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Oral Examination:

Identification of the cellular mechanisms underlying the contribution of stress and glucocorticoids to Alzheimer's disease pathology

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Identification of the cellular mechanisms underlying the contribution of stress and glucocorticoids to Alzheimer's disease pathology

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PhD Dissertation

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To my parents, *Theodoros and Konstantina*

for teaching me to feel, to love, to dream,
to try for a better world...
to live as a Human!!

Στους γονείς μου, *Θοδωρή και Κωνσταντίνα*

που με δίδαξαν να νιώθω, να αγαπώ, να ονειρεύομαι
και να προσπαθώ για έναν καλύτερο κόσμο...
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TABLE OF CONTENTS

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ABBREVIATIONS

Aβ: amyloid beta peptide
Ac-Tub: acetylated tubulin
ACC: anterior cingulate cortex
AD: Alzheimer's disease
ADDLs: amyloid-derived diffusible ligands
ADX:adrenalectomised
ADX: adrenalectomy
AF1 and AF2: transcription-activation functions
AF2: transactivation
AICD: APP intracellular C-terminal domain
APLP: amyloid precursor-like protein
APOE: apolipoprotein E
APP: amyloid precursor protein
α7nAChRs: α7 nicotinic acetylcholine receptors
BACE: β-site APP cleaving enzyme
βCTF: βC-terminal fragment
BV: brain volume
CaMKII: Ca^{+2}/Calmodulin-dependent kinase II
cdk5: cyclin-dependent kinase 5
Cho: choline-containing compounds
CON: controls
CORT: corticosterone
Cr: total creatinine
CTFs: carboxy-terminal fragments
CT100: C-100 amyloid fragment
C99: C-terminal fragment 99
DBD: DNA-binding domain
DEPC: diethylpyrocarbonate
DEX: dexamethasone
DHT: dihydrotestosterone
EPM: elevated plus maze
ERK1/2: extracellular signal-regulated kinases 1/2
FAD: familial Alzheimer's disease
FTDP-17: frontotemporal dementia and parkinsonism linked to chromosome 17
GC: glucocorticoids
Glu: glutamate

GPCR: G protein-coupled cell surface receptor
GR: glucocorticoid receptor(s)
GRE(s): glucocorticoid response element(s)
GSK-3ß: glycogen synthetase kinase 3β
HPA: hypothalamic-pituitary-adrenal axis
HV: hippocampal volume
i.c.v.: intracerebroventricular
ISHH: In situ hybridization
JNK: c-Jun N-terminal kinase
KO: knockout
KPI: Kunitz protease inhibitor
LHV: left hippocampal volume
LBD: ligand-binding domain
LTD: long-term depression
LTP: long-term potentiation
MANOVA: multivariate analysis of variance
MAP kinase/MAPK: mitogen activated protein kinase
MARK: microtubule affinity-regulating kinase
MCI: mild cognitive impairment
MEK-1: mitogen-activated kinase (MAP) kinase 1
mI: *myo*-inositol
MR: mineralocorticoid receptor(s)
MRI: magnetic resonance imaging
MRS: proton magnetic resonance spectroscopy
MT: microtubule
NAA: N-acetyl aspartate
NF-κB: nuclear factor κB
NFT: neurofibrillary tangles
NLS: nuclear localization signal
NO: nitric oxide
NP: neuritic plaques
NSAIDS: non-steroidal anti-inflammatory drugs
NT: neuropil threads
OD: optical density
O_2^-: superoxide
PFA: paraformaldehyde
PFC: prefrontal cortex
PHF: paired helical filaments

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PKC: protein kinase C

PP1: protein phosphatase 1

PP2A: protein phosphatase 2A

PRESS: point-resolved spectroscopy sequence

PSEN/PS: Presenilin

PYK2: Proline-rich tyrosine kinase 2

RARE: rapid-acquisition relaxation-enhancement

RHV: right hippocampal volume

RSC: retrosplenial cortex

RU38486: mifepristone

sAPP: secreted APP

SARK: stress-activated protein kinases

SEM: standard error of the mean

Ser: serine

SD: standard deviation

SDAT: senile dementia of Alzheimer type

SF: straight filaments

SFv: shrinkage factor

SPECT: single photon emission computed tomography

SSC: saline sodium citrate

Thr: threonine

TE: trans-entorhinal

Abstract

Clinical evidence suggests the involvement of stress and glucocorticoids (GC) in the etiopathogenesis of Alzheimer's disease (AD), a disease marked by severe memory impairments as well as alterations in mood and emotional state. The experiments described in this dissertation represent an attempt to establish the cellular mechanisms through which stress and GC may impact on the development of AD. These studies focused on the hippocampus and prefrontal cortex (PFC), brain areas that are severely affected in AD; both areas were found to respond to stress and GC hypersecretion with neuronal atrophy and death, effects that are mediated by glucocorticoid receptors (GR). Furthermore, stress and GC were shown to induce impairments in cognitive behaviors which depend on the structural integrity of the hippocampus and PFC. The neurodegeneration which accompanies AD is characterized by mis-processing of the amyloid precursor protein (APP) and abnormal hyperphosphorylation of the microtubule-associated protein tau; the latter leads to cytoskeletal disruption and synaptic loss. Experiments in laboratory animals and cell systems established that stress/GC can contribute to AD pathology by triggering the amyloidogenic pathway of APP processing by increasing the expression of APP-cleaving enzymes (BACE and nicastrin), giving rise to the APP C-terminal fragment 99 (C99) which, after further processing, yields amyloid beta (Aβ). While Aβ is the APP product to which AD pathology is usually ascribed, C99 also has deleterious effects on neuronal survival and cognition. The present studies showed that stress/GC-induced changes in APP processing were paralleled by impairments in hippocampus- and PFC-dependent behaviors and anxiety (in animals), as well as by increased abnormal hyperphosphorylation of tau, decreased tau degradation, and reduced stability of microtubules; additional evidence obtained suggests that synaptic loss may also be a consequence of stress/GC-induced alterations in APP processing and tau biochemistry. A more detailed analysis of the mechanisms through which GC (the main physiological response to stress) might influence tau revealed the involvement of a number of kinases (GSK3β, cdk5 and MAPK), all of which have been implicated in AD pathology. Since normal laboratory species do not develop AD pathology unless they are engineered to express human forms of APP or tau, one part of this work examined whether GC could induce their harmful effects in a rat neuronal cell line that had been stably transfected with *htau*. The results of those experiments confirmed the hypothesis that *htau* is responsible for making neurons sensitive to the AD-like pathological effects of GC; specifically, as compared to wild-type cells, those expressing *htau* showed increased sensitivity to GC and Aβ, while at the same time increasing APP mis-processing to C99 and Aβ, as well as tau hyperphosphorylation. In summary, these experiments describe, for the first time, the processes through which stress/GC can lead to AD-like pathology. They raise the hypothesis that stress/GC primarily trigger APP mis-processing, which then leads to tau kinase activation and tau pathology; the tau kinases then feeds back to exacerbate the pathobiochemistry in a positive feedback loop. Lastly, the results reported herein suggest new preventative and therapeutic strategies, and also suggest that the AD-related proteins tau and APP may be general key players in mediating the effects of stress and GC in stress-related disorders.

Kurzfassung

Es gibt klinische Anhaltspunkte dafür, dass Stress und Glucocorticoide (GC) an der Entstehung der Alzheimer-Krankheit (AD) beteiligt sind, einer Krankheit, deren Hauptmerkmale schwere Beeinträchtigungen des Gedächtnisses sowie eine Verschlechterung von Motivation und Emotionalität sind. Die Experimente, die im Rahmen dieser Dissertation durchgeführt wurden, stellen den Versuch dar, die zellulären Mechanismen zu ermitteln, durch die sich Stress und GC auf die Entstehung von AD auswirken könnten. Diese Studien konzentrieren sich auf den Hippocampus und den präfrontalen Cortex (PFC), also auf Hirnareale, die bei AD schwer betroffen sind. Bei beiden Arealen stellte sich heraus, dass sie auf Stress und eine übermäßigen Ausschüttung von GC mit Atrophie und Tod von Neuronen reagierten. Diese Auswirkungen wurden von Glucocorticoid-Rezeptoren (GR) vermittelt. Des Weiteren konnte gezeigt werden, dass Stress und GC eine Minderung der kognitiven Fähigkeiten, die auf einen intakten Hippocampus und PFC angewiesen sind, bewirkten. Die Neurodegeneration bei der Alzheimer-Krankheit ist durch die fehlerhafte Spaltung des Amyloid-Vorläuferproteins (APP) gekennzeichnet sowie durch eine abnorme Hyperphosphorylierung des Mikrotubuli-assoziierten Tau-Proteins. Letzteres führt zu zytoskelettaler Störung und Rückbildung von Synapsen. In Experimenten an Versuchstieren und mit Nervenzellkulturen konnte gezeigt werden, dass Stress und GC an der Entstehung von AD beteiligt sein können, und zwar durch das Auslösen des amyloid-induzierten Signalwegs. Dies geschieht durch die Erhöhung der Expression von APP-spaltenden Enzymen (BACE und Nicastrin), was die Bildung des APP C-Terminal-Fragments 99 (C99) zur Folge hat, das nach weiterer Verarbeitung zu Amyloid beta (Aβ) wird. Auch wenn Aβ allgemein als das APP-Produkt gesehen wird, auf das die Entstehung der Alzheimer-Krankheit zurückzuführen ist - auch C99 hat schädigende Auswirkungen auf Neuronen und Kognition. Die hier durchgeführten Studien zeigen, dass Stress und GC Änderungen in der Verarbeitung von APP verursachen, was zu Beeinträchtigungen im vom Hippocampus und PFC abhängigen Verhalten und zu erhöhter Ängstlichkeit führt. Des Weiteren führen diese Änderungen zu einer erhöhten abnormen Hyperphosphorylierung von Tau, zu vermindertem Tau-Abbau und zu einer verminderten Stabilität der Mikrotubuli. Erste Ergebnisse deuten darauf hin, dass der Rückbildung von Synapsen auch eine Folge der durch Stress oder GC induzierten Veränderungen in der APP-Spaltung und im Tau-Stoffwechsel sein könnte. Eine detailliertere Analyse der Wirkungsweisen, durch die GC (die hauptsächliche physiologische Antwort auf Stress) Tau beeinflussen könnten, zeigte die Involvierung mehrerer Kinasen (GSK3β, cdk5 und MAPK), von denen man annimmt, dass sie an der Entstehung von AD beteiligt sind. Da nur transgene Labortiere, die die humanen Formen von APP oder Tau exprimieren, die Alzheimer-Krankheit entwickeln, wurde in einem Experiment untersucht, ob GC schädliche Folgen in einer neuronalen Ratten-Zelllinie, die stabil mit *htau* transfiziert war, haben könnten. Die Ergebnisse dieser Experimente bestätigten die Hypothese, dass *htau* dafür verantwortlich ist, dass Neurone für die Alzheimer-ähnlichen pathologischen Folgen von GC sensitiviert werden. *htau* exprimierende Zellen zeigten im Vergleich zu Wildtyp-Zellen eine erhöhte Sensitivität gegenüber GC und Aβ, während gleichzeitig die fehlerhafte Spaltung von APP zu C99 und Aβ, aber auch die Tau-Hyperphosphorylierung anstiegen. Diese Experimente beschreiben – zum ersten Mal – wie Stress und Glucocorticoide zu einer Alzheimer-artigen Symptomatik führen können. Daraus lässt sich die Hypothese ableiten, dass Stress und Glucocorticoide primär die falsche APP-Spaltung auslösen, die dann zur Aktivierung der Tau-Kinase und zur Tau-Pathologie führt. Die Tau-Missbildung führt zu einer weiteren Verstärkung der

Pathobiochemie des Tau-Proteins. Letztlich geben die hier dargelegten Ergebnisse Hinweise für neue präventive und therapeutische Maßnahmen und auch darauf, dass die Alzheimer-spezifischen Proteine Tau und APP die allgemeinen „Key Players" sein könnten, die die Auswirkungen von Stress und GC auf stressbedingte Krankheiten vermitteln.

Chapter 1

General Introduction

- What is Alzheimer's disease?
- Alzheimer's disease – progression and neuroanatomy
- Pathobiochemistry
- Predisposing factors

GENERAL INTRODUCTION

1.1. What is Alzheimer's disease?

Alzheimer's disease (AD) is a progressive age-related disorder that was first described in 1906 by the German physician Alois Alzheimer whose first case, Auguste D., a woman aged 51, showed impairments of mental function that were characterized by unique neurohistopathological features at post mortem (Möller and Graeber 1998). Today, Alzheimer's disease is the fourth leading cause of death in the elderly. Since AD can only be definitively diagnosed upon autopsy and neuropathological examination, the term *senile dementia of the Alzheimer type* (SDAT) is used to describe patients suspected to be suffering from AD. Diagnosis of SDAT, based on neurological examination and the exclusion of other causes of dementia, applies to 7-10% of the population aged over 65 years (Sisodia 2000).

Clinically, AD is characterized by a progressive loss of cognitive functions. The disease is especially marked by a decline in memory and language functions as well as impaired learning abilities, apathy and relatively severe mood disturbances; at later stages of the disease, psychosis and agitation become manifest and daily living activities that require sensory-motor co-ordination become challenges for the patient. Many late-stage patients may also develop gait disturbances or seizures.

The condition mild cognitive impairment (MCI) is now believed to be a forerunner of AD, with 10-15% of affected individuals progressing to SDAT as compared to 1-2% conversion in healthy individuals (Petersen et al 2003). MCI patients show slow, but progressive impairments in certain cognitive domains (usually one at a time, e.g. language, attention, critical thinking, writing, reading, but most commonly, memory) *(*DeCarli 2003*)*. As shown in Fig. 1.1, however, a significant number of MCI, but not AD, patients may "revert" to normal (Petersen 2003).

