away from plaques (by 58.95% at 12 months (651.72 ± 54.58 μm^2 vs. 410.00 ± 69.28 μm^2, $P < 0.0030$) and by 66.65% at 18 months (702.16 ± 44.61 μm^2 vs. 421.30 ± 40.91 μm^2, $P < 0.0044$; Fig. 3F).


A
10 µm
B
10 µm
C
30 µm
D
E
10 µm
20 µm
F
Away
Around
GFAP Surface (µm²)
Age (months)
I
GFAP Surface (µm²)
Age (months)
G
GFAP Volume (µm³)
Age (months)
J
GFAP Volume (µm³)
Age (months)
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GFAP Somata Volume (µm³)
Age (months)
K
GFAP Soamta Volume (µm³)
Age (months)

This difference in surface was paralleled by a higher volume of GFAP of 65.18% (183.33 ± 20.75 μm^3 vs. 110.98 ± 23.27 μm^3, $P < 0.0037$) and 71.06% (196.32 ± 17.07 μm^3 vs. 114.76 ± 11.50 μm^3, $P < 0.0034$) at 12 and 18 months of age respectively when compared with astrocytes situated away from plaques (Fig. 3G). Similarly, we observed significant higher volume of somata of astrocytes associated with plaques when compared with astrocytes distant to the plaques by 46.95% at 12 months (89.32 ± 7.79 μm^3 vs. 60.78 ± 10.32 μm^3, $P < 0.0046$) and by 21.59% at 18 months (84.84 ± 7.49 μm^3 vs. 69.77 ± 6.55 μm^3, $P < 0.0137$; Fig. 3H). Age did not affect the GFAP surface, volume and body volume of away and around astrocytes in either 3xTg-AD and non-Tg conditions ($p > 0.05$).

Astrocytes surrounding plaques in the DG showed similar structural changes at 18 months of age when the burden of Aβ plaques becomes evident within this region. These reactive astrocytes associated with Aβ plaques in DG also showed significantly higher GFAP surface by 48.06% (327.82 ± 24.19 μm^2 vs. 221.40 ± 15.66 μm^2, $P < 0.0013$; Fig 3I), volume by 57.10% (72.27 ± 6.77 μm^3 vs. 46.00 ± 4.85 μm^3, $P < 0.0032$; Fig 3J), as well as the somatas volume by 110.70% (31.10 ± 3.25 μm^3 vs. 14.76 ± 1.36 μm^3, $P < 0.0005$; Fig. 3K) when compared with control animals. At the same time, the astrocytes positioned away from the plaques kept showing atrophic characteristics in both the CA1 and DG areas (Fig. 3A).

Figure 3. (←) *Confocal dual labelling images (GFAP in green and Aβ in red) in 3xTg-AD mice showing the accumulation of astrocytes around the Aβ plaques and vascular Aβ deposits (A-D). Astrocytes surrounding Aβ plaques (A, B) and Aβ loaded blood vessel (C), undergo astrogliosis and in some cases accumulate intracellular Aβ (yellow; A, B). Occasionally, one could observe some distant astrocytes sending reactive processes towards Aβ plaques (D). (F–K) Bar graphs showing GFAP-positive astrocytic surface (F), volume (G), and soma volume (H) differences between astrocytes located around the amyloid plaques (Aβ) and those distant to the plaques in the CA1 of 3xTg-AD animals. (I–K): Similar astrocytic surface (I), volume (J), and soma volume (K) differences are observed in the DG at 18 months of age. Bars represent mean ± SEM ($P < 0.05$).*

Discussion

The astroglial cells, which form the ultimate homeostatic system in the CNS, are intimately involved in many forms of neurological diseases. At the same time, the reaction of astrocytes during the early stages of chronic neurological disorders and their pathogenetic role remain virtually unknown. The reactive astrogliosis is a characteristic feature of terminal stages of neurodegenerative processes, including the AD (Nagele et al., 2004; Rodriguez et al., 2009b; Rossi and Volterra, 2009; Simpson et al., 2010).

Here, we analysed the GFAP-positive astroglial profiles in the hippocampus of transgenic AD animals of different age. To the best of our knowledge, this is the first attempt to characterise the age-dependent changes in the morphology of GFAP-positive astrocytes in the relevant model of family AD. For our analysis, we have chosen hippocampus because the majority of astroglial cells in this brain region are GFAP positive (Kimelberg, 2004). First, we found that overall density of GFAP-positive astroglial cells is affected neither by age nor by the AD conditions. This shows that neither ageing nor AD pathology are associated with cell loss; similarly, it argues against the prominent astroglial proliferative response in the AD brain. Second, contrary to the general assumption that the AD is associated with reactive astrogliosis with overall increase in GFAP expression and astroglial proliferation (Nagele et al., 2004), we found that in both DG and the CA1 areas there is a generalised atrophy of GFAP-positive astrocytes. This atrophy is manifested by a lower surface and volume of GFAP-positive profiles and appears at the early stages of the disease. Indeed, already at 6 months (when the hippocampus is virtually free from the neuritic plaques) both volume and surface area of astrocytes in DG were lesser. This overall hypotrophy of GFAP-positive profiles in DG remains at 12 and 18 months. The similar hypotrophy of GFAP-positive astrocytes in CA1 region becomes apparent only at 18 months of age.

During the later stages of AD, the changes in astroglial morphology is complex. The appearance of the neuritic plaques in AD, similarly to other brain injuries (Wilhelmsson et al., 2006), results in astrogliosis in both hippocampal areas. In parallel, ageing is also accopained by astrogliosis in the CA1 area. Importantly, the astrocytes of 3xTg-AD mice demonstrating clear signs of reactivity (hypertrophy, thick processes, large cell bodies, and overall elevated volume and surface area of GFAP-positive profiles) were

exclusively associated with the Aβ plaques. Astrocytes localised distantly (>50 μm) from the borders of the plaques were atrophic as reflected by lesser volumen of cell bodies, reduced number of main processes, their arborisation, and overall GFAP surface and volume. The existence of two distinct populations of astrocytes in Aβ plaques-infested brains of transgenic AD animals was also demonstrated in the in vivo Ca^{2+} imaging experiments, which showed increased Ca^{2+} excitability of the astrocytes contacting neuritic plaques (Kuchibhotla *et al.*, 2009).

The early atrophy of astroglial cells in the progression of family-like AD (and indeed the transgenic AD models reproduce the FAD type pathology) can be pathologically relevant. The atrophic changes were not only associated with the thick primary proximal processes but also with secondary medial and occasionally distal thin processes, which may indicate the reduction in overall astroglial arborisation and hence possible decrease in glial synaptic coverage (Rodriguez et al., 2009b). The reduction of synaptic coverage by astroglial membranes together with overall atrophic changes in astroglia can be instrumental in the early imbalance of neurotransmission, reduced glial homeostatic function, and in disrupted synaptic connectivity. The atrophic or degenerative changes in astroglia have been recently observed in several types of chronic neurodegeneration, including amyotrophic lateral sclerosis (Rossi et al., 2008; Rossi and Volterra, 2009), in Wernicke encephalopathy (Hazell, 2009), in frontotemporal dementia (Broe et al., 2004), in schizophrenia and major depression (Gosselin et al., 2009; Rajkowska et al., 2002; Si et al., 2004). The degenerative/ atrophic changes in astroglia may represent the common mechanism in early disruptions of synaptic connectivity in all forms of neurodegeneration. In AD, the early astroglial asthenia may result in inadequate support of synapses, leading to the extinction of the latter and disruption of connectivity within neural circuitry.

Indeed, the synaptic loss at the early stages of AD is well documented (Scheff et al., 2007; Terry, 2000) and the underlying mechanisms are unclear. The results of our morphological analysis may indicate that it is the astroglial failure which produces early synaptic disorders, and hence early cognitive deficits, in the Alzheimer type neuropathology.

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Chapter 4

Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: Mechanism for deficient glutamatergic transmission?

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Abstract

Astrocytes are fundamental for brain homeostasis and the progression and outcome of many neuropathologies including Alzheimer's disease (AD). In the triple transgenic mouse model of AD (3xTg-AD) generalised hippocampal astroglia atrophy precedes a restricted and specific β-amyloid (Aβ) plaque-related astrogliosis. Astrocytes are critical for CNS glutamatergic transmission being the principal elements of glutamate homeostasis through maintaining its synthesis, uptake and turnover via glutamate-glutamine shuttle. Glutamine synthetase (GS), which is specifically expressed in astrocytes, forms glutamine by an ATP-dependent amination of glutamate. Here, we report changes in GS astrocytic expression in two major cognitive areas of the hippocampus (the dentate gyrus, DG and the CA1) in 3xTg-AD animals aged between 9 and 18 months. We found a significant reduction in N_v (number of cell/mm^3) of GS immunoreactive (GS-IR) astrocytes starting from 12 months (28.59%) of age in the DG, and sustained at 18 months (31.65%). CA1 decrease of GS-positive astrocytes N_v (33.26%) occurs at 18 months. This N_v reduction of GS-IR astrocytes is paralleled by a decrease in overall GS expression (determined by its optical density) that becomes significant at 18 months (21.61% and 19.68% in DG and CA1, respectively). GS-IR N_v changes are directly associated with the presence of Aβ deposits showing a decrease of 47.92% as opposed to 23.47% in areas free of Aβ. These changes in GS containing astrocytes and GS-immunoreactivity indicate AD-related impairments of glutamate homeostatic system, at the advanced and late stages of the disease, which may affect the efficacy of glutamatergic transmission in the diseased brain that may contribute to the cognitive deficiency.

Introduction

The central nervous system relies on astrocytes for its correct functioning. Astroglia is critical for metabolic support to neurones by providing glucose and lactate (Magistretti, 2009; Pellerin et al., 2007), regulates ion environment, i.e. K+ and water movements and provides reactive-oxygen-species scavengers like glutathione (Kofuji and Newman, 2004; Simard and Nedergaard, 2004; Verkhratsky et al., 2011).

Astrocytes, as a component of the tripartite synapse, modulate neurotransmission and control the extracellular level of neurotransmitters (Araque *et al.*, 1999; Danbolt, 2001; Grosche *et al.*, 1999; Halassa and Haydon, 2010; Perea *et al.*, 2009; Wilhelmsson *et al.*, 2006). Therefore, astrocytes are essential for glutamatergic transmission being key elements for "*de novo*" synthesis of glutamate and for the glutamate-glutamine cycle; which, in addition, are fundamental for the synaptic plasticity associated to cognitive processes (Kvamme, 1998; McKenna, 2007). The bulk of glutamate release during neurotransmission is taken up by astroglia through Na^+-dependent glutamate transporters (Danbolt, 2001; Kirischuk *et al.*, 2007). In astrocytes glutamate is converted to glutamine by glutamine synthetase (GS) (Danbolt, 2001) (which is considered astrocytic-specific enzyme although some recent studies have shown some degree of oligodendroglial and microglial GS expression under some pathological situations (Gras *et al.*, 2011; Takasaki *et al.*, 2010)). Subsequently, astrocytic glutamine is transported back to neurones for its further conversion into glutamate (Danbolt, 2001; Deitmer *et al.*, 2003). Thus, the glutamate-glutamine shuttle makes both astrocytes and GS essential for glutamatergic neurotransmission (Walton and Dodd, 2007). At the same time astrocytic glutamate uptake prevents glutamate excitotoxicity (Danbolt, 2001); disturbance of astroglial-based glutamate homeostasis may lead to neurotransmitter imbalance, neuronal malfunction and death, as well as impaired cognition (Choi, 1992; Walton and Dodd, 2007).

Astroglia is fundamental for the onset, progression and outcome of neuropathological processes by limiting the damage and promoting the revascularisation of the surrounding tissue through reactive astrogliosis (Giaume *et al.*, 2007; Heneka *et al.*, 2010; Nedergaard *et al.*, 2010; Verkhratsky *et al.*, 2010) and by contributing to

neuroinflammation by release of various pro-inflammatory factors, such as interleukins (Aloisi *et al.*, 1992; Nedergaard and Dirnagl, 2005; Samland *et al.*, 2003).

Alzheimer's disease (AD) is a highly malignant neurodegenerative process characterised by anomalous intraneuronal and extracellular accumulation of β-amyloid protein (Aβ) (Walsh and Selkoe, 2007) and hyperphosphorilated cytoskeleton Tau protein in neurons (Braak *et al.*, 1999). As a consequence of this anomalous protein formation and by a yet unknown mechanism, severe loss of specific ACh neurons and synapses appear at middle and advanced stages of the disease (Yankner, 1996). As a result, the CNS reacts by both neuronal compensation and glial reactivity (Noristani *et al.*, 2010; Pekny and Nilsson, 2005). Recently, in a GFAP based study, we have described that the associated reactive astrogliosis observed in the triple transgenic animal model (3xTg-AD) is preceded by a generalised atrophy of astrocytes that occurs at the middle stages of the disease (9-12 months of age). Formation of the senile plaques triggers secondary astrogliosis in astrocytes associated with Aβ depositions, and the later stages of the pathology are characterised by concomitant astroglial atrophy and astrogliosis that in any case is not associated with astrocytic density alterations (12-18 months of age) (Olabarria *et al.*, 2010; Rodriguez *et al.*, 2009b). Furthermore, and even if the two main pathological hallmarks have to be considered when studying AD, astrocytic involvement, as recently demonstrated by us (Olabarria *et al.*, 2010; Rodriguez *et al.*, 2009b), is mainly related with Aβ pathology, since astrocytes modulate extracellular volume and components and Aβ directly affects the extracellular space, while tau pathology remains intraneuronal throughout AD (Braak *et al.*, 1999).

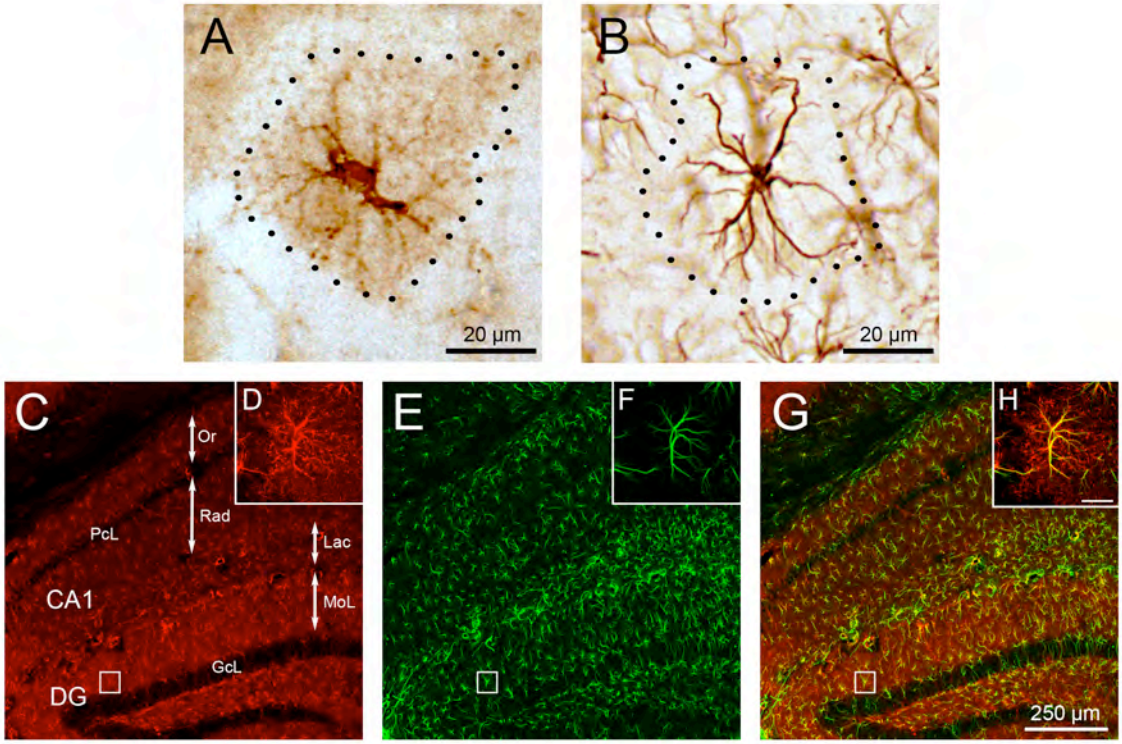

Figure 1. *Light and confocal micrographs of different astrocytic phenotypes according to their enzyme content and cytoskeleton component in the hippocampus of 3xTg-AD mice. (A-B) Light microscopy images of GS (A) and GFAP (B) positive astrocytes showing their differential anatomical characteristics but similar domains. (C,E,G) Hippocampal confocal images evidencing astrocytic GS (C, red) and GFAP (E, green) expression pattern and their co-localisation (G, yellow). (D,F,H) High magnification confocal images illustrating the majority dual expression and co-existence of GS and GFAP (inserts D, F, H) in a representative astrocyte of the molecular layer of the DG. DG, dentate gyrus; GcL, granule cell layer; MoL, molecular layer; Lac, stratum lacunosum moleculare; Or, stratum oriens; PcL, pyramidal layer; Rad, stratum radiatum.*

Materials and methods

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice

Experiments were performed on male 3xTg-AD mice, which harbours the mutant genes for amyloid precursor protein (APPSwe), for presenilin 1 PS1M146V and for tauP301L (Oddo et al., 2003a; Oddo et al., 2003b) and their background-matching controls as described in detail previously (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008; Rodriguez et al., 2009a).

Fixation and tissue processing

Animals of different age groups (9, 12 and 18 months; n = 4-8) were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75 % acrolein (25 ml, TAAB, UK) in a solution of 2 % paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2 % paraformaldehyde (75 ml). Brains were then removed and cut into 4 - 5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodriguez et al., 2008). The brain sections were post-fixed in 2 % paraformaldehyde for 24 hours and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40 – 50 μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25 % sucrose and 3.5 % glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels -1.58 mm/-2.46 mm (hippocampus) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).

Antibodies

A mouse antiserum generated against GS (anti-GS; Millipore, UK; MAB302) was used for the determination of GS positive astrocytes. A rabbit anti-GFAP IgG fraction of antiserum (Sigma-Aldrich, UK; #G9269) was used for the determination of glial cytoskeleton and comparison to GS labelling distribution. A monoclonal antibody

against amyloid beta conjugated with Alexa 488 (Convance, USA; SIG-39347) was employed to label neuritic plaques. The immunolabelling pattern that we obtained with these antibodies is equivalent to that obtained previously (Olabarria et al., 2010; Wilhelmsson et al., 2004) and their specificity has also been previously demonstrated by western blot (Aksenov et al., 1996; Eng et al., 2000; Hensley et al., 1995; Rodriguez et al., 2008). To assess for non-specific background labelling or cross reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and/or secondary antibodies from the incubation solutions resulted in a total absence of target labelling (data not shown).

Immunohistochemistry

To minimise methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using precisely the same experimental conditions. For this procedure, vibratome sections were first incubated for 30 min in 30% methanol in 0.1M PB and 3% hydrogen peroxide (Sigma, UK). Sections were rinsed with 0.1 M PB for 5 mins and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. Subsequently the sections were washed with PB profusely before rinsing in 0.1 M TS for 10 minutes. Brain sections were then incubated with 0.5% albumin bovine serum (BSA, Sigma, Dorset, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, Dorset, UK,) for 30 minutes. For the single labelling, sections were incubated for 48 hours at room temperature with primary antibody (mouse anti-GS, 1:500, cat# MAB302, Millipore, UK).

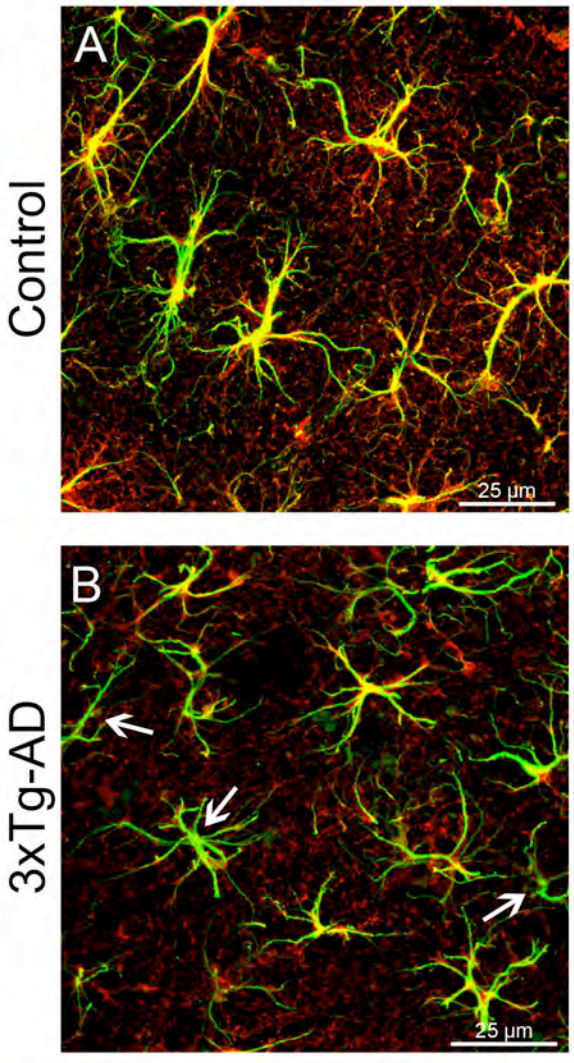

Figure 2. *Confocal micrographs showing GFAP (green) and GS (red) labelling in the hippocampus of either control (A) or 3xTg-AD mice (B). Majority of astrocytes co-express (yellow) GFAP and GS in control mice, whilst some of GFAP positive astrocytes of the 3xTg-AD mice fail to express GS (arrows).*

Sections were rinsed in 0.1 M TS for 30 minutes and incubated in 1:200 dilution of biotinylated horse anti-mouse IgG (Vector laboratories, Peterborough, UK) for 1 hour at room temperature. Sections were rinsed in 0.1 M TS for 30 minutes, followed by incubation for 30 minutes in avidin-biotin peroxidase complex (Vector Laboratories Ltd, Peterborough, UK). The peroxidase reaction product was visualised by incubation in a solution containing 0.022 % of 3,3'diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003 % H_2O_2 for 1.5 minutes as described previously (Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2009a). The reaction was stopped by rinsing the tissue in 0.1 M TS for 6 minutes followed by 0.1 M PB for 15 minutes. Brain sections were permanently mounted onto gelatinised slides. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100 %) followed by xylene; and then permanently coverslipped.

For dual immunofluorescence labelling, the sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (1) mouse anti-GS (1:500) and (2) rabbit anti-GFAP (1:30,000) simultaneously. Subsequently, GS and GFAP were detected in a sequential manner on the same sections by incubation with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen, Paisley, UK), respectively.

For triple immunofluorescence labelling, the sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (1) mouse anti-GS (1:500) and (2) rabbit anti-GFAP (1:30,000) simultaneously. Subsequently, GS and GFAP were detected in a sequential manner on the same sections by incubation with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 633-conjugated goat anti-rabbit (Invitrogen, Paisley, UK), respectively. Then, the sections were washed in 0.1M TS for 30 min and incubated in 0.5% BSA in 0.1 M TS and 0.25% Triton X-100 for 30 minutes. Subsequently, sections were incubation in mouse anti-amyloid beta Alexa 488-conjugated antibody (1:2000) for 20 hours at room temperature.

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

GS positive cell count in hippocampus

We determined the numerical density (N_v; # / mm^3) of GS positive astrocytes at 9, 12 and 18 months of age in both 3xTg-AD and non-Tg mice in the DG and CA1 subfields of the hippocampus. For this, 3 - 4 representative non-consecutive coronal sections throughout the dorsal hippocampus at levels -1.70/-2.18 (Paxinos and Franklin, 2004) were quantified accounting for an analysed volume of approximately 6,000,000 μm^3 in the DG and 15,000,000 μm^3 in CA1. The specific analysed areas were the molecular layer (MoL) in the DG and all the strata of the CA1 apart from the pyramidal cell layer that is practically devoid of GS expression due to the dense packing of pyramidal somata and almost no presence of astrocytic cell bodies. GS positive astrocytes were intensely labelled against lighter background that made them easy to identify with equal chance of being counted. A single observer determined the number of GS positive astrocytes blindly; therefore, counting bias was kept to a minimum.

Optical Density (OD) Measurement

Using computer-assisted imaging analysis (Image J 1.32j, NIH, USA), we analysed the expression and density of GS labelling at 9, 12 and 18 months of age in both 3xTg-AD and non-Tg mice by measuring their optical density (OD) as described previously (Cordero et al., 2005). Briefly, to exclude any experimental errors and/or bias, all images were taken at constant light intensity. Optical filters were used to ensure the specificity of the signal recorded by the camera. The staining was observed throughout the thickness of the section (40 μm) using light microscopy (Nikon Eclipse 80i). No differences were observed in GS immunoreactivity throughout the thickness of the section between 3xTg-AD and non-Tg control animals; hence the changes in OD were used as measure of increased GS expression. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with readout of 255 corresponding to the area with very low GS expression and 0 corresponding to the densest area of labelling. The calibration density was kept constant for measuring all section to avoid experimental variances. Sections background OD was determined from the corpus callosum (CC) that was considered as blank since GS labelling in the CC is virtually absent. GS density of the entire DG MoL and CA1 (with the exception of the pyramidal cell layer) were measured independently and a single measurement was obtained from every sub-region in each hemisphere. To analyse the change in GS density against constant control, the 255 was divided by control region (CC) and the obtained factor was multiplied by the

region of interest in every given section. Inverse optical density was obtained by subtracting from the obtained background level (set at 255). Measurement of mean density were taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each slice. The results are shown as inverse GS density (IOD/pixel).

GS and GFAP positive cell count in relation to Aβ plaques in CA1

Triple labelling pictures of the CA1 at 18 months of age in both 3xTg-AD and non-Tg mice were taken using confocal scanning microscopy (Leica SP2, inverted), recording layers at every 0.5 μm. Both GS and GFAP positive cells were counted separately and taking in to account their localisation regarding Aβ plaques. We considered all cells with the somata within 50 μm from the plaque border of the plaque-associated, and cell with somata positioned more distantly as cells not associated with plaques.

Statistical analysis

Data were expressed as mean ± SEM. One-way and two-way analysis of variance (ANOVA) was used to determine the changes in N_v and OD of GS within and between each experimental group (3xTg-AD or non-Tg), followed by Bonferroni post-hoc test. Unpaired t-tests were used to examine differences between 3xTg-AD and non-Tg animals at different time points and differences between away and around conditions in 3xTg-AD. Significance was accepted at $p \leq 0.05$. The data were analysed using Minitab (Minitab 15 Software) and GraphPad Prism (GraphPad Software).

Results

GS immunoreactive (GS-IR) astrocytes were widely distributed throughout the subdivisions of the hippocampus in both non-Tg and 3xTg-AD mice. This distribution was similar to the GFAP immunoreactivity in both DG and CA1 (Fig. 1C, 1E, 1G, 2A). GS-IR astrocytes show typical protoplasmic morphology characterised by small round cell bodies with few primary and several secondary processes extending radially in random fashion. GFAP-IR astrocytes were characterised by primary and secondary cytoskeleton processes extending radially from the cell body and frequently co-localising with GS-IR main processes. (Fig. 1A, 1B, 1D, 1F, 1H). However, and differently to GFAP-positive astrocytes, GS-IR astrocytes showed a clear and profuse labelling not only in the cell body and primary processes but also throughout the fine and thin distal processes (Fig. 1A, 1D, 3C-F).

GS immunoreactivity in non-Tg animals

In non-Tg animals, GS-IR showed a uniform pattern in the DG and in the CA1 being constant at all age groups. The GS-IR Nv at all ages was significantly lower in the DG compared to the CA1 (5,599 ± 603 cells/mm^3 vs. 9,700 ± 1,041 cells/mm^3, 42.27%, P = 0.009; 4,852 ± 306 cells/mm^3 vs. 10,456 ± 1,788 cells/mm^3, 53.59%, P = 0.021; 5,064 ± 511 cells/mm^3 vs. 10,727 ± 1,083 cells/mm^3, 52.78%, P = 0.001; at 9, 12 and 18 months respectively; Fig 3A, 3B). The overall GS-IR, as determined by the IOD, was slightly higher in the DG at 9 and 12 months of age when compared to CA1 (10.67% and 14.38%); but just being significantly higher in the advanced age (18 months, 16.34%, P = 0.046; Fig. 4A, 4B).