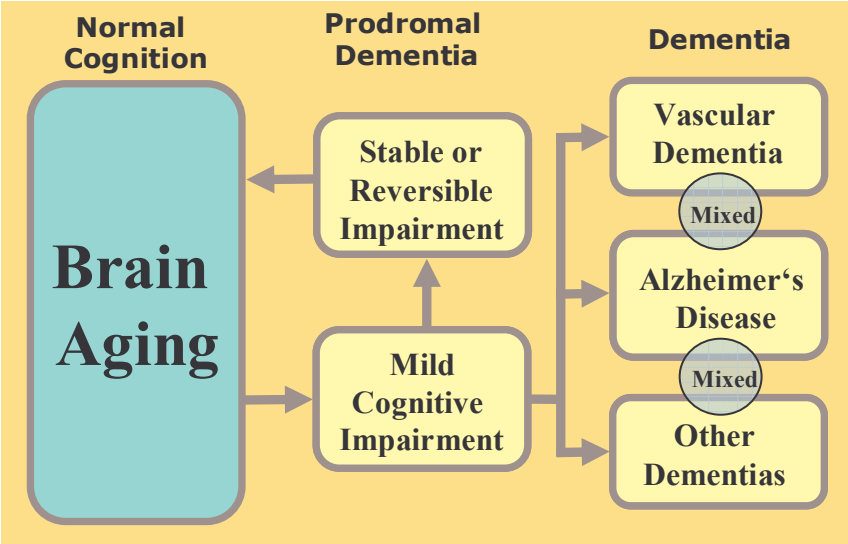

Figure 1.1 Progression of dementia and SDAT. Note that some individuals with Mild Cognitive Impairment (MCI) will not develop dementia or even exhibit reversible impairment (*from:* J.C. Morris, *Geriatrics*, 2005, Suppl: 9-14)

As already alluded to, a definitive diagnosis of AD can only be made *post mortem*, based on neuropathological lesions in the neocortex, hippocampus, and basal forebrain. The lesions include:

i) the extracellular accumulation of *senile plaques*, which consist mainly of deposits of amyloid β peptide [Aβ, a cleavage product of the transmembrane protein *Amyloid Precursor Protein* (APP)];

ii) intracellular *neurofibrillary tangles* (*NFTs*) that result from the aggregation of abnormally hyper-phosphorylated *tau*, a microtubule-associated protein [2, 3, 4]. More information on APP, Aβ, tau and NFT can be found in Section 1.3.

Although the etiology and pathogenesis of AD remain unclear, our understanding of some of the molecular and cell biological processes underlying the disease has grown enormously in the last decade. It is also now recognized that a variety of factors, including genetic mutations, and exposure to gonadal and stress hormones are contributory to the development and progress of the disease. However, advanced age is the one common and predominating risk factor in all cases, with the prevalence of dementia doubling approximately every 5 years between the ages of 65 and 95 (Fratiglioni et al 1991). Genetic inheritance is responsible for just some 7% of AD cases (referred to as familial Alzheimer's disease (FAD) cases). Mutations

in the *APP* and *apolipoprotein E* (*APOE*) genes increase the risk of developing AD (Corder et al 1993)**.** Gender differences in prevalence of AD are quite pronounced, suggesting that women are at a greater risk for developing AD; this sex difference is most commonly associated with the post-menopausal (age-dependent) decline in estrogen production (Fratiglioni et al 1997; Monk and Brodaty 2000). Lastly, stress and corticosteroids, the hormones released in response to stress, are thought to play a causative role in AD since several studies describe elevated cortisol levels in AD patients, and because hippocampal degeneration and memory impairments are found in patients with disturbed adrenocortical activity (Lupien et al 1998; Sterkman et al 1999; Umegaki et al 2000; Rasmuson et al 2001).

1.2. AD progression and neuroanatomy of disease

Rather than being a single-cause disorder, AD is clearly a multifactorial disease resulting from the interplay of genetic and environmental factors. Identification of the molecular and cellular targets of these factors could eventually serve to prevent or delay the progression of AD pathology. To this end, it is important to be able to monitor the temporal and spatial development of AD-like pathology since the build-up of Aβ-containing senile plaques and NFTs occurs over years (even decades), eventually causing lesions that involve neuronal dystrophy and death and loss of synaptic connections, in particular within neuronal circuits in the neocortex, hippocampus, and basal forebrain cholinergic system which are essential in attention and memory processes. *Post mortem* observations suggest that patients may be largely asymptomatic (preclinical phase) even after such lesions are rather advanced, i.e. cognitive impairment may not be evident until well after disease onset (see Fig 1.2). However, it remains unclear as to whether a certain threshold needs to be reached before symptoms are observable or whether such a threshold varies between individuals. Interestingly, the distribution, shape and size of amyloid deposits varies widely between patients. Thus, it seems likely that individual genetic differences or lifetime experiences, or a combination thereof, may interact to determine when these supposed thresholds are reached. Based on the

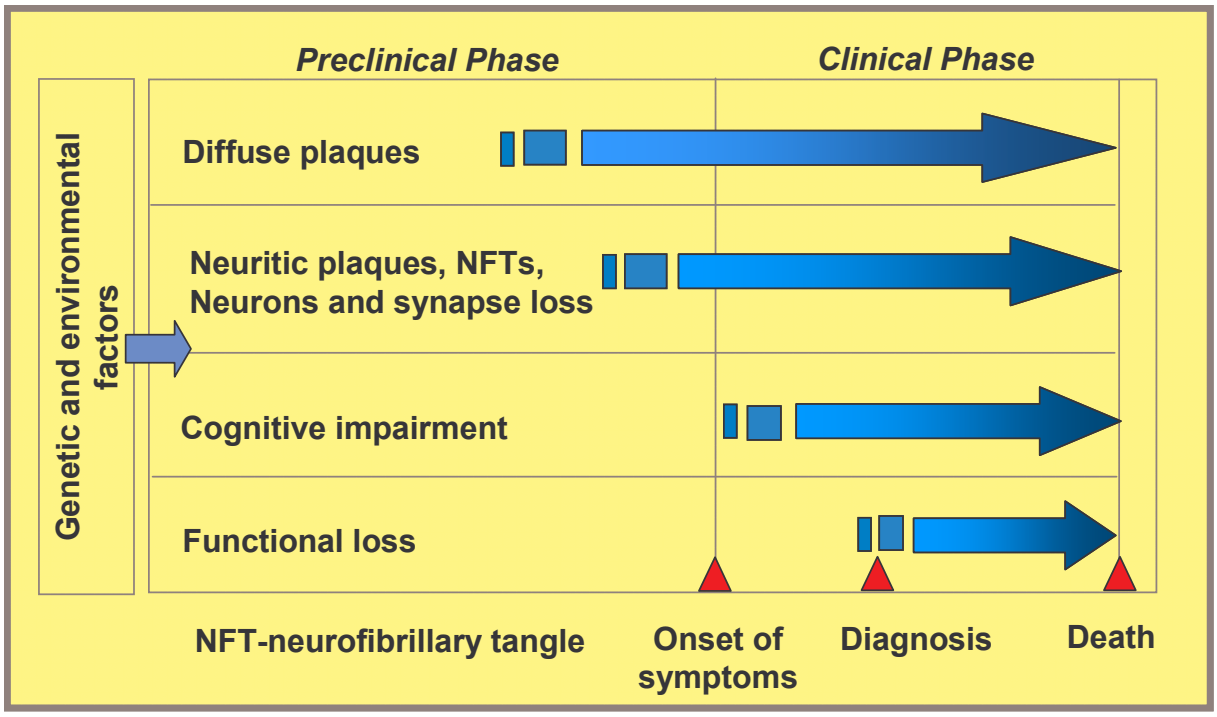

Figure 1.2 Chronic disease model of AD. As a long-lasting disease likely evolving over decades, AD progression can be divided in 2 phases: I) the preclinical (asymptomatic) and II) the clinical (symptomatic) one. Note that diffused amyloid plaques and NFT are formed far before the onset of symptoms (*from:* J.C. Morris, *Geriatrics,* 2005 Suppl: 9-14).

literature, factors other than genetic ones, such as stress and physiological or pharmacological exposure to adrenal and gonadal steroids could also be important factors. On the other hand, epidemiological studies strongly indicate that that low education status or reduced intellectual activities are major risk factors for the development of AD.

In contrast to *senile plaques* which are heterogeneously distributed throughout the brain, *neurofibrillary lesions* develop at predictable sites and affect specific cell types in given cell layers and brain regions; thus, tau pathology spreads in a stereotypic, sequential, and hierarchical manner. This uniform pattern formed the basis of Braak and Braak's (1991) description of the 6 stages of the progression of tau pathology; these stages, summarized below, show good correlation with impairment of memory and mental status (Henderson 1994).

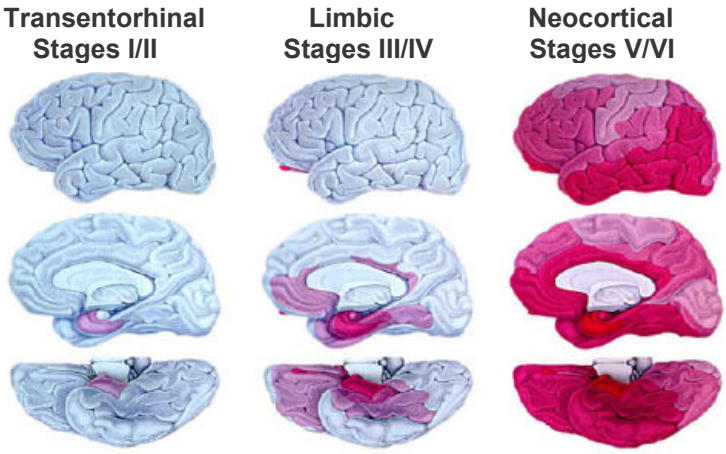

Figure 1.3 Neuropathological staging according to Braak & Braak (1991)

- *Stage I*: Projection neurons of the a-layer (large pyramidal neurons) of the *trans-entorhinal* (TE) region display neurofibrillary changes, notably in the *absence* of amyloid deposits. Patients are asymptomatic and, if at all, show mild changes in the hippocampal CA1 subfield (see Figs. 1.3 and 1.4).
- *Stage II*: The TE becomes more severely affected and mild changes occur in the *pre*-a layer of the entorhinal region. As with Stage I patients, subjects in this phase of disease show no signs of cognitive impairment and only minor neurofibrillary disturbances in the CA1 region.
- *Stages III and IV*, also known as the *limbic stages*, are characterized by mild cognitive impairment (MCI). Both *pre*-a layers of the entorhinal and TE regions are severely lesioned and lesions become more evident in the CA1 area of Stage III patients. Stage III patients also start developing mild lesions in the magnocellular forebrain nuclei, the anterodorsal nucleus of the thalamus and the amygdala. Numerous NFT also occur in the CA1 area at Stage IV.
- *Stages V and VI*, also referred to as *the neocortical stages*, represent fully developed AD. While primary sensory areas are relatively spared, subcortical nuclei show pronounced changes during Stage V. Finally, in Stage VI, the hippocampus is infected with neurofibrillary changes and CA1 neuronal degeneration is rife; all cortical association areas as well as the primary sensory areas and subcortical nuclei are also markedly affected (see Figs. 1.3 and 1.4).

Interestingly, plaques and tangles are observed in many non-demented aged individuals at autopsy. While supporting the view that the pre-clinical phase of AD is protracted, these observations also indicate that plaque and tangle formation are normal events during aging; most likely, pathology only becomes overtly manifest (amnesic type of memory impairment, deterioration of language, and visuospatial deficits) once these lesions reach a 'critical mass' (Arriagada et al 1992; Braak et al 1999; Guillozet et al 2003).

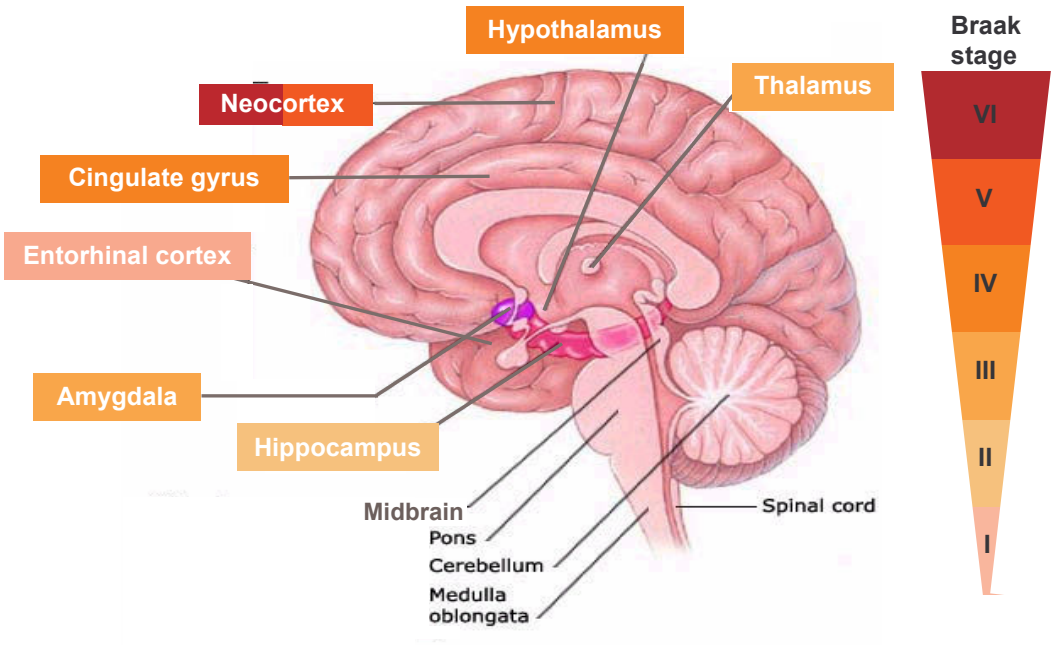

Figure. 1.4 Temporal and spatial development of tau pathology during AD progression. According to Braak & Braak (1991), affected brain areas are classified into six stages, starting with large pyramidal neurons of entorhinal cortex (stage I), the class of neurons most likely to develop tangles are large cortico-cortical projection neurons in limbic system and association cortices, up to stage VI where hippocampal degeneration is rife and neocortical areas as well as subcortical nuclei are markedly affected (*drawn by* I. Sotiropoulos & O.F.X. Almeida).

1.3. Pathobiochemistry – Molecular and cellular mechanisms of AD

As already mentioned, AD is associated with several typical histopathological hallmarks. These include selective, but widespread, neuronal degeneration, synaptic loss and dystrophic neurons, extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) that eventually result in diffuse cerebral atrophy.

1.3.1. Senile plaques

Also known as *amyloid plaques*, senile plaques are located in the neuropil of AD patients (Fig. 1.4). The plaques consist of a central extracellular core of aggregated amyloid β-peptide, arranged as β-sheet filaments surrounded by distended neurites (dystrophic axons and dendrites).

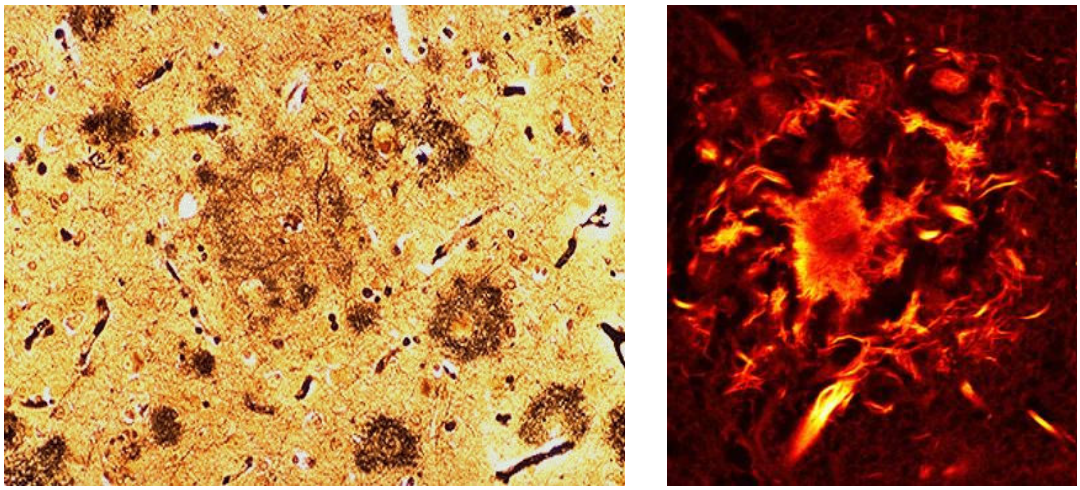

Fig 1.5. Senile plaques stained with silver (left) or TR 450 dye (right) (by Brian J. Bacskai; http://cellscience.bio-rad.com/moviesandimages/pathology.htm.)

Amyloid plaques are commonly surrounded by microglia and reactive astrocytes. While their central core contains numerous proteins, a 39-43 amino acid peptide, known as amyloid β-peptide (Aβ) is the principal component; Aβ is a cleavage product of the "parental" protein, *amyloid-precursor protein (APP)* (Allsop et al 1983). Aβ is also frequently deposited in the small blood vessels of the meninges and cerebral cortex, mostly in the outer walls of arterioles and capillaries (Fig. 1.4). Additionally, soluble oligomeric forms of Aβ are found throughout the brain, probably representing precursors of the insoluble senile plaques described in Section 1.3.1.3.

1.3.1.1. Amyloid Precursor Protein (APP) and its processing: The main component of senile plaques, Aβ, is derived from the proteolytic processing of amyloid-precursor protein (APP). APP is a type-I trans-membrane sialo-glycoprotein. It has a single membrane-spanning domain, a large extracellular glycosylated N-terminus and a shorter cytoplasmic C-terminus. APP occurs in many membranous structures; like other proteins, it is synthesized in the endoplasmic reticulum, transported to the Golgi network from where, after

post-translational modifications, it is transported via the secretory pathway to the cell membrane, as described later in this section (for review, see Mattson 2004).

The *APP* gene, located on chromosome 21, is expressed in many cell and tissue types including endothelia, glial and neuronal cells. Through alternative splicing of exons 7, 8 and 15, the *APP* gene can give rise to at least 8 protein products. Neurons always express *APP* isoforms that contain exon 15. These neuronal APP isoforms, of which there are 3 (APP695, APP751 and APP770, consisting of 695, 751 and 770 amino acid residues, respectively), are more amyloidogenic than other isoforms, thus increasing the chances of plaque formation; both APP751 and APP770 contain the 56-amino acid Kunitz protease inhibitor (KPI) domain, which is encoded by exon 7. In addition, APP770 contains a 19-amino acid insert that codes for an OX-2 domain. A splice variant, which lacks exon 15, is termed L-APP. Here, it is important to note that there are two other amyloid precursor-like proteins (APLP1 and 2) which have APP-like extracellular and intracellular domains and seem to function similarly to APP in many biochemical pathways (Heber et al 2000); importantly, however, these proteins do not contain the amyloidogenic Aβ region found in APP (for reviews, see Coughlan and Breen 2000; Turner et al 2003).

As mentioned earlier, APP matures through the secretory pathway; it may be N- and O-glycosylated, sulphated and/or phosphorylated. Additionally, it undergoes post-translational proteolytic cleavage via two different pathways. The first, the 'amyloidogenic pathway' results in the generation of the Aβ peptide; the other, referred to as 'anti-amyloidogenic pathway' does not result in the generation of Aβ peptides (see Fig. 1.6).

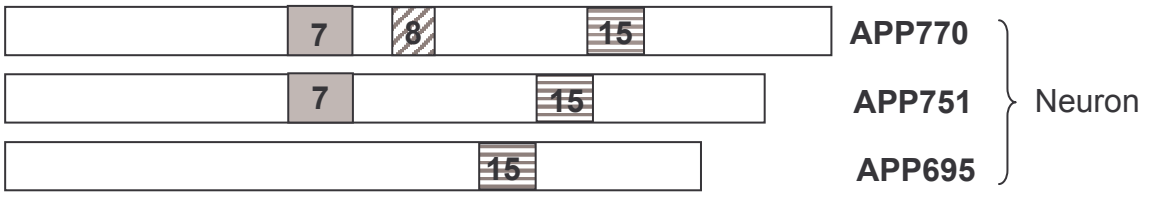

Figure 1.6. The three APP isoforms found in neurons. APP770, APP751, APP695 consist of 770, 751 and 695 amino acid residues, respectively.

The amyloidogenic pathway is initiated by APP cleavage through sequential enzymatic cleavages (see Table 1). First, β-secretase (BACE1) cleaves APP at the N-terminus of the Aβ-containing domain. BACE1 is a type-I membrane-bound aspartyl protease preferentially located in the Golgi apparatus and endosomes. This cleavage generates soluble sAPPβ, which is released from the membrane, and a C-terminal fragment (C99) that remains associated with the membrane; the latter undergoes a second cleavage by γ-secretase within the transmembrane domain of APP and results in production of Aβ; it is this peptide which subsequently aggregates and deposits in the brain parenchyma (and around blood vessels) of AD patients. There are several potential γ-secretase cleavage sites, giving rise to Aβ peptides consisting of between 39 and 43 amino acid residues. Recently, two other cleavage sites, namely the *ε*- and *ζ*-cleavage sites, which result in the generation of $A\beta_{49}$ and $A\beta_{46}$ have been identified (Weidemann et al 2002; Zhao et al 2004).

Table 1.1 Characteristics of enzymes involved in APP cleavage

ENZYME		*REFERENCE*
α-secretase	i) ADAM 10, a member of the ADAM (disintegrin and metalloprotease) family of proteases	• Lammich et al. 1999
	ii) ADAM 9/MDC9 can influence sAPPα concentrations	• Koike et al. 1999
	iii) Tumor necrosis factor A-converting enzyme (TACE/ ADAM17) is co-localized with senile-plaques and NFT in hippocampus and cortex of AD patients.	• Buxbaum et al.1998
β-secretase	i) BACE-1, highly expressed in neurons and in astrocytes can cleave APP at both β-secretase cleavage sites.	• Vassar et al. 1999; • Rossner et al 2004
	ii) BACE-2, like BACE-1, can cleave APP at Aβ sites, but it is not highly expressed in the brain	• Farzan et al. 2000 • Bennett et al. 2000
	Carboxypeptidase B, found in the cytosol of various neurons and some microglial cells, can cleave APP in vitro and in vivo	• Matsumoto et al. 2000
γ-secretase	A heteromeric protein complex consisting of presenilin 1 or 2, nicastrin, presenilin enhancer-2 (PEN-2), anterior pharynx defective-1 (APH-1) and CD147	• Francis et al. 2002 • De Strooper 2003 • Zhou et al. 2005
Caspase 8 and 9	Caspases 8 and 9 can cleave APP, APLP1 and, possibly, APLP2	• Lu et al 2000 • Galvan et al 2002

Buxbaum et al. 1998 J Biol Chem 273:27765-7; Koike et al. 1999 Biochem J 343:371-5; Lammich et al. 1999 Proc Natl Acad Sci U S A 96:3922-7; Vassar et al. 1999 Science 286:735-41; Bennett et al 2000 J Biol Chem 275:20647-51; Farzan et al. 2000 Proc Natl Acad Sci U S A 97:9712-7; Lu et al 2000 Nat Med 6:397-404; Matsumoto et al 2000 Eur J Neurosci. 12:227-38; Francis et al 2002 Dev Cell 3:85-97; Galvan et al 2002 J Neuroch 82:283-294; De Strooper 2003 Neuron 38:9-12; Rossner et al 2005 J Neurochem 92:226-34; Zhou et al PNAS 2005 102:7499-7504.

The anti-amyloidogenic pathway starts with APP cleavage by α-secretase (see Table 1). α-secretase cleaves within the Aβ domain, thus precluding the generation of the neurotoxic Aβ peptide. The residual C-terminal fragment (C83) undergoes γ-secretase cleavage to yield the p3 peptide (Fig. 1.7). APP processing can also be influenced by the presence of polymorphic mutations in the *APP* gene and in the *Presenilin 1* (*PSEN1,* on chromosome 14) and *Presenilin 2* (*PSEN2*, on chromosome 1) genes. The products of the later two genes are parts of an enzymatic complex that regulates the production of Aβ from APP (see Table 1.1). To date, at least twelve AD-associated mutations have been identified in the *APP* gene, more than one hundred in that of *PSEN1*, and a few in that of *PSEN2* (Snider et al 2005).

1.3.1.2 Neural functions of APP and its cleavage products: The precise functions of APP are still poorly understood. APP has been implicated in brain development and maturation, with its mRNA first being detected at embryonic day 9.5 in the neural tube of the mouse (Salbaum and Ruddle 1994). The presence of immunoreactive APP in radial glia cells during early development suggests that it may have a role in neural cell differentiation (Trapp et al 1994). Adding support to a neurodevelopmental role for APP are the observations that knockout (KO) of the *APP* gene in mice leads to reduced brain weight and size of the forebrain commissures (Zheng et al 1995; Magara et al 1999), and that KO of APP as well as its homologues, APLP1 and 2, results in abnormal layering of the cerebral cortex (Herms et al 2004).

It is noteworthy that APP binds to the protein Numb, which seems to link APP to cell-fate decisions via the Notch signaling-mediated pathway. Further, APP has been implicated in neural cell adhesion through its collagen-, laminin-binding domains, as well as its binding to telencephalin (a neural cell adhesion molecule)**.** APP also associates with Fe65 (an adapter protein which couples APP to actin filament formation), indicating its role in motility, in particular in the morphological remodeling of neurites: APP activity promotes neurite formation, probably due to its heparin-binding domain. Full-length APP also appears to function as a G protein-coupled cell surface receptor (GPCR), transducing signals from the extracellular matrix to the inner cell (for review see Turner et al 2003); it influences numerous signaling cascades such as

adenylyl cyclase, phospholipase C, voltage-dependent calcium channels and apoptotic pathways after binding to G_o. On the other hand, intracellular APP seems to serve the role of a 'cargo receptor' for the axonal transport and recycling of synaptic vesicles (Kamal et al 2000).