3xTg-AD mice astrocytes showed lower N_v *of GS-IR*

In non-Tg animals ageing did not affect the N_v of GS-IR astrocytes in the DG nor CA1. However, 3xTg-AD mice showed a significant decrease in the DG ($F_{2,13}$=8.449 ; P = 0.006; 34.32%; P < 0.05 for 12 months and 36.02%; P < 0.05 for 18 months compared to 3xTg-AD animals at 9 months of age). GS-IR N_v in CA1 was not affected by genotype. This was further confirm by the two-way ANOVA analysis, which showed that GS-IR cell N_v is affected by age and genotype ($F_{2,22}$=5.29; P = 0.013 and $F_{2,22}$=9.41; P = 0.006) in the DG, whereas not the CA1. From 12 months of age the 3xTg-AD mice showed a significant reduction of the N_v of GS-IR cells in the DG

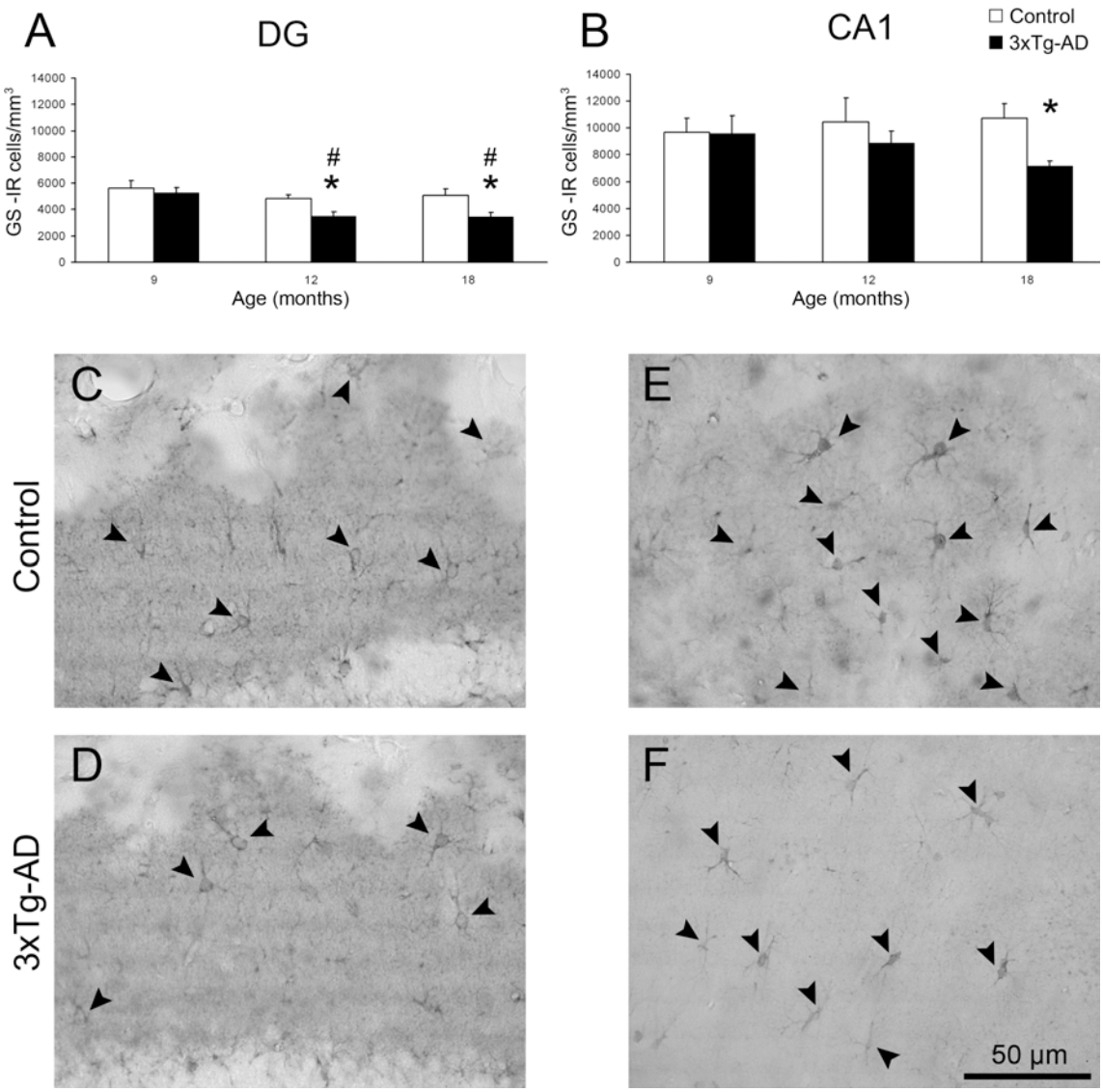

Figure 3. *Bar graphs illustrating GS-IR N_v (number of cells/mm^3) in the DG (A) and CA1 (B) of 3xTg-AD mice compared with non-Tg control animals. Bars represent mean ± SEM. ∗ =P<0.05 when compared to age match control. # = P<0.05 when compared to 9 months of age 3xTg-AD mice. Light micrographs illustrating the difference in GS positive astrocytes (arrowheads) between non-Tg control mice and 3xTg-AD mice in either DG (C,D) or CA1 (E,F).*

(4,852 ± 306 cells/mm^3 vs. 3,465 ± 344 cells/mm^3; 28.59%, P = 0.016; Fig 3A) compared to the non-Tg control animals, whereas no apparent difference was found in CA1. At 18 months of age GS-IR cell N_v was significantly lower in both the DG (5,064 ± 511 cells/mm^3 vs. 3,462 ± 321 cells/mm^3; 31.65%, P = 0.036) and in the CA1 (10,727 ± 1083 cells/mm^3 vs. 7,159 ± 400.78 cells/mm^3; 33.26%, P = 0.026; Fig 2, 3B, 3C-3F).

3xTg-AD mice exhibit a decrease in GS expression

In parallel to the alteration in the Nv of GS-IR cells, we observed a decrease in the expression of GS in the hippocampus of 3xTg-AD mice with no apparent regional differences, as shown by the decrease of the inverse optical density in both DG ($F_{2,13}$=5.734 ; P = 0.0197; 35.97% and 37.93% ; P < 0.05 for 18 months when compared to 9 months and 12 months respectively) and CA1 ($F_{2,13}$=4.510 ; P = 0.0371; 36.07% ; P< 0.05 for 18 months when compared to 12 months of age) (IOD; Fig. 4). The 3xTg-AD mice, when compared to non-Tg controls, showed a significantly decreased GS expression in both DG (66 ± 3 vs. 52 ± 4; 21.62%; P < 0.05) and CA1 (57 ± 2 vs. 46 ± 2; 19.69%; P = 0.010) at 18 months of age (Fig. 4), but not at the earlier ages (Fig. 4A, 4B). As overall, it was shown that GS expression is affected by age in both DG and CA1 ($F_{2,22}$=7.09; P = 0.004 and $F_{2,22}$=6.44; P = 0.006 respectively) regardless the genotype.

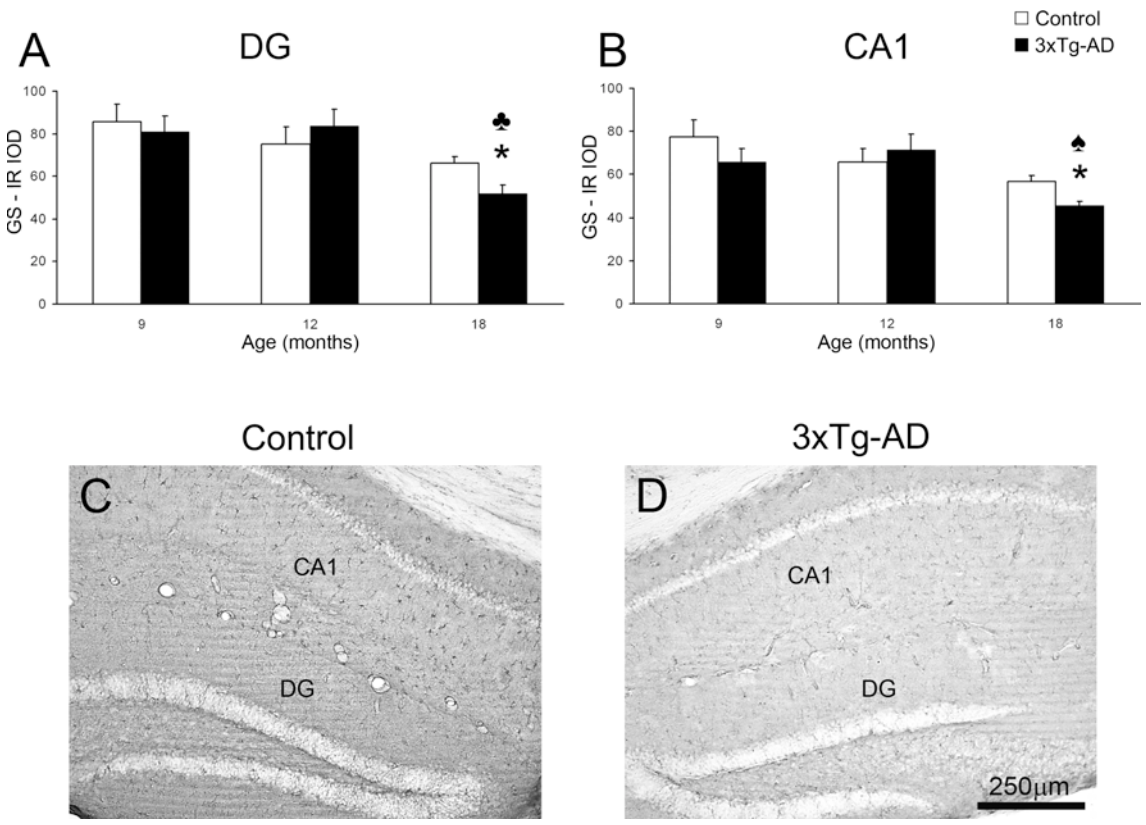

Figure 4. *Bar graphs showing GS content as determined by its inverted optical density (IOD) in DG (A) and CA1 (B) of 3xTg-AD mice compared with control non-Tg animals. Bars represent mean ± SEM. ∗ =P<0.05 when compared to age match control. ♣ = P<0.05 when compared to 9 and 12 months of age 3xTg- AD groups. ♠ =P<0.05 when compared to 12 months of age mice. Illustrative light micrographs of control mice (C) and 3xTg-AD mice (D) showing the GS expression in both the DG and CA1.*

N_v of GS-IR astrocytes decrease in 3xTg-AD mice is associated, although no exclusive, with Aβ plaques.

GS-IR decrease was not homogenous throughout the CA1 parenchyma in 3xTg-AD. GS-IR N_v was compared with GFAP-IR Nv in either the vicinity or distant of Aβ plaques in 3xTg-AD at 18 months of age, to asses whether GS expression changes were related to Aβ plaques. No GFAP-IR N_v related changes were observed in any of the conditions (Fig 5). However, GS-IR astrocyte N_v was significantly diminished in CA1 areas free of Aβ (10,069 ± 572 cells/mm^3 vs. 7,704 ± cells/mm^3; 23.49%, P = 0.010) when compared to GFAP-IR N_v density in same areas, being this reduction more patent in the vicinities of neuritic plaques (10,314 ± 922 cells/mm^3 vs. 5,338 ± 685 cells/mm^3; 48.24%, P = 0.002). In addition, GS-IR N_v associated to neuritic plaques was significantly lower to GS-IR N_v away from Aβ deposits (by 24.24%, P = 0.022).

GS-IR and GFAP-IR N_v was almost identical in non–Tg controls, showing 99.22% of co-localisation (data not shown).

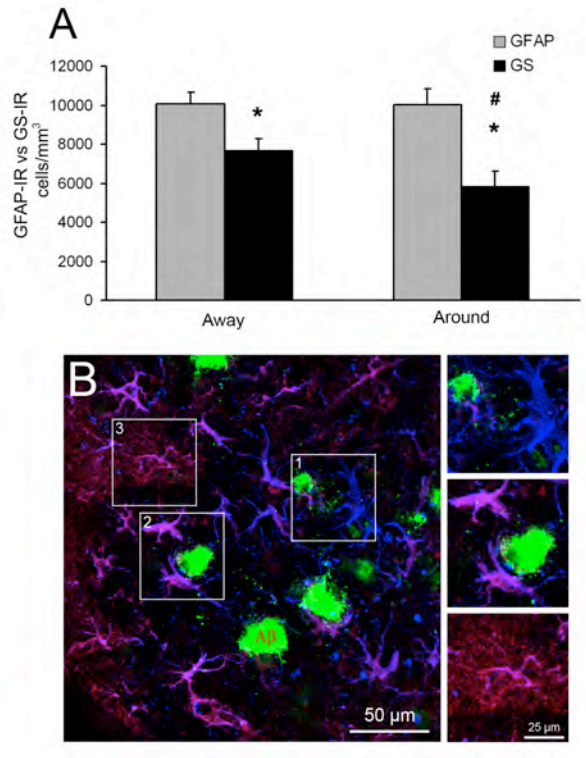

Figure 5. *GFAP and GS astrocytes in the hippocampus of 3xTg-AD and their relationship with Aβ plaques. (A) Bar graph showing GFAP-IR and GS-IR astrocytes N_v differences in the distance (away; >50µm) and vicinities (around; <50µm) of Aβ deposits. Bars represent mean ± SEM. ∗ = P ≤ 0.05 compared to correspondent GFAP-IR cell N_v; # = P ≤ 0.05 compared to GS-IR cell N_v away. (B-E) Confocal micrographs illustrating GFAP (blue), GS (red) and Aβ (green) labelling. Several GFAP positive astrocytes in surrounding Aβ plaques (<50µm) lack of GS immunoreactivity (1), whilst others, co-express GFAP and GS, without the typical extended GS domain (2). (3) Astrocytes > 50µm away from Aβ deposits co-expressing GFAP and GS in their cell bodies and main processes (pink). The distal fine processes express just GS (red).*

Discussion

In the present study we analysed the functional status of astroglia in the triple transgenic AD animal model by determining the expression of the GS at different ages. Our results in this animal model demonstrate that the expression of astroglial GS in hippocampus is affected at the human equivalent advanced and late stages of AD, as indicated by a reduction in both the N_v of GS-IR astrocytes and GS expression (as determined by IOD) in the 3xTg-AD when compared to the control animals. These changes in Nv appear in both DG and CA1, albeit they initially occur in the DG at 12 months of age. Such reduction becomes more evident in the surroundings of Aβ plaques at 18 months of age. The GS expression is also decreased in the 3xTg-AD in both DG and CA1 but at a later age (18 months).

Astrocytes are known to be involved in most neurological diseases including AD (Giaume *et al.*, 2007; Heneka *et al.*, 2010; Rodriguez *et al.*, 2009b). AD is characterised by a profound cognitive impairment due to a severe loss of synapses and neurones; which is generally believed to be associated with reactive astrogliosis (Alzheimer, 1910; Braak *et al.*, 1999). Our recent studies in the 3xTg-AD animal model, however, have found a more complex astroglial reactions in the late stages; which might show a similar pattern during human AD progression. At the early stages of the pathology astrocytes undergo generalised atrophy and down-regulation of GFAP expression, which may indicate early compromise of astrocytic homeostatic functions (Olabarria *et al.*, 2010; Rodriguez *et al.*, 2009b; Verkhratsky *et al.*, 2010). Several studies have previously described that GFAP abnormalities are associated with impaired glutamate homeostasis (Hughes *et al.*, 2004; Pekny *et al.*, 1999; Sullivan *et al.*, 2007; Weir and Thomas, 1984). In a hypoxic animal model, astrocytes in the affected areas showed an abnormal GFAP cytoskeleton characterised with very short processes; which was associated with redistribution of GFAP and GLAST to the cell body, suggesting an impaired glutamate homeostasis (Sullivan *et al.*, 2007). In addition, in GFAP knock-out mice, the lack of GFAP compromised the trafficking of the GLT-1 to the cell membrane, affecting glutamate uptake (Hughes *et al.*, 2004). Furthermore, "*in vitro*" studies have shown an inverse correlation between astroglial expression of GFAP and GS (Weir and Thomas, 1984). Recently has been demonstrated that, as opposed to what was thought, 3xTg-AD model shows some degree of neuronal loss as a consequence of the pathological burden

(Fuhrmann *et al.*, 2010), strengthening the relevance of this AD model and allowing the comprehension of many described synapse-related pathological events like the current one.

Studies in AD brains have shown a decrease of cortical astrocytic GS expression (Le Prince *et al.*, 1995; Robinson, 2000) that is accompanied by a "*de novo*" neuronal expression of GS as a compensatory reaction (Robinson, 2000, 2001). This direct effect on astrocytic GS expression is also present in other neurodegenerative diseases, such as Huntington's disease, which is associated with GLT-1 glutamate transporter down-regulation (Jacob *et al.*, 2007; Lievens *et al.*, 2001). Further studies show that GS is oxidised due to Aβ in AD brains causing reduction of enzymatic activity (Castegna *et al*., 2002) and that GS reduced expression is related to neuritic plaques in AD brain cortex (Robinson, 2000). On the contrary, human studies have shown increased levels of GS immunoreactivity in the prefrontal cortex in AD brains (Burbaeva *et al.*, 2005). Obviously, phylogenetic proximity, tissue preservation and medical history play crucial role in the interpretation of human vs rodent data. Our current work shows that astrocytes at the late stages (plaque formation and stabilisation, 12-18 months) of AD-like pathology, in agreement with the above-mentioned studies, are functionally compromised as shown by the reduction of GS expression and therefore their capability to master the glutamate-glutamine cycle.

Thus, we could hypothesise that astrocytes fail to support neurones and control synapses from the appearance of the disease pathological burden. Furthermore, and considering astrocytic physiological function, one could consider that these changes result in an impaired glutamate homeostasis that is manifested by deficient glutamate/glutamine turnover and restricted supply of glutamine to neurones, being this failure somehow more dramatic in the proximities of Aβ plaques. Furthermore, GS deficiency could also be a consequence of impaired astroglial glutamate uptake; inducing a potential neurotoxic process by abnormal glutamate metabolism. Hence GS deficiency may reflect an altered glutamatergic neurotransmission of AD, at the advanced and late stages of the disease, which can account for a global hippocampal neurotransmitter imbalance underlaying the mnesic and cognitive impairments observed in the disease.

Abbreviations:

3xTg-AD: Triple transgenic mouse of Alzheimer's disease; ACh neurons: Acetylcholinergic neurons; AD: Alzheimer's disease; ATP: Adenosine triphosphate; CA1: Cornus ammonis 1; CC: Corpus callosum; CNS: Central nervous system; DG: Dentate gyrus; GcL Granule cell layer; GFAP: Glial fibrillary acidic protein; GLAST: Glutamate aspartate transporter; GLT-1: Glutamate transporter; GS: Glutamine synthetase; GS-IR: Glutamine synthetase immunoreactivity/-reactive; IgG: Immunoglobulin G; IOD: Inversed optical density; Lac: Stratum lacunosum moleculare; MoL: Molecular layer; N_V: Numerical density (cell number/ mm^3); OD: Optical density; Or: Stratum oriens; PB: Phosphate buffer; PcL: Pyramidal layer; Rad: Stratum radiatum; TS: Trizma® base saline.

Competing interests:
The authors declare that they have no competing interests

Authors' contributions:
M.O. carried out the immunohistochemical study and contributed to the writing of the manuscript. H.N.N. contributed to the immunhistochemical study. A.V. participated in the conception of the study and writing. J.J.R. participated in the conception and design of the study and writing of the manuscript as well as coordinated the study. All authors read and approved the final manuscript.

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Chapter 5

Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease

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Cell Death and Disease. **1**:e1.

Abstract

The formation of cerebral senile plaques composed of amyloid β peptide (Aβ) is a fundamental feature of Alzheimer's disease (AD). Glial cells and more specifically microglia become reactive in the presence of Aβ. In a triple transgenic model of AD (3xTg-AD), we found a significant increase in activated microglia at 12 (by 111%) and 18 (by 88%) months of age when compared with non-transgenic (non-Tg) controls. This microglial activation correlated with Aβ plaque formation, and the activation in microglia was closely associated with Aβ plaques and smaller Aβ deposits. We also found a significant increase in the area density of resting microglia in 3xTg-AD animals both at plaque-free stage (at 9 months by 105%) and after the development of amyloid plaques (at 12 months by 54% and at 18 months by 131%). Our results show for the first time that the increase in the density of resting microglia precedes both plaque formation and activation of microglia by extracellular Aβ accumulation. We suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting a microglia increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

Introduction

Microglial cells, which were discovered by Pio Del Rio Hortega (Del Rio-Hortega, 1919), are the resident macrophages of the CNS. These cells of myeloid origin (Ransohoff and Perry, 2009) enter the CNS shortly alter birth, and disseminate over the brain and the spinal cord, where they rapidly transform into the resting microglia with a peculiar morphological appearance (Kreutzberg, 1996). The resting microglial cells have small somata and multiple fine processes, with each cell occupying the defined domain that does not overlap with the neighbouring microglia. In the healthy CNS, microglial processes are on a constant move, scanning the microenvironment in their territorial domains (Davalos et al., 2005; Nimmerjahn et al., 2005). Insults to the nervous system trigger a complex and multi-stage activation of microglia (Hanisch and Kettenmann, 2007), which results in both phenotypic and functional changes.

Being the intrinsic CNS defence system, microglia are intimately involved in all forms of neuropathology (Giaume *et al.*, 2007), including various types of neurodegeneration. Alzheimer's disease (AD); (Alzheimer, 1907) is the most common form of neurodegeneration, which results in severe and irreversible cognitive decline and dementia (Walsh and Selkoe, 2004). The loss of neurones and synaptic connectivity in AD has been specifically linked to the extracellular deposition of amyloid β-peptide (Aβ), with its progressive aggregation to form insoluble amyloid plaques as well as intracellular Tau neurofibrillary tangles (Selkoe, 2001, 2002). Microglial activation in AD has been discovered two decades ago (McGeer *et al.*, 1987), and activated microglia are generally believed to be involved in AD-associated inflammatory response (Heneka and O'Banion, 2007), although the precise role of microglial cells in AD pathogenesis remains to be elucidated and characterised (Hickman *et al.*, 2008; Streit *et al.*, 2004).

Some studies have shown that activated microglia contribute to neurodegeneration in AD, either via the chronic secretion of pro-inflammatory peptides (Zilka *et al.*, 2006) or by being involved in the formation of Aβ plaques (Stalder *et al.*, 1999). However, other reports have suggested a neuroprotective role of microglia in AD through, for example, Aβ clearance (Bard *et al.*, 2000; Hickman *et al.*, 2008). At any

rate the concentration of activated microglia in the vicinity of Aβ plaques is routinely observed (Frautschy *et al.*, 1998; Jimenez *et al.*, 2008; Simard *et al.*, 2006; Wegiel *et al.*, 2003), although the changes in the resting microglia population in AD remain unknown.

The aim of the present study was to quantify the density of resting and activated microglia in the CA1 subfield of the hippocampus in a triple transgenic mouse model (3xTg-AD) of AD. Developed by Oddo et al. (Oddo *et al.*, 2003a; Oddo *et al.*, 2003b), 3xTg-AD mice express mutant versions of the human presenilin 1 (PS1M146V) and tau (P301L) genes on a Tg2576 (amyloid precursor protein $(APP)_{Swe}$ mutant) background, resulting in the formation of both Aβ plaques and neurofibrillary tangles with a similar spatial and temporal distribution to that observed in human AD patients. We provide further evidence for the localisation of reactive microglia to amyloid plaques within CA1; in addition, we discovered a significant increase in the density of resting microglial cells, which precedes the massive activation of microglia.

Materials and Methods

All animal procedures were carried out in accordance with the United Kingdom Animals (for Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice.

The procedure for generating 3xTg-AD mice has been described previously (Oddo et al., 2003a; Oddo et al., 2003b). Briefly, human APP cDNA harbouring the Swedish mutation (KM670/671NL) and human P301L mutated four-repeat Tau were co microinjected into single-cell embryos of homozygous PS1M146V knock-in mice. The background of the PS1 knock-in mice is a hybrid 129/C57BL6. The control mice were also from the same strain and genetic background as the PS1 knock-in mice, but they express the endogenous wild-type mouse PS1 gene. All 3xTg-AD and control mice were obtained by crossing homozygous breeders. Mice were independently group housed and kept on a daily 12-h light–dark cycle dark schedule. All mice were given *ad libitum* access to food and water.

Fixation and tissue processing

Male 3xTg-AD and their respective on-transgenic controls (non-Tg) of different ages (9, 12 and 18 months of age; n=3–5) were anaesthetised with an intraperitoneal injection of sodium pentobarbital. Brains were fixed by perfusion through the aortic arch with 3.8% acrolein (TAAB, UK) in a solution of 2% paraformaldehyde and 0.1M phosphate buffer (PB), pH 7.4, followed by 2% paraformaldehyde. The brains were removed from the cranium and cut into 4–5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus. These slabs were subsequently post-fixed for 30 min in 2% paraformaldehyde before sectioning at 40–50 mm on a vibrating microtome (VT1000, Leica, Milton Keynes, UK). To remove excess reactive aldehyde groups, sections were treated with 1% sodium borohydride in PB for 30 min. Coronal vibratome sections at levels -1.58 mm/-2.46 mm (hippocampus) posterior to the bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).

Antibody and reagents

A polyclonal affinity-purified rat antiserum raised against CD11b (Mac-1; Serotec, Kidlington, UK) was used for the determination of activated microglial cells. A Mac-1 antibody was used to selectively label activated microglia in our brain sections. Mac-1 has a specific affinity to the microglial surface membrane receptor CD11b, which is highly expressed by reactive microglia (Solovjov et al., 2005), and is therefore routinely used as a specific marker of reactive microglia. Although we identified a small amount of low-intensity labelling for resting microglia in our tissue sections, the staining was observed to be largely specific for reactive microglia. For the identification of β-amyloid (Aβ) plaques, we used a monoclonal mouse antiserum that reacts with abnormally processed isoforms, as well as precursor forms of Aβ, recognising an epitope within amino acids 3–8 (EFRHDS; anti- Aβ 6E10 [SIG-39320]; Signet Laboratories, Dedham, MA, USA). To assess for nonspecific background labelling or cross-reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and/or secondary antibodies from the incubation solutions resulted in a total absence of target labelling (data not shown). Resting microglia was identified by using biotinylated tomato lectin (Sigma-Aldrich, Poole, UK). Tomato lectin is a sugar-binding glycoprotein that has a specific affinity for poly-N-acetyl lactosamine residues occurring on the surface membranes of microglia and endothelial cells (Acarin et al., 1994).

Immunohistochemistry

To optimise the detection of Mac-1 and tomato lectin (resting microglia), sections through the hippocampus of all control and transgenic animals were processed by using the highly sensitive avidin–biotin peroxidise complex method (Hsu et al., 1981). For this procedure, vibratome sections were first incubated for 30 min in 0.5% bovine serum albumin in 0.1M Tris-buffered saline (TBS), pH 7.6, containing 0.25% Triton X-100 to minimise non-specific labelling. The tissue sections were then incubated for 48 h at room temperature in 0.1% bovine serum albumin in TBS containing 0.25% Triton X-100 that in turn contained either rat anti-Mac-1 (1 mg/ml) or biotinylated tomato lectin (2 mg/ml). Briefly, following repeated washing in TBS, sections for Mac-1 were incubated in 1:200 dilutions of biotinylated donkey anti-rat IgG followed by avidin-biotin complex (Elite kit; Vector Laboratories, Peterborough,

UK). For tomato lectin identification the sections were directly incubated in the avidin-biotin complex. The peroxidase reaction product was visualised by incubation in a solution containing 0.022% 3,30-diaminobenzidine (Aldrich, Gillingham, UK) in TBS containing 0.003% H_2O_2 for 6 min.

For the detection and determination of Mac-1-positive cells and their relationship with Aβ senile plaques, we used dual indirect immunofluorescence labelling. The sections were incubated for 48 h at room temperature in a primary antibody cocktail containing (1) mouse anti-β amyloid monoclonal antibody (Aβ; 1:2000; Covance, Emeryville, CA, USA) and (2) rat anti-Mac-1 (1:30.000; Sigma, Saint Louise, MO, USA) simultaneously. Subsequently, Aβ and Mac-1 were detected in a sequential manner on the same sections by incubation with rhodamine (TRITC)-conjugated goat anti-mouse and FITC-conjugated goat anti-rat (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector Laboratories).

Cell counting

The S_v (number/mm^2) of Mac-1 (Mac-1-IR) and TL-IR cells were determined from four non-consecutive coronal sections, separated by at least 80 mm, which were taken from representative sections of the dorsal hippocampus. The Image J 1.41 image processing software was used to outline and determine the area of CA1, by performing the quantification; then the number of either Mac-1-IR or TL-IR was determined. Cells were counted and images obtained using a Nikon E-80i widefield microscope and a confocal microscope (Leica SP5 upright). To ensure consistency and reproducibility, samples were counted blindly.

Statistical analysis

Data were expressed as mean ± S.E. Analysis of variance (one-way and two-way) was used to examine differences in the mean number of Mac-1- IR and TL-IR cells in the 3xTg-AD and non-Tg animals during ageing followed by a Bonferroni's post-hoc test where appropriate. Whenever two groups were compared, an unpaired t-test was applied.

Results

The populations of resting and activated microglia were analysed in the hippocampi of 3xTg-AD mice at three different ages, at 9, 12 and 18 months. Importantly, at 9 months of age the hippocampal tissue of these animals is virtually plaque-free, whereas the plaque load becomes substantial at 12 and 18 months (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008). The resting microglial cells were identified by specific staining with tomato lectin (Acarin et al., 1994), whereas activated microglia were stained with Mac-1 antibody raised against CD11b (Solovjov et al., 2005).

In the dorsal hippocampus, and more specifically within the CA1 of both non-Tg and 3xTg-AD mice, we observed two different phenotypes of microglial cells (Figures 1 and 2). The resting, tomato-lectin immunoreactive (TL-IR) cells were characterised by a small cell body equipped with thin to- medium ramified processes extending to the surrounding neuropil (Figure 1B and C), typical of resting microglia. Occasionally, resting microglia was also observed in the vicinity of dense deposits that were likely to be potential Aβ aggregates (data not shown). The other type of microglial cells, which were reactive for Mac-1, showed enlarged cell bodies from which processes with an enlarged and thicker appearance emanated (Figure 2D and E), thus being consistent with a reactive phenotype. Reactive microglia was predominant in 3xTg-AD mice (Figure 2A-C).