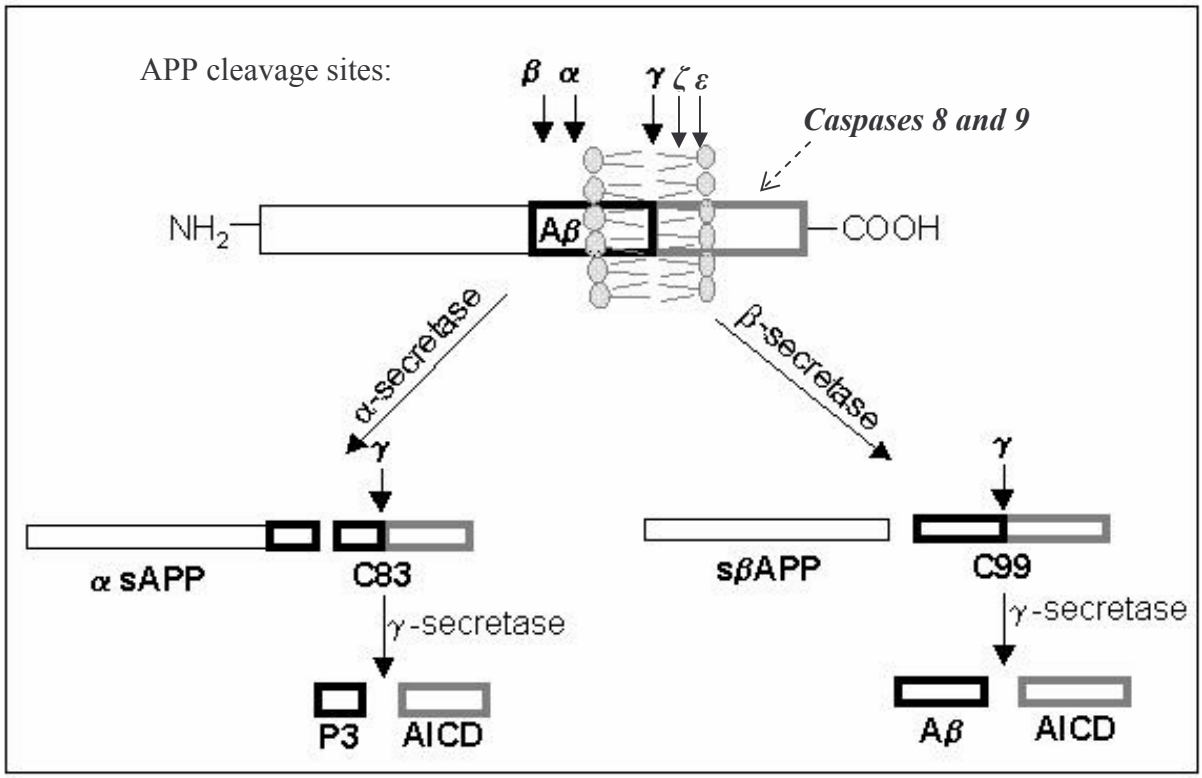

Figure 1.7. Principal APP-processing pathways. Cleavage by the membrane-associated metalloprotease α-secretase enzyme occurs within the Aβ domain (between residues 16 and 17), thereby preventing the formation of Aβ, and instead resulting in the release of the large soluble extracellular N-terminal portion of APP (sαAPP) and a C-terminal fragment consisting of 83 residues (C83). C83 can undergo further processing by γ-secretase to release a peptide called p3. Aβ is generated through 2 sequential endoproteolytic steps involving distinct enzymatic activities: β- and γ-secretase. β-secretase, also known as BACE1, cleaves APP at the N-terminal region of the Aβ sequence. Cleavage by β-secretase generates a slightly shorter soluble N-terminus peptide (sβAPP) and the amyloidogenic C-terminal fragment (C99). The cleavage of C99 by γ-secretase liberates the C-terminal 50 residues of APP, the APP intracellular domain (AICD) and Aβ. Additionally, both Caspase 8 and 9 can cleave APP, thus releasing a C-terminal peptide, C31 (not shown), which exhibits pro-apoptotic role by activating caspases 8 and 9 and amplifying the apoptotic response (for refs. see *Box 1*) (*adapted by* I. Sotiropoulos & C. Catania).

The various APP-derived products also have multiple roles in cell homeostasis and viability. *In vitro* and *in vivo* studies have shown that secreted APP (sAPPα) influences cell proliferation, differentiation and survival. sAPPα increases the *in vitro* proliferation of embryonic neural stem cells (Hayashi et al 1994) through its regulation of EGF-induced proliferation of progenitors in the subventricular zone of the lateral ventricle, the most prominent

neurogenic area of the adult brain (Caille et al 2004), and astrocyte-released sAPPα is important for normal axonal development (Perez et al 1997). sAPPα can also modulate membrane excitability (Furukawa et al 1996), calcium homeostasis (Mattson et al 1993) and NMDA receptor-mediated currents (Furukawa and Mattson 1998), in addition to altering neuronal morphology by increasing the number of presynaptic terminals in the frontoparietal region and thus, probably facilitating memory (Roch et al 1994). Further support for the beneficial effects of APP-derived peptides comes from animal studies showing that the intracerebroventricular (i.c.v.) infusion of sAPPα (or smaller peptides containing growth-promoting domains) can improve learning and memory (Meziane et al 1998). Interestingly, impaired cognitive function in aged rats is accompanied by reduced CSF levels of sAPPα (Anderson et al 1999) and a similar negative correlation has been reported in humans with FAD (Swedish mutation) (Almkvist et al 1997). These findings agree with other clinical studies that demonstrated that the levels of sAPPα, α-secretase ADAM 10 and α-secretase activity were reduced in the CSF of patients with sporadic AD (Colciaghi et al 2002; Tyler et al 2002).

Very little is known about the intracellular signaling pathways that are activated by sAPPα, but its cysteine-rich N-terminal domains (highly similar to those of the EGF and TGF-α family members) are thought to serve as a receptor(s) that stimulate downstream pathways, e.g. the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway, as well as ERK1/2 (p42/p44) mitogen-activated protein (MAP) kinases and nuclear factor κB (NF-κB) (Cheng et al 2002; Barger et al 1996). Additionally, in association with other proteins such as Fe65 and/or Tip60, and upon nuclear translocation, the intracellular C-terminal domain of APP (AICD, see Fig. 1.7) derived from γ-secretase-mediated cleavage, can regulate the transcription of several genes, including APP itself, BACE (von Rotz et al 2004), as well as glycogen synthase kinase-3β (which can stimulate formation of tau-rich neurofibrillary tangles, as discussed later) (Kim et al 2003). Thus, nuclear translocation of intracellular domains generated from membrane proteins by γ-secretase represents a pathway for signaling from the cell surface to the nucleus, including the activation of signaling cascades such as Notch and N-cadherin (Schroeter et al 1998; Marambaud et al 2003).

Lastly, it should be noted that the intracellular carboxy-terminal fragments (CTFs), that are reportedly even more neurotoxic than Aβ (see Section 1.3.1.3) can contribute to apoptosis by lowering the threshold to other apoptotic stimuli (such as glutamate) or by altering calcium release (for review see Chang et al 2005). Deficits in memory and learning functions have been observed after i.c.v. injection of CT105 and in transgenic mice over-expressing the CT100 (C-100 amyloid fragment) (Choi et al 2001).

1.3.1.3. Aβ, a peptide with opposing actions

Since their discovery in the early 1980s, Aβ peptides have been shown to have both neurotrophic and toxic properties through their actions on different cellular pathways and signaling cascades. This apparent paradox may be explained by the fact that Aβ is generated during normal cellular metabolism (Haas et al 1992) and that its actions differ according to its intracellular vs. extracellular localization; the latter promotes its aggregation and toxicity. Because of their ability to self-aggregate, Aβ monomers eventually dimerize and oligomerize; larger aggregates result in the successive formation of protofibrils and β-sheet fibrils, with the latter forming the core of senile plaques. Senile plaques are comprised of the more hydrophobic $A\beta_{1-42}$ which aggregates more readily than other forms of Aβ (such as $A\beta_{1-40}$ which is the main component of vascular amyloid).

Neurotoxic properties of Aβ peptides

Much earlier work focused on the neurotoxic effects of fibrillary Aβ, assuming that fibril formation was a key step in Aβ-induced neuronal damage and eventual neurodegeneration (Lorenzo et al 1994). However, the degree of amyloid deposition correlates only poorly with cognitive decline in AD; in fact, dementia best correlates with another neuropathological hallmark, namely loss of synaptic terminals, possibly caused by soluble Aβ (Lue et al 1999, Mucke et al. 2000; Roselli et al 2005). Further, new studies have shown that small oligomers, the so-called amyloid-derived diffusible ligands (ADDLs), and protofibrils also exert neurotoxic effects; they can rapidly block long-term potentiation (LTP, the electrophysiological correlate of memory) in the hippocampus, increase oxidative stress partly by activating Fyn signaling

pathways, and stimulate GSK3β-mediated phosphorylation of tau. Interestingly, Fyn activity and soluble Aβ levels are concomitantly high in tau tangle-positive neurons of AD brains (for review see Klein et al 2001).

In vivo studies have shown that acute injections of soluble Aβ into the brain can produce short-term amnesia (Flood et al 1991) and acute and transient deficits in learning and memory deficits without impairment of recall of previously consolidated information. In contrast, the long-lasting damaging effects of Aβ (impairment of synaptic transmission, plasticity and memory) seem to require an aggregated conformation of Aβ and extended exposure to the peptide (Nakamura et al 2001; Stephan et al 2003).

Of particular importance and relevance to neuronal signaling is the ability of extracellular Aβ to impair calcium homeostasis, and thus, affecting nerve cell excitability by facilitation of membrane depolarisation and Ca^{2+} influx. Aβ can alter Ca^{2+} concentrations by binding to either α7 nicotinic acetylcholine receptors (α7nAChRs) or to L-type voltage-dependent Ca^{2+} channels and, thereby synaptic plasticity and transmission, and LTP and long-term depression (LTD) (Ekinci et al 1999). Aβ can also modulate LTP and LTD and alter dendritic architecture by remodeling the cytoskeleton through its activation of extracellular-signal-regulated kinase/mitogen activated protein kinase (ERK/MAP kinase), (for review see Small et al 2001). Certain stress-associated mitogen-activated protein (MAP) kinases are known to be associated with microglial activation in AD; interestingly, inhibition of p38 MAP kinase and c-Jun N-terminal kinase (JNK) relieves Aβ-induced blockade of LTP, implicating these kinases in the cellular mechanisms of Aβ action (for review see Rowan et al 2004).

Cholinergic, serotonergic and glutamatergic systems are severely damaged in AD patients. Through its effects on both, pre-synaptic activity and post-synaptic activation of nicotinic acetylcholine receptors, Aβ can regulate cholinergic neurotransmission. Aβ can also modulate NMDA (but not AMPA) receptor function in a manner that depends on cell type and Aβ concentrations (for review see Turner et al 2003). Oxidative stress is another mechanism through which Aβ can produce its neurotoxic effects. The peptide stimulates

production of free radicals and inflammatory mediators through aberrant microglial activation and astrocyte-mediated stimulation of nitric oxide (NO) generation, subsequently resulting in neuronal cell death (Weldon et al 1998).

Numerous studies have shown that exogenous administration of Aβ has toxic effects in cultured cells of various types; the activation of multiple cellular pathways which can lead to cell death have been reported (for review see Small et al 2001). One interesting finding is that Aβ can induce abnormal phosphorylation of tau protein (Takashima et al 1998) which consequently disrupts the cytoskeleton; these observations provide a link between amyloid peptide and tau pathology.

Because of the apparent ease with which Aβ can oligomerize (under conditions that are not completely defined), the results of most published studies are difficult to interpret because it is not always apparent to what extent soluble or insoluble Aβ might be responsible for neurodegneration and behavioral impairments seen in AD. Nevertheless, one may speculate that some of the paradoxical phenotypes observed in transgenic models of AD (e.g. cognitive impairment in the absence of amyloid plaques) may be explicable in terms of the toxic effects of both soluble and insoluble forms of Aβ. More information on the biological actions of soluble and insoluble forms of Aβ over acute and chronic time frames will be essential for gaining knowledge about the mechanisms, course and consequences of early stage of AD where cell death alone cannot account for the deficits in cognitive performance (Selkoe 2002). It increasingly appears that the build-up of Aβ fibrils is a protracted event, but that diffusible forms of Aβ may already be causing synaptic dysfunction and that other processes (e.g. oxidative stress and inflammation) are only recruited at much later stages of the disease, i.e. synaptic disruption with significant impact on cognition occurs well in advance of full-blown neurodegeneration (for review see Walsh et al 2002).

Neurotrophic effects of Aβ peptide

Despite its above-mentioned potentially deleterious effects, soluble Aβ is a normal, physiological product, detectable in the blood plasma and cerebrospinal fluid of young and old healthy individuals (Seubert et al 1992);

thus, the secretion of soluble Aβ *per* se does not imply pathology, but rather this form of the peptide may have a role in the normal functioning of nerve cells. Kameretz et al (2003) showed that activated healthy neurons secrete Aβ which, in turn, downregulates excitatory synaptic transmission, thus illustrating that Aβ can serve a homeostatic role in the maintenance of neuronal activity. Other data that directly or indirectly support a role of Aβ in the physiological regulation of synaptic plasticity include demonstrations that (i) increases of synaptic APP occur concomitantly with improved learning capacity in rats (Huber et al 1997); (ii) $A\beta_{1-42}$ induces an up-regulation of a synaptic vesicle protein transcript (Hesse et al 2001); (iii) a transient increase of synaptic Aβ occurs after perforant pathway lesions (Lazarov et al 2002); (iv) APP and its regulatory protein, Fe65, are detectable in growth cones and synapses both *in vitro* and *in vivo* (Sabo et al 2003); and (v) neurotransmitters, including acetylcholine (Isacson and Lin 2000), glutamate and serotonin (Nitsch et al 1997) can modulate APP processing.

It should be mentioned here that the nature of Aβ plays an important role as illustrated by the lower protection provided by $A\beta_{1-42}$ *vs.* $A\beta_{1-40}$ and $A\beta_{25-35}$ (Plant et al 2003). Interestingly, the ratio of $A\beta_{1-40}$ *vs.* $A\beta_{1-42}$ changes from 90% to 50% in AD patients (Mehta et al 2001). Moreover, *in vitro* studies have shown that the dose of Aβ is an important factor in determining the neuroprotective *vs.* toxic effects of Aβ. Thus, low doses (10pM to 1nM, similar to the concentrations found in the CSF of normal individuals are clearly neuroprotective against specific neurotoxins (Mehta et al 2001). In this context, it is interesting to note that Aβ reverts from being anti-apoptotic to pro-apoptotic as its concentrations are increased, apparently because of the peptide's physiochemical properties which endow it with the potential to act as either an anti-oxidant and pro-oxidant: when chelated to Cu^{2+} (which catalyzes the dismutation of superoxide [O_2^-] to H_2O_2), Aβ acts as an antioxidant and monomeric Aβ can inhibit the reduction of Fe^{3+} and generation of O_2^- and prevent the lipid peroxidation induced by Fe^{2+}; on the other hand, oligomeric or aggregated Aβ loses its antioxidant activity and reduces Fe^{3+}, subsequently contributing to the oxidative stress and lipid peroxidation – besides causing cell death, the latter can also lead to tau phosphorylation (Gomez-Ramos et al 2003).

1.3.2. Neurofibrillary tangles (NFT)

Besides senile plaques and Aβ deposits, *neurofibrillary tangles* (NFT), whose major component was shown by immuno- and protein chemistry to be tau, a cytoskeletal protein are another characteristic feature of AD. NFT are comprised of highly insoluble paired helical filaments (PHF) (Lee et al 1991) that appear as left-handed double helices and straight filaments (SF); both of them consist of *abnormally hyperphosphorylated* filaments of tau. Being insoluble, NFT cannot be easily proteolyzed *in vivo* and are seen in tissue sections as "ghost" or "tombstone" tangles long after the death of the parental neuron (see Fig. 1.6). There is a relatively good correlation between cognitive performance and the number of NFT (Arriagada et al 1992).

NFT are found in the cell body. Intraneuronal neurofibrillary lesions observed in AD brain include (i) *neuropil threads* (NT) which are widely distributed in neuritic processes, and (ii) *neuritic plaques* (NP) which are argyrophilic dystrophic neurites often associated with extracellular amyloid plaques (Goedert et al 1998). Generally, NP develop at later stages of AD and show an irregular distribution. Both, NFT and NT, can apparently develop in the absence of amyloid deposits, giving rise to a class of neurodegenerative diseases known as "tauopathies" (Sergeant et al 2005).

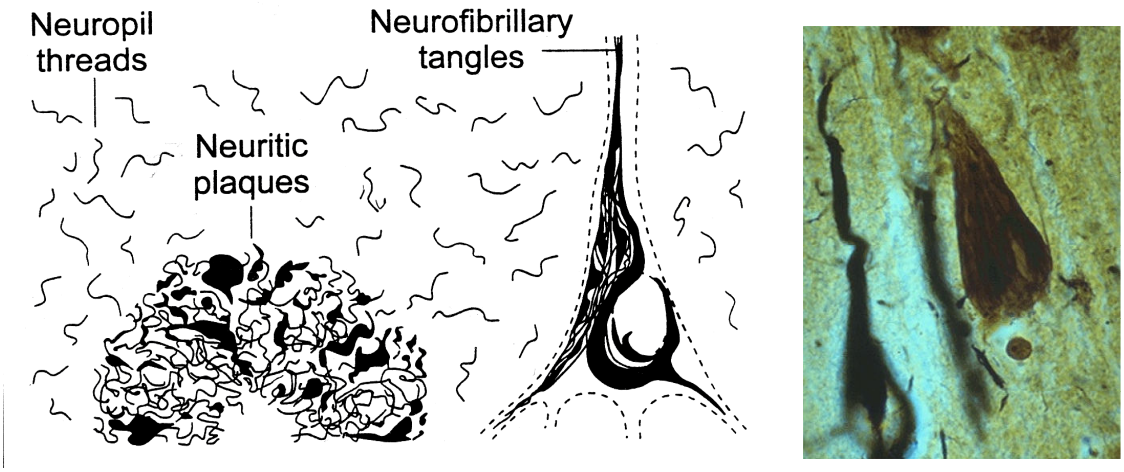

Figure 1.8. Development of neurofibrillary tangles. Neurofibrillary tangles (NFT), neuropil threads (NT) and dystrophic neurites of neuritic plaques (NP) represent different forms of the argyrophylic changes in the neuronal cytoskeleton. NFT are localized primarily in large pyramidal neurons. (from "*Cell Death and Diseases of the Nervous System*, 1999 Humana Press, USA).

1.3.2.1 Tau protein isoforms and their differential expression

Since its discovery in 1975 (Weingarten et al 1975), much has been learnt about tau and its isoforms, and the temporal and spatial distribution and function of the latter in different cell types. Tau is a soluble low M_r microtubule (MT)-associated protein, predominantly expressed in axons of mature and growing neurons. Astrocytes and oligodendrocytes also express tau protein at low levels and tau is found in the cell body, excreances and nucleus of a variety of non-neuronal tissues (Gu et al 1996; Vanier et al 1998).

The human tau gene, which consists of at least 16 exons - of which 8 are alternative spliced forms (Fig. 1.9), is located on chromosome 17q. It generates three transcripts (2, 6 and 9 kb) which are differentially expressed in the nervous system, depending on the neuronal type and state of maturation. The 2 kb transcript encodes tau in the nucleus, and the 6 and 9 kb tau isoforms in the retina and peripheral nervous system, respectively (for review, see Andreadis 2005). As an indirect consequence of alternative splicing of the tau gene and the fact that this process is developmentally regulated, the 352 amino acid residue-long tau isoform (Fig 1.7) is only expressed in fetal rodent and human brain, whereas the 3 isoforms encoded by exon 10 are expressed in adult rodent brain. In the adult human brain, 6 tau isoforms (352 to 441 amino acids in length) are expressed, seen as a series of closely-spaced bands (58-66 kDa) when resolved on SDS-polyacrylamide gels (Takuma et al 2003). Tau isoform expression may be highly specific to particular neuronal subpopulations, e.g. tau mRNA containing exon 10 is not found in granule cells of the dentate gyrus (Goedert et al 1990). In the peripheral nervous system, the inclusion of exon 4a in the N-terminal half results in the additional expression of higher M_r proteins, called 'big tau' (110-125 kDa) (Goedert et al 1992). In view of the work to be presented in this thesis, it is relevant to mention that 'big tau' is found in the rat phaeochromacytoma cell line, PC12. Other tau isoforms (e.g. in muscle) may be generated by the inclusion of exon 6. Interestingly, exon 6 itself can also undergo alternative splicing: in addition to its canonical site, exon 6 has two additional 3' splice sites which, by frameshifting, can generate C-truncated tau molecules (Fig 1.7), e.g. in neuroblastoma cells and in the hippocampus of normal and AD-affected individuals (Luo et al 2004). The

canonical exon 6 is found in fetal human brain and in adult spinal cord and skeletal muscle. Finally, excision of the intron between exons 13 and 14 in mouse fetal brain yields a tau protein that has an extended C-terminus.

Several mutations in the tau gene of patients with frontotemporal dementia and Parkinson's disease (FTDP-17) have been identified. These patients exhibit extensive neuronal loss and tau deposition, indicating that such

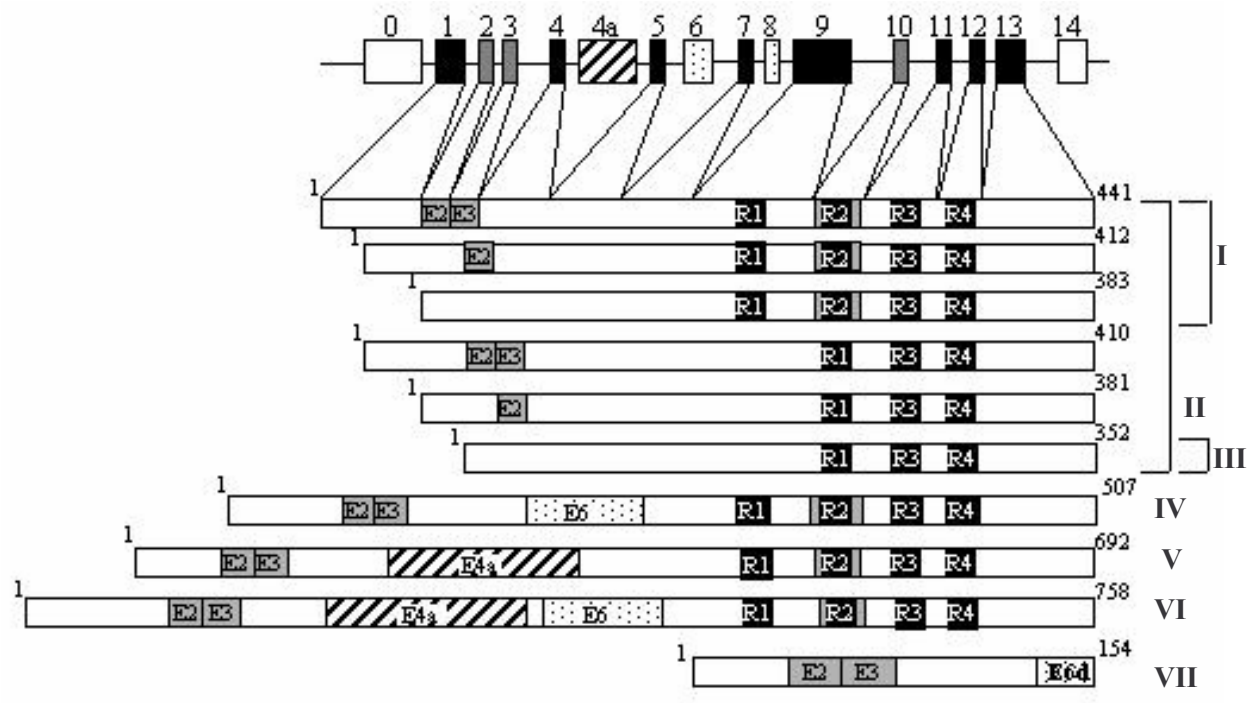

Figure 1.9 Schematic representation of the tau gene, main tau isoforms and their expression in different tissues. Different tau isoforms are generated by alternative mRNA splicing of different exons (E2, E3, E4a, E6, E10). To date, exon 8 has only been identified in bovine tau mRNA. The roman numbers (**I-VII**) on the right indicate the different tissues in which these tau isoforms are expressed: **I**, Adult rodent brain; **II**, Adult human brain; **III**, Fetal rodent and human brain; **IV**, Longest possible isoform with E6; **V**, Longest Peripheral Nervous System isoform; **VI**, Longest muscle isoform; **VII**, Longest possible isoform with E6d (part of E6 – see also text above) (*modified from* M. Forman et al 2000 J Chem Neuroanat 20:225-244 and Andreadis 2005).

mutations can contribute to tauopathy. Most tau mutations are missense, deletion, silent mutations in the coding region of tau gene, or intronic mutations located in the intron following exon 10 (see Fig. 1.7). Briefly, *in vitro* studies have shown that tau mutations in exons 1, 9, 11, 12 and 13 modulate tau-MT binding, thus reducing the ability of tau to promote MT assembly. Other exon 10 and intronic mutations influence tau mRNA splicing, resulting in the expression of different isoforms of soluble tau (for review see Schraen-Maschke et al 2004).

1.3.2.2 Tau function depends on interactions with other proteins

Tau can influence cell morphology during division, growth, differentiation and migration. The generation of *tau* transgenic mice has helped us understand the role of tau in these cellular processes in the brain, especially during development. Mice lacking the *tau* gene (*tau*$^{-/-}$ mice) are viable but show reduced microtubule (MT) density and stability in some small-caliber axons (e.g. cerebellar parallel fibers) (Harada et al 1994), muscle weakness, and learning deficits (Ikegami et al 2000). On the other hand, another *tau*$^{-/-}$ mouse was phenotypically normal, but primary cultures of hippocampal neurons prepared from this mouse exhibited a significant delay in axonal and dendritic development, effects that could be rescued by the expression of exogenous human tau proteins (Dawson et al 2001). Interestingly, in the first *tau*$^{-/-}$ mouse mentioned, expression levels of another microtubule-associated protein (MAP) family member, MAP1A, were increased. On the other hand, when both *tau and MAP1B* were deleted, mice died within 4 weeks of birth, and primary neuronal cultures from these double-KO mice displayed poor axonal elongation (Takei et al 2000), suggesting an essential and synergistic relationship between tau and MAPs in neuronal development.

Many studies have focused on domains in the tau molecule that allow interactions with other proteins. All tau isoforms have 2 major domains, the *projection domain* at the amino-terminal and the *MT-binding domain* at the carboxy-terminal end of the molecule (Fig. 1.10). Several distinct roles have been proposed for the projection domain, including the determination of spacing between axonal microtubules and interactions with other cytoskeletal proteins (e.g. spectrin and actin filaments and, subsequently, neurofilaments). There is also evidence that tau proteins interact with cytoplasmic organelles such as mitochondria and plasma membrane-associated proteins concerned with signal transduction (e.g. Fyn tyrosine kinase [see also section 1.3.1.3.1] and phospholipase.

The carboxy-terminal segment of tau contains 3-4 tandem repeat sequences (31 of 32 amino acids each, encoded by exons 9, 10, 11, 12), which make up

the MT-binding domain (Fig. 1.8). The MT-binding capacity of tau is higher in 4-repeat (4R, with exon 10) isoforms in comparison to 3-repeat (3R, without exon 10) isoforms (Goedert et al 1990), i.e. with those expressed in fetal brain, presumably providing the necessary plasticity for the pronounced cytoskeletal remodelling that occurs during development. The increased MT-binding capacity of 4R-tau results from the inter-region between repeats 1 and 2 (absent in 3R-tau) which shows the greatest potency for inducing MT polymerisation (Goode et al 1994). Tau facilitates tubulin assembly by binding to MT, thus stabilizing polymerised MT, nucleating and orienting MT; in addition, tau inhibits the rate of MT depolymerization, i.e. reduces their dynamic instability. By regulating MT growth and contraction dynamics, tau plays an essential role in establishing neuronal cell polarity and axonal outgrowth during development and for maintaining axonal morphology and axonal transport in mature cells (for review see Shahani et al 2002).