Resting microglia in AD

There was no difference between the morphological characteristics of TL-IR resting microglia in either 3xTg-AD or the respective non-Tg controls, independent of the age (Figure 1B and C). The area density (S_v, number/mm^2) of resting microglia was significantly larger in 3xTg-AD animals when compared with the control at all ages ($P<0.01$; Figure 1A). When the S_v was compared between 3xTg-AD and non-Tg controls, it was significantly larger at 9 (105%, P=0.058), 12 (54%, P=0.0198) and 18 (131%, P=0.0116; Figure 1A) months. In non-Tg animals, ageing (9–18 months) did not affect the S_v of resting microglia, whereas in 3xTg-AD animals we found a clear age-dependent increase in S_v between 9 and 18 months ($F_{2,13}=7.078$; P=0.0106). Furthermore, a more complete two-way ANOVA analysis showed that both age and

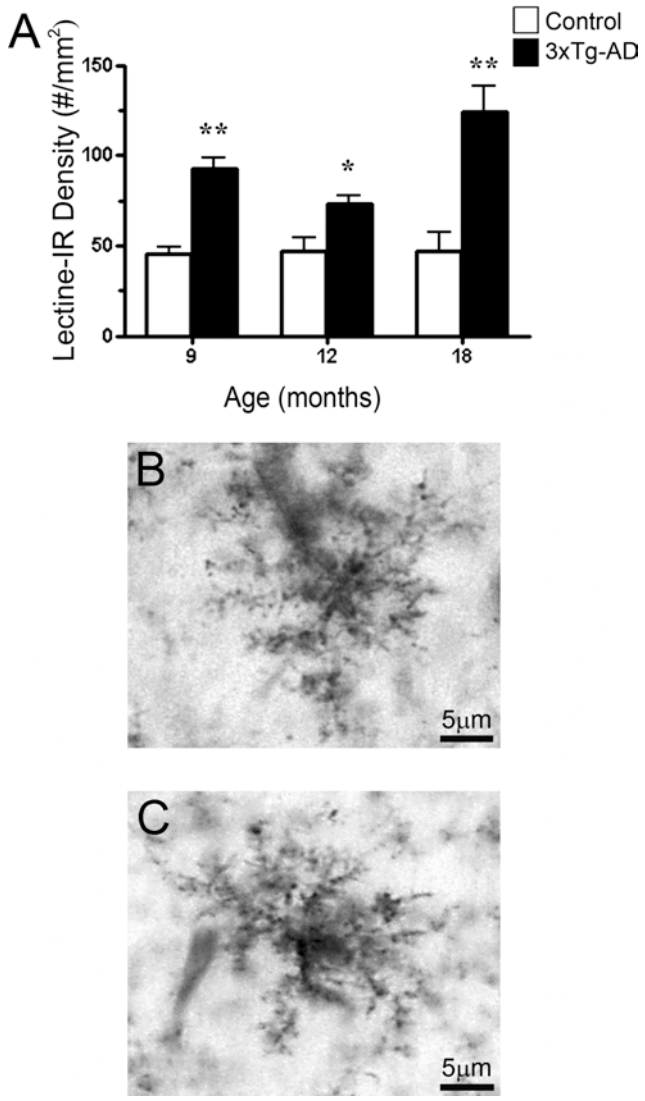
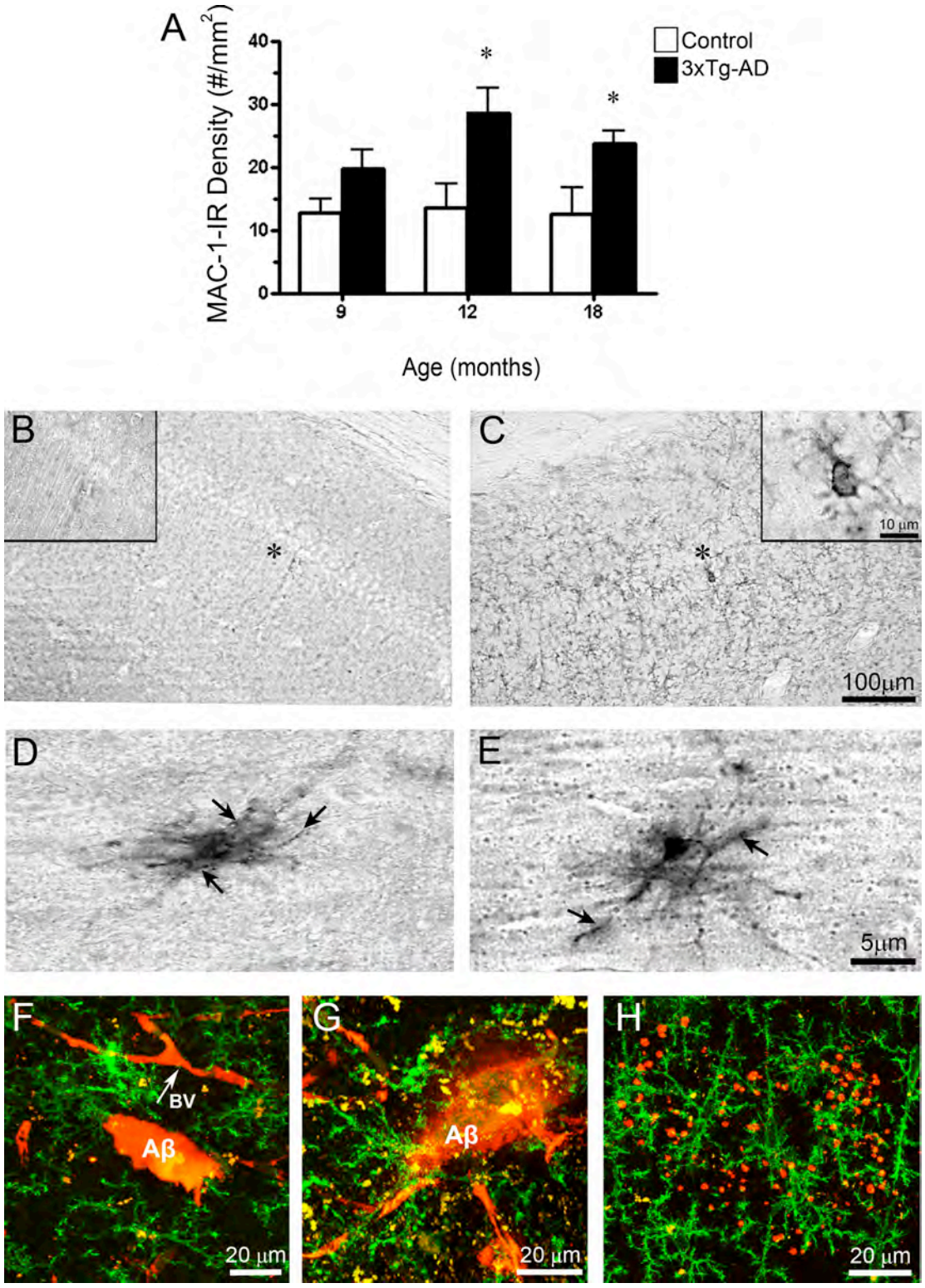


Figure 1. *Visualisation and quantification of resting microglia in the hippocampi of 3xTg-AD animals. (A) Bar graph showing the area density of resting microglia (S_v; number/mm^2) in the hippocampal CA1 of 3xTg-AD mice and non-Tg control mice. (B,C) Brightfield micrographs showing the characteristic morphology of resting TL-IR microglia with small cell body equipped with thin to medium ramified processes extending to the surrounding neuropil in the CA1 subfield of the hippocampus of 9- and 18-month-old 3xTg-AD mouse, respectively, which is not modified either by age or by Aβ amyloid plaques. Scale bar = 5 µm.*

Figure 2 (→)*: Microglial activation in plaque-infested hippocampal tissue. (A) Bar graph showing the effect of age-related changes on MAC-1-IR reactive microglia within the CA1 subfield of the hippocampus at 9, 12 and 18 months between non-Tg control and 3xTg-AD mice. (B, C) Brightfield micrographs showing the increase in MAC-1-IR reactive microglia (asterisk) in 3xTg-AD (C) versus non-Tg control mice (B) in the hippocampal CA1. (D, E) High-magnification micrographs illustrating the characteristic morphology of reactive microglia within the CA1 subfield of the hippocampus of an 18-month-old 3xTg-AD mouse. Reactive microglia appear with most enlarged cell bodies from which a greater number of numerous processes emanated, but with an enlarged and thicker (arrows) appearance (F, G). Confocal images showing recruitment of MAC-1- IR microglia (green) in the vicinity of Aβ amyloid plaques (red; F,G) and aggregates (H) in the CA1 subfield of the hippocampus of an 18-month-old 3xTg-AD mouse. In (f) we can also observe this reactive microglia surrounding a blood vessel (BV) attaint of Aβ deposits. Scale bars: (B, C) 100 µm, (D, E) 5 µm and (F) 20 µm.*


A
MAC-1-IR Density (#/mm2)
40
30
20
10
0
9
12
18
Age (months)
Control
3xTg-AD
*
*
B
C
10 μm
100μm
D
E
5μm
F
BV
Aβ
20 μm
G
Aβ
20 μm
H
20 μm

genotype had an effect on S_v of resting microglia ($F_{2,19}$=5.26; P = 0.0015 and $F_{2,19}$=43.03; P < 0.001 respectively).

Activated microglia in AD

We found no change in the typical morphology of Mac-1-IR reactive microglia in all age groups in both control and 3xTg-AD animals. In control animals the area density of Mac-1-IR was stable at all ages, showing no significant changes at 9, 12 and 18 months (Figure 2A). In contrast, in the 3xTg-AD mice the area density of Mac-1-IR (total activated microglial cells) was significantly increased throughout the whole extension of CA1 (Figure 2B and C) at 12 months (111%; P=0.0311) and 18 months (88%; P=0.0330), but showed only a non-significant rise at 9 months (55.44%, P=0.1163). In addition, one can see that the genotype had a significant effect on the area density of MAC-1-IR ($F_{2,19}$=16.92; P = 0.001). The Mac-1 activated microglia were almost exclusively localised in close proximity to Aβ plaques and/or aggregates (Figure 2F-H). The cell bodies of these reactive microglial cells were distributed in a circular shape around the plaque periphery (Figure 2F-H). The Mac-1-IR activated microglial cells were normally present in multiple clusters, revealing an active phagocytic activity as shown by a high degree of co-localisation with Aβ (Figure 2F, G). This phagocytic function was effective in removing the Aβ load, as shown by the presence of small packs of broken Aβ aggregates surrounded by hypertrophic and multiprocess MAC-1-IR cells (Figure 2H). Reactive microglia were also present around vascular elements that accumulated Aβ, which is typical of AD-associated angiopathy (Figure 2F).

Discussion

Although it has long been known that activated microglia are associated with compact Aβ plaques (McGeer and McGeer, 1995), their role in the development of AD is not completely understood (Ransohoff and Perry, 2009; Streit et al., 2004).

Although numerous studies have attempted to characterise microglial function in AD, few have aimed to quantify the changes in the two types of microglia population (resting and reactive). In the present study, we investigated the changes in the density of microglial cells in 3xTg-AD mice, with particular focus on the CA1 subfield of the hippocampus.

The CA1 area was shown to be highly susceptible to Aβ pathology in both human AD patients and 3xTg-AD mice, which is also important in the context of memory impairment. Within the hippocampus the CA1 is the major component of the Schaffer collateral pathway, acting as a neural conduit between the hippocampal subfields, subiculum and entorhinal cortex (Amaral and Witter, 1989). As such, neuronal activity in CA1 has been strongly linked to memory formation, an aspect of cognitive function, which is typically compromised in AD (Walsh and Selkoe, 2004).

We found that the reactive microglia was densely populated with regions of high Aβ deposition, with the processes of many of these cells appearing to be in contact with Aβ aggregates. Some microglial cells contained an intracellular deposition of Aβ, thus indicating active phagocytosis. Similar observations were made in the brains of Tg2576 mice (expressing human mutated APPswe), in which activated microglia were concentrated in close proximity to amyloid plaques (Frautschy *et al.*, 1998). Similarly, the aggregation of activated microglia around Aβ plaques was shown in vivo in 3 – 4-month-old double-transgenic mice expressing a mutant APP and PS1; chronic imaging experiments demonstrated that the number of plaque-associated microglia increased at the rate of about three cells per plaque per month (Bolmont *et al.*, 2008).

We found an increase in activated microglia in the CA1 area at 12 and 18 months, which correlates with the age of the appearance and development of Aβ plaques in

the hippocampus of the 3xTg-AD animal model, even though in cortical areas they appear as early as at 6 months of age (Oddo *et al.*, 2003a; Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2009).

We therefore suggest that the activation of microglia occurs in response to Aβ plaque formation and stabilisation. The activated microglia surrounded not only the fully formed plaques but also smaller deposits of Aβ (Figure 2H). This observation may be interpreted in either of two ways: (i) a result of plaque destruction and clearance, or (ii) the formation of small soluble aggregates before plaque consolidation. Activated microglia associated with Aβ plaques have been implicated in the phagocytosis of cerebral Aβ (Bard *et al.*, 2000; Hickman *et al.*, 2008) with reports indicating that these cells may be involved in the stabilisation or clearance of Aβ plaques in the brain (Christie *et al.*, 2001). Our results corroborate these suggestions, as we found the accumulation of Aβ by activated microglia, as shown by MAC-1/ Aβ double staining (Figure 2H and G). Incidentally, we did not observe a substantial activation of microglia at a pre- and/or early plaque stage at 9 months of age, indicating that microglial reactivity does not develop until the plaques are formed and/or consolidated. Similarly, a recent longitudinal study using multiphoton microscopy to image the brains of 5–6-month-old B6C3-YFP transgenic mice (bearing APPswe and PS1d9x- YFP genes) has also alluded to this, reporting that microglia are recruited to Aβ plaques only after they have been formed (Meyer-Luehmann *et al.*, 2008).

Apart from confirming the AD-associated activation of microglia, we found the disease-specific increase in the density of resting microglial cells. To the best of our knowledge, this is the first attempt to characterise resting microglia in the AD. The immunoreactivity against tomato lectin, which is used as a specific probe for resting microglia, increased at all ages in 3xTg-AD animals, whereas there were no age-dependent changes in the control brains. An increase in the density of resting microglia was quite substantial – the number of cells had roughly doubled. This increase preceded both the plaque formation and the activation of microglia by extracellular Aβ accumulation. The mechanism of microglial proliferation and the origin of the additional resting microglial cells remain obscure and require further study. In particular, we cannot rule out the role for blood-borne macrophages, which

have been shown to migrate across the blood–brain barrier in response to perivascular amyloid deposition, as well as the secretion of cytokines and chemoattractant peptides by microglia and astrocytes in these areas (Simard *et al.*, 2006; Wegiel *et al.*, 2003). Upon entering the CNS, these cells function as reactive microglia, localising as phagocytes to amyloid plaques, secreting pro-inflammatory peptides and effectively accumulating Aβ (Boche and Nicoll, 2008; Simard *et al.*, 2006). We cannot exclude, however, that a similar infiltration can start before the active inflammatory Aβ -associated phase; at that stage, the intruding cells may transform to the resting microglia. All in all, we may suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting microglia increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

An increase in the density of resting microglia associated with atrophic changes in astrocytes (Rodriguez *et al.*, 2009) may also lead to a remodelling of the glial-related domain structure of the grey matter (Figure 3). This in turn can affect synaptic connectivity, because of both reduced astroglial coverage and the possible synaptolitic effects of multiplied microglia. Thus, early changes of neuroglia can represent a pathologically relevant step in the cognitive decline observed at the early stages of AD.

Several therapeutic strategies have attempted to target microglia activity as a means of treating AD, each with limited success. Of these, immunotherapy protocols aimed to up-regulate the microglial clearance of cerebral Aβ (Bard *et al.*, 2000; Schenk, 2002), whereas an alternative strategy used anti-inflammatory agents to reduce microglial neurotoxicity (Gasparini *et al.*, 2004). These attempts reflect the well-known dichotomy of microglial function, which may be both neuroprotective and neurotoxic (Ransohoff and Perry, 2009). Nonetheless, without a comprehensive understanding of microglial changes and roles in AD, and especially the balance between different stages of microglial activation, these attempts at therapeutic intervention may continue to be futile. Furthermore, an early increase in resting microglia, which precedes the Aβ plaque formation, may also have certain diagnostic significance.

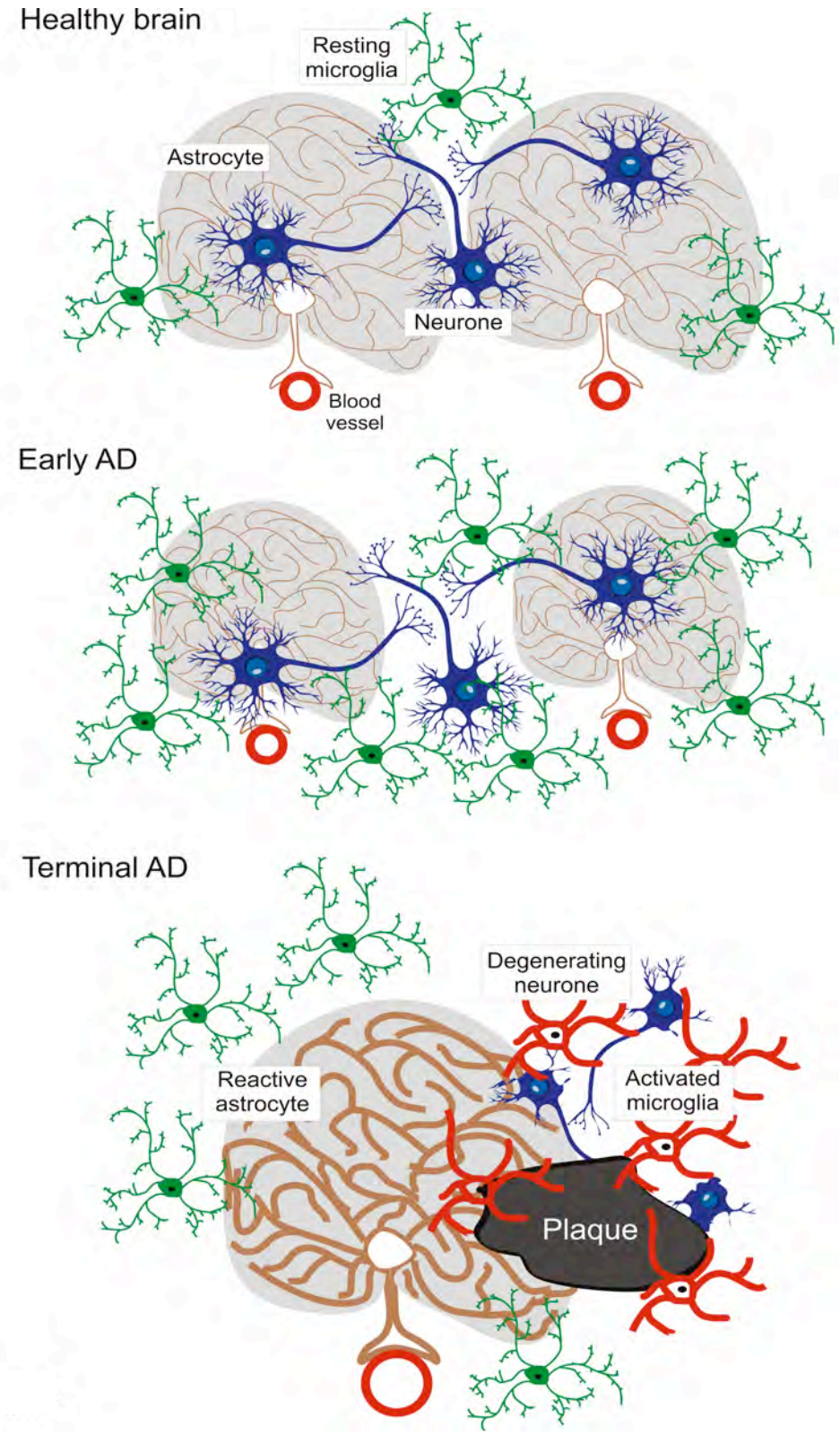


Figure 3. *Glial reactions at the different stages of Alzheimer's disease. At the early, A plaque-free stages of AD, an increase in the density of resting microglia coincides with the initial atrophy of astrocytes (Rodriguez et al., 2009). Consolidation of plaques at the later stages of AD triggers rapid and massive activation of microglia, reactive astrogliosis and neurodegeneration.*

Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

General Discussion

Chapter 6 – General Discussion

1. General overview

Alzheimer's disease is a neurodegenerative disease that causes a severe intellectual decline which progresses until the fatal phase is reached. Although over 100 years has passed since Alois Alzheimer made the first observations about the disease, no effective treatment exists (Alzheimer, 1907; Patel and Grossberg, 2011). In this thesis we focus our work on studying a particular neural cell population; **glial cells**. Glia are composed by different types of cells such as astrocytes, microglia and oligodendrocytes, and they perform many of the most essential functions of the brain raging from structural and defence to metabolic and homeostatic. In this thesis, majority of the experimental work is centred in alterations of astroglial cells (**chapter 3 and 4**). Firstly, cytoskeleton morphological alterations are studied (**chapter 3**) and later on, in **chapter 5**, functional alterations related to glutamatergic homeostasis. Finally, the last experimental chapter (**chapter 5**) shows alterations of microglial cell in AD pathology as well as their interaction with astroglia. In addition, we will suggest some possible future work to expand these findings that will enlighten the understandings of AD pathology & hopefully reveal some new therapeutical approach.

2. Astrocytes in Alzheimer disease

Pathologically, AD occurs with accumulation of β-amyloid and hyperphosphorylated tau accumulation in the form of plaques and tangles, respectively. Severe neuronal and synaptic loss also occurs and these are the main factors responsible for the cognitive decline associated to the disease. Other neural cells such as astrocytes and microglia also play a role in the disease becoming reactive, although not much is known yet.

Astrocytes are the most numerous cells in the brain and are essential for the well being of the CNS. They perform a number of fundamental functions such as extracellular homeostasis, neural metabolic support, control of the blood-brain barrier and modulation of synaptic function. In addition, astrocytes are known to be involved in most neurological diseases including AD (Giaume *et al.*, 2007; Heneka *et al.*, 2010; Rodriguez *et al.*, 2009). However, the potential of astrocytes in brain pathology and in particular dementia, is poorly understood. Based on the limited literature, we may

conclude that astrocytic reactions in various types of dementia can range from proliferation and astrogliosis to apoptosis and dystrophy (Broe *et al.*, 2004; Rodriguez *et al.*, 2009).

For the work carried out in this thesis, we have used the 3xTg-AD mouse model that is one of the most advanced and relevant models of AD because it shows temporal- and region-specific Aβ and tau pathology, which closely resembles what is seen in human AD brains. The work of this thesis have been mainly focused on the hippocampus that is one of the earliest and most affected brain regions in AD, which plays an essential role in cognition and memory; aspect that is deeply affected in AD.

2.1. Astroglial morphological changes in the 3xTg-AD

Our first experimental study was the analysis of the GFAP-positive astroglial profiles in the 3xTg-AD animals of different age in the dentate gyrus of the hippocampus, which receives the majority of the input from the first affected region in AD; the entorhinal cortex. GFAP expressing astrocytes are numerous throughout the hippocampus including the dentate gyrus and they represent virtually the totality of astrocytes in this region (Kimelberg, 2004). Up-to-date and to the best of our knowledge, this was the first attempt to characterise the age-dependent changes in the morphology of GFAP-positive astrocytes in the 3xTg-AD. Very surprisingly, contrary to the general assumption that the AD is associated with reactive astrogliosis with overall increase in GFAP expression and astroglial proliferation (Nagele *et al.*, 2004), our analysis showed that astrocytes in 3xTg-AD were actually atrophic in comparison to control animals of the same age. This atrophy was statistically becomes significant at 12 months of age, although it was already patent the tendency towards atrophy from the sixth month of age (**chapter 3,** Fig 2). This novel evidence made us reconsider the classical role that astrocytes has in AD and also made us study this phenomenon in more depth. Indeed, this early atrophy could mean that astroglia processes may have reduced synaptic contact and therefore, synaptic modulation would be compromised. Furthermore, this astroglia early atrophy could count for the early synaptic dysfunction observed in AD patients. In this way it is conceivable that the earliest possible change could take place at 6 months of age, which can reflect a real earlier astroglial alteration even though it skipped to the sensitivity of the techniques applied to address this matter.

In any case, observed astroglial atrophy need to be explored. For that, we expanded the previous analysis including animals of 18 months of age in both 3xTg-AD and control groups, and also extended the analysis to the hippocampal CA1 sub-region, which shows the most prominent and earliest Aβ burden within the hippocampus during the progression of the disease. Our new results reinforced what we observed previously. We found that in both DG and CA1 hippocampal sub-regions the GFAP-positive astrocytes are significantly atrophic from early stages of the disease. As observed previously, already from 6 months of age, when the pathology is nearly absent, GFAP-positive astrocytes in the DG show a tendency towards atrophy. However it is not until 12 months of age that this atrophy becomes marked and the GFAP-positive astrocytes remain atrophic at 18 months of age. GFAP-positive atrophy in CA1 arises at 18 months of age too. However, this GFAP profile atrophy was not accompanied by a decrease of GFAP-positive astrocytes density, being completely independent to age and AD pathology. These findings argue against the general prominent of astrogliotic and astroglial proliferative response in the AD brain.

Early astroglial morphological dwindle can be pathologically significant in the progression of the familial type of AD (and indeed the transgenic AD models reproduce the FAD type pathology). These astroglial alterations were not only affecting primary thick processes but also thin and distal processes most likely indicating the reduction in astroglial arborisation and consequently decreasing glial synaptic coverage (Rodriguez *et al.*, 2009). Such synaptic coverage reduction by astrocytes can be involved in the early neurotransmission imbalance and in disrupted synaptic connectivity. Interestingly, similar astroglial alterations have been described in other types of neurodegenerative processes such as amyotrophic lateral sclerosis (Rossi *et al.*, 2008; Rossi and Volterra, 2009), Wernicke's encephalopathy (Hazell, 2009), frontotemporal dementia (Broe *et al.*, 2004), schizophrenia and major depression (Gosselin *et al.*, 2009; Rajkowska *et al.*, 2002; Si *et al.*, 2004), suggesting atrophic astroglial alterations can be common feature of different neurodegenerative pathologies. In the case of AD, early astroglial alterations could fail to provide adequate support to synapses, leading towards a neural circuit disruption.

In this sense, several studies have shown that early stages of AD is indeed associated with synaptic loss (Scheff *et al.*, 2007; Terry, 2000), although the underlying

mechanism is not uncovered. Based in our data, we could firmly consider astroglial failure could produce such early synaptic disorder in AD-like pathology.

However, the existence or not of astrogliosis needed to be verified in the 3xTg-AD, to confirm whether the astrogliosis, which has been reported countless time in both AD brains and animal models (Beach and McGeer, 1988; Beach *et al.*, 1989; Ingelsson *et al.*, 2004), was also taking place in the 3xTg-AD model. In order to address this question, similar GFAP analysis to the previous one was performed in animals of 12 and 18 months for CA1 and 18 months for DG, distinguishing, this time, astrocytes that were surrounding Aβ plaques (<50 μm) and astrocytes that were distant from Aβ plaques (>50 μm). For this proposes, Aβ plaques were also immunolabelled. We observed astrocytes that were in the vicinities of the Aβ showed clear signs of reactivity (thick processes, enlarged cell bodies, and overall higher volume and surface area of GFAP-positive processes), while astrocytes localised distantly from the borders of the Aβ plaques were atrophic showing decreased volume of cell bodies, reduced number of primary and specially secondary processes, and an overall reduction in both GFAP volume and surface. The existence of two distinct populations of astrocytes in relation to Aβ plaque presence in brains of transgenic AD animals was also demonstrated in the *in vivo* Ca^{2+} imaging experiments, which showed increased Ca^{2+} excitability of the astrocytes contacting neuritic plaques (Kuchibhotla *et al.*, 2009).

So this shows that during the later stages of AD, the changes in astroglial morphology is complex and that these two reactions develop in parallel. This could represent the underlying mechanism for loss in synaptic connectivity and plasticity, which in turn underlies cognitive deficits associated with AD. Astroglial alterations observed in this work happen before plaques and tangles are manifest in the hippocampus. It is then conceivable to speculate that dystrophic astrocytes fail to provide adequate support for synaptic contacts, which may lead to a distorted balance between excitatory and inhibitory synapses), with ensuing disruption of neuronal circuitry – and indeed the synaptic loss is observed in early stages of AD when brain parenchyma remains relatively free from neuritic plaques (Scheff *et al.*, 2007; Terry, 2000).

A possible mechanism behind synaptic disruption caused by astrocytes could be the

defective extracellular homeostasis and in particular reduced ability for glutamate uptake, making neurons more vulnerable for glutamate excitotoxicity.

2.2. Astroglial homeostatic alterations in the 3xTg-AD

GFAP-based morphological evidences show astrocytes are compromised during the development of AD. However, knowing that it was likely that other aspects of astrocytes could be also affected and considering the importance of astrocytes in synaptic modulation, we analysed the involvement of astrocytes in glutamatergic transmission. Indeed, astroglial role in glutamate homeostasis is one of the major functions they perform in the CNS. Glutamate-glutamine shuttle is fundamental for glutamatergic neurotransmission and astrocytes are an essential component, which exclusively re-uptake glutamate from the synaptic cleft and subsequently transform it to its inactive form glutamine, by the astroglial-specific glutamine synthetase. Then, glutamine is passed to neurons where it will be stored in vesicles until a new action potential triggers their release to the synaptic cleft.

Therefore, we analysed whether astrocytes are also functionally compromised in AD by determining the expression of the GS in 3xTg-AD mice of different ages. We found that expression of astroglial GS in the hippocampus is altered during the progression of AD, as shown by a lower N_v of GS-IR astrocytes and GS expression (as determined by IOD) in the 3xTg-AD when compared to the control animals. These changes in N_v were initially observed in the DG at 12 months of age and continued at 18 months of age as well as expanded to CA1. The GS expression, measured by OD, was also decreased in the 3xTg-AD in both DG and CA1 but at a later age (18 months).

Similarly to the GFAP analysis in relation to Aβ plaques, we determined N_v of GS-IR astrocytes in the distance (>50μm) and vicinities (<50μm) of Aβ plaque and we found that the above-mentioned lower N_v of GS-IR cells was more marked in the vicinities of neuritic plaques.

These results show a spatial- and temporal correlation with the observations we made in GFAP alterations, perfectly coinciding hippocampal sub-regions with the onset of the alterations (DG at 12 months and CA1 at 18 months of age) of both GFAP and GS analysis (see Fig. 1). Very interestingly, a number of studies had previously found

association between GFAP alterations and glutamatergic homeostasis imbalance. A hypoxic rat model showed that GFAP cytoskeleton is essential for GLAST anchoring in astrocytic membrane, suggesting GFAP processes retraction could lead to excitotoxic vulnerability (Sullivan *et al.*, 2007). Similarly, in GFAP knock-out mice, the absence of GFAP showed altered GLT-1 trafficking to cell membrane (Hughes *et al.*, 2004). Contrarily, an "*in vitro*" study has been evidenced an inverse correlation between astroglial expression of GFAP and GS (Weir and Thomas, 1984).

Studies in AD brains have shown a decrease of cortical astrocytic GS expression (Le Prince *et al.*, 1995; Robinson, 2000) that was compensated by a "*de novo*" neuronal expression of GS (Robinson, 2000, 2001). Similar effect on astrocytic GS expression was also observed in Huntington's disease, another neurodegenerative disease that is associated with GLT-1 glutamate transporter down-regulation (Jacob *et al.*, 2007; Lievens *et al.*, 2001). In addition, there are evidences that make Aβ presence responsible for GS oxidation (Lievens *et al.*, 2001) as well as for decreased expression of GS (Robinson, 2000). Contrarily, other study showed increased GS immunoreactivity in prefrontal cortex in AD brains (Burbaeva *et al.*, 2005). Data from **Chapter 4** shows that astrocytes at the earlier middle-late stages (plaque formation and stabilisation, 12-18 months) of AD-like pathology, in agreement with the above-mentioned studies, are functionally compromised as shown by the reduction of GS expression and therefore their capability to master the glutamate-glutamine cycle.

Therefore, these results reinforce our hypothesis that astrocytes fail to support neurones and control synapses from the first manifestations of the pathological burden. GS deficiency could reflect astroglial impaired ability to re-uptake glutamate; triggering a potential neurotoxic wave. However, considering astrocytic physiological function, this GS scarcity could also lead to a deficient glutamate-to-glutamine turnover and restricted supply of glutamine to neurones. Thus GS deficiency may reflect an altered glutamatergic neurotransmission of AD, being responsible for a global hippocampal neurotransmitter imbalance, underlying the so characteristic cognitive impairments observed in the disease.