Interestingly, presenilin 1 (PS1, also involved in APP processing and Notch signaling – see Table 1.1) can directly bind to the MT-binding domain of tau; PS1 also binds to GSK3β (see Box 1.1.), a kinase for which tau is a substrate. PS1 is thought to bring tau and GSK3β in close proximity, thus facilitating tau phosphorylation (Takashima et al 1998). Together with the fact that increased phosphorylation levels of tau interfere with the ability of tau to bind effectively to MT (and *vice versa*), recent evidence showing direct and competitive binding on tau between MT and protein phosphatase 2A (PP2A) indicates a role for the MT-binding domain in inhibiting tau phosphorylation (Sontag et al 1999).

1.3.2.3. Phosphorylation regulates tau function

Although tau is subject to several types of post-translational modification, including glycosylation, ubiquitination, glycation and phosphorylation, which influence the protein's structure, function(s) and further cellular processing, phosphorylation has received most attention. Tau pathology is thought to, at least partly, arise from the impaired ability of hyperphosphorylated tau to bind efficiently to MT. It should be noted, however, that phosphorylated tau is found in healthy cells, i.e. its occurrence is not restricted to pathological

states.

In its longest expressed (CNS) form (441 amino acids), tau bears 80 putative serine and threonine residues at which phosphorylation can occur (Fig. 1.8); some 60% of these are associated with neuropathology (Buee et al 2000). While tau dephosphorylation promotes rapid and extensive MT polymerization, phosphorylation at certain sites results in conformational changes of tau and, subsequently, decreased MT binding assembly and stability (Gong et al 2000). Phosphorylation at specific residues (e.g. Ser 262 or 396, located in the repeat domain and in the C-terminal region, respectively) can completely abolish MT-binding *in vitro* (Biernat et al 1993) although other studies have shown that phosphorylation of Ser 262 alone is insufficient to eliminate tau binding to MT (Seubert et al 1995). Furthermore, phosphorylation of sites outside the MT-binding domain (e.g. within the proline-rich region - see Fig. 1.8) results in decreased *de novo* nucleation of MT in cell-free assembly reactions (Brandt et al 1994).

The phosphorylation state of tau seems to influence the protein's distribution in the developing neuron. Tau phosphorylated in its proline-rich region is mainly localized in the somatodendritic compartment, whereas dephosphorylation in this region or phosphorylation in the C-terminal domain of tau is associated with predominant localization in the distal region of the axon (Dotti et al 1987; Mandell et al 1996). A gradient of phosphorylated to dephosphorylated tau can be observed as one moves from the soma to the axon and down to the growth cone. Interestingly, however, tau appears to be strongly associated with MT in the distal axon, close to the growth cone, where MT are less stable and in a dynamic state (Black et al 1996), suggesting that tau in the growing axon may have functions other than simply increasing microtubule stability.

Before reviewing the evidence for links between tau phosphorylation and AD, consideration will be given to the enzymatic mechanisms that contribute to the regulation of tau phosphorylation and dephosphorylation.

1.3.2.4. Key kinases and phosphatases that regulate the phosphorylation state of tau

A large number of protein kinases can phosphorylate tau *in vitro*, sometimes at multiple sites (Shahani et al, 2002; http://www.lille.inserm.fr/u422/TauPhosphoSeq.htm). Most of these kinases are either (i) *proline-directed kinases*, e.g. glycogen synthetase kinase 3β (GSK-3ß or tau protein kinase I), cyclin-dependent kinase 5 (cdk5 or tau protein kinase II), and the mitogen-activated protein kinase (MAPK) family or (ii) *non-proline-directed kinases*, e.g. protein kinase A (PKA), protein kinase C (PKC), calmodulin kinase II (CamKII) and microtubule affinity-regulating (MARK) kinase (Morishima-Kawashima et al 1995). Below, those kinases that have received special attention in the context of AD are described in some detail (see Box 1.1).

Worthy of special mention are the proline-directed kinases, GSK3β and cdk5, which phosphorylate tau at many sites, most of which are common to the two enzymes (Shahani et al 2002). As both enzymes are highly expressed in the brain, they are associated with all stages of neurofibrillary pathology in AD, and although direct correlations between the activity of these enzymes and AD pathology have not been established, the colocalization of one or both kinases with hyperphosphorylated tau and NFT and evidence of their active forms in pretangle neurons of AD brains strongly implicates them in tau pathology (Pei et al 1999).

Besides phosphorylation at serine/threonine-proline motifs, recent studies have shown that tau can also be phosphorylated at tyrosine motifs by Fyn. Fyn also indirectly induces tau phosphorylation through phosphorylation and subsequent activation of GSK3, which, in turn, phosphorylates tau (see also Box 1.1). It is noteworthy that Aβ can activate Fyn as well as several other kinases of which tau is a substrate. It is also important to note that the phosphorylation state of tau depends on the dynamic interplay between kinases and phosphatases. Of particular interest to AD pathology are protein phosphatases 1, 2A [PP1 and PP2A]. The activities of PP-2A and PP-1 are compromised by 20-30% in AD brain (Gong et al 1995). Tau phosphorylation is regulated by PP-2A, but not by PP-2B (Gong et al 2000). In fact, inhibition of PP2A activity by okadaic acid results in abnormal tau phosphorylation at

sites that are found in AD. A transgenic mouse expressing a dominant negative PP2A mutation has been reported to exhibit a 34% reduction in phosphorylation at sites that are found in AD. A transgenic mouse expressing a dominant negative PP2A mutation has been reported to exhibit a 34% reduction of PP2A activity, accompanied by some early aspects of tau pathology, e.g AT8-positive neurons and redistribution of tau into the somatodendritic compartment (Kins et al 2001). Interestingly, PP1 seems to regulate the activities of GSK3β and cdk5, whereas PP2B plays a relatively minor role in tau phosphorylation.

1.3.2.5 Abnormally hyperphosphorylated tau in AD

As mentioned earlier, tau phosphorylation levels are increased in several tauopathies such as AD. At least, 25 phosphorylation sites have been identified in paired helical filaments (PHF-tau) in AD brains (Hanger et al 1998); most of these sites are clustered in the region flanking the MT-binding domain. While many of the phosphorylation sites are common to PHF-tau and native tau, the extent of phosphorylation as well as the number of phosphorylation sites in the affected brain far exceeds that in normal brain. PHF-tau and fetal tau share several phosphorylated sites, but PHF-tau shows a greater degree of phosphorylation than the fetal protein (Kanemaru et al 1992). Fetal tau in the CNS shows more phosphorylation than adult brain tau exhibiting a gradient of tau phosphorylation (Goedert et al 1993). Expectedly, PHF-tau has a strongly reduced capacity for MT-binding as compared to normal tau; dephosphorylation of PHF-tau results in normal MT-binding (Yoshida and Ihara 1993; Hanger et al 1998). A series of antibodies recognizing different phosphorylation sites (phospho-dependent antibodies), conformational epitopes of tau protein (conformation-dependent antibodies), or a combination of both, is now available (see Fig. 1.10).

Box 1.1. Kinases of significance in AD pathology

GSK3β

- Overexpression of GSK3β in cellular and animal models results in tau hyperphosphorylation patterns and reduced tau-MT binding capacity reminiscent of the situation in AD.[1,2]
- GSK3β shows preference for primed substrates, i.e. those previously phosphorylated by another kinase (e.g. cdk5), thus potentiating its own phosphorylation capacity.[7,8]
- Inhibition of GSK3β by LiCl rescues tau hyperphosphorylation and enhances tau association with MT.[3]
- GSK3β-induced phosphorylation attenuates tau degradation.[4]
- Even in the absence of Aβ, over-expression or activation of GSK3β leads to tau hyperphosphorylation (without fibrillar tau) and impairs spatial memory.[5,6]
- Other signalling pathways can activate GSK3β through (i) phosphorylation of Ser9 and Tyr216 (resulting in, respectively, inhibition or stimulation of GSK3β activity), sometimes with the involvement of other tyrosine kinases such as Fyn,[9,10] or (ii) modulation of protein-protein interactions (e.g. in the Wnt pathway). Calcium also regulates GSK3β activity though a calcium-sensitive enzyme, tyrosine kinase 2 (PYK2), which phosphorylates GSK3β at Tyr216.[11] However, it was recently suggested that almost all tyrosine phosphorylation of GSK3β results from auto-phosphorylation, independently of other kinases.[12]
- Aβ can activate GSK3β through Akt, leading to phosphorylation of microtubule–associated proteins such as tau and MAP1B; interestingly, GSK3β promotes Aβ production.[13] Microtubule dynamics may also be modulated through GSK3β-mediated phosphorylation of light chains of kinesin, a motor protein that moves proteins towards the plus-end of microtubules, promoting release of cargo and influencing axonal transport.
- GSK3β activity is modulated by cellular stressors (e.g. heat shock, glutamate, H_2O_2) and various apoptotic stimuli,[14] indicating its role in cell death.[15,16]
- GSK3β regulation *via* protein-protein interactions is well exemplified by the Wnt/β-catenin signaling pathway, thus serving as a link between Aβ and GSK3β. β-catenin has a role in neuronal homeostasis; when directed to synapses it can modulate synaptic strength in response to depolarization.[17,18]
- In familial AD, PS1 proteins form complexes with β-catenin, whose levels are markedly reduced in AD patients carrying PS1 mutations[19,20]; since PS1 also interacts with GSK3β, it may function as a docking-regulator in the interaction between GSK3β and tau.
- The Wnt signaling pathway regulates APP trafficking and processing.[21] This pathway may thus link amyloidogenesis and neurofibrillary changes. Wnt-1 signaling favors non-amyloidogenic processing and is neuroprotective against Aβ-induced toxicity. Lithium, which inhibits GSK3β, mimics many of the actions of Wnt.[22-24]

In conclusion, phosphorylation of a wide range of substrates and triggering signal transduction events that participate in cell death and loss of neuronal and synaptic plasticity,[25] may contribute to GSK3β's role in AD pathology.

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cdk5

- Activity largely restricted to post-mitotic neurons where its activators, p35 and p39 (or their truncated forms p25 and p29, respectively) are located; thought to play an important role in brain development and function.[1]
- p25 levels are elevated in AD brains, facilitating cdk5-induced hyperphosphorylation of tau, [2,3] and cdk5 staining intensity is more intense in pre-tangle neurons and during early stages of NFT formation.[4]

/cont'd

- When complexed with p25, cdk5 phosphorylates tau at sites similar to those that are phosphorylated during mitosis, suggesting similar processes in mitosis and AD pathology.[5]
- Tau, phosphorylated by cdk5 is less potent at promoting MT assembly and expression of cdk5/p25 in primary neuronal cultures results in increased tau phosphorylation, cytoskeletal and MT disruption and apoptosis;[2] mice over-expressing p25 exhibit hyperphosphorylation of tau and neurofilaments.[6]
- cdk5 may also phosphorylate tau through its regulation of other kinases and/or phosphatases. Thus, in a context-dependent manner, cdk5 phosphorylates two inhibitors of protein phosphatase 1, (I1 and I-2) thereby, respectively, activating and inhibiting them.[7,8]
- cdk5 can be phosphorylated by Fyn tyrosine kinase (which also influences GSK3β activity and phosphorylates tau) at Tyr15.[9]
- Glutamate can induce cdk5 activity in a casein kinase-I-dependent fashion.[10]
- m- and μ-calpain-dependent proteoloysis of p35 and p39 into p25 and p29, respectively, serves as a further mechanism which influences cdk5 activity[2,11]. [p25 and p29 appear to be more stable than their precursors]
- cdk5 is involved in neuronal death (necrosis and apoptosis) induced by a range of stimuli, including Aβ. Some of the underlying mechanisms include phosphorylation of NMDA receptors, resulting in increased Ca^{2+} influx and apoptosis, or Ca^{2+}-induced activation of calpain and necrosis.[12,13] Interestingly, calpain stimulates cleavage of p35 to p25, thereby prolonging the activation of cdk5 which, in turn, triggers neuronal death.[14] Further, cdk5 can increase the expression of the proto-oncogene p53, which activates Bax-mediated apoptosis.[15,16]
- APP can be phosphorylated by cdk5 at Thr668, thus directing APP away from the soma into the axon.[17]
- Together with GSK3β, cdk5 is implicated in Aβ-induced increases in tau phosphorylation and p53-mediated apoptosis.[18]
- A cytoprotective role for cdk5 is attested to by its ability to phosphorylate and inhibit the apoptotic actions of Jun N-terminal kinase 3 (JNK3) by enhancing PI3K/Akt activity.[19]
- cdk5 regulates other kinase signal transduction pathways; for example, it downregulates the activity of mitogen-activated kinase (MAP) kinase 1 (MEK-1), the upstream modulator of the extracellular signal-regulated kinases 1/ 2 (ERK1/2).[20]

References: [1]Shelton S et al 2004 J Neuroch 88: 1313-1326; [2]Patrick G et al 1999 Nature 402: 615-622; [3]Tandon A et al 2003 J Neuroch 86: 572-581; [4]Augustinack J et al 2002 J Neuropath Exp Neurol 61: 557-564; [5]Hamdane M et al 2003 JBC 278(36): 34026-34034; [6]Ahlijanian M et al PNAS 2000 97: 2910-2915; [7]Huang et al 2000 PNAS 97: 5824-5829; [8]Agarwal-Mawal A et al 2001 JBC 276: 23712-23718; [9]Sasaki Y et al 2002 Neuron 35: 907-920; [10]Liu F et al 2002 JBC 277: 45393-45399; [11]Patzke H et al 2002 JBC 177: 8054-8060; [12]Li B et al 2001 PNAS 98: 12742-12747; [13]Wang J et al 2003 Nat Neurosc 6: 1039-1047; [14]Gong X et al 2003 Neuron 38: 33-46; [15]Morrison R et al 2003 Neuroch Res 28: 15-27; [16]Weishaupt et al 2003 Mol Cell Neurosc 24: 489-502; [17]Iijima K et al 2000 J Neuroch 75: 1085-1091; [18]Ferreira A et al 1997 Mol Cell Neurosc 9: 220-234; [19]Zhang Y et al 2002 JBC 156: 519-529; [20]Tanaka T et al 2001 J Neurosc 21: 550-558.