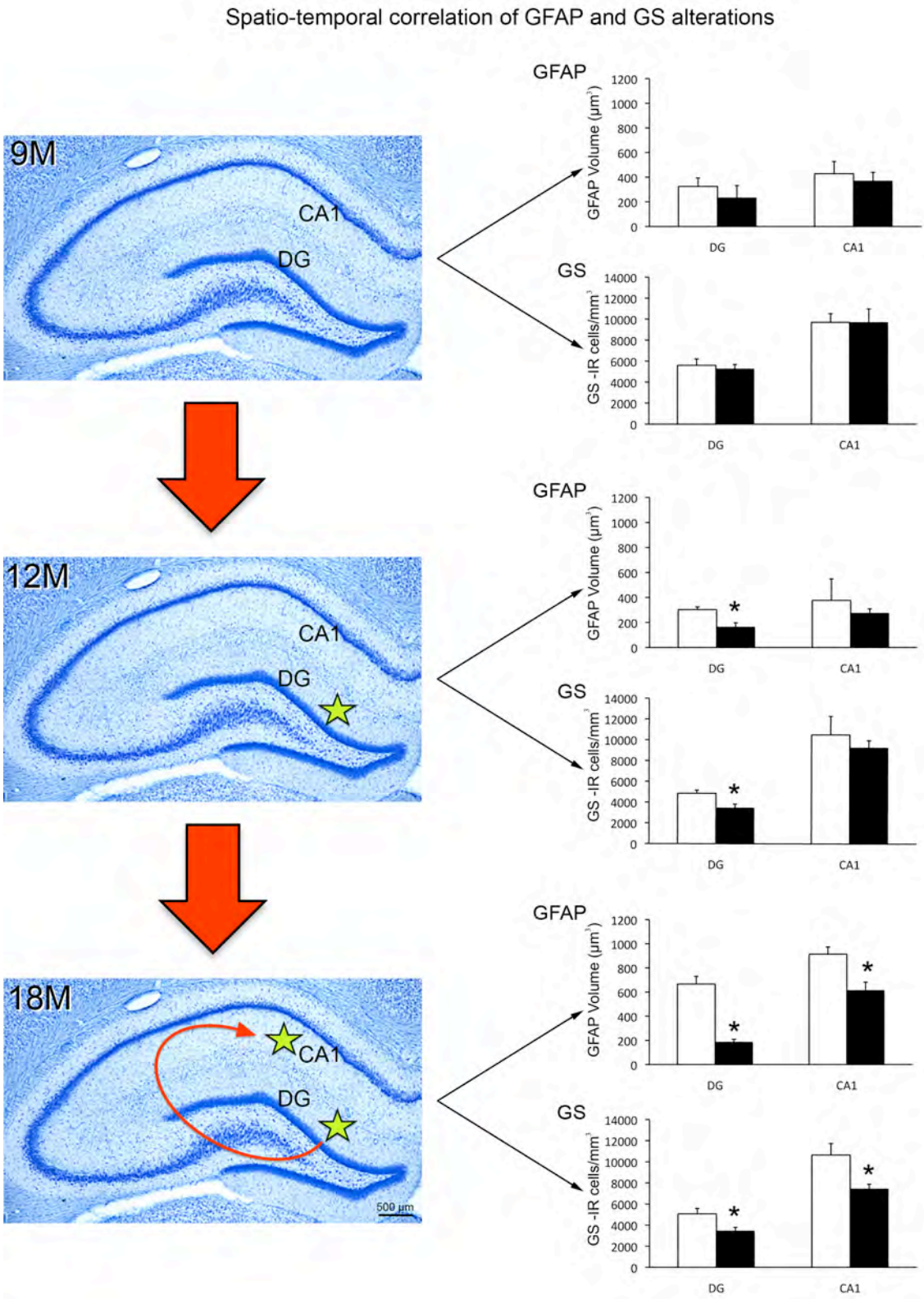


Figure 1. *Spatio-temporal correlation of astroglial alterations in the hippocampus of the 3xTg-AD. Subtle initial astroglial alterations are observed at 9 months of age. Later on at 12 months of age astrocytes in the DG show both GFAP and GS alterations. At 18 months of age, both DG and CA1 manifest astroglial cytoskeleton atrophy and astrogliosis as well as functional deficit.*

3. Microglia in Alzheimer's disease

In parallel to astroglial analysis, we also studied microglial cell populations in the hippocampus during the progression of the disease. Microglia is the immunocompetent cells of the brain and is tightly associated with pathological processes. When the brain suffers an insult, microglia undergoes morphological alterations from a resting to a reactive state, being this activation common in all brain pathologies (Kettenmann *et al.*, 2011). Regarding AD, although it is well known that activated microglia are associated with Aβ plaques (McGeer and McGeer, 1995), their role in the onset and progression of AD is poorly understood (Ransohoff and Perry, 2009; Streit *et al.*, 2004). Many studies have aimed to characterise microglial involvement in AD, though very few focused on the quantification of the changes in resting and activated microglia during the progression of AD-like pathology; the two types of microglia population. In **chapter 5**, we aimed to investigate the changes in the density of microglial cells in the CA1 sub-region of the hippocampus of the 3xTg-AD mice. We paid particular attention to the CA1 because it is the first and the most affected hippocampal area affected by Aβ pathology in both human AD patients and 3xTg-AD mice, which in turn is also fundamental for memory.

We found that the areas with Aβ plaques, and in particular the immediate surroundings, were densely populated by activated microglia that were sending processed to Aβ aggregates and some of them had a significant degree of intracellular Aβ accumulation, suggesting contribution to the clearance of the pathological Aβ built up. Studies performed in other AD models, Tg2576 and APP/PS1 mice, showed similar microglial reaction around Aβ plaques (Bolmont *et al.*, 2008). In addition we found that activated microglia was not only associated to Aβ plaque deposits but also to smaller Aβ aggregations (see chapter 5, Fig. 2h). In the case of the later, whether this small Aβ aggregates are the result of activated microglia mediated plaque destruction or soluble aggregates before plaque consolidation is unclear. However some studies have reported that microglia may have an active role in Aβ clearance (Bard *et al.*, 2000; Bolmont *et al.*, 2008; Christie *et al.*, 2001). Interestingly, such increase of activated microglia in the CA1 of 3xTg-AD perfectly correlates the age of appearance and development, 12 and 18 months, of Aβ plaques in 3xTg-AD animal model, even though in cortical areas they appear as early as at 6 months of age (Oddo *et al.*, 2003a; Oddo *et al.*, 2003b; Rodriguez

et al., 2008). In addition, we did not find considerable activation of microglia at 9 months of age where plaque formation is still subtle, indicating that microglial reactivity does not develop until the plaques are formed and/or consolidated and agreeing with previous reports in regarding this matter (Meyer-Luehmann *et al.*, 2008).

Furthermore, our results evidenced that resting microglia also responses to AD-like pathology. However, unlike activated microglia, resting microglia population was already increased in the 3xTg-AD mice before substantial Aβ plaque formations at 9 months of age while no change was found in normal aged control animals. This was based on the increase of tomato lectin immunoreactivity, a resting microglial marker. The mechanism involved in the resting microglia proliferation prior to Aβ load is unclear. However, the role of blood-borne macrophages should not be ruled out as a potential source of immunoactive cells in AD brains due to the possibility of their infiltration into the brain parenchyma as a consequence of perivascular Aβ accumulation. Although infiltrated peripheral immune cells function as reactive microglia, we cannot exclude that similar infiltration can start before the Aβ-derived phase and that these cells can differentiate into resting microglia. All in all, we may suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting microglia increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

4. General Conclusion

Alzheimer's disease, over hundred years after its discovery, still remains as one of the biggest pathological threats to human condition. Many efforts have been invested in understanding this devastating pathology. However, far from being able to cure it, we are still battling to slow down the irreversible progression of the decline. Neuronal research has been the focus of most of the scientific research, achieving valuable discoveries that allowed the development of the only pharmacological approaches available nowadays, and certainly will contribute to the therapeutical strategies in the future. However, fundamental questions remain unanswered.

The first manifestation of AD is the cognitive decline and it happens prior the appearance of the senile plaque and tau tangles (Coleman *et al.*, 2004; Elias *et al.*, 2000). Cognitive decline is strongly correlated with synaptic malfunction and disruption of neural connectivity (Samuel *et al.*, 1994). Indeed, the loss of synaptic contacts is accepted as the earliest pathological event in AD (Elias *et al.*, 2000; Terry, 2000). In addition, the correlation of Aβ plaques and tau tangles with cognitive early cognitive decline is poor (Terry, 2000; Verkhratsky *et al.*, 2011). Synaptic disruption has permitted the development of different hypothesis explaining the mechanisms of AD pathology. All these hypothesis, of course, have as common fetish the neuron and that inclination is reflected in the consideration of AD, like many other brain pathologies, as a ***neurodegenerative*** disease.

Auspiciously, over the last decades we got to understand the vital relevance of glia in the nervous system, opening an immense research field to explore. Today, we are beginning to understand the importance of glia in the CNS and thanks to this the perception of the nervous system has vividly evolved. Astroglia has a tight control of the overall homeostasis of the brain (Verkhratsky and Butt, 2007). Majority of synapses are modulated by astrocytes and progressive astroglial deterioration would have a direct impact on synaptic connectivity. Therefore astroglial inefficient support could be the underlying mechanism in the early cognitive dysfunction of AD (Olabarria *et al.*, 2011; Rodriguez *et al.*, 2009; Verkhratsky *et al.*, 2011).

Our data shows that the 3xTg-AD mice bear astroglial atrophy and reduced capability of glutamate re-cycling, independently to senile plaques and tau tangles appearance. First astroglial alteration signs take place in the molecular layer of the dentate gyrus in the hippocampus, which shows the first atrophic alterations at 12 months of age. Interestingly, at the time of such alteration the molecular layer of the DG is free of Aβ and tangles (Fig. 2). However, despite of the lack of plaque and tangles, the consequences of the lack of support of astrocytes towards synapse in the dentate gyrus could have an enormous impact on hippocampal function and severe implication in cognitive function. Firstly, majority of synapses in the molecular layer of the DG are glutamatergic and astroglial presence is essential to prevent excitotoxicity. Reduction of astroglial profiles reaching glutamatergic synapses would reduce dramatically glutamate up-take rate of astrocytes causing prolonged high concentration of glutamate in the synapses triggering excitotoxic damage to granular cell neurons (Choi, 1992; Danbolt, 2001; Kirischuk *et al.*, 2007; Nicotera *et al.*, 2007). Secondly, concomitant reduction of glutamate-glutamine cycle capacity would affect synaptic efficiency by the decreased availability of glutamate in axon terminals, having a direct impact on cognitive function (Olabarria *et al.*, 2011). Thirdly, inefficient synaptic connectivity would disrupt the main route for cognitive processing. The molecular layer of the DG receives massive input from the layer II of EC, which is the main information influx to the hippocampus (Amaral *et al.*, 2007; Witter and Amaral, 2004). Therefore, synaptic disruption in the molecular layer of the DG would partially mutilate the hippocampus and disconnect it in a significant degree from the information processing routes. Very interestingly, the 3xTg-AD mice have also shown reduced neurogenesis the subgranular layer of the DG (Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2011). This happens in parallel (9-12 months of age) to the astroglial alteration and would inevitably contribute to the impoverishment of the capacity of the DG to provide adequate neuronal pool to respond to the cognitive demands of the hippocampus (Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2011). Thus, hippocampal capability to perform adequate cognitive tasks would be disturbed and could explain the early cognitive decline that precedes appearance of senile plaques and tangles.

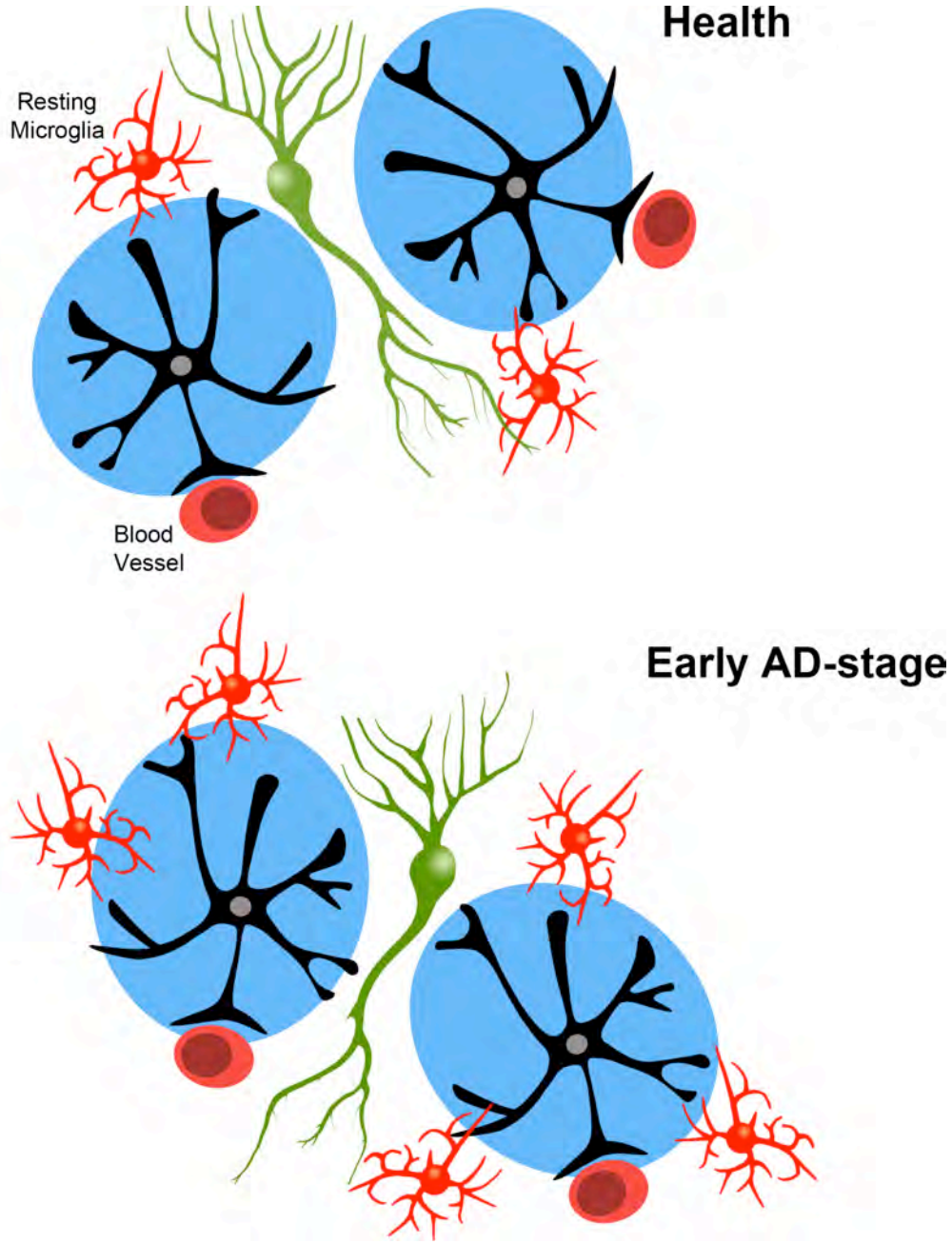
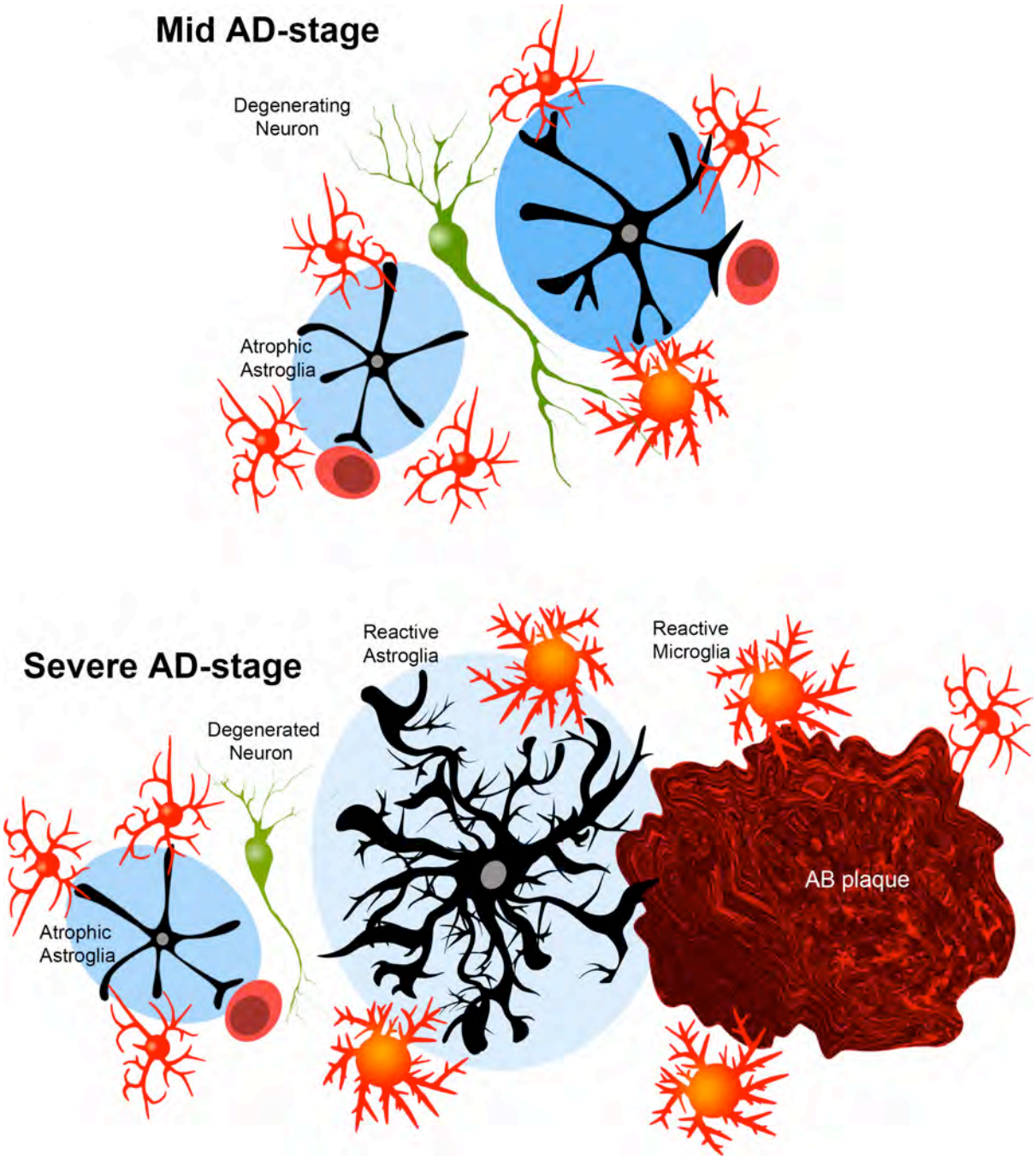


Figure 2. *Summary drawing representing different stages of the AD progression from healthy brain to severe AD pathology. Note: Astroglial GS expression intensity is represented by a hallo (blue) surrounding the GFAP cytoskeleton (black).* ***Health:*** *Adequate neuro-glial-vascular unit. Astrocytes provide adequate support to neurons and maintain contact within their domain with blood vessels and neighbouring astrocytes. Resting microglia surveys the environment searching for "on" signals.* ***Early AD-stage:*** *Early resting microglia proliferation occurs probably due to subtle early pathological events. Neurons are also slightly affected.*



Mid AD-stage: *Neurons show evident degenerative signs due to the deficitarian support of atrophic astrocytes (atrophic cytoskeleton and deficient glutamate homeostasis). In addition microglia becomes reactive and releases pro-inflammatory molecules contributing to the damage.* ***Severe AD-Stage:*** *Aberrant astrogliosis and microglial activation becomes evident in the vicinities of Aβ plaques. Astrocytes distant from neuritic plaques undergo atrophy and fail to support neurons appropriately. Glutamate homeostatic deficiency is ubiquitous, being more evident in astrocytes around the senile plaques. Consequently disruption of neuronal circuitry takes places.*

Astroglial alterations observed in the molecular layer of the DG could be the consequence of the very early (1 month of age) astroglial alterations of the EC (Yeh *et al.*, 2011). The EC is the first brain region affected in AD and that is also represented in the 3xTg-AD. Very early astroglial cytoskeleton atrophy, similar to the one observed in the hippocampus, takes place in EC (Yeh *et al.*, 2011). Parallel synaptic disruption could be the consequence of such astroglial alteration and this could modify the connectivity between the EC and the hippocampal DG, perturbing the balanced environment. As a consequence, astrocytes in the molecular layer of the DG could develop a non-ideal condition leading to the subsequent astroglial alterations. However, the triggering factor causing astroglial atrophy in the EC in not known.

Same astroglial alterations take place in the CA1 later at 18 months of age. The delay between the DG astroglial alteration and the one in the CA1 could be due the synaptic organisation of the hippocampal circuitry. The hippocampus is organised in what is known as the trisynaptic circuit (for more details see hippocampus anatomy section) and due to this, the DG, the CA3 and the CA1 are connected unidirectional in a sequential mode (Amaral *et al.*, 2007; Witter and Amaral, 2004). Therefore significant astroglial alterations in DG could be diluted throughout the intense neuronal wiring and synaptic contacts for a period of time until it affects astroglial population in CA1. In fact, same process would the one explaining the delay between the astroglial alterations in the EC and DG (1 and 12 months respectively). Therefore, is it reasonable to consider that neuronal functions by this stage are deeply affected if CA1 astroglia can detect remotely (distance and time) the alterations of the DG through the hippocampal neuronal network.

However other factors are also involved in astroglial response in AD. The appearance of Aβ in from of plaques and aggregates triggers the most known astroglial pathological reaction: astrogliosis. Interestingly the Aβ burden does not follow the regional pattern of astroglial dystrophic alterations (Fig. 3). Indeed, CA1 is the first hippocampal area showing Aβ plaques (12 months of age). This causes astroglial reaction in the CA1 before the astroglial dystrophy is manifested. Of course, this does not mean that astroglia in CA1 first becomes hypertrophic to later on become dystrophic. Astroglial in the CA1 reacts to the presence of Aβ accumulation and only those astrocytes in the vicinities of Aβ would develop astrogliotic phenotype. The rest

of astroglial population becomes dystrophic at more advance stage. Aβ presence also has the same effect in astrocytes of the DG. However, the manifestation of astrogliosis happens at 18 months of age. It was proposed that astrogliosis walls up Aβ accumulation to protect the rest of the parenchyma from Aβ toxicity (Eng and Ghirnikar, 1994)

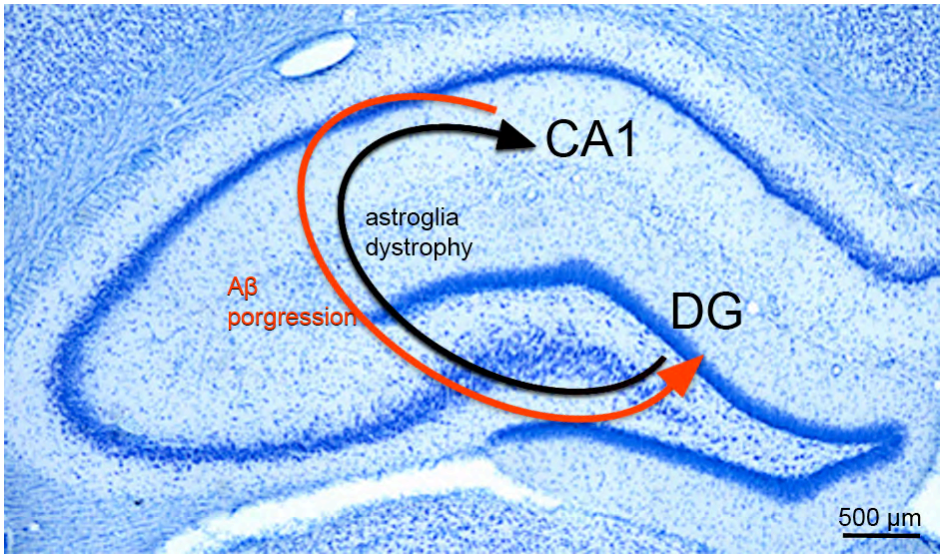


Figure 3. *Representation of the opposite progression of astroglial dystrophy and Aβ. Astroglial dystrophy starts in the DG (12 months) and passes to the CA1 (18 months), whereas Aβ plaque appearance begins in the CA1 (12 months) and later on also appear in the DG (18 months).*

Other studies have shown that astrocytes can clear Aβ accumulation and internalise it by removing it from the extracellular space (Wyss-Coray et al., 2003). Nevertheless, the specific role of astrogliosis still remains unclear and it is not known whether it is beneficial or detrimental (Rothermundt et al., 2003).

The involvement of microglial in the development of AD is known. Microglial activation is the common adaptation of microglia cell in different pathologies including AD. Activated microglia has a very delicate task in brain defence and often goes far beyond the protection role and passes into the dark side contributing to the damage. Our results, however, show a novel microglial behaviour. Already at 9 months of age, before Aβ plaques appearance, resting microglia proliferates in a dramatic way preceding microglial activation that takes place at 12 months of age. This microglial response denotes that earlier pathological events happen before Aβ burden that require microglial

preparation and recruitment for upcoming pathological events. However, microglia does not become reactive until Aβ is present in the CA1 parenchyma. From this stage on, both resting and activated microglial are high in number. Activate microglia is commonly found in close contact to plaques and is believed to phagocyte Aβ (DeWitt *et al.*, 1998). In contrast, microglia can also release pro-inflammatory molecules weakening even more the already affected hippocampus (Kettenmann et al, 2011). One worth mentioning aspect is that astrocytes close to Aβ plaques inhibit microglia to clear Aβ (DeWitt *et al.*, 1998).

All in all, these evidences of glial participation in AD demonstrate that synaptic disconnectivity and loss, does not merely concern neurons. Indeed, we have shown that glial cells have a great impact on hippocampal synaptic contacts and neuronal circuitry. Therefore it is conceivable that neuropathologies like AD could be considerer beyond the ***neurodegenerative*** point of view, and expand the consideration also to ***gliodegeneration,*** which happens in parallel or even earlier, and in this way making a more faithful description of the pathology and the involved parts. Therefore, therapeutical strategies should not neglect glial participation in AD.

5. Future directions

The results of this thesis should be expanded to further clarify the precise consequences of astroglial and microglia participation in AD. Regarding astroglia, it would be essential to investigate the synaptic coverage at electron microscopy level to verify that astroglia synaptic presence is affected due to the cytoskeleton atrophy. This particular aspect will be covered in the period before I move to my next lab (January 2012). In addition, the role of astrogliosis should be investigated in depth to understand its involvement and its possible modulation to control the intrinsic damaging potential. In this way, it would be interesting to study the beneficial impact of rutinnary activities like enriched mental exposure and exercise on the astroglia (this also is planed to do in the above mentioned period). This particular aspect would be also interesting to observe regarding microglial. In addition, it would be essential to understand astroglial-microglia interaction during the disease, because as we hypothesise is a fundamental contributor factor to the pathology of AD.

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Chapter 1

General Introduction

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Chapter 2

Materials and Methods

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Chapter 6

General Discussion

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Appendix 1

GLIA

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Concomitant Astroglial Atrophy and Astrogliosis in a Triple Transgenic Animal Model of Alzheimer's Disease

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KEY WORDS
astroglia; Alzheimer's disease; hippocampus; plasticity; β-amyloid

ABSTRACT
Astrocytes are fundamental for brain homeostasis and are at the fulcrum of neurological diseases including Alzheimer's disease (AD). Here, we monitored changes in astroglia morphology throughout the age-dependent progression of AD. We used an immunohistochemical approach that allows us to determine the domain of glial cytoskeleton, by measuring the surface, volume, and the relationship between astrocytes and neuritic plaques. We investigated astroglia in the hippocampus of a triple transgenic mouse model of AD (3xTg-AD) that mimics the progression of the human disease. The numerical density of astrocytes is affected neither by AD nor by age. We found reduction of surface and volume of GFAP profiles from early ages (6 months; 43.84 and 52.76%, respectively), persisting at 12 (40.73 and 45.39%) and 18 months (64.80 and 71.95%) in the dentate gyrus (DG) of 3xTg-AD, whereas in CA1 it appears at 18 months (29.42 and 32.74%). This cytoskeleton atrophy is accompanied by a significant reduction of glial somata volume in DG at 12 and 18 months (40.46 and 75.55%, respectively), whereas in CA1 it is significant at 18 months (42.81%). However, while astroglial atrophy appears as a generalized process, astrocytes surrounding plaques are clearly hypertrophic as revealed by increased surface (48.06%; 66.66%), and volume (57.10%; 71.06%) of GFAP profiles in DG and CA1, respectively, at 18 months. We suggest differential effects of AD on astroglial populations depending on their association with plaques accounting for the progressive disruption of neural networks connectivity and neurotransmitters imbalance which underlie mnesic and cognitive impairments observed in AD.

INTRODUCTION

Astrocytes are the main homeostatic cellular elements of the central nervous system (CNS), which define its structure and provide for numerous aspects of CNS function (Nedergaard et al., 2003; Oberheim et al., 2006, 2009; Verkhratsky, 2006, 2009; Wang and Bordey, 2008). Astrocytes generate the blood–brain barrier, control extracellular ion homeostasis and neurotransmitter balance, and maintain synaptic connectivity, strength and plasticity (Abbott et al., 2006; Bundgaard and Abbott, 2008; Halassa et al., 2007; Haydon and Carmignoto, 2006; Perea et al., 2009; Volterra and Meldolesi, 2005).

Neurological diseases result from disruption of a fine equilibrium among neural cell damage, neuroprotection, and regeneration. Astrocytes, being homeostatic cells in the CNS are invariably involved in every kind of neuropathology (Giaume et al., 2007; Nedergaard and Dirnagl, 2005; Seifert et al., 2006). Astroglia forms the first line of brain defense through its wide-ranged homeostatic activity. Astrocyte regulates the volume and composition of extracellular space (Kofuji and Newman, 2004), contain extracellular levels of glutamate (Danbolt, 2001; Kirischuk et al., 2007), thereby limiting the intrinsic excitotoxicity of the latter, regulate fluid movements, and provide the main antioxidant system (Dringen, 2000; Simard and Nedergaard, 2004). However, there is an intrinsic dichotomy in the function of astroglia, which can contribute to neuronal damage through failure or reversal of various homeostatic molecular cascades (Giaume et al., 2007; Nedergaard and Dirnagl, 2005). Pathological insults to the CNS trigger a specific astrocytic reaction known as reactive astrogliosis, which aims at protecting the brain parenchyma, isolating the damaged area, reconstructing the blood–brain barrier, and promoting the remodeling of the neural circuitry through isomorphic gliosis (Wilhelmsson et al., 2006).

Alzheimer disease (AD, Alzheimer, 1907; Kraepelin, 1919) is one of the most common form of dementia (Braak et al., 1999), clinically manifested by cognitive deficits such as memory and learning impairment, and histopathologically by substantial neuronal and synaptic loss associated with extracellular β-amyloid (Aβ) accumulation in the form of plaques and appearance of intraneuronal hyperphosphorilated tau protein fibrillary tangles (Selkoe, 2001). The first and most affected areas in AD are those directly responsible for cognitive tasks;

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these areas include entorhinal cortex, hippocampus, basal forebrain, and nucleus basalis of Meynert (Yankner, 1996).

The pathological potential of glia in dementia was originally suggested by Alois Alzheimer in 1910 (Alzheimer, 1910). Several studies have shown that both aggregated Aβ protein and the intact cores of Aβ plaques isolated from human AD brain tissue, as well as Aβ fragments can stimulate astrogliosis, which is also observed in human AD brains (Nagele et al., 2004). Experiments *in vitro*, have demonstrated that Aβ can trigger astroglial Ca^{2+} signaling, mitochondrial depolarization, and oxidative stress (Abramov et al., 2003, 2004); the Aβ was also reported to trigger apoptosis in cultured embryonic astrocytes (Assis-Nascimento et al., 2007) and in human AD brains (Kobayashi et al., 2004). Reactive astrocytes were postulated to accumulate large amounts of Aβ (Nagele et al., 2003, 2004) forming astroglial Aβ deposits, although this ability has not been conclusively confirmed. Here, we report the results of detailed analysis of astroglial morphology in the hippocampus of transgenic AD animal model of different ages.

MATERIALS AND METHODS

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice

Experiments were performed on male 3xTg-AD mice and their background-matching controls as described in detail previously (Oddo et al., 2003a,b; Rodriguez et al. 2008, 2009a).

Fixation and Tissue Processing

3xTg-AD animals of different age groups (3, 6, 9, 12, and 18 months; n = 6, 6, 7, 7, 3; respectively) and their equivalent (3, 6, 9, 12, and 18 months; n = 6, 7, 7, 4, 3; respectively) non-Tg controls were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75% acrolien (25 mL, TAAB, UK) in a solution of 2% paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75 mL). Brains were then removed and cut into 4–5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodriguez et al., 2008). The brain sections were postfixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40–50 μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels −1.58 mm/–2.46 mm (hippocampus) posterior to Bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (2004).

Antibodies

A monoclonal mouse antiserum generated against GFAP from pig spinal cord (anti-GFAP; Sigma-Aldrich, UK; #G3893) was used for the determination of glial cytoskeleton properties and change and rabbit anti-GFAP IgG fraction of antiserum (Sigma-Aldrich, UK; #G9269) for the analysis of glial cytoskeleton in relationship with neuritic plaques. For the identification of beta amyloid (Aβ) plaques, we used a monoclonal mouse antiserum that reacts with abnormally processed isoforms, as well as precursor forms of Aβ, recognizing an epitope within amino acids 3–8 (EFRHDS; anti-Ab 6E10 [SIG-39320]; Signet Laboratories, Dedham, MA). The immunolabeling pattern we obtained with this antibody is equivalent to that obtained previously in different brain regions (Oddo et al., 2003a). The specificity of the antibodies has been previously reported previously using immunohistochemistry and western blot (Eng et al., 2000; Halliday et al., 1996; Rodriguez et al., 2008). To assess for nonspecific background labeling or cross-reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and secondary antibodies from the incubation solutions resulted in a total absence of target labeling (data not shown).

Immunohistochemistry

For the detection and determination of GFAP cells and its relationship with Aβ senile plaques we used both, single, and dual indirect immunofluorescence labeling. To minimize methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using precisely the same experimental conditions. However, since the same animals have been used for both experiments we could only use sequential sections that did not allow an exact matching in anatomical levels for both single and dual labeling. Nevertheless, this was minimized by collecting sections that ranged between 40 and 100 μm apart therefore guaranteeing the maximum possible anatomical proximity. For this procedure, the vibratome sections were first incubated in 1% sodium borohydride (Aldrich, UK) for 30 min. The sections were then washed with PB profusely before rinsing in 0.1 M TS for 10 min. Brain sections were then incubated with 0.5% albumin bovine serum BSA (Sigma, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, UK) for 30 min. For the single labeling, sections were incubated for 48 h at

room temperature in primary antibody (mouse anti-GFAP, 1:30,000, SIGMA, Saint Louise, MI). The sections were rinsed in 0.1 M TS for 30 min and incubated in 1:200 dilution of fluorescein (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Baltimore Pike, PA) for 1 h at room temperature and finally rinsed in 0.1M TS for 30 min.

For dual labeling, the sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (1) mouse anti-beta amyloid monoclonal antibody (Aβ; 1:2,000; Covance, Emeryville, CA) and (2) rabbit anti-GFAP (1:30,000; SIGMA, Saint Louise, MI) simultaneously. Subsequently, Aβ and GFAP were detected in a sequential manner on the same sections by incubation with Rhodamine (TRITC)-conjugated goat anti-mouse and (FITC)-conjugated goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).

Morphological Analysis of Astrocyte Cytoskeleton

Astrocytes (n = 30–35, in both single and dual labeling experiments) were imaged using confocal scanning microscopy (Leica SP5 upright), recording layers at every 0.2 μm. Parallel confocal planes were superimposed and morphological analysis was carried out by cell analyst (Chvatal et al., 2007) using digital filters (average 3 × 3, convolution, gauss 5 × 5, despeckle, simple objects removal) to determine the surface (S) and the volume (V) of the GFAP-stained cytoskeleton of astrocytes. When analyzing astroglial morphology in relation to Aβ plaques, we considered all cells with somata within 50 μm from the plaque border as plaque-associated, and cell with somata positioned more distantly as cells not associated with plaques.

GFAP-Positive Cell Count in Hippocampus

To determine whether the changes in hippocampal GFAP cytoskeleton surface and volume are linked with changes in the number of astrocytes expressing GFAP, we determined the total number of GFAP-positive astrocytes in the dentate gyrus (DG) and CA1 subfields in five representative nonconsecutive sections throughout the dorsal hippocampus (analyzed in an area of 152,000 μm^2 for DG and 235,000 μm^2 for CA1 in coronal sections of 40 μm thickness). The specific analyzed areas were the molecular layer in the DG and stratum radiatum and stratum lacunosum-moleculare in the CA1. Confocal stack images were used to this propose. GFAP-positive astrocytes were intensely labeled against dark background that made them easy to identify with equal chance of being counted. The number of GFAP-positive astrocytes was determined blindly by a single observer; therefore, counting bias was kept to a minimum.

Statistical Analysis

Data were expressed as mean ± SE. Paired or unpaired t-test were used to examine differences in the number, surface, and volume of GFAP labeled cells between the 3xTg-AD and non-Tg animals, as well as was to analyze astrocytic cytoskeleton differences in relationship with Aβ senile plaques.

RESULTS

Ageing and AD Conditions Are Not Associated with Changes in Numbers of GFAP-Positive Astrocytes

We analyzed the astroglial morphology in the hippocampus, where the majority (∼80%) of astrocytes express GFAP (Kimelberg, 2004). Numerous GFAP immunoreactive cells (GFAP-IR), showing stellate shape and multiple branched processes that are typical of astrocytes (Figs. 1–3), were widely distributed throughout the hippocampus in both non-Tg and 3xTg-AD mice; some of them having the typical morphology of perivascular astrocytes (Fig. 3C). When we calculated the density of astrocytes within the DG and CA1 of the 3xTg-AD mice and compared it with the non-Tg, we found no changes in the density of GFAP-IR astrocytes at all ages (Fig. 1).

Astroglial Cytoskeletal Modifications in the 3xTg-AD

From 6 months of age, astrocytes in the 3xTg-AD mice showed a clear tendency to reduce their GFAP surface by 43.84% (781.11 ± 87.44 μm^2 vs. 1390.89 ± 340.01 μm^2; n.s) and volume by 52.76% (254.61 ± 36.62 μm^3 vs. 559.08 ± 152.39 μm^3; n.s), when compared with their non-TG controls in the DG (Fig. 2A,B, respectively) but not in the CA1 subfield where similar changes only appears at later age (18 months; Figs. 2C and 3D). However, these changes in the DG become significant only at 12 and 18 months of age in 3xTg-AD mice when compared with controls. The surface decreases by 40.73% (575.04 ± 89.65 μm^2 vs. 970.33 ± 54.84 μm^2, $P < 0.0001$) and 64.79% (631.25 ± 78.17 μm^2 vs. 1793.84 ± 138.53 μm^2, $P = 0.0019$), respectively (Fig. 2A). Likewise, there was a significant decrease in volume by 45.39% (165.18 ± 32.32 μm^3 vs. 302.48 ± 21.72 μm^3, $P = 0.0162$) and 71.98% (186.76 ± 22.19 μm^3 vs. 665.95 ± 64.18 μm^3, $P = 0.0103$), respectively (Fig. 2B). These changes in GFAP surface and volume were accompanied by a reduction in glial branching, indicative of the process of atrophy (Fig. 2F,H). In the CA1 subfield, the decrease in surface and volume of GFAP profiles in 3xTg-AD animals was significant only at 18 months (Fig. 2C,D). The surface decrease by 29.42% (1586.00 ± 106.97 μm^2 vs. 2247.19 ± 120.76 μm^2, $P = 0.0095$) whereas the volume decreased by 32.74% (615.02 ± 66.92 μm^3 vs. 914.44 ± 58.88 μm^3, $P = 0.0237$; Fig. 2C,D).

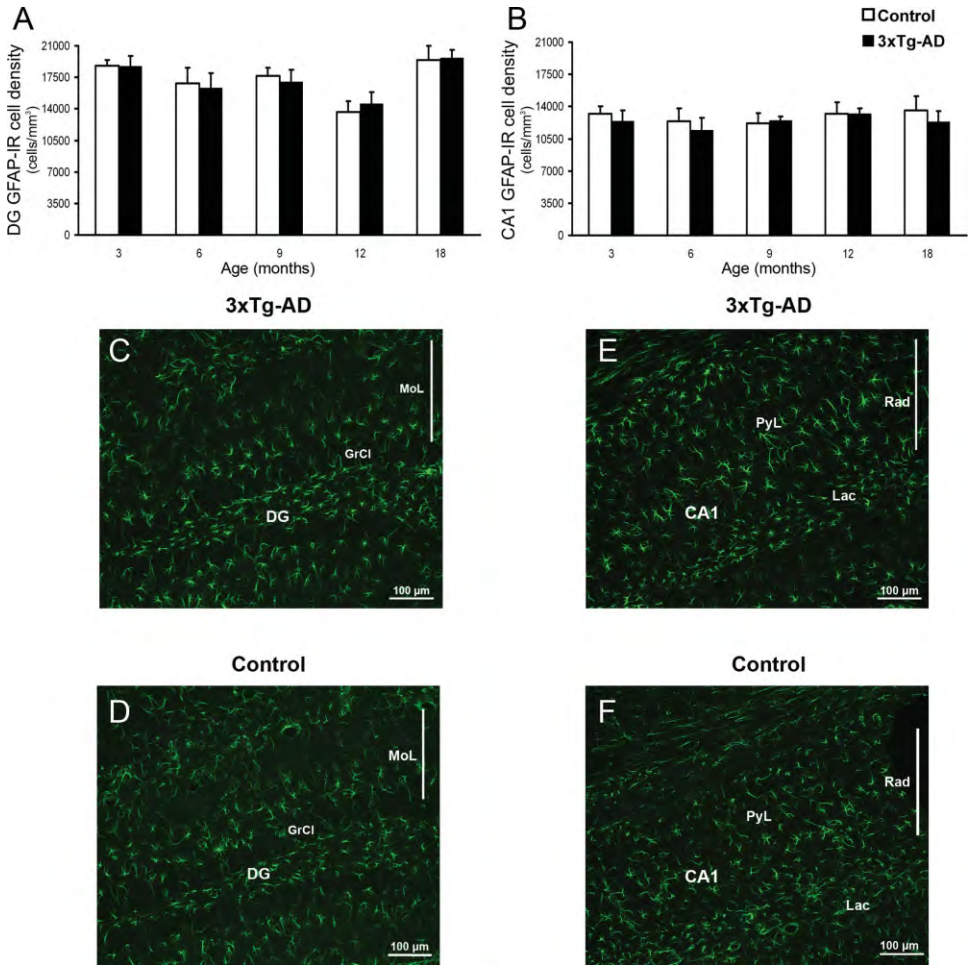

Fig. 1. (**A**,**B**) Bar graphs showing the numerical density (number of cells/mm^3) of GFAP-IR cells in the DG (A) and CA1 (B) of 3xTg-AD mice compared with control animals. Bars represent mean ± SEM. (**C**–**F**) Confocal images illustrating the expression and number of GFAP-IR cells in the DG (C) and CA1 (E) of 3xTg-AD mice compared with non-Tg animals (D and F, respectively). DG, dentate gyrus; GrCL, granule cell layer; Mol, molecular layer; PyL, pyramidal layer; Rad, stratum radiatum; Lac, stratum lacunosum moleculare.

Decrease in the Volume of Astrocytes Somata in the 3xTg-AD

Hippocampal astrocytic somata were normally separated between each other determining their different cellular domains. The astroglial processes were extending outward from the somata in multiple directions; generally, proximal primary processes had a tendency to extend radially whereas distal and secondary order extended in random fashion (Figs. 2E–H and 3A,B). From our results, it is evident that the astroglial somata was rarely placed in the center of the astrocytic extensions and domain (Figs. 2E–H and 3A,B). As happened with the overall GFAP profiles, the glial somatic volumes also decreased significantly, but without any evident change in their shape (Fig. 2F,H). This decrease (by 40.46%, 106.53 ± 16.14 μm^3 vs. 178.93 ± 57.83 μm^3, n.s) appeared already at 6 months of age in the DG, but was significant only at 12 and 18 months; when the volumes of somata decreased by 40.46%, (74.14 ± 14.97 μm^3 vs. 124.53 ± 11.72 μm^3, $P = 0.0476$) and by 75.55% (66.97 ± 8.88 μm^3 vs. 274.03 ± 25.90 μm^3, $P = 0.0016$), respectively (Fig. 2I). In CA1, this effect was evident at 18 months of age (decrease by 42.81%, 197.19 ± 28.87 μm^3 vs. 264.18 ± 7.26 μm^3, $P = 0.0081$; Fig. 2J).

Differential Changes in Morphology of Astrocytes Depending on Their Relation to Aβ Plaques

The accumulation of Aβ plaques in the hippocampus of 3xTG-AD animals starts at 12 months, with a clear prevalence in the CA1 compared with the DG. These plaques are surrounded by astrocytes, which display typical astrogliotic characteristics such as hypertrophy and increased branching (Fig. 3B–E). Some of these reactive astrocytes are also associated with blood vessels highly loaded with Aβ (Fig. 3C).

Reactive astrocytes surrounding Aβ plaques in CA1 had significantly larger GFAP surface when compared with astrocytes situated away from plaques (by 58.95% at 12 months (651.72 ± 54.58 μm^2 vs. 410.00 ± 69.28

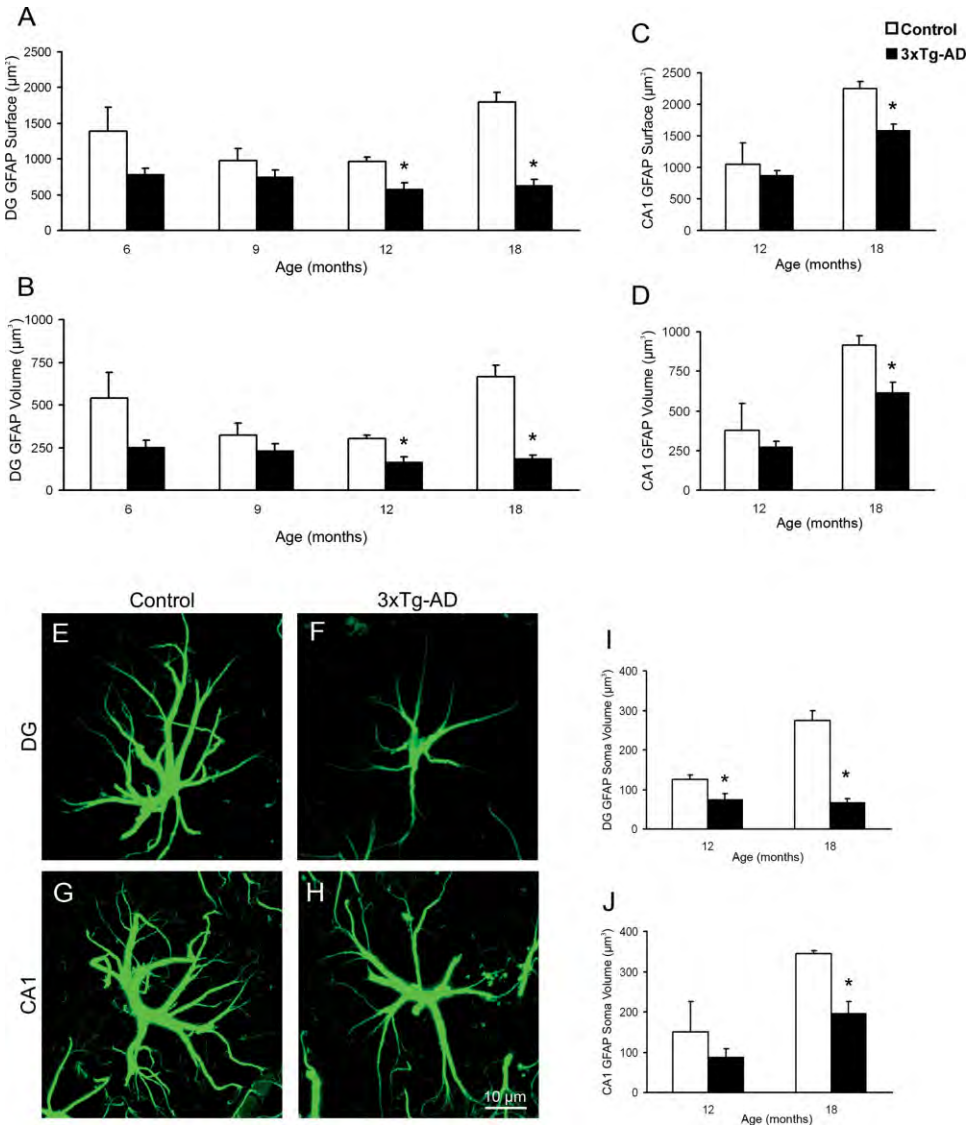

Fig. 2. Bar graphs showing a decreased GFAP surface, volume, and soma volume in both the DG (**A**, **B**, **I**) and the CA1 (**C**, **D**, **J**) of the hippocampus of the 3xTg-AD mice when compared with control animals. Bars represent mean ± SEM ($P < 0.05$. (G–J) Confocal micrographs illustrating the astrocytic atrophy in 3xTg-AD mice in the DG (**F**) and CA1 (**H**) compared with control animals (**E** and **G**).

μm^2, $P = 0.0030$) and by 66.65% at 18 months (702.16 ± 44.61 μm^2 vs. 421.30 ± 40.91 μm^2, $P = 0.0044$; Fig. 3F). This increase in surface was paralleled by an increase in volume by 65.18% (183.33 ± 20.75 μm^3 vs. 110.98 ± 23.27 μm^3, $P = 0.0037$) and 71.06% (196.32 ± 17.07 μm^3 vs. 114.76 ± 11.50 μm^3, $P = 0.0034$) at 12 and 18 months of age respectively when compared with astrocytes situated away from plaques (Fig. 3G). Similarly, we observed significant increase in the volume of somata of astrocytes associated with plaques when compared with astrocytes distant to the plaques (by 46.95% at 12 months (89.32 ± 7.79 μm^3 vs. 60.78 ± 10.32 μm^3, $P = 0.0046$) and by 21.59% at 18 months (84.84 ± 7.49 μm^3 vs. 69.77 ± 6.55 μm^3, $P = 0.0137$; Fig. 3H).

Astrocytes surrounding plaques in the DG showed similar structural changes at 18 months of age when the burden of Aβ plaques becomes evident within this region. These reactive astrocytes associated with Aβ plaques in DG also increased significantly their GFAP surface (by 48.06%, 327.82 ± 24.19 μm^2 vs. 221.40 ± 15.66 μm^2, $P = 0.0013$; Fig 3I), volume (by 57.10%, 72.27 ± 6.77 μm^3 vs. 46.00 ± 4.85 μm^3, $P = 0.0032$; Fig 3J), as well as the somatas volume by 110.70% (31.10 ± 3.25 μm^3 vs. 14.76 ± 1.36 μm^3, $P = 0.0005$; Fig. 3K) when compared with control animals, At the same time, the astrocytes positioned away from the plaques kept showing atrophic characteristics in both the CA1 and DG areas (Fig. 3A).

DISCUSSION

The astroglial cells, which form the ultimate homeostatic system in the CNS, are intimately involved in

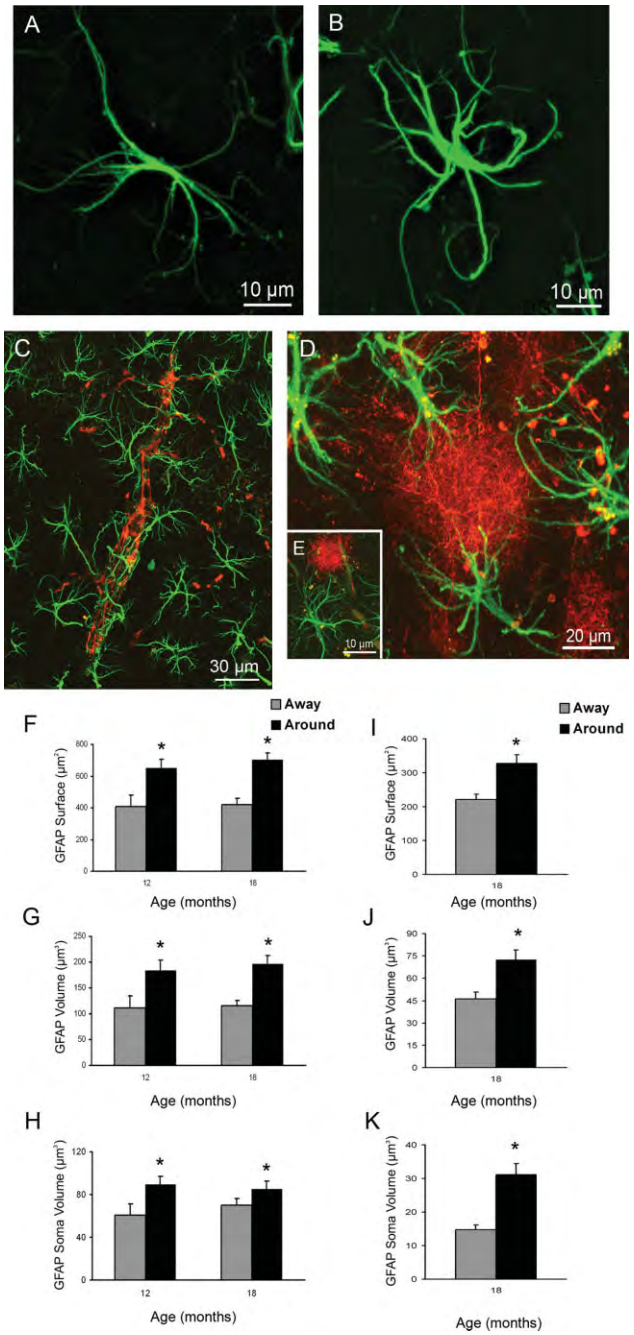

Fig. 3. (**A**,**B**) Confocal images of hippocampal preparations dually labeled by GFAP and by anti-β amyloid monoclonal antibody illustrating differential changes in GFAP profiles in astrocytes distant to the plaques (A) and associated with the Aβ plaques (B). (**C**–**E**) Confocal dual labelling images (GFAP in green and Aβ in red) in 3xTg-AD mice showing the accumulation of astrocytes around the Aβ plaques and vascular Aβ deposits. Astrocytes surrounding Aβ plaques (D, E) and Aβ loaded blood vessel (C), undergo astrogliosis. (**F**–**K**) Bar graphs showing GFAP-positive astrocytic surface (F), volume (G), and soma volume (H) differences between astrocytes located around the amyloid plaques (Aβ) and those distant to the plaques in the CA1 of 3xTg-AD animals. (I–K): Similar astrocytic surface (I), volume (J), and soma volume (K) differences are observed in the DG at 18 months of age. Bars represent mean ± SEM ($P < 0.05$).

many forms of neurological diseases. At the same time, the reaction of astrocytes during the early stages of chronic neurological disorders and their pathogenetic role remain virtually unknown. The reactive astrogliosis is a characteristic feature of terminal stages of neurodegenerative processes, including the AD (Nagele et al., 2004; Rodriguez et al., 2009b; Rossi and Volterra, 2009; Simpson et al., 2008).

Here, we analyzed the GFAP-positive astroglial profiles in the hippocampus of transgenic AD animals of different age. To the best of our knowledge, this is the first attempt to characterize the age-dependent changes in the morphology of GFAP-positive astrocytes in the relevant model of family AD. For our analysis, we have chosen hippocampus because the majority of astroglial cells in this brain region are GFAP positive (Kimelberg, 2004). First, we found that overall density of GFAP-positive astroglial cells is affected neither by age nor by the AD conditions. This shows that neither ageing nor AD pathology are associated with cell loss; similarly, it argues against the prominent astroglial proliferative response in the AD brain.

Second, contrary to the general assumption that the AD is associated with reactive astrogliosis with overall increase in GFAP expression and astroglial proliferation (Nagele et al., 2004), we found that in both DG and the CA1 areas there is a generalized atrophy of GFAP-positive astrocytes. This atrophy is manifested by decreased surface and volume of GFAP-positive profiles and appears at the early stages of the disease. Indeed, already at 6 months (when the hippocampus is virtually free from the neuritic plaques) both volume and surface area of astrocytes in DG were decreased. This overall hypotrophy of GFAP-positive profiles in DG remains at 12 and 18 months. The similar hypotrophy of GFAP-positive astrocytes in CA1 region becomes apparent only at 18 months of age.

During the later stages of AD, the changes in astroglial morphology is complex. The appearance of the neuritic plaques in AD, similarly to other brain injuries (Wilhelmsson et al., 2006), results in astrogliosis in both hippocampal areas. Importantly, the astrocytes demonstrating clear signs of reactivity (hypertrophy, thick processes, enlarged cell bodies, and overall increase in the volume and surface area of GFAP-positive profiles) were exclusively associated with the Aβ plaques. Astrocytes localized distantly (>50 μm) from the borders of the plaques were atrophic as reflected by decreased volume of cell bodies, reduced number of main processes, their arborization, and overall GFAP surface and volume. The existence of two distinct populations of astrocytes in Aβ plaques-infested brains of transgenic AD animals was also demonstrated in the *in vivo* Ca^{2+} imaging experiments, which showed increased Ca^{2+} excitability of the astrocytes contacting neuritic plaques (Kuchibhotla et al., 2009).

The early atrophy of astroglial cells in the progression of family-like AD (and indeed the transgenic AD models reproduce the FAD type pathology) can be pathologically relevant. The atrophic changes were not only associated with the thick primary proximal processes but also with secondary medial and occasionally distal thin processes, which may indicate the reduction in overall astroglial

arborisation and hence possible decrease in glial synaptic coverage (Rodriguez et al., 2009b). The reduction of synaptic coverage by astroglial membranes together with overall atrophic changes in astroglia can be instrumental in the early imbalance of neurotransmission, reduced glial homeostatic function, and in disrupted synaptic connectivity. The atrophic or degenerative changes in astroglia have been recently observed in several types of chronic neurodegeneration, including amyotrophic lateral sclerosis (Rossi et al., 2008; Rossi and Volterra, 2009), in Wernicke encephalopathy (Hazell, 2009), in frontotemporal dementia (Broe et al., 2004), in schizophrenia and major depression (Gosselin et al., 2009; Rajkowska et al., 2002; Si et al., 2004). The degenerative/atrophic changes in astroglia may represent the common mechanism in early disruptions of synaptic connectivity in all forms of neurodegeneration. In AD, the early astroglial asthenia may result in inadequate support of synapses, leading to the extinction of the latter and disruption of connectivity within neural circuitry. Indeed, the synaptic loss at the early stages of AD is well documented (Scheff et al., 2007; Terry, 2000), and the underlying mechanisms are unclear. The results of our morphological analysis may indicate that it is the astroglial failure which produces early synaptic disorders, and hence early cognitive deficits, in the Alzheimer type neuropathology.

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MOLECULAR NEURODEGENERATION

RESEARCH ARTICLE Open Access

Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: mechanism for deficient glutamatergic transmission?

Markel Olabarria[1], Harun N Noristani[1], Alexei Verkhratsky[1,2,3,4] and José J Rodríguez[2,3,4*]

Abstract

Astrocytes are fundamental for brain homeostasis and the progression and outcome of many neuropathologies including Alzheimer's disease (AD). In the triple transgenic mouse model of AD (3xTg-AD) generalised hippocampal astroglia atrophy precedes a restricted and specific β-amyloid (Aβ) plaque-related astrogliosis. Astrocytes are critical for CNS glutamatergic transmission being the principal elements of glutamate homeostasis through maintaining its synthesis, uptake and turnover via glutamate-glutamine shuttle. Glutamine synthetase (GS), which is specifically expressed in astrocytes, forms glutamine by an ATP-dependent amination of glutamate. Here, we report changes in GS astrocytic expression in two major cognitive areas of the hippocampus (the dentate gyrus, DG and the CA1) in 3xTg-AD animals aged between 9 and 18 months. We found a significant reduction in N_v (number of cell/mm^3) of GS immunoreactive (GS-IR) astrocytes starting from 12 months (28.59%) of age in the DG, and sustained at 18 months (31.65%). CA1 decrease of GS-positive astrocytes N_v (33.26%) occurs at 18 months. This N_v reduction of GS-IR astrocytes is paralleled by a decrease in overall GS expression (determined by its optical density) that becomes significant at 18 months (21.61% and 19.68% in DG and CA1, respectively). GS-IR N_v changes are directly associated with the presence of Aβ deposits showing a decrease of 47.92% as opposed to 23.47% in areas free of Aβ. These changes in GS containing astrocytes and GS-immunoreactivity indicate AD-related impairments of glutamate homeostatic system, at the advanced and late stages of the disease, which may affect the efficacy of glutamatergic transmission in the diseased brain that may contribute to the cognitive deficiency.