MAPK family members

- Signaling cascades involve at least 3 kinases that act sequentially, e.g. MAPK kinase kinase → MAPK kinase → MAPK; A family of proline-directed protein kinases that allow propagation of extracellular signals to intracellular targets.
- Comprises ERK1/2, the JNKs, and p38. The JNKs and p38 are also referred to as stress-activated protein kinases (SARK).

(i) ERK1/2

- Activated ERK (and its upstream activators, e.g. Ras) are increased in AD brains, in neurons showing hyperhphosphorylated tau and early tau deposition[1] (but see also ref. 2).
- Tau is an ERK substrate;[3] inhibition of ERK attenuates the cytotoxicity induced by a number of agents, including Aβ.[4,5] On the other hand, activated ERK is reportedly cytoprotective and may mediate the actions of sAPPα.[6,7]
- ERK pathway influences APP production and secretion. Activation of ERK by numerous stimuli, (e.g. sex steroids) leads to increased production of neuroprotective sAPPα[8,9] which, by activating ERK (in a feedback loop), can phosphorylate tau.[10, 11]
- ERK mediates Aβ-induced tau hyperphosphorylation, resulting in altered hippocampal dendritic architecture, synaptic plasticity and LTP.[12-15]
- Expression of both wildtype *APP* and mutated (FAD-type) *APP* activates a range of MAPK.[16,17]
- The above observations show that activated ERK can either exacerbate or improve the progress of AD pathology; ERKs can synergize with activated JNKs (increased in Braak stages I/ II).[18]

(ii) JNK
- JNKs phosphorylate various transcription factors (e.g. p53) and cytoplasmic substrates such as tau.[19,20]
- A number of upstream activators (e.g. Cdc42/Rac1) and downstream effectors (e.g. c-Jun) of the JNK cascade are activated and/or elevated in AD brains, associating with neurofibrillary lesions and degenerating neurons.[21,22]
- Activated JNKs are implicated in the mediation of apoptosis induced by various stimuli, including Aβ [23]
- JNK can regulate APP metabolism through phosphorylation at Thr668 [21, 24]. In the presence of lipid peroxidation products, JNK and p38 can up-regulate expression of BACE-1 (involved in β-secretase clevage of APP and Aβ production).[25]

(iii) p38
- p38 pathway is activated by various extracellular stimuli[23].
- Absolute and activated levels of p38 are elevated in AD brains, in association with neurofibrillary pathology and senile plaques.[26]
- A role of p38 in LTP has been suggested.[27]

References: [1]Perry G et al 1999 Neuroreport 10: 2411-2415; [2]Lovestone S et al 1994 Curr Biol 4: 1077-1086; [3]Latimer D et al 1995 FEBS Lett 365: 42-46; [4]Murray B et al 1998 PNAS 95: 11975-11980; [5]Seo S et al 2001 J Neuroch 78: 600-610; [6]Guyton K et al 1996 JBC 271(8) 4138-4142; [7]Cheng G et al 2002 Exp Neurol 175: 407-414; [8]Manthey D et al 2001 Eur J Biochem 268: 4285-4291; [9]Goodenough S et al Neurosc lett 296: 49-52; [10]Greenberg et al 1994 PNAS 91: 7104-7108; [11]Cheng G et al 2002 Exper Neurol 175: 407-414; [12]Sato et la 1997 Bioch Biophys Res Commun 232: 637-642; [13]Ferreira A et al 1997 Mol Cell Neurosc 9:220-234; [14]Sweat J et al 2004 Curr Opin Neurob 14: 1-7; [15]Zhu X et al 2002 Neurosignals 11: 270-281; [16]Murayama Y et al 1996 Gerontology 42 Suppl 1:2-11; [17]Grant S et al 1999 Brain Res Mol Brain Res 72: 115-120; [18]Rowan M et al 2004 Exp Geront; [19]Mielke K et al 2000 Progr Neurob 61: 45-60; [20]Reynolds C et al 1997 J Neuroch 68: 1736-1744; [21]Standen C et al 2001 J Neuroch 76: 316-320; [22]Matsuda S et al 2001 J Neurosc 21: 6597-6607; [23]Harper S et al 2001 Cell Signal 13:299-310; [24]Mudher A et al 2001 J Neurosc 21:4987-4995; [25]Tamagno E et al 2005 J Neuroch 92: 628-636; [26]Ferrer I 2001 J Neural Transm 108:1397-1415; [27]Coogan A et al 1999 Neurosc 93: 57-69.

The progression of tau aggregation to NFT formation can be histopathologically divided into three stages, i) pre-tangle stage, ii) mature tangle stage, and iii) the co-called "ghost" tangle stage (see section 1.3.2 & Fig 1.6). The cellular events believed to lead to NFT formation include hyperphosphorylation and conformational changes in tau protein which result in reduced affinity of tau for MT; these changes lead to disruption of the neuronal cytoskeleton and polymerization of hyperphosphorylated tau in the form of PHF in neuronal the perikarya (NFT) and their dendritic processes (seen as neuropil threads) (Morishima-Kawashima et al 1995). PHF can be formed from all six known isoforms of human CNS tau, as well as some truncated forms; the C-terminal portion, which contains the MT-binding domain, which forms the core of the C-terminal portion and which contains the MT-binding domain, forms the core of detergent-insoluble PHF (Lee et al 1991).

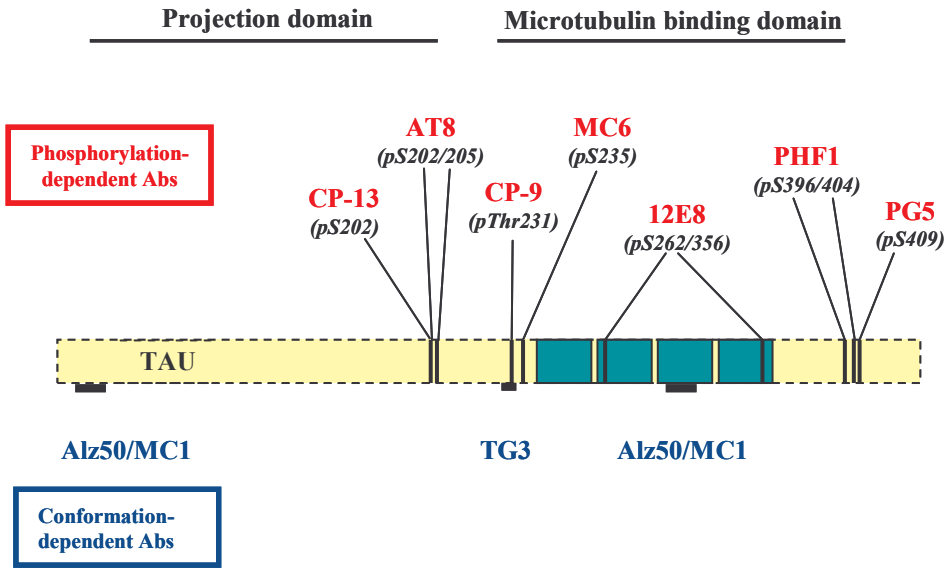


Fig. 1.10 Schematic drawing of Tau protein showing some threonine (T) or serine (S) residues that are phosphorylated; some antibodies that recognize specific phosphorylation-tau as well as conformation-dependent epitopes are shown (drawn by I. Sotiropoulos).

Specifically, two hexapeptide motifs in the second and third repeat of tau are thought to be crucial for conformational changes from a mostly random coil to a β-sheet structure, promoting tau self-aggregation and PHF formation; these features explain why conformationally-altered forms of tau, even as oligomeric fibrils, are resistant to proteolytic digestion and bind thioflavin-S (Von Bergen et al 2005). Recently, an α-helical form of tau was reported in *ex vivo* PHF and in immunopurified PHF (Barghorn et al 2004); these findings raise new questions about the relative roles of β-sheet- and α-helix-mediated tau aggregation NFT formation.

PHF-tau is thought to exert a neurotoxic function by interfering with MT stability and assembly, thereby compromising defective dendritic plasticity and axonal transport (Salehi et al 2003). In addition, studies involving over-expression of human tau indicated that PHF-tau can affect normal cell metabolism by disrupting intracellular compartments (Spittaels et al 1999). Interestingly, however, NFT-bearing neurons appear to survive for decades (Morsch et al 1999) and microtubule reduction in AD occurs independently of tau filament and NFT formation (Cash et al 2003). Moreover, correlations between (i) age-at-death and hippocampal neuron number or the amount of NFT and (ii) apoptotic morphology and DNA fragmentation and tau deposition (Broe et al 2001) do not exist. These observations, together with the fact NFT-

bearing neurons undergo oxidative stress, suggest that NFT may not be obligatory for neuronal death in AD and that, rather than triggering neuronal degeneration, NFT may elicit oxidative stress (Lee et al 2005).

Interestingly, tau hyperphosphorylation can also result in neurotoxicity despite an absence of filaments: by mimicking the structural/functional properties of hyperphosphorylated tau with high-stoichiometric tau phosphorylation (pseudo-phosphorylated tau), Fath et al (2004) demonstrated the onset of apoptosis in the absence of tau aggregates. Furthermore, Wittmann et al (2001) and Jackson et al (2002) demonstrated degeneration and cell death in *Drosophila* and in various cell lines simply after overexpressing tau (without filament formation), apparently by interfering with organelle and vesicular trafficking and therefore, cellular homeostasis. Importantly, tau depletion was found to abrogate Aβ-induced neurodegeneration, indicating that the availability of tau may prime cells for vulnerability to Aβ (Rapoport et al 2002).

1.4. Factors predisposing to AD

As AD is a multifactorial disease, the investigation of risk factors and their influence on different parameters of AD pathology is a very promising research field with great potential for identifying new treatment targets. Besides aging, which represents the highest risk factor for developing AD, its accompanying systemic (e.g. diabetes, hyperinsulinaemia, hypothyroidism, hypertension, hypercholesterolaemia, obesity and cardiovascular disease) and brain disorders are also thought to represent significant risks. In addition, (i) genetics, (ii) gender and sex hormones, and (iii) stress and corticosteroids have more recently been recognized as vulnerability factors.

1.4.1. Genetic mutations

Mutations in 3 genes, *amyloid precursor protein* (*APP*), *presenilin 1* (*PSEN1*) and *presenilin 2* (*PSEN2*) genes, have been causally linked to early-onset familial AD (FAD) (Lendon et al 1997). However, Mendelian inheritance of the disease accounts for only 5-7% of all AD cases (Shastry et al 1999), indicating

that the majority of cases may have a complex etiology due to environmental and genetic factors which are individually insufficient to lead to pathology. The *apolipoprotein E* (*APOE*) gene is now recognized to be involved in sporadic late-onset cases of AD, but only <50% of non-FAD cases are carriers of the *ApoE ε4* allele, the variant associated with AD (Corder et al 1993); it therefore seems likely that polymorphisms in other susceptibility genes must be involved in the pathogenesis of AD (Shastry et al 1999) and synergistic effects between different susceptibility genes cannot be ruled out.

1.4.2. Gender and sex hormones

The role of gender and sex hormones has been intensively studied in the last decade since women seem to be at a greater risk for dementia, including AD, as compared to men; this gender divergence becomes more pronounced beyond the age of 85 (Fratiglioni et al 2000). The most likely basis underlying these differences is the differing sex hormone profiles in men and women but particularly, the post-menopausal loss of estrogens in women (Monk and Brodaty 2000).

Both, aged animals and animals subjected to different lesion models, respond to treatment with estradiol with improved neuronal survival and recovery (Wise et al 2000; Garcia-Segura et al 2001). Supporting a neuroprotective role of estrogen, it has been observed that rodent ovariectomy increases Aβ levels in brain, an effect that can be partly reversed with either low-dose or high-dose estradiol replacement therapy (Petanceska et al 2000). Other studies have shown that estradiol decreases Aβ production *in vitro* (Xu et al 1998) and in both normal mice and transgenic mouse models of AD (Zhang et al 2001). Further, estrogens stimulate neurogenesis within the adult hippocampal formation (Gould et al 2000) and display antioxidant properties (Behl et al 1995).

Many studies report that estrogens can preserve and even restore cognitive functions in older women and reduce a woman's risk of developing SDAT/AD (Henderson 1994; Seeman et al 1997)**.** However, population-based studies indicate that, besides increasing the risk of gynecological cancers (Archer 2004), estrogens might actually be detrimental to cognitive performance

insofar that they accelerate cognitive decline, increase risk of dementia and stroke, and lead to hippocampal atrophy (Den Heijer 2003) Moreover, the issue of estrogen supplements for menopausal women has become a highly-charged topic with a recent Women's Health Initiative Consensus Statement highlighting potential risks of exposing menopausal women to estrogen-therapy (Turgeon et al 2004).