Keywords: Astroglia, Alzheimer?'?s disease, glutamine synthetase, GFAP, amyloid beta, excitotoxicity, hippocampus, plasticity

Introduction

The central nervous system relies on astrocytes for its correct functioning. Astroglia is critical for metabolic support to neurones by providing glucose and lactate [1,2], regulates ion environment, i.e. K^+ and water movements and provides reactive-oxygen-species scavengers like glutathione [3-5].

Astrocytes, as a component of the tripartite synapse, modulate neurotransmission and control the extracellular level of neurotransmitters [6-11]. Therefore, astrocytes are essential for glutamatergic transmission being key elements for "*de novo*" synthesis of glutamate and for the glutamate-glutamine cycle; which, in addition, are fundamental for the synaptic plasticity associated to cognitive processes [12,13]. The bulk of glutamate release during neurotransmission is taken up by astroglia through Na^+-dependent glutamate transporters [9,14]. In astrocytes glutamate is converted to glutamine by glutamine synthetase (GS) [9] (which is considered

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astrocytic-specific enzyme although some recent studies have shown some degree of oligodendroglial and microglial GS expression under some pathological situations [15,16]). Subsequently, astrocytic glutamine is transported back to neurones for its further conversion into glutamate [9,17]. Thus, the glutamate-glutamine shuttle makes both astrocytes and GS essential for glutamatergic neurotransmission [18]. At the same time astrocytic glutamate uptake prevents glutamate excitotoxicity [9]; disturbance of astroglial-based glutamate homeostasis may lead to neurotransmitter imbalance, neuronal malfunction and death, as well as impaired cognition [18,19].

Astroglia is fundamental for the onset, progression and outcome of neuropathological processes by limiting the damage and promoting the revascularisation of the surrounding tissue through reactive astrogliosis [20-23] and by contributing to neuroinflammation by release of various pro-inflammatory factors, such as interleukins [24-26].

Alzheimer's disease (AD) is a highly malignant neurodegenerative process characterised by anomalous intraneuronal and extracellular accumulation of β-amyloid protein (Aβ) [27] and hyperphosphorilated cytoskeletal Tau protein in neurons [28]. As a consequence of this anomalous protein formation and by a yet unknown mechanism, severe loss of specific ACh neurons and synapses appear at middle and advanced stages of the disease [29]. As a result, the CNS reacts by both neuronal compensation and glial reactivity [30,31]. Recently, in a GFAP based study, we have described that the associated reactive astrogliosis observed in the triple transgenic animal model (3xTg-AD) is preceded by a generalized atrophy of astrocytes that occurs at the middle stages of the disease (9-12 months of age). Formation of the senile plaques triggers secondary astrogliosis in astrocytes associated with Aβ depositions, and the later stages of the pathology are characterized by concomitant astroglial atrophy and astrogliosis that in any case is not associated with astrocytic density alterations (12-18 months of age) [32,33]. Furthermore, and even if the two main pathological hallmarks have to be considered when studying AD, astrocytic involvement, as recently demonstrated by us [32,33], is mainly related with Aβ pathology, since astrocytes modulate extracellular volume and components and Aβ directly affects the extracellular space, while tau pathology remains intraneuronal throughout AD [28].

Materials and methods

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice

Experiments were performed on male 3xTg-AD mice, which harbours the mutant genes for amyloid precursor protein (APPSwe), for presenilin 1 PS1M146 V and for tauP301 L [34,35] and their background-matching controls as described in detail previously [34-37].

Fixation and tissue processing

Animals of different age groups (9, 12 and 18 months; n = 4-8) were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75% acrolein (25 ml, TAAB, UK) in a solution of 2% paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75 ml). Brains were then removed and cut into 4 - 5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously [36]. The brain sections were post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40 - 50 μm thickness using a vibrating microtome (VT1000 S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at levels -1.58 mm/-2.46 mm (hippocampus) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin [38].

Antibodies

A mouse antiserum generated against GS (anti-GS; Millipore, UK; MAB302) was used for the determination of GS positive astrocytes. A rabbit anti-GFAP IgG fraction of antiserum (Sigma-Aldrich, UK; #G9269) was used for the determination of glial cytoskeleton and comparison to GS labelling distribution. A monoclonal antibody against amyloid beta conjugated with alexa 488 (Convance, USA; SIG-39347) was employed to label neuritic plaques. The immunolabelling pattern that we obtained with these antibodies is equivalent to that obtained previously [32,39] and their specificity has also been previously demonstrated by western blot [36,40-42]. To assess for non-specific background labelling or cross reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and/or secondary antibodies from the incubation solutions resulted in a total absence of target labelling (data not shown).

Immunohistochemistry

To minimise methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using precisely the same experimental conditions. For this procedure, the vibratome sections were first incubated for 30 min in 30% methanol in 0.1 M PB and 3% hydrogenperoxide (Sigma, UK). Sections were rinsed with 0.1 M PB for 5 mins and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. Subsequently the sections were washed with PB profusely before rinsing in 0.1 M TS for 10 minutes. Brain sections were then incubated with 0.5% albumin bovine serum (BSA, Sigma, Dorset, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, Dorset, UK,) for 30 minutes. For the single labelling, sections were incubated for 48 hours at room temperature with primary antibody (mouse anti-GS, 1:500, cat# MAB302, Millipore, UK). The sections were rinsed in 0.1 M TS for 30 minutes and incubated in 1:200 dilution of biotinylated horse anti-mouse IgG (Vector laboratories, Peterborough, UK) for 1 hour at room temperature. Sections were rinsed in 0.1 M TS for 30 minutes, followed by incubation for 30 minutes in avidin-biotin peroxidase complex (Vetor Laboratories Ltd, Peterborough, UK). The peroxidase reaction product was visualized by incubation in a solution containing 0.022% of 3,3'diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003% H_2O_2 for 1.5 minutes as described previously [36,37]. The reaction was stopped by rinsing the tissue in 0.1 M TS for 6 minutes followed by 0.1 M PB for 15 minutes. Brain sections were permanently mounted onto gelatinized slides. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) followed by xylene; and then permanently coverslipped.

For dual immunofluorescence labelling, the sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (1) mouse anti-GS (1:500) and (2) rabbit anti-GFAP (1:30,000) simultaneously. Subsequently, GS and GFAP were detected in a sequential manner on the same sections by incubation with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen, Paisley, UK), respectively.

For triple immunofluorescence labelling, the sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (1) mouse anti-GS (1:500) and (2) rabbit anti-GFAP (1:30,000) simultaneously. Subsequently, GS and GFAP were detected in a sequential manner on the same sections by incubation with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 633-conjugated goat anti-rabbit (Invitrogen, Paisley, UK), respectively. Then, the sections were washed in 0.1 M TS for 30 min and incubated in 0.5% BSA in 0.1 M TS and 0.25% Triton X-100 for 30 minutes. Subsequently, sections were incubation in mouse anti-amyloid beta Alexa 488-conjugated antibody (1:2000) for 20 hours at room temperature.

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

GS positive cell count in hippocampus

We determined the numerical density (N_v; #/mm^3) of GS positive astrocytes at 9, 12 and 18 months of age in both 3xTg-AD and non-Tg mice in the DG and CA1 subfields of the hippocampus. For this, 3 - 4 representative non-consecutive coronal sections throughout the dorsal hippocampus at levels -1.70/-2.18 [38] were quantified accounting for an analyzed volume of approximately 6,000,000 μm^3 in the DG and 15,000,000 μm^3 in CA1. The specific analysed areas were the molecular layer (MoL) in the DG and all the strata of the CA1 apart from the pyramidal cell layer that is practically devoid of GS expression due to the dense packing of pyramidal somata and almost no presence of astrocytic cell bodies. GS positive astrocytes were intensely labelled against lighter background that made them easy to identify with equal chance of being counted. A single observer determined the number of GS positive astrocytes blindly; therefore, counting bias was kept to a minimum.

Optical Density (OD) Measurement

Using computer-assisted imaging analysis (Image J 1.32j, NIH, USA), we analyzed the expression and density of GS labelling at 9, 12 and 18 months of age in both 3xTg-AD and non-Tg mice by measuring their optical density (OD) as described previously [43]. Briefly, to exclude any experimental errors and/or bias, all images were taken at constant light intensity. Optical filters were used to ensure the specificity of the signal recorded by the camera. The staining was observed throughout the thickness of the section (40 μm) using light microscopy (Nikon Eclipse 80i). No differences were observed in GS immunoreactivity throughout the thickness of the section between 3xTg-AD and non-Tg control animals; hence the changes in OD were used as measure of increased GS expression. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with readout of 250 corresponding to the area with very low GS expression and 0 corresponding to the densest area of labelling. The calibration density was kept constant for measuring all section to avoid experimental variances. Sections background OD was determined from the corpus callosum (CC) that was considered as blank since GS labelling in the CC is virtually absent.

GS density of the entire DG MoL and CA1 (with the exception of the pyramidal cell layer) were measured independently and a single measurement was obtained from every sub-region in each hemisphere. To analyze the change in GS density against constant control, the 255 was divided by control region (CC) and the obtained factor was multiplied by the region of interest in every given section. Inverse optical density was obtained by subtracting from the obtained background level (set at 255). Measurement of mean density were taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each slice. The results are shown as inverse GS density (IOD/pixel).

GS and GFAP positive cell count in relation to Aβ plaques in CA1

Triple labelling pictures of the CA1 at 18 months of age in both 3xTg-AD and non-Tg mice were taken using confocal scanning microscopy (Leica SP2, inverted), recording layers at every 0.5 μm. Both GS and GFAP positive cells were counted separately and taking in to account their localisation regarding Aβ plaques. We considered all cells with the somata within 50 μm from the plaque border of the plaque-associated, and cell with somata positioned more distantly as cells not associated with plaques.

Statistical analysis

Data were expressed as mean ± SEM. Unpaired t-tests were used to examine differences between 3xTg-AD and non-Tg animals at different time points and differences between away and around conditions in 3xTg-AD. Significance was accepted at $p \leq 0.05$. The data were analyzed using GraphPad Prism (GraphPad Software).

Results

GS immunoreactive (GS-IR) astrocytes were widely distributed throughout the subdivisions of the hippocampus in both non-Tg and 3xTg-AD mice. This distribution was similar to the GFAP immunoreactivity in both DG and CA1 (Figure 1C, E, G, 2A). GS-IR astrocytes show typical protoplasmic morphology characterized by small round cell bodies with few primary and several secondary processes extending radially in random fashion. GFAP-IR astrocytes were characterised by primary and secondary cytoskeleton processes extending radially from the cell body and frequently co-localising with GS-IR main processes. (Figure 1A, B, D, F, H). However, and differently to GFAP-positive astrocytes, GS-IR astrocytes showed a clear and profuse labelling not only in the cell body and primary processes but also throughout the fine and thin distal processes (Figure 1A, D, 3C-F).

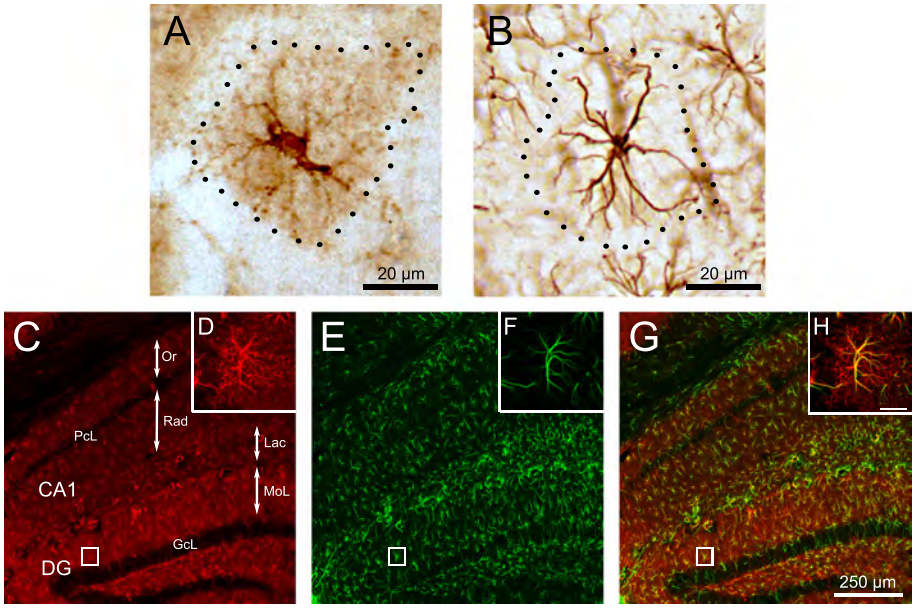

Figure 1 Light and confocal micrographs of different astrocytic phenotypes according to their enzyme content and cyoskeletal component in the hippocampus of 3xTg-AD mice. (A-B) Light microscopy images of GS (A) and GFAP (B) positive astrocytes showing their differential anatomical characteristics but similar domains. (C,E,G) Hippocampal confocal images evidencing astrocytic GS (C, red) and GFAP (E, green) expression pattern and their co-localisation (G, yellow). (D,F,H) High magnification confocal images illustrating the majoritary dual expression and co-existence of GS and GFAP (inserts D, F, H) in a representative astrocyte of the molecular layer of the DG. DG, dentate gyrus; GcL, granule cell layer; MoL, molecular layer; Lac, stratum lacunosum moleculare; Or, stratum oriens; PcL, pyramidal layer; Rad, stratum radiatum.

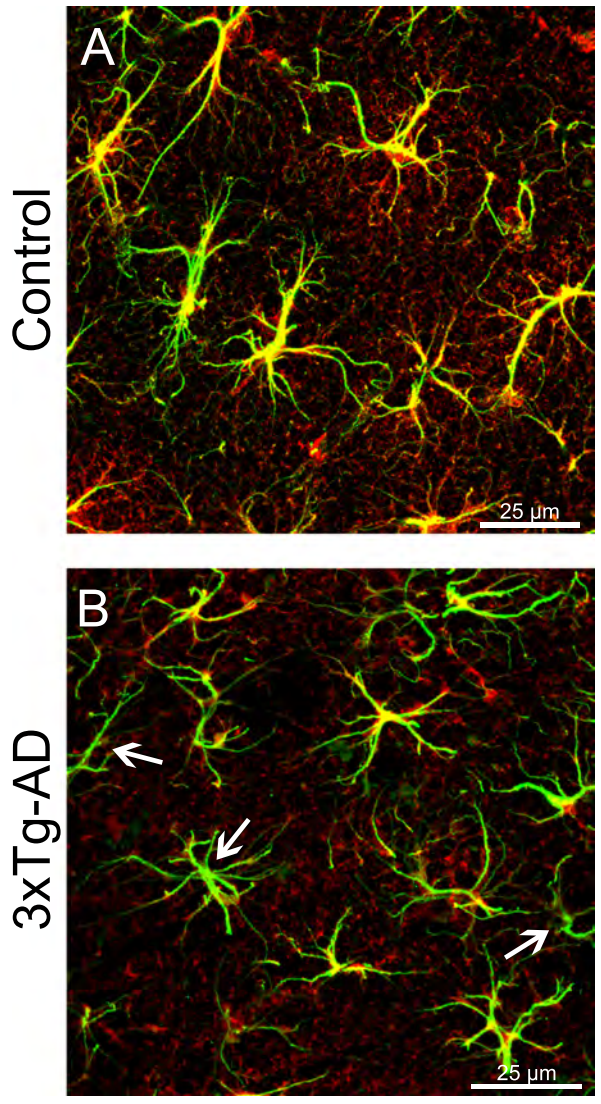

Figure 2 Confocal micrographs showing GFAP (green) and GS (red) labelling in the hippocampus of either control (A) or 3xTg-AD mice (B). Majority of astrocytes co-express (yellow) GFAP and GS in control mice, whilst some of GFAP positive astrocytes of the 3xTg-AD mice fail to express GS (arrows).

GS immunoreactivity in non-Tg animals

In non-Tg animals, GS-IR showed a uniform pattern in the DG and in the CA1 being constant at all age groups. The GS-IR N_v at all ages was significantly lower in the DG compared to the CA1 (5,599 ± 603 cells/mm^3 vs. 9,700 ± 1,041 cells/mm^3, 42.27%, p = 0.009; 4,852 ± 306 cells/mm^3 vs. 10,456 ± 1,788 cells/mm^3, 53.59%, p = 0.021; 5,064 ± 511 cells/mm^3 vs. 10,727 ± 1,083 cells/mm^3, 52.78%, p = 0.001; at 9, 12 and 18 months respectively; Figure 3A, B). The overall GS-IR, as determined by the IOD, was slightly higher in the DG at 9 and 12 months of age when compared to CA1 (10.67% and 14.38%); but just being significantly higher in the advanced age (18 months, 16.34%, p = 0.046; Figure 4A, B).

N_v of GS-IR astrocytes decreases in 3xTg-AD mice

From 12 months of age the 3xTg-AD mice showed a significant reduction of the N_v of GS-IR cells in the DG (4,852 ± 306 cells/mm^3 vs 3,465 ± 344 cells/mm^3; 28.59%, p = 0.016; Figure 3A) compared to the non-Tg control animals, whereas no apparent difference was found in CA1. At 18 months of age the decrease in GS-IR cell N_v was significant in both the DG (5,064 ± 511 cells/mm^3 vs 3,462 ± 321 cells/mm^3; 31.65%, p = 0.036) and in the CA1 (10,727 ± 1083 cells/mm^3 vs 7,159 ± 400.78 cells/mm^3; 33.26%, p = 0.026; Figure 2, 3C-3F).

3xTg-AD mice exhibit a decrease in GS expression

In parallel to the decrease in the N_v of GS-IR cells, we observed a decrease in the expression of GS in the hippocampus with no apparent regional differences, as shown by the decrease of the inverse optical density (IOD; Figure 4). The 3xTg-AD mice, when compared to non-Tg controls, showed a significantly decreased GS expression in both DG (66 ± 3 vs 52 ± 4; 21.62%; p < 0.05) and CA1 (57 ± 2 vs 46 ± 2; 19.69%; p = 0.010) at 18 months of age (Figure 4), but not at the earlier ages (Figure 4A, B).

N_v of GS-IR astrocytes decrease in 3xTg-AD mice is associated, although no exclusive, with Aβ plaques

GS-IR decrease was not homogenous throughout the CA1 parenchyma in 3xTg-AD. GS-IR N_v was compared with GFAP-IR N_v in either the vicinity or distant of Aβ plaques in 3xTg-AD at 18 months of age, to asses whether GS expression changes were related to Aβ plaques. No GFAP-IR N_v related changes were observed in any of the conditions (Figure 5). However, GS-IR astrocyte N_v was significantly diminished in CA1 areas free of Aβ (10,069 ± 572 cells/mm^3 vs 7,704 ± 558 cells/mm^3; 23.49%, p = 0.010) when compared to GFAP-IR N_v density in same areas, being this reduction more patent in the vicinities of neuritic plaques (10,314 ± 922 cells/mm^3 vs 5,338 ± 685cells/mm^3; 48.24%, p = 0.002). In addition, GS-IR N_v associated to neuritic plaques was significantly lower to GS-IR N_v away from Aβ deposits (by 24.24%, p = 0.022).

GS-IR and GFAP-IR N_v was almost identical in non-Tg controls, showing 99.22% of co-localisation (data not shown).

Discussion

In the present study we analysed the functional status of astroglia in the triple transgenic AD animal model by determining the expression of the GS at different ages. Our results in this animal model demonstrate that the

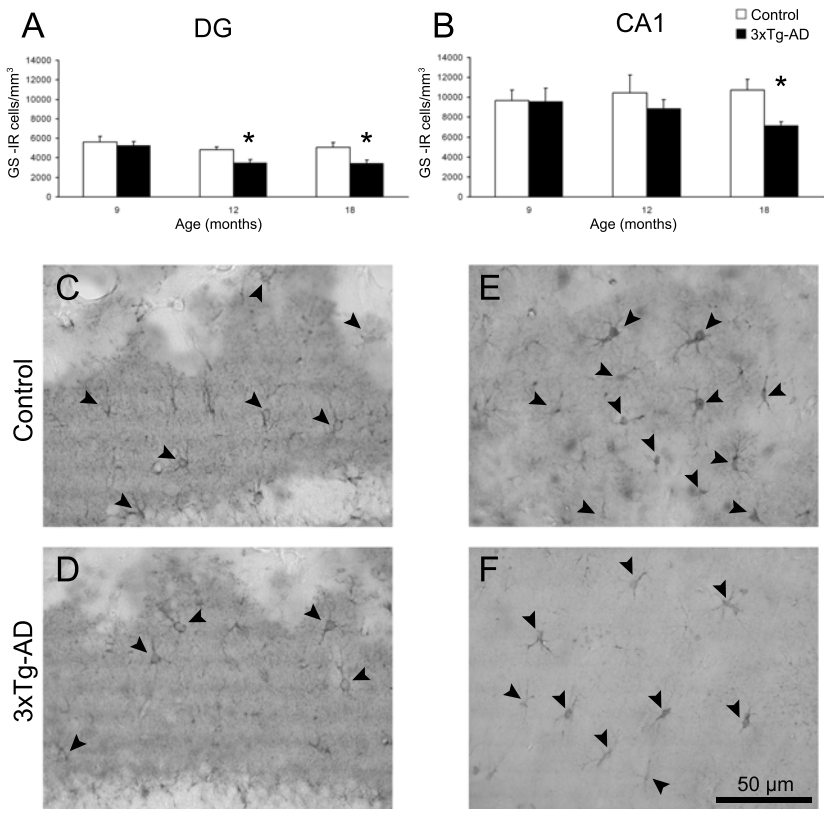

Figure 3 Bar graphs illustrating GS-IR N_v (number of cells/mm^3) in the DG (A) and CA1 (B) of 3xTg-AD mice compared with non-Tg control animals. Bars represent mean ± SEM. Light micrographs illustrating the difference in GS positive astrocytes (arrowheads) between non-Tg control mice and 3xTg-AD mice in either DG (C,D) or CA1 (E,F).

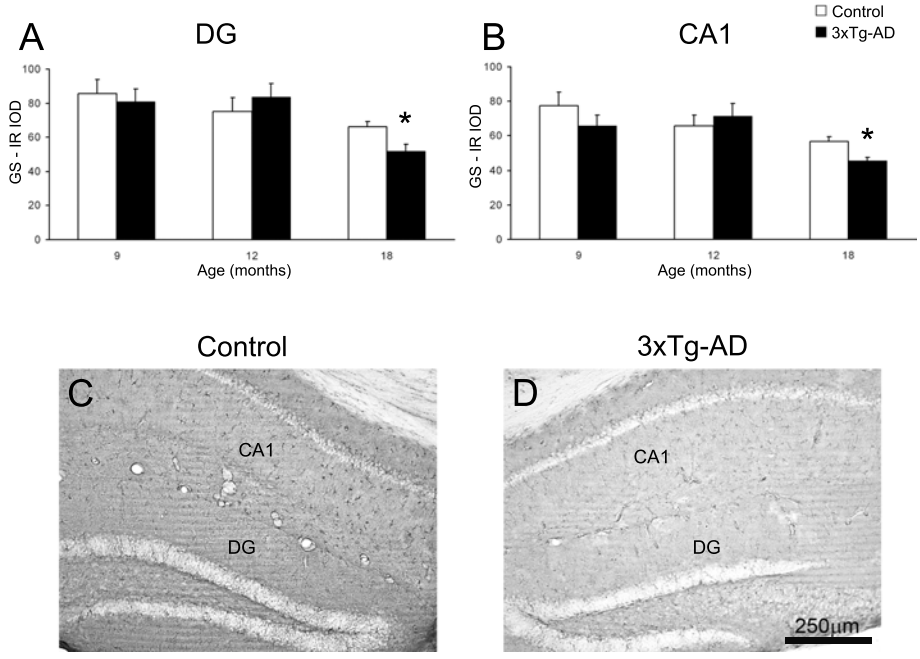

Figure 4 Bar graphs showing GS content as determined by its inverted optical density (IOD) in DG (A) and CA1 (B) of 3xTg-AD mice compared with control non-Tg animals. Bars represent mean ± SEM. Illustrative light micrographs of control mice (C) and 3xTg-AD mice (D) showing the GS expression in both the DG and CA1.

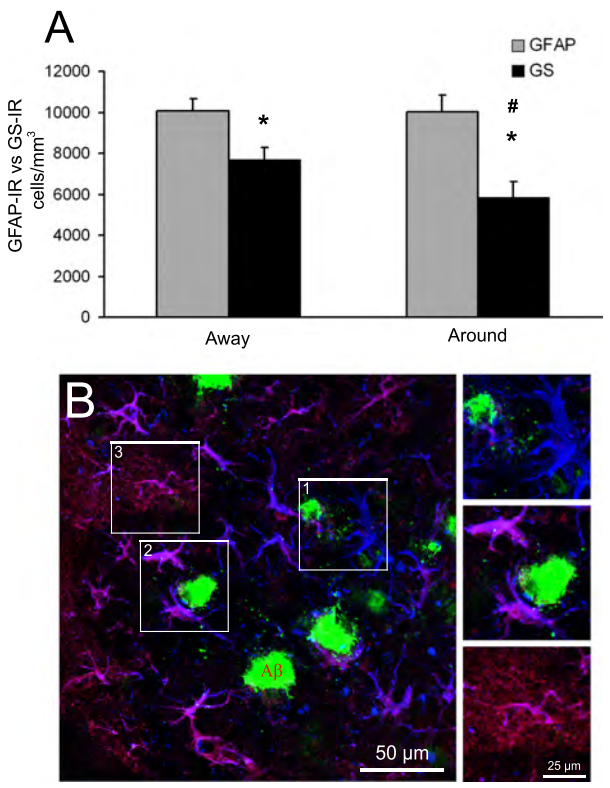

Figure 5 GFAP and GS astrocytes in the hippocampus of 3xTg-AD and their relationship with Aβ plaques. (A) Bar graph showing GFAP-IR and GS-IR astrocytes N_v differences in the distance (away; > 50 μm) and vicinities (around; < 50 μm) of Aβ deposits. Bars represent mean ± SEM. * $p \leq 0.05$ compared to correspondent GFAP-IR cell N_v; # $p \leq 0.05$ compared to GS-IR cell N_v away. (B-E) Confocal micrographs illustrating GFAP (blue), GS (red) and Aβ (green) labelling. Several GFAP positive astrocytes in surrounding Aβ plaques (< 50 μm) lack of GS immunoreactivity (1), whilst others, co-express GFAP and GS, without the typical extended GS domain (2). (3) Astrocytes > 50 μm away from Aβ deposits co-expressing GFAP and GS in their cell bodies and main processes (pink). The distal fine processes express just GS (red).

expression of astroglial GS in hippocampus is affected at the human equivalent advanced and late stages of AD, as indicated by a reduction in both the N_v of GS-IR astrocytes and GS expression (as determined by IOD) in the 3xTg-AD when compared to the control animals. These changes in N_v appear in both DG and CA1, albeit they initially occur in the DG at 12 months of age. Such reduction becomes more evident in the surroundings of Aβ plaques at 18 months of age. The GS expression is also decreased in the 3xTg-AD in both DG and CA1 but at a later age (18 months).

Astrocytes are known to be involved in most neurological diseases including AD [20,22,33]. AD is characterised by a profound cognitive impairment due to a severe loss of synapses and neurones; which is generally believed to be associated with reactive astrogliosis [28,44]. Our recent studies in the 3xTg-AD animal model, however, have found a more complex astroglial reactions in the late stages; which might show a similar pattern during human AD progression. At the early stages of the pathology astrocytes undergo generalised atrophy and down-regulation of GFAP expression, which may indicate early compromise of astrocytic homeostatic functions [23,32,33]. Several studies have previously described that GFAP abnormalities are associated with impaired glutamate homeostasis [45-48]. In a hypoxic animal model, astrocytes in the affected areas showed an abnormal GFAP cytoskeleton characterised with very short processes; which was associated with redistribution of GFAP and GLAST to the cell body, suggesting an impaired glutamate homeostasis [45]. In addition, in GFAP knock-out mice, the lack of GFAP compromised the trafficking of the GLT-1 to the cell membrane, affecting glutamate uptake [46]. Furthermore, "*in vitro*" studies have shown an inverse correlation between astroglial expression of GFAP and GS [48]. Recently has been demonstrated that, as opposed to what was thought, 3xTg-AD model shows some degree of neuronal loss as a consequence of the pathological burden [49], strengthening the relevance of this AD model and allowing the comprehension of many described synapse-related pathological events like the current one.

Studies in AD brains have shown a decrease of cortical astrocytic GS expression [50,51] that is accompanied by a "*de novo*" neuronal expression of GS as a compensatory reaction [51,52]. This direct effect on astrocytic GS expression is also present in other neurodegenerative diseases, such as Huntington's disease, which is associated with GLT-1 glutamate transporter down-regulation [53,54]. Further studies show that GS is oxidized due to Aβ in AD brains causing reduction of enzymatic activity [55] and that GS reduced expression is related to neuritic plaques in AD brain cortex [51]. On the contrary, human studies have shown increased levels of GS immunoreactivity in the prefrontal cortex in AD brains [56]. Obviously, phylogenetic proximity, tissue preservation and medical history play crucial role in the interpretation of human vs rodent data. Our current work shows that astrocytes at the late stages (plaque formation and stabilisation, 12-18 months) of AD-like pathology, in agreement with the above-mentioned studies, are functionally compromised as shown by the reduction of GS expression and therefore their capability to master the glutamate-glutamine cycle.

Thus, we could hypothesise that astrocytes fail to support neurones and control synapses from the appearance of the disease pathological burden. Furthermore, and considering astrocytic physiological function, one could consider that these changes result in an impaired

glutamate homeostasis that is manifested by deficient glutamate/glutamine turnover and restricted supply of glutamine to neurones, being this failure somehow more dramatic in the proximities of Aβ plaques. Furthermore, GS deficiency could also be a consequence of impaired astrogial glutamate uptake; inducing a potential neurotoxic process by abnormal glutamate metabolism. Hence GS deficiency may reflect an altered glutamatergic neurotransmission of AD, at the advanced and late stages of the disease, which can account for a global hippocampal neurotransmitter imbalance underlaying the mnesic and cognitive impairments observed in the disease.

Abbreviations

3xTg-AD: Triple transgenic mouse of Alzheimer's disease; ACh neurons: Acetylcholinergic neurons; AD: Alzheimer's disease; ATP: Adenosine triphosphate; CA1: Cornus ammonis 1; CC: Corpus callosum; CNS: Central nervous system; DG: Dentate gyrus; GcL Granule cell layer; GFAP: Glial fibrillary acidic protein; GLAST: Glutamate aspartate transporter; GLT-1: Glutamate transporter; GS: Glutamine synthetase; GS-IR: Glutamine synthetase immunoreactivity/-reactive; IgG: Immunoglobulin G; IOD: Inversed optical density; Lac: Stratum lacunosum moleculare; MoL: Molecular layer; N_V: Numerical density (cell number/mm^3); OD: Optical density; Or: Stratum oriens; PB: Phosphate buffer; PcL: Pyramidal layer; Rad: Stratum radiatum; TS: Trizma®® base saline.

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Authors' contributions

MO carried out the immunohistochemical study and contributed to the writing of the manuscript. HNN contributed to the immunhistochemical study. AV participated in the conception of the study and writing. JJR participated in the conception and design of the study and writing of the manuscript as well as coordinated the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease

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The formation of cerebral senile plaques composed of amyloid β peptide (Aβ) is a fundamental feature of Alzheimer's disease (AD). Glial cells and more specifically microglia become reactive in the presence of Aβ. In a triple transgenic model of AD (3 $\times$ Tg-AD), we found a significant increase in activated microglia at 12 (by 111%) and 18 (by 88%) months of age when compared with non-transgenic (non-Tg) controls. This microglial activation correlated with Aβ plaque formation, and the activation in microglia was closely associated with Aβ plaques and smaller Aβ deposits. We also found a significant increase in the area density of resting microglia in 3 $\times$ Tg-AD animals both at plaque-free stage (at 9 months by 105%) and after the development of A plaques (at 12 months by 54% and at 18 months by 131%). Our results show for the first time that the increase in the density of resting microglia precedes both plaque formation and activation of microglia by extracellular Aβ accumulation. We suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting microglia increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

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Microglial cells, which were discovered by Pio Del Rio Hortega,[1] are the resident macrophages of the CNS. These cells of myeloid origin[2] enter the CNS shortly after birth, and disseminate over the brain and the spinal cord, where they rapidly transform into the resting microglia with a peculiar morphological appearance.[3] The resting microglial cells have small somata and multiple fine processes, with each cell occupying the defined domain that does not overlap with the neighbouring microglia. In the healthy CNS, microglial processes are on a constant move, scanning the microenvironment in their territorial domains.[4,5] Insults to the nervous system trigger a complex and multi-stage activation of microglia,[6] which results in both phenotypic and functional changes.

Being the intrinsic CNS defence system, microglia are intimately involved in all forms of neuropathology,[7] including various types of neurodegeneration. Alzheimer's disease (AD[8]) is the most common form of neurodegeneration, which results in severe and irreversible cognitive decline and dementia.[9] The loss of neurones and synaptic connectivity in AD has been specifically linked to the extracellular deposition of amyloid β-peptide (Aβ), with its progressive aggregation to form insoluble amyloid plaques as well as intracellular Tau neuro-fibrillary tangles.[10,11] Microglial activation in AD has been discovered two decades ago,[12] and activated microglia are generally believed to be involved in AD-associated inflammatory response,[13] although the precise role of microglial cells in AD pathogenesis remains to be elucidated and characterised[14,15]

Some studies have shown that activated microglia contribute to neurodegeneration in AD, either via the chronic secretion of pro-inflammatory peptides[16] or by being involved in the formation of Aβ plaques.[17] However, other reports have suggested a neuroprotective role of microglia in AD through, for example, Aβ clearance.[15,18] At any rate the concentration of activated microglia in the vicinity of Aβ plaques is routinely observed,[19–22] although the changes in the resting microglia population in AD remain unknown.

The aim of the present study was to quantify the density of resting and activated microglia in the CA1 subfield of the hippocampus in a triple transgenic mouse model (3 $\times$ Tg-AD)

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Abbreviations: 3xTg-AD, triple-transgenic mice model of AD; AD, Alzheimer's disease; Aβ, amyloid β peptide; APP, Amyloid precursor protein; CNS, central nervous system; CA1, CA1 (*Cornu Ammonis* 1) region of hippocampus; TL, tomato-lectin; Tg2576, transgenic mice, expressing (expressing human mutated APP_{swe})
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of AD. Developed by Oddo *et al*.,[23,24] 3 × Tg-AD mice express mutant versions of the human presenilin 1 ($PS1_{M146V}$) and tau (P_{301L}) genes on a Tg2576 (amyloid precursor protein $(APP)_{Swe}$ mutant) background, resulting in the formation of both Aβ plaques and neurofibrillary tangles with a similar spatial and temporal distribution to that observed in human AD patients. We provide further evidence for the localisation of reactive microglia to amyloid plaques within CA1; in addition, we discovered a significant increase in the density of resting microglial cells, which precedes the massive activation of microglia.

Results

The populations of resting and activated microglia were analysed in the hippocampi of 3 × Tg-AD mice at three different ages, at 9, 12 and 18 months. Importantly, at 9 months of age the hippocampal tissue of these animals is virtually plaque-free, whereas the plaque load becomes substantial at 12 and 18 months.[23,25,26] The resting microglial cells were identified by specific staining with tomato lectin,[27] whereas activated microglia were stained with Mac-1 antibody raised against CD11b.[28]

In the dorsal hippocampus, and more specifically within the CA1 of both non-Tg and 3 × Tg-AD mice, we observed two different phenotypes of microglial cells (Figures 1 and 2). The resting, tomato-lectin immunoreactive (TL-IR) cells were characterised by a small cell body equipped with thin-to-medium ramified processes extending to the surrounding neuropil (Figure 1b and c), typical of resting microglia. Occasionally, resting microglia were also observed in the vicinity of dense deposits that were likely to be potential Aβ aggregates (data not shown). The other type of microglial cells, which were reactive for Mac-1, showed enlarged cell bodies from which processes with an enlarged and thicker appearance emanated (Figure 2d and e), thus being consistent with a reactive phenotype. Reactive microglia were predominant in 3 × Tg-AD mice (Figure 2a–c).

Resting microglia in AD. There was no difference between the morphological characteristics of TL-IR resting microglia in either 3 × Tg-AD or the respective non-Tg controls, independent of the age (Figure 1b and c). The area density (S_v, number/mm^2) of resting microglia was significantly larger in 3 × TG-AD animals when compared with the control at all ages ($P<0.01$; Figure 1a). When the S_v was compared between 3 × Tg-AD and non-Tg controls, it was significantly larger at 9 (105%, $P=0.058$), 12 (54%, $P=0.0198$) and 18 (131%, $P=0.0116$; Figure 1a) months. In non-Tg animals, ageing (9–18 months) did not affect the S_v of resting microglia, whereas in 3 × Tg-AD animals we found a clear age-dependent increase in S_v between 9 and 18 months ($F_{2,13}=7.078$; $P=0.0106$).

Activated microglia in AD. We found no change in the typical morphology of Mac-1-IR reactive microglia in all age groups in both control and 3 × Tg-AD animals. In control animals the area density of Mac-1-IR was stable at all ages, showing no significant changes at 9, 12 and 18 months (Figure 2a). In contrast, in the 3 × Tg-AD mice the area

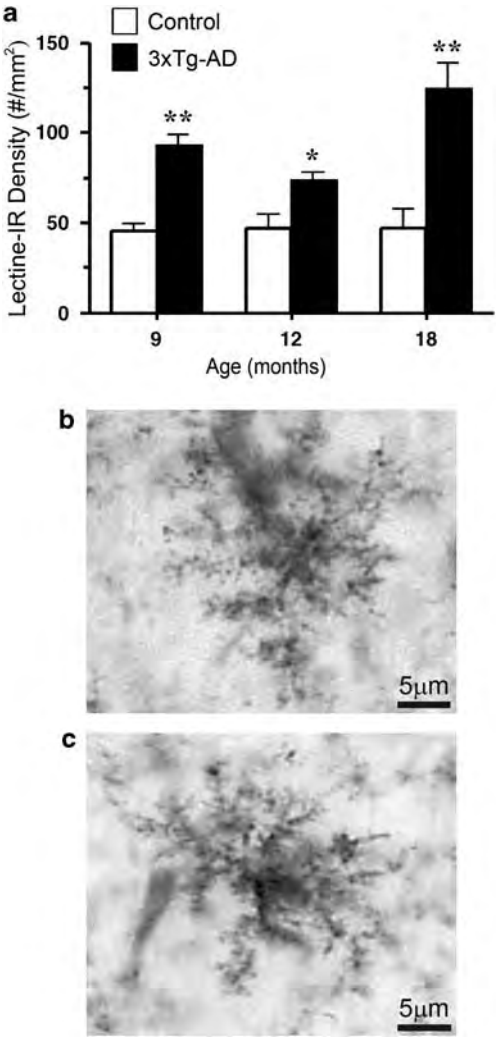

Figure 1 Visualisation and quantification of resting microglia in the hippocampi of 3 × Tg-AD animals. (**a**) Bar graph showing the area density of resting microglia (S_v; number/mm) in the hippocampal CA1 of 3 × Tg-AD mice and non-Tg control mice. (**b**, **c**) Brightfield micrographs showing the characteristic morphology of resting TL-IR microglia with small cell body equipped with thin to medium ramified processes extending to the surrounding neuropil in the CA1 subfield of the hippocampus of 9- and 18-month-old 3 × Tg-AD mouse, respectively, which is not modified either by age or by Aβ amyloid plaques. Scale bar = 5 μm

density of Mac-1-IR (total activated microglial cells) was significantly increased throughout the whole extension of CA1 (Figure 2b and c) at 12 months (111%; $P=0.0311$) and 18 months (88%; $P=0.0330$), but showed only a non-significant rise at 9 months (55.44%, $P=0.1163$). The Mac-1 activated microglia were almost exclusively localised in close proximity to Aβ plaques and/or aggregates (Figure 2f–h). The cell bodies of these reactive microglial cells were distributed in a circular shape around the plaque periphery (Figure 2f–h). The Mac-1-IR activated microglial cells were normally present in multiple clusters, revealing an active phagocytic activity as shown by a high degree of co-localisation with Aβ (Figure 2f, g). This phagocytic function was effective in removing the Aβ load, as shown by the presence of small packs of broken Aβ aggregates surrounded by hypertrophic and multiprocess MAC-1-IR cells (Figure 2h). Reactive microglia were also

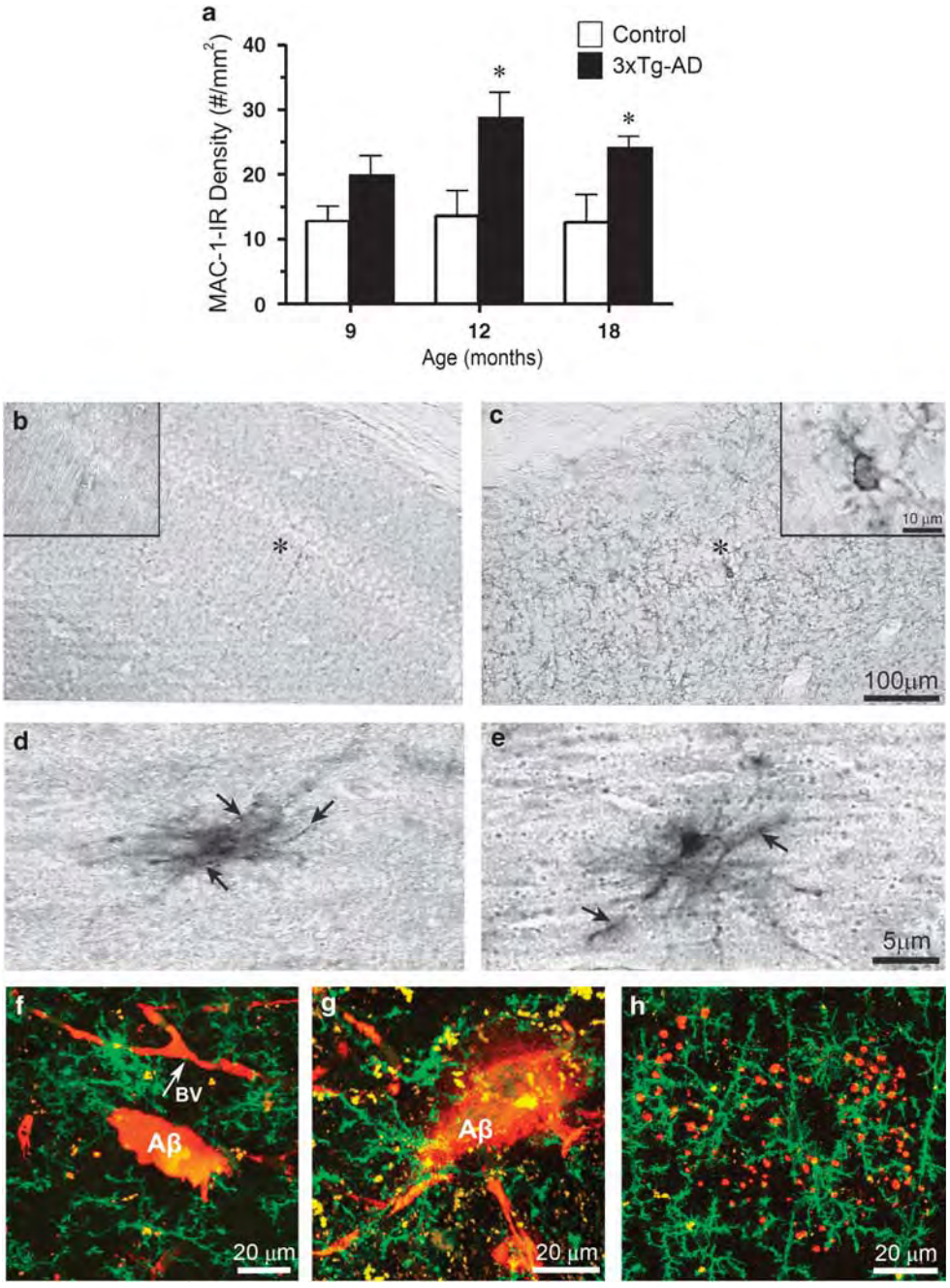

Figure 2 Microglial activation in plaque-infested hippocampal tissue. (**a**) Bar graph showing the effect of age-related changes on MAC-1-IR reactive microglia within the CA1 subfield of the hippocampus at 9, 12 and 18 months between non-Tg control and 3 × Tg-AD mice. (**b**, **c**) Brightfield micrographs showing the increase in MAC-1-IR reactive microglia (asterisk) in 3 × Tg-AD (**c**) *versus* non-Tg control mice (**b**) in the hippocampal CA1. (**d**, **e**) High-magnification micrographs illustrating the characteristic morphology of reactive microglia within the CA1 subfield of the hippocampus of an 18-month-old 3 × Tg-AD mouse. Reactive microglia appear with most enlarged cell bodies from which a greater number of numerous processes emanated, but with an enlarged and thicker (arrows) appearance (**f**, **g**). Confocal images showing recruitment of MAC-1-IR microglia (green) in the vicinity of Aβ amyloid plaques (red; **f**, **g**) and aggregates (**h**) in the CA1 subfield of the hippocampus of an 18-month-old 3 × Tg-AD mouse. In (**f**) we can also observe this reactive microglia surrounding a blood vessel (BV) attaint of Aβ deposits. Scale bars: (**b**, **c**) 100 μm, (**d**, **e**) 5 μm and (**f**) 20 μm

present around vascular elements that accumulated Aβ, which is typical of AD-associated angiopathy (Figure 2f).

Discussion

Although it has long been known that activated microglia are associated with compact Aβ plaques,[29] their role in the development of AD is not completely understood.[2,14] Although numerous studies have attempted to characterise microglial function in AD, few have aimed to quantify the changes in the two types of microglia population (resting and reactive). In the present study, we investigated the changes in the density of microglial cells in 3 × Tg-AD mice, with particular focus on the CA1 subfield of the hippocampus.

The CA1 area was shown to be highly susceptible to Aβ pathology in both human AD patients and 3 $\times$ Tg-AD mice, which is also important in the context of memory impairment. Within the hippocampus the CA1 is the major component of the Schaffer collateral pathway, acting as a neural conduit between the hippocampal subfields, subiculum and entorhinal cortex.[30] As such, neuronal activity in CA1 has been strongly linked to memory formation, an aspect of cognitive function, which is typically compromised in AD.[9]

We found that the reactive microglia was densely populated with regions of high Aβ deposition, with the processes of many of these cells appearing to be in contact with Aβ aggregates. Some microglial cells contained an intracellular deposition of Aβ, thus indicating active phagocytosis. Similar observations were made in the brains of Tg2576 mice (expressing human mutated APP_{swe}), in which activated microglia were concentrated in close proximity to amyloid plaques.[19] Similarly, the aggregation of activated microglia around Aβ plaques was shown *in vivo* in 3–4-month-old double-transgenic mice expressing a mutant APP and PS1; chronic imaging experiments demonstrated that the number of plaque-associated microglia increased at the rate of about three cells per plaque per month.[31]

We found an increase in activated microglia in the CA1 area at 12 and 18 months, which correlates with the age of the appearance and development of Aβ plaques in the hippocampus of the 3 $\times$ Tg-AD animal model, even though in cortical areas they appear as early as at 6 months of age.[23,25,26]

We therefore suggest that the activation of microglia occurs in response to Aβ plaque formation and stabilisation. The activated microglia surrounded not only the fully formed plaques but also smaller deposits of Aβ (Figure 2h). This observation may be interpreted in either of two ways: (i) a result of plaque destruction and clearance, or (ii) the formation of small soluble aggregates before plaque consolidation. Activated microglia associated with Aβ plaques have been implicated in the phagocytosis of cerebral Aβ,[15,18] with reports indicating that these cells may be involved in the stabilisation or clearance of Aβ plaques in the brain.[32] Our results corroborate these suggestions, as we found the accumulation of Aβ by activated microglia, as shown by MAC-1/Aβ double staining (Figure 2f and g). Incidentally, we did not observe a substantial activation of microglia at a pre- and/or early plaque stage at 9 months of age, indicating that microglial reactivity does not develop until the plaques are formed and/or consolidated. Similarly, a recent longitudinal study using multiphoton microscopy to image the brains of 5–6-month-old B6C3-YFP transgenic mice (bearing *APP_{swe}* and *PS1d9x-YFP* genes) has also alluded to this, reporting that microglia are recruited to Aβ plaques only after they have been formed.[33]

Apart from confirming the AD-associated activation of microglia, we found the disease-specific increase in the density of resting microglial cells. To the best of our knowledge, this is the first attempt to characterise resting microglia in the AD. The immunoreactivity against tomato lectin, which is used as a specific probe for resting microglia, increased at all ages in 3 $\times$ Tg-AD animals, whereas there were no age-dependent changes in the control brains. An increase in the density of resting microglia was quite substantial – the number of cells had roughly doubled. This increase preceded both the plaque formation and the activation of microglia by extracellular Aβ accumulation. The mechanism of microglial proliferation and the origin of the additional resting microglial cells remain obscure and require further study. In particular, we cannot rule out the role for blood-borne macrophages, which have been shown to migrate across the blood–brain barrier in response to perivascular amyloid deposition, as well as the secretion of cytokines and chemoattractant peptides by microglia and astrocytes in these areas.[21,22] Upon entering the CNS, these cells function as reactive microglia, localising as phagocytes to amyloid plaques, secreting pro-inflammatory peptides and effectively accumulating Aβ.[21,34] We cannot exclude, however, that a similar infiltration can start before the active inflammatory Aβ-associated phase; at that stage, the intruding cells may transform to the resting microglia. All in all, we may suggest that AD pathology triggers a complex microglial reaction: at the initial stages of the disease the number of resting microglia increases, as if in preparation for the ensuing activation in an attempt to fight the extracellular Aβ load that is characteristic of the terminal stages of the disease.

An increase in the density of resting microglia associated with atrophic changes in astrocytes[26] may also lead to a remodelling of the glial-related domain structure of the grey matter (Figure 3). This in turn can affect synaptic connectivity, because of both reduced astroglial coverage and the possible synaptolitic effects of multiplied microglia. Thus, early changes of neuroglia can represent a pathologically relevant step in the cognitive decline observed at the early stages of AD.

Several therapeutic strategies have attempted to target microglia activity as a means of treating AD, each with limited success. Of these, immunotherapy protocols aimed to up-regulate the microglial clearance of cerebral Aβ,[18,35] whereas an alternative strategy used anti-inflammatory agents to reduce microglial neurotoxicity.[36] These attempts reflect the well-known dichotomy of microglial function, which may be both neuroprotective and neurotoxic.[2] Nonetheless, without a comprehensive understanding of microglial changes and roles in AD, and especially the balance between different stages of microglial activation, these attempts at therapeutic intervention may continue to be futile. Furthermore, an early increase in resting microglia, which precedes the Aβ plaque formation, may also have certain diagnostic significance.

Materials and Methods

All animal procedures were carried out in accordance with the United Kingdom Animals (for Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3R's.

Mice. The procedure for generating 3 $\times$ Tg-AD mice has been described previously.[23,24] Briefly, human APP cDNA harbouring the Swedish mutation (KM670/671NL) and human P301L mutated four-repeat Tau were co-microinjected into single-cell embryos of homozygous PS1M146V knock-in mice. The background of the PS1 knock-in mice is a hybrid 129/C57BL6. The control mice used were also from the same strain and genetic background as the PS1 knock-in mice, but they express the endogenous wild-type mouse *PS1* gene. All 3 $\times$ Tg-AD and control mice were obtained by crossing homozygous breeders. Mice were independently group housed and kept on a daily 12-h light–dark cycle dark schedule. All mice were given *ad libitum* access to food and water.

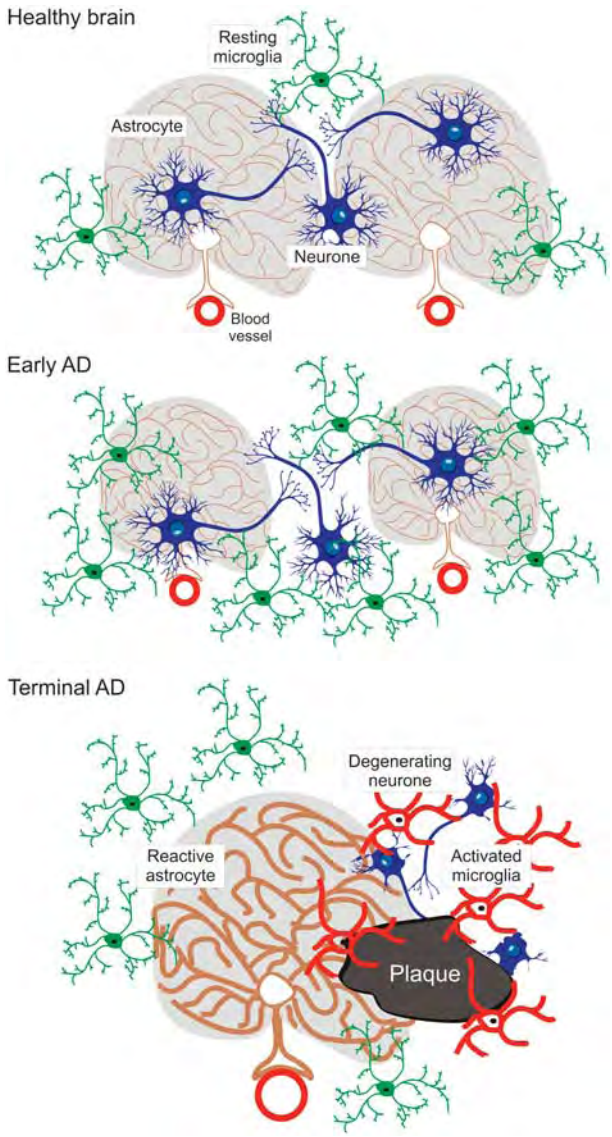

Figure 3 Glial reactions at the different stages of Alzheimer's disease. At the early, A plaque-free stages of AD, an increase in the density of resting microglia coincides with the initial atrophy of astrocytes.[26] Consolidation of plaques at the later stages of AD triggers rapid and massive activation of microglia, reactive astrogliosis and neurodegeneration

Fixation and tissue processing. Male 3 × Tg-AD and their respective non-transgenic controls (non-Tg) of different ages (9, 12 and 18 months of age; $n = 3$–5) were anaesthetised with an intraperitoneal injection of sodium pentobarbital. Brains were fixed by perfusion through the aortic arch with 3.8% acrolein (TAAB, UK) in a solution of 2% paraformaldehyde and 0.1 M phosphate buffer (PB), pH 7.4, followed by 2% paraformaldehyde. The brains were removed from the cranium and cut into 4–5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the SVZ. These slabs were subsequently post-fixed for 30 min in 2% paraformaldehyde before sectioning at 40–50 μm on a vibrating microtome (VT1000, Leica, Milton Keynes, UK). To remove excess reactive aldehyde groups, sections were treated with 1% sodium borohydride in PB for 30 min. Coronal vibratome sections at levels −1.58 mm/−2.46 mm (hippocampus) posterior to the bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin.[37]

Antibody and reagents. A polyclonal affinity-purified rat antiserum raised against CD11b (Mac-1; Serotec, Kidlington, UK) was used for the determination of activated microglial cells. A Mac-1 antibody was used to selectively label activated microglia in our brain sections. Mac-1 has a specific affinity to the microglial surface membrane receptor CD11b, which is highly expressed by reactive microglia,[28] and is therefore routinely used as a specific marker of reactive microglia. Although we identified a small amount of low-intensity labelling for resting microglia in our tissue sections, the staining was observed to be largely specific for reactive microglia. For the identification of β-amyloid (Aβ) plaques, we used a monoclonal mouse antiserum that reacts with abnormally processed isoforms, as well as precursor forms of Aβ, recognizing an epitope within amino acids 3–8 (EFRHDS; anti-Aβ 6E10 [SIG-39320]; Signet Laboratories, Dedham, MA, USA). To assess for non-specific background labelling or cross-reactivity between antibodies derived from different host species, a series of control experiments were performed. Omission of primary and/or secondary antibodies from the incubation solutions resulted in a total absence of target labelling (data not shown). Resting microglia were identified by using biotinylated tomato lectin (Sigma-Aldrich, Poole, UK). Tomato lectin is a sugar-binding glycoprotein that has a specific affinity for poly-*N*-acetyl lactosamine residues occurring on the surface membranes of microglia and endothelial cells.[27]

Immunohistochemistry. To optimise the detection of Mac-1 and tomato lectin (resting microglia), sections through the hippocampus of all control and transgenic animals were processed by using the highly sensitive avidin–biotin peroxidise complex method.[38] For this procedure, vibratome sections were first incubated for 30 min in 0.5% bovine serum albumin in 0.1 M Tris-buffered saline (TBS), pH 7.6, containing 0.25% Triton X-100 to minimise non-specific labelling. The tissue sections were then incubated for 48 h at room temperature in 0.1% bovine serum albumin in TBS containing 0.25% Triton X-100 that in turn contained either rat anti-Mac-1 (1 mg/ml) or biotinylated tomato lectin (2 mg/ml). Briefly, following repeated washing in TBS, sections for Mac-1 were incubated in 1:200 dilutions of biotinylated donkey anti-rat IgG followed by avidin-biotin complex (Elite kit; Vector Laboratories, Peterborough, UK). For tomato lectin identification the sections were directly incubated in the avidin-biotin complex. The peroxidase reaction product was visualised by incubation in a solution containing 0.022% 3,3′-diaminobenzidine (Aldrich, Gillingham, UK) in TBS containing 0.003% H_2O_2 for 6 min.

For the detection and determination of Mac-1-positive cells and their relationship with Aβ senile plaques, we used dual indirect immunofluorescence labelling. The sections were incubated for 48 h at room temperature in a primary antibody cocktail containing (1) mouse anti-β amyloid monoclonal antibody (Aβ; 1:2000; Covance, Emeryville, CA, USA) and (2) rat anti-Mac-1 (1:30.000; Sigma, Saint Louise, MO, USA) simultaneously. Subsequently, Aβ and Mac-1 were detected in a sequential manner on the same sections by incubation with rhodamine (TRITC)-conjugated goat anti-mouse and FITC-conjugated goat anti-rat (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1 M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector Laboratories).

Cell counting. The S_v (number/mm^2) of Mac-1 (Mac-1-IR) and TL-IR cells was determined from four non-consecutive coronal sections, separated by at least 80 mm, which were taken from representative sections of the dorsal hippocampus. The ImageJ 1.41 image processing software was used to outline and determine the area of CA1, by performing the quantification; then the number of either Mac-1-IR or TL-IR was determined. Cells were counted and images obtained using a Nikon E-80i widefield microscope and a confocal microscope (Leica SP5 upright). To ensure consistency and reproducibility, samples were counted blindly.

Statistical analysis. Data were expressed as mean ± S.E. Analysis of variance (one-way) was used to examine differences in the mean number of Mac-1-IR and TL-IR cells in the 3 × Tg-AD and non-Tg animals during ageing, followed by a Bonferroni's post-hoc test where appropriate. Whenever two groups were compared, an unpaired *t*-test was applied.

Conflict of interest

The authors declare no conflict of interest.

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Appendix 2

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Review

Astroglia in dementia and Alzheimer's disease

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Astrocytes, the most numerous cells in the brain, weave the canvas of the grey matter and act as the main element of the homoeostatic system of the brain. They shape the microarchitecture of the brain, form neuronal-glial-vascular units, regulate the blood–brain barrier, control microenvirionment of the central nervous system and defend nervous system against multitude of insults. Here, we overview the pathological potential of astroglia in various forms of dementias, and hypothesise that both atrophy of astroglia and reactive hypertrophic astrogliosis may develop in parallel during neurodegenerative processes resulting in dementia. We also show that in the transgenic model of Alzheimer's disease, reactive hypertrophic astrocytes surround the neuritic plaques, whereas throughout the brain parenchyma astroglial cells undergo atrophy. Astroglial atrophy may account for early changes in synaptic plasticity and cognitive impairments, which develop before gross neurodegenerative alterations.
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Astrocytes, for the first time visualised by Otto Deiters[1] and Christianised by Michael von Lenhossek[2] (for historical overview, see also references[3,4]), are the main type of glia in the central nervous system (CNS). The astrocytes, which dwell in both grey and white matter, are the main element of brain homoeostatic system, being responsible for all aspects of metabolic support, nutrition, control of ion and neurotransmitter environment, regulation of brain-blood barrier and defence of the CNS.[5,6] In addition, astroglial cells are endowed with numerous signalling cascades, which include a wide array of plasmalemmal neurotransmitter receptors and intracellular second messenger pathways.[7–10] These pathways in combination with *trans*-cellular communication routes represented by gap junctions[11,12] and regulated gliotransmitter release[13] support information processing within glial networks, the mechanisms and functional relevance of which remains essentially enigmatic.