The importance of androgens in the causality or prevention of AD is unclear at present. A number of authors have correlated lower circulating testosterone levels in AD vs. non-demented individuals (Hogervorst et al 2001), suggesting that androgen depletion in males may promote AD pathogenesis; moreover, there is some evidence that testosterone may facilitate cognitive function (Barret-Conner et al 1999). Since testosterone can be 5α-reduced to the pure androgen dihydrotestosterone (DHT) or, in the brain aromatized to estradiol, an immediate question that arises is whether androgens directly account for the beneficial effects observed, or whether their effects occur secondarily to conversion to estrogen. In fact, while testosterone can promote the non-amyloidogenic processing of amyloid precursor protein (APP) in cultured neurons (Gouras et al 2000), subsequent experiments ascribed this effect to estrogen since aromatase inhibitors abolished the protective effects of testosterone (Goodenough et al 2000). With respect to sex steroid effects on tau, testosterone can, independently of its aromatization to estradiol, prevent tau hyperphosphorylation by modulating the activity of GSK3β (Papasozomenos and Shanavas 2002).

1.4.3. Stress and stress hormones

1.4.3.1. Physiology of stress: Living organisms need to continuously maintain the integrity of their 'internal environment' (or *milieu*) using homeostatic mechanisms. Stress may be defined as a disruption of homeostasis following the impact of internal or external challenges ('stressors'). The stress response represents a process of 'maintaining stability through change,' also referred to as allostasis, a process involving the activation of the autonomic, neuroendocrine and immune systems. These systems, through their physiological mediators such as adrenalin from adrenal medulla, glucocorticoids from the adrenal cortex and cytokines from immune

cells, act at various tissues to produce the 'adaptive response.' The stress response (and adaptations to it) is governed by neural circuits in the brain; the brain regions especially involved are the cortex, hippocampus and the locus coeruleus (Fig. 1.11). Here, particular focus will be placed on the corticosteroids (adrenocortical hormones) secreted from the adrenal glands since the work in this thesis is concerned with their role in AD.

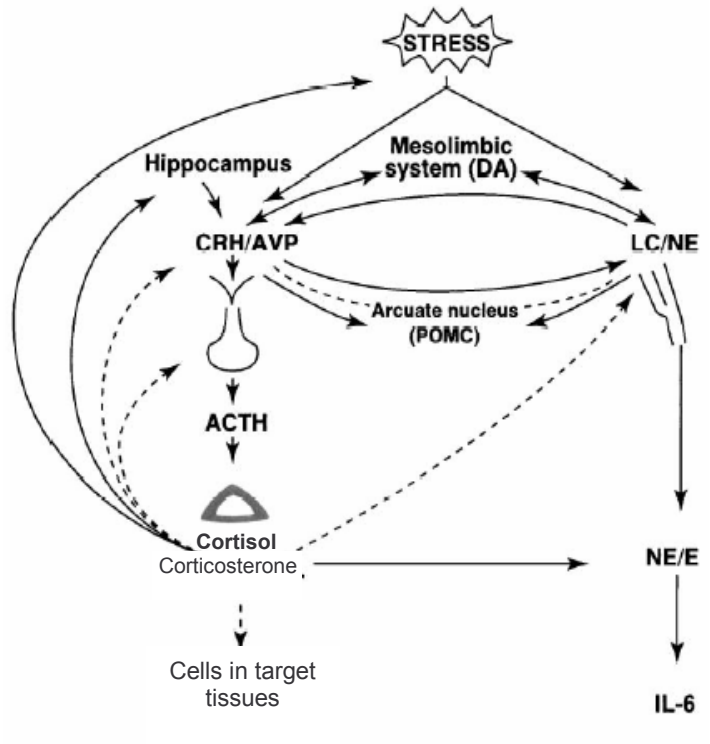


Fig. 1.11 A simplified schematic representation of the neural and endocrine regulation of the stress response. *(adapted from Tsigos & Chrousos, 2002: J Psychos Res 53: 865-871*).
Abbreviations:DA, dopamine; CRH, corticotropin releasing hormone; AVP, arginine vasopressin; LC, locus coeueleus; NE, norepinephrine; POMC, pro-opiomelanocortin (precursor of ACTH; ACTH, adrenocorticotropin; IL-6, interleukin 6.

1.4.3.2 HPA axis, corticosteroids, and their mechanisms of action:

The major glucocorticoids in humans and rodents are cortisol and corticosterone, respectively. During stress, glucocorticoids stimulate gluconeogenesis and supply energy to challenged tissues, as well as suppress the immune response and other functions that would be interfere with the adaptive response, e.g. reproduction. As shown in Fig. 1.12, homeostasis in the HPA axis is restored when glucocorticoids feed back upon the pituitary and brain to curtail their own secretion (Pariante et al 2004). This negative feedback loop is crucial since chronic hypersecretion of adrenocortical hormones can have deleterious effects, e.g. excessive loss of body mass, hypo-immunity, induction of diabetes. As will be discussed later, chronic exposure of the brain to corticosteroids also compromises neuronal birth, survival and plasticity and impairs mood, emotional control and cognitive

functions such as learning and memory (Sousa and Almeida 2002). The physico-chemical nature of glucocorticoids (small, lipophilic molecules that can easily penetrate the plasma membrane as well as the blood-brain barrier) and their ability to regulate neuronal excitability account for some of these effects.

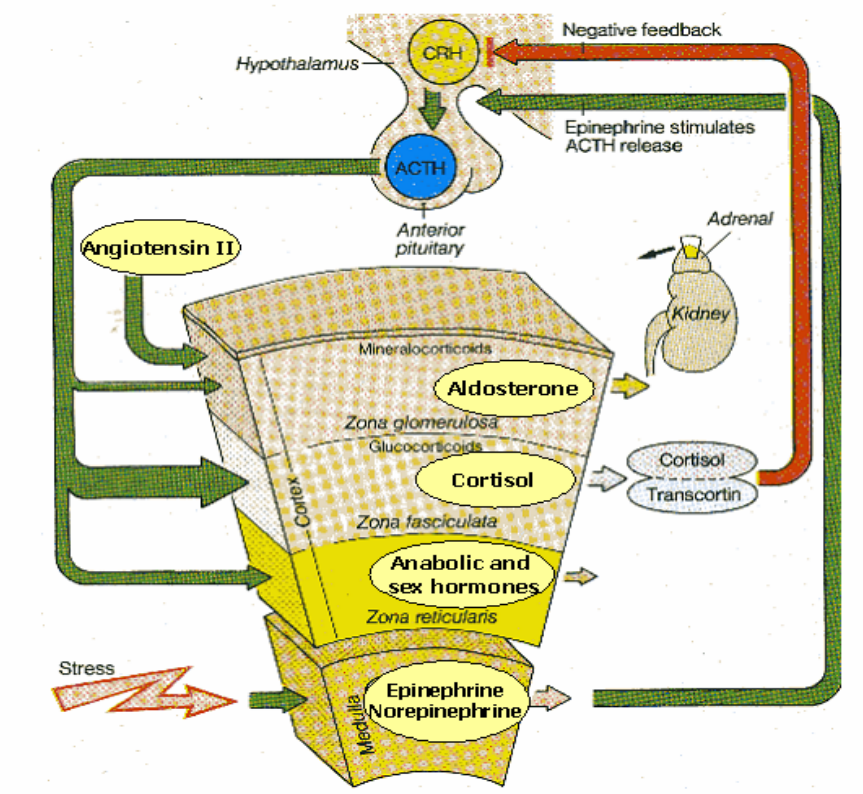


Fig. 1.12. Organization of the hypothalamic-pituitary-adrenal (HPA) axis Stressful stimuli trigger the cascade release of hormones: corticotropin releasing hormone (CRH) and vasopressin (AVP) from the paraventricular nucleus (hypothalamic PVN) are secreted into the pituitary portal vessel system, activate the synthesis of proopiomelanocortin (POMC) from which adreno-corticotropin hormone (ACTH) is derived. ACTH secretion into the bloodstream stimulates gluco-corticoid synthesis and secretion from the *zona fasciculata* and *reticularis* of the adrenal cortex. *(adapted from A. Despotopoulos & S. Silbernagl, "Color Atlas of Physiology" (Georg Thieme Verlag, DE).*

Corticosteroids are synthesized from cholesterol (Fig. 1.13), bound to carrier proteins and transported to their target cells; they exert their biological effects by binding to and activating one or two nuclear hormone receptors: the mineralocorticoid (MR) and glucocorticoid (GR) receptors (Fig. 1.14). GR are expressed ubiquitously throughout the brain (with highest levels in the hippocampus), while MR expression is restricted to the hippocampus, hypothalamus and septum (Fig 1.14); in some cases, MR and GR may be co-localized in individual cells (van Steensel et al 1996). Of particular relevance to the work presented in this thesis, GR are found in several brain areas that are relevant to cognition, namely the hippocampus, the amygdala and the prefrontal cortex. The hippocampus is especially important for declarative or spatial memory (Eichenbaum et al 1999) while the amygdala is critical for emotional memory (LeDoux 2000) as well as for the emotional modulation of

other types of memory (McGaugh and Roozendaal 2002). Prefrontal cortex is critical for working memory (this term refers to active, short-term storage) (Baddeley 2001).

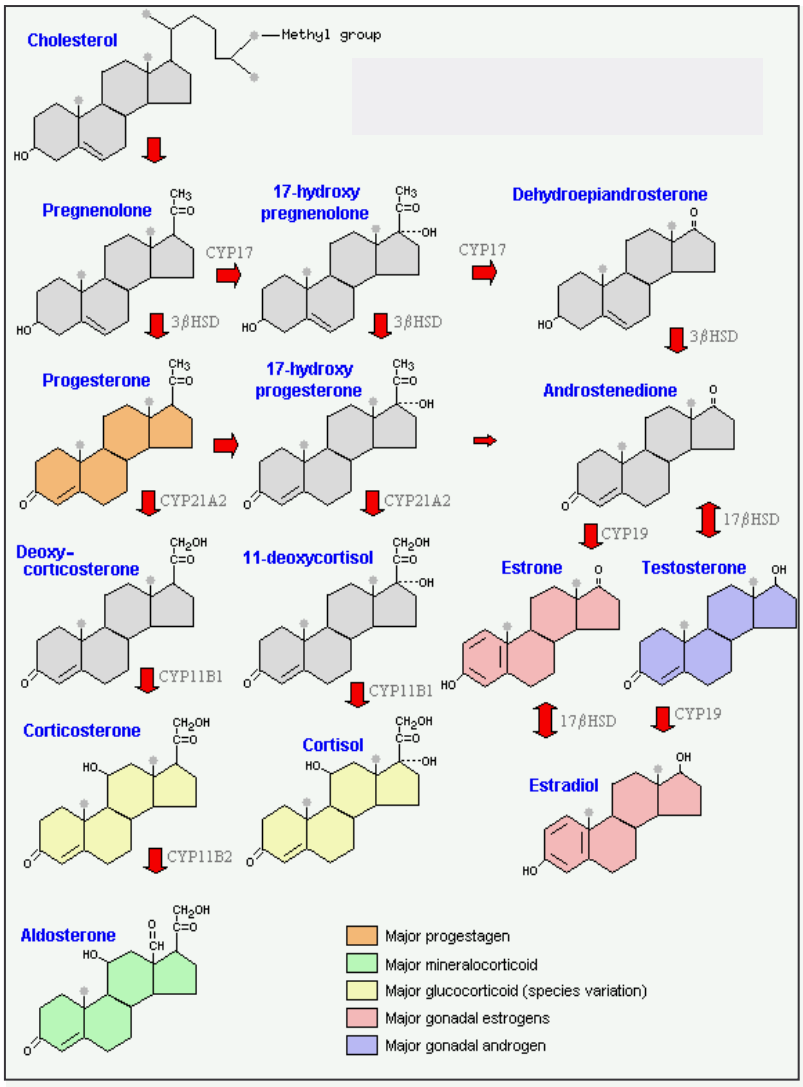


Fig. 1.13 The major steroidogenic pathways in mammals. *(illustration from: http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/basics/steroidogenesis.html).*

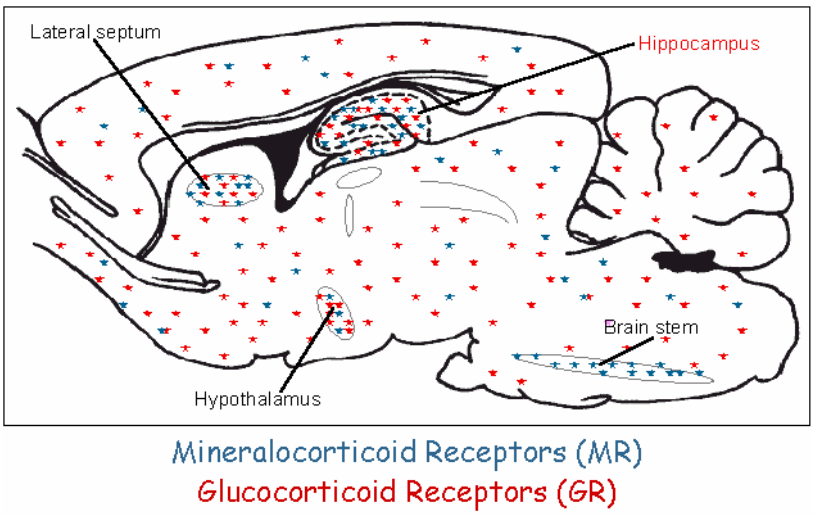
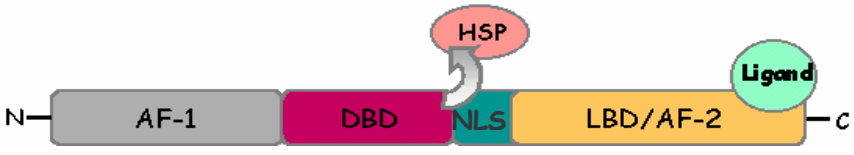


Fig. 1.14 Schematic drawing of the rat brain in sagittal section showing the distribution of MR and GR. The map is based on data obtained by immuno-cytochemical methods