The main type of astroglia in the grey matter, the protoplasmic astrocytes, are characterised by complex morphology with elaborated processes, which cover neuronal membranes, residing within the astrocytic territories; the latter being defined by the arborisation of a single astrocyte. Importantly, a single-astroglial microanatomical domain is essentially free from the processes of other astrocytes, giving the grey matter in the brain and in the spinal cord a regular parcellating structure.[14] A single astrocyte in the rodent brain has a volume $\sim 66\,000\,\mu m^3$, and the membrane of this astrocyte covers around 140 000 synapses lying within the astroglial domain;[15] human astrocytes are considerably larger and more complex and their processes wrap up to 2 millions of synapses.[16] Protoplasmic astrocytes send processes to neighbouring blood vessels, where these processes form endfeet plastering the capillary wall and participating in formation of blood–brain barrier. As a result, astroglial cells integrate neurones and blood vessels into relatively independent neuronal-glial-vascular units. Astroglial endfeet are capable of releasing vasoactive substances that control local blood flow and coordinate the latter with neuronal activity.[17–19]

Individual astroglial domains are further integrated into an internally continuous superstructure, because the peripheral processes of astroglial cells are coupled through gap junctions, which form a specific pathway for intercellular communications binding astroglia into a functional syncytium. This coupling most likely has anatomical specificity, providing for specific organisation of glial networks: for example, in somato-sensory cortex astroglial coupling is restricted to the

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Keywords: astrocyte; Glia/Alzheimer's disease; dementia

Abbreviations: 3xTg-AD, triple-transgenic mice model of AD; AD, Alzheimer's disease; APP, Amyloid precursor protein; APP/ApoE-KO, Double-transgenic mice overexpressing a human mutated form of APP (APP^{V717F}) in combination with ApoE gene knockout; APP_{751SL}, Single-transgenic mice expressing APP mutation APP Ser^{751} to Leu; $APP_{751SL}/PS1_{M146L}$, Double-transgenic mice expressing APP_{751SL} and $PS1_{M146L}$; APP_{SWE}, Single-transgenic mice expressing the 'Swedish' APP double-point mutations Lys^{670} to Asn and Met^{671} to Leu; $APP_{SWE}/PS1_{dE9}$, Double-transgenic mice expressing APPswe and $PS1_{dE9}$; CNS, central nervous system; FAD, Familial form of Alzheimer's disease; GFAP, glial fibrillary acidic protein; HAD, HIV-1-associated dementia; nAChR, nicotinic cholinoreceptor; nbm, nucleus basalis magnocellularis; PDAPP, Single-transgenic mice expressing the 'Swedish' and 'Indiana' APP mutations Asp^{664} to Ala (PDAPP(D664A); PS1, Preseniline 1; $PS1_{dE9}$, Single-transgenic mice (line S-9) expressing human PS1 carrying the exon 9-deleted variant associated with FAD; $PS1_{M146L}$, Single-transgenic mice expressing PS1 mutation Met^{146} to Leu; Tau_{P301L}, Single-transgenic mice expressing the Pro^{301} to Leu tau mutation (P301L)

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barrel superstructure.[20] Gap junctions provide for intercellular diffusion of variety of substances, including ions, second messengers and metabolites such as glucose and ATP.[21–23] This intercellular route underlies long-distance communications within glial networks; one of the examples of such communication is represented by glial calcium waves;[21,24,25] yet propagating waves of other molecules can also be relevant for intercellular signalling and information processing.[26]

Functional integration between neuronal and glial networks is accomplished mainly through chemical transmission, although there are incidental appearances of neuronal–glial gap junctions.[27,28] Synaptic transmission in neuronal–glial circuitry occurs either at the level of tripartite synapse (when perisynaptic astroglial processes are directly exposed to neurotransmitter released from the neuronal terminals,[29,30]) or in neuronal–glial synapses.[31,32] The mechanisms of glia to neurones signalling are more complex; at least in part they may involve the exocytotic release of gliotransmitters.[13]

Astroglia in neurological diseases

Progression and outcome of neurological diseases are determined by the balance between destruction, neuroprotection and regeneration. In this context, glial cells are invariably involved in every kind of neuropathology, which, to a very large extent, is shaped by glial performance.[6,33] Astrocytes, in accordance with their homoeostatic function, are deeply involved in neural diseases. Astroglia forms the first line of brain defence by controlling the volume and composition of extracellular space. Astrocytes buffer an excess of extracellular K^+, thus maintaining neuronal excitability,[34] control extracellular levels of glutamate,[35,36] thereby limiting the intrinsic excitotoxicity of the latter, regulate fluid movements and provide the main antioxidant system in the brain.[37] At the very same time astroglial cells can contribute to neuronal damage, when, for example, severe insults compromise astrocyte metabolism, which leads to a depolarisation and reversal of glutamate uptake system, the latter underlying the release of additional glutamate, thus exacerbating the brain damage.[6]

Acute and chronic brain insults trigger a specific glial reaction, generally known as reactive astrogliosis, represented by a complex morphofunctional remodelling of astrocytes.[38] Reactive astrogliosis is a defensive brain reaction which is aimed at (i) isolation of the damaged area from the rest of the CNS tissue, (ii) reconstruction of the blood–brain barrier and (iii) facilitation of the remodelling of brain circuits in areas surrounding the lesioned region.[39,40] As a result, astrogliosis comes in different guises. Astrocytes surrounding the lesion undergo a robust hypertrophy and proliferation, which ultimately ends up in complete substitution of previously existing tissue architecture with a permanent glial scar; this process is called anisomorphic (i.e., changing the morphology) astrogliosis. In astrocytes more distal to the damage, the reactive changes are much milder and, although astroglial cells modify their appearance and undergo multiple biochemical and immunological changes, they do not distort the normal architecture of CNS tissue, but rather permit growth of neurites and synaptogenesis, thus facilitating the remodelling of neuronal networks. This type of astrocyte reaction is defined as isomorphic (i.e., preserving morphology) astrogliosis.

Astroglia and dementia

Dementia is the ultimate scourge of mankind, being generally absent in every other animal species; and as such it may be considered as the most appalling and horrible disease, because it effectively robs human beings from their intelligence and turns them into helpless entities. The causes of dementia are many, ranging from traumatic injuries (mechanical, chemical or as a result of irradiation) and viral infections, which may predispose to dementia-related chronic neurodegenerative conditions, to the intrinsic neurodegenerative processes associated with genetic factors, predispositions and other yet unknown reasons. The pathological role of astroglia in dementiae begun to be explored only very recently; however, some generalisations can already be drawn.

First and foremost, both astrogliosis and astroglial dystrophy are manifest in different types of dementia; furthermore, both these processes may develop in parallel depending on the disease form and/or stage. The frontotemporal dementias (the clinical term covering several types of sporadic non-Alzheimer cognitive disruptions, which include e.g., Pick's disease and frontotemporal lobar degeneration), is, for example, associated with a very early and profound apoptotic death and dystrophy of astrocytes.[41] The degree of astroglial loss directly correlates with the severity of dementia. Conversely, other study, using postmortem tissues from 21 frontotemporal dementia cases, found prominent astrogliosis in the frontal and temporal cortices at the very early stages of the disease, with astrocyte densities increasing by 4–5 times of the control.[42]

Astrogliosis also assumes the leading pathological role in thalamic dementia (the rather rare form of specific dementia of unknown aetiology). Numerous alterations of protoplasmic astrocytes, manifested by highly localised proliferation of perivascular and peryneuronal astroglial processes, believed to be a primary pathological change, which can produce dementia even without severe neuronal loss.[43] Similarly, in HIV-1-associated dementia (HAD) significant astroglial hypertrophy and increase in GFAP (glial fibrillary acidic protein) expression is observed in entorhinal cortex and hippocampus.[44] Furthermore, there are indications that HIV-1 infected reactive astrocytes, together with activated microglial cells, may constitute a primary cause of neuronal damage, through the release of various inflammatory and death factors.[45,46] At the same time, however, HAD also leads to a prominent apoptotic death of astrocytes especially in the patients with rapidly progressing cognitive deficits.[47]

Astroglia and Alzheimer's disease

Alzheimer's disease (AD), existing in both genetic (familial AD or FAD) and sporadic forms,[48] is characterised by severe neurodegeneration associated with an occurrence of specific histopathological markers represented by focal extracellular deposits of fibrillar β-amyloid (also called neuritic plaques) in

brain parenchima and in the wall of blood vessels and intraneuronal accumulation of neurofibrillary tangles composed from abnormal hyperphosphorylated tau filaments. The progression of AD is rather stereotypic with initial neurodegenerative events appearing in the transentorhinal cortex, which afterwards spread to the entorhinal cortex and hippocampus. Subsequently, the spreading wave of neurodegeneration swallows the rest of the temporal, frontal and parietal lobes.[49,50] At the advanced stage of the AD, the grey matter undergoes severe damage manifested by a profound loss of neurones and synaptic contacts.

The pathological potential of glial cells in progression of dementia (as well as in other types of brain pathology) was originally suggested by Alois Alzheimer himself in 1910.[51] Many studies of the AD-related pathological potential of astroglia have focused on the investigations of the effects of β-amyloid on astrocytes. The treatment of cultured glial cells with aggregated β-amyloid or with amyloid plaques isolated from human AD brains triggered reactive astrogliosis.[52]

In mixed astroglial-neuronal cultures β-amyloid peptide (Aβ 1–42) and its toxic fragment (Aβ 25–35) provoked sporadic increases and/or oscillations in cytosolic calcium $[Ca^{2+}]_i$, which lasted for many hours.[53] These $[Ca^{2+}]_i$ responses were observed solely in astrocytes and never in neurones and were generated by Ca^{2+} influx from extracellular space, most likely through transmembrane channels formed by Aβ proteins. Importantly, astroglial $[Ca^{2+}]_i$ fluctuations were somehow linked to neuronal death, which occurred 24 h after Aβ treatment; inhibition of Ca^{2+} influx had a clear neuroprotective effect.[53]

In the AD human tissue the main astroglial reaction found hitherto is represented by prominent astrogliosis, mostly observed in the cells surrounding amyloid plaques.[54] Importantly, activated astrocytes are capable of accumulating large amounts of Aβ;[55] the later being taken up by astrocytes in association with neuronal debris. In addition, reactive astrocytes seem to accumulate large amounts of neuronal subtype of nicotinic cholinoreceptor (α7nAChRs), which is known to have an exceptionally high affinity to β-amyloid. Astroglial β-amyloid deposits are clearly associated with plaques, as astrocytes positioned away from the plaques show no β-amyloid burden.[54,55] Processes of activated astrocytes were also reported to participate in plaques formation.[54,55]

At the very same time astroglial changes during AD progression remain virtually unexplored. The main reason for this is purely experimental as for many years the suitable animal models for AD were absent.

Animal models of AD

Alzheimer's disease, as every other dementia, is a sole prerogative of humans; no animal suffers from AD.[56] Hence, substantial efforts were invested in producing relevant animal models of AD (for a comprehensive review of literature, see references[57,58]). Initial models of AD were simply normal aged animals,[59,60] which showed cholinergic involution associated (in monkeys) with β-amyloid deposition.[61] As the AD is manifested by a loss of cholinergic neurones,[62] several cholinergic models of the disease were created. Among these, the most relevant were the rodent models with lesions in the nucleus basalis magnocellularis (nbm) that is the equivalent of the nucleus of Meynert in humans.[56,63,64] These models offered the possibility to investigate the differences in structure, function and behaviour of the cholinergic systems in young and aged animals.[63,64] The majority of these models, however, were created by using non-selective excitotoxic toxins such as NMDA, ibotenic acid, quisqualic acid and certain alkaloid substances.[56] More refined lesion models used more selective and specific toxins that affected only cholinergic neurones in the relevant areas, such as septum, nbm and the diagonal band of Broca, but preserved non-cholinergic neurones.[56,65] These models were produced by using AF64A cholinotoxin, which binds to the high affinity choline uptake system, and 192 IgG-saporin that binds selectively and irreversibly to low affinity nerve growth factor receptor interrupting cholinergic neuronal protein synthesis. Both these interventions lead to selective impairment and death of cholinergic neurones.

Table 1 Summary of the transgenic AD animal models and the observed neuropathology

Transgenic mouse model	Neuropathology	Reference
APP_{SWE}	Plaques	68
PDAPP	Plaques	69
APP_{751SL}	Plaques	70
$PS1_{M146L}$	Plaques	70
$APP_{SWE}/PS1_{dE9}$	Plaques	71
APP/apoE-ko	Plaques	72
TgCRND8 $\beta APP6_{95}$	Plaques	73
$APP_{751SL}/PS1_{M146L}$	Plaques	70
THY-Tau22	Tangles	74
Tau_{P301L}	Tangles	68
3xTg-AD	Plaques and tangles	75

None of the models mentioned above, however, mimicked the histopathology (plaques and tangles) and progression of AD. Therefore several experimental transgenic animals that replicate various neuropathological features of AD have been developed (see Table 1 and reference[58]). Initially, simple transgenic models harbouring single-mutated β-amyloid-related proteins (amyloid precursor protein (APP) or presenilines, PS) or mutated tau were created. The very first APP transgenic animal showing an AD-like pathology was developed in 1995 by Games *et al*.[66] This model was called PDAPP (single-transgenic mice expressing the 'Swedish' and 'Indiana' APP mutations Asp^{664} to Ala (PDAPP(D664A)) and showed many pathological features of AD, including extracellular β-amyloid deposits, neuritic dystrophy, astrogliosis and memory impairments, yet the latter did not correlate with the degree of β-amyloid deposition.[58,66] Subsequently, Hsiao *et al*.[67] developed the transgenic mice carrying APP_{swe} mutation (Tg2576 mice) using a prion protein vector. These animals had abundant Aβ plaques as well as a memory and learning impairment from 9 months of age. Subsequently, the double-APP mutation, resulting the APP23 mouse was created. This model showed a 14% loss of hippocampal CA1 pyramidal neurons.

Mutations in PS proteins account for the majority of cases of FAD.[58] To address the role of genetic risk factors in APP

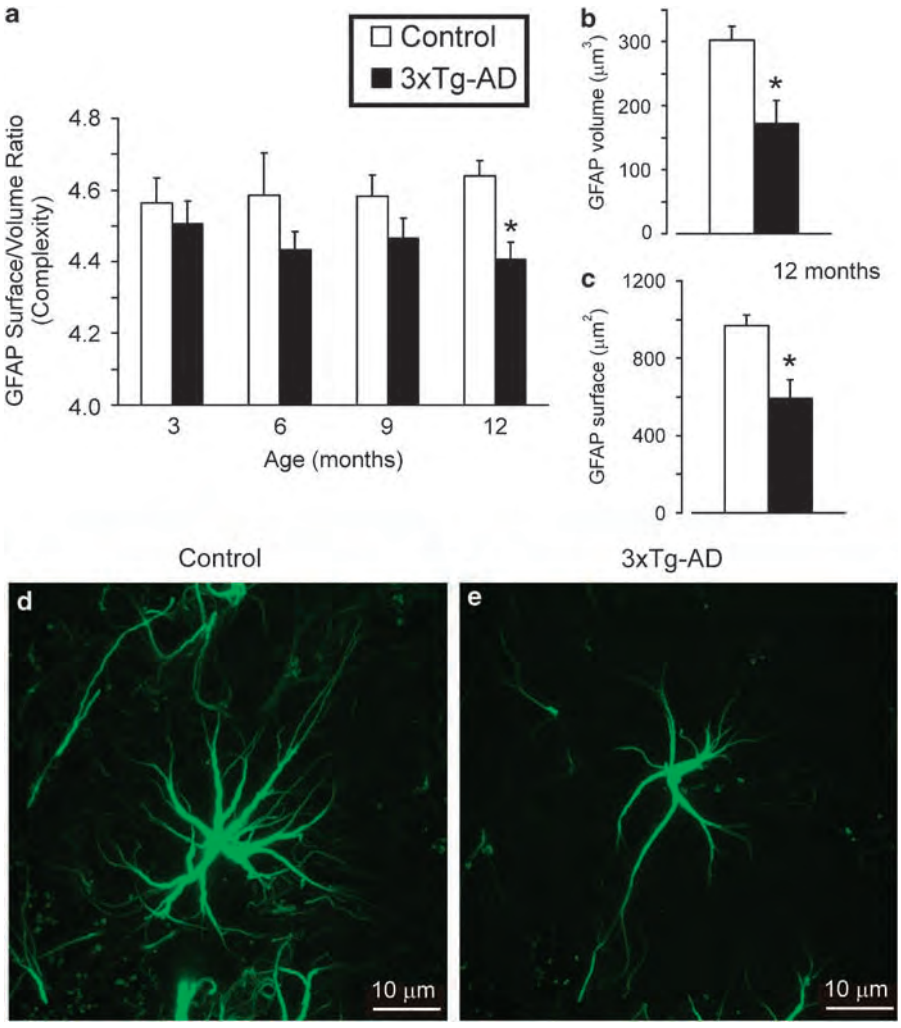

Figure 1 Bar graphs showing the complexity (**a**) of glial cytoskeleton by measuring the GFAP area coverage *versus* volume ratio of within the molecular layer of the dorsal dentate gyrus of both control and 3xTg-AD mice at different ages. (**b** and **c**) Bar graphs showing the coverage area (**b**) and volume (**c**) of GFAP at 12 months of age in control and 3xTg-AD mice; $^*P < 0.05$ compared with age matching controls. (**d** and **e**) Fluorescence photomicrographs showing GFAP-positive cells in control (**d**) and 3xTg-AD mice (**e**) at 12 months of age. There is an evident decrease in arborisation, surface area and volume in 3xTg-AD mice when compared with matching controls. All animal procedures were performed according to the Animal Scientific Procedures Act of 1986 under the licence from the United Kingdom Home Office. The animals group were housed and kept on daily 12 h light-dark cycles dark schedule. All mice were given ad libitum access to food and water

processing and memory, double transgenic have been created. Co-expression of $PS1_{dE9}$ with APP resulted in a viable model that showed accelerated Aβ deposition and memory deficits without tangle formation.[71] Expression of mutant APP on an ApoE knockout background did not affect the age of onset of Aβ plaques but instead of fibrillar Aβ mature plaques, only diffuse plaques were formed, suggesting that ApoE affects fibrillogenesis and/or clearance of Aβ.[76]

The pathological tau animals were developed in parallel, the first model being created in 1995;[77] these transgenic animals showed somatodendritic localisation of hyperphosphorlation of tau, but have not developed neurofibrillary tangle pathology (NFT). After identification of the pathogenic mutations of tau in FTDP-17, different transgenic models with clear neuronal NFT were produced.[57,58] The Tau_{P301L} (single-transgenic mice expressing the Pro^{301} to Leu tau mutation (P301L)) mutation is the most common associated mutation linked with FTDP-17.[78] Transgenic mice overexpressing Tau_{P301L} exhibit neurofibrillary tangles without Aβ pathology and/or neuronal loss, except for the spinal cord (Table 1; [58,78]).

The triple-transgenic (3xTg-AD) AD animal model. One of the most advanced AD animal models is represented by the triple-transgenic mice developed in 2003 by Salvatore Oddo and Frank LaFerla. These animals (the 3xTg-AD mouse model) harbour the mutant genes for amyloid precursor protein (APP_{Swe}), for presenilin 1 $PS1_{M146V}$ and for tau_{P301L}.[75,79] These mice are recognised as relevant AD model because they show temporal- and region-specific Aβ and tau pathology, which closely resembles that seen in the human AD brain. As well as progressively developing plaques and tangles, the 3xTg-AD animals also show clear functional and cognitive impairments including LTP, spatial memory and long-term memory deficits. These all are

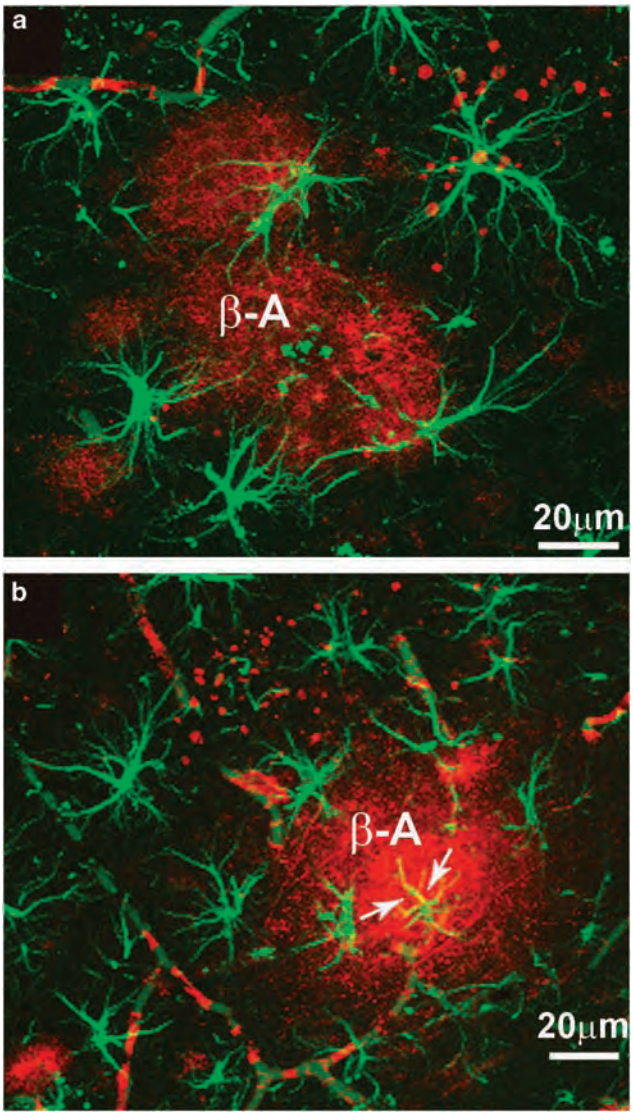

Figure 2 Confocal image showing GFAP-positive (green) reactive astrocytes surrounding β-amyloid plaques (red; **a**). (**b**) Reactive astrocytes (green) and an astrocyte showing cytoplasmic β-amyloid accumulation (indicated by arrows; colocalisation, yellow) near a neuritic plaque (red)

manifest in an age-related manner; most importantly functional deficits precede the appearance of histological markers.[75,79] Cognitive deficits in the 3xTg-AD model correlate with the accumulation of intraneuronal Aβ.[80,81]

It has to be noted, however, that all animal models of AD developed so far have some limitations, the most serious one being the absence of significant neuronal loss. This may reflect some intrinsic differences between human and animal brain, shorter lifespan of experimental animals or influence of other yet unknown factors. At the same time absence of neuronal loss in animals undergoing severe amyloidosis may also question the toxic potential of neuritic plaques.

Glial atrophy and astrogliosis in transgenic AD animals. We characterised morphology of astroglia in the hippocampus of male 3xTG-AD mice using single and dual immunohistochemical labelling.[82] For this labelling, we used the antibodies to GFAP an astroglia cytoskeleton-specific protein which expression varies between different brain regions and is upregulated upon reactive astrogliosis, or combined GFAP immunostaining with antibodies against β-amyloid. Morphology of GFAP-positive astrolglial profiles was reconstructed from series of optical confocal planes taken from 50 μm thick fixed brain slices. After the reconstruction of individual astrocytes the following parameters were determined: surface area, volume and cellular complexity (area *versus* volume; for the detailed description of imaging/analysis techniques, see references[83,84]).

Morphological characterisation of GFAP-positive astroglial cells was performed on animals of different ages, from 3 to 12 months. To our surprise we found consistent reduction in the GFAP expression (determined as surface and volume of GFAP-positive profiles), which progressed with age. In very young animals (3 months old), which did not have any signs of AD pathology, astroglial morphology was already somewhat altered. There was a slight reduction in astrocyte complexity (by 1.29%; Figure 1a) that was directly associated with a decrease in the number of processes and overall decrease in both surface (12.62%) and volume (14.25%; data not shown) of GFAP-labelled structures. Similar reduction in GFAP-labelled profiles was observed at all ages, although this decrease becomes significant only at 12 months of age. In 12-month-old animals the complexity was reduced by 5.04% ($P=0.0105$; Figure 1a, d and e), whereas GFAP-positive surface decreased by 38.99% and volume by 42.93% ($P=0.0211$, 0.0292, respectively; Figure 1). On a cellular level, this decrease in complexity was reflected by reduced number of main processes and their arborisation (Figure 1c).

Incidentally, the same decrease in astrocytes complexity was found in the postmortem brains of dementia patients. In this study,[85] astroglial cells from neocortical area 11 were Golgi impregnated and their complexity was characterised by a fractal dimension. Astrocytes from adult and healthy aged participants had similar complexity, whereas astrocytes from patients with dementia showed significant decrease in fractal dimension, thus indicating the decreased complexity. Furthermore, the volume of the brain parenchima occupied by the processes of single astrocyte was smaller in demented brains.

At the very same time confocal images revealed that the specific population of astrocytes surrounding amyloid plaques display the typical reactive characteristics (Figure 2), showing thick processes and enlarged cell bodies. Some of these astrocytes closely associated with amyloid plaques showed β-amyloid accumulation, as revealed by specific antibodies (Figure 2b). Conversely, astrocytes positioned away from the plaques did not show β-amyloid burden, which is consistent with previous observations.[54,55]

The 3xTg-AD animals show other changes that could be associated with glial malfunction. For example, we observed a transient increase in the number of asymmetric excitatory synapses in AD mice at 2 months of age, which subsequently dropped to control levels at 6–9 months. Nonetheless, this increase in excitatory synaptic contacts re-emerged again at 12 months of age when the brain parenchyma was infested with plaques, and neurones showed tangles.[86] In addition, we

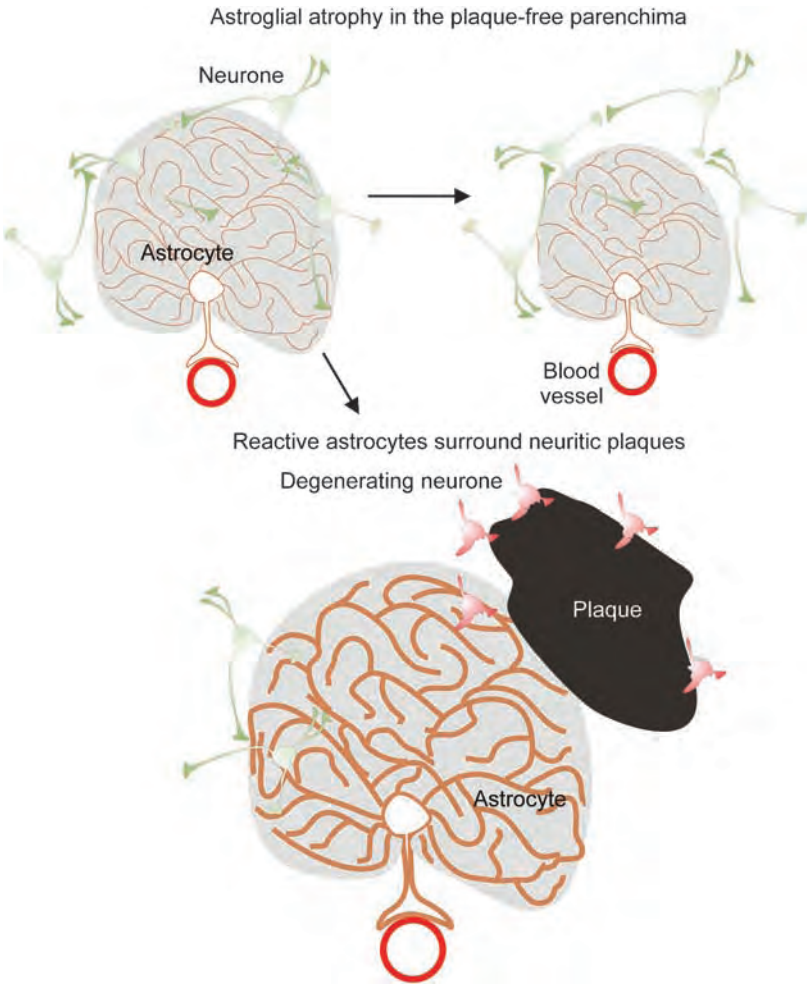

Figure 3 Astroglial hypothesis of Alzheimer's disease

also found significant decrease in the neurogenic capacity of 3x-Tg-AD mice at 12 months of age,[87] which may reflect degenerative changes in 'stem-cell'-like astrocytes.

Astroglial theory of AD?

The pivotal role of astroglia in various types of brain pathology is widely accepted.[6,88,89] Nevertheless, the pathological potential of astrocytes in human dementias remains virtually unexplored. From the scarce data available in the literature, we may conclude that astrocytic reactions in various types of dementia can be manifold, ranging from proliferation and astrogliosis to apoptosis and dystrophy.

In AD, these two reactions develop in parallel and may represent the underlying mechanism for loss in synaptic connectivity and plasticity, which in turn determine cognitive deficits. The reaction of astrocytes to the AD progression is spatially distinct (Figure 3): astroglial cells surrounding the plaques undergo gliosis, whereas astrocytes distant from the amyloid deposits develop atrophy. The first signs of this atrophy appear early in the genetic form of AD pathology in mice; and these signs precede the formation of both plaques and tangles. It is then conceivable to speculate that dystrophic astrocytes fail to provide adequate support for synaptic contacts, which may lead to their remodelling (e.g., distorted balance between excitatory and inhibitory synapses), with ensuing disruption of neuronal circuitry – and indeed the synaptic loss is observed in early stages of AD when brain parenchyma remains relatively free from neuritic plaques.[90,91] Furthermore, reduced astroglial coverage may significantly affect extracellular brain homoeostasis. In particular, dystrophic astrocytes may have reduced ability for glutamate uptake, thus increasing the overall brain vulnerability to glutamate excitotoxicity. In contrast, the reactive astroglial cells may serve another function – the function of neuronal killers. Indeed, the main pillar of β-amyloid hypothesis of AD, the toxicity of the neuritic plaques has been considerably shaken by experiments on transgenic animals. The latter, despite acquiring a heavy burden of amyloid plaques, show very little (if any) neuronal death.[58] This discrepancy is fundamental, as massive neuronal death assumes the main role in the development of severe dementia in humans. Naturally, the absence of neuronal extinction in the animal model can be explained by a variety of reasons, yet it may also hint that alternative mechanisms of the neurodegenerative process are in operation. These mechanisms could be

associated with both astroglial atrophy (reduced glutamate uptake with increased glutamate excitotoxicity) and astrogliosis, as reactive astrocytes and activated microglia may secrete numerous death-promoting molecules.

The AD, as well as other types of dementia, are complex processes which engulf all cellular elements in the brain. Can astrocytes assume the central stage in these pathologies? This question requires further detailed and insightful investigation.

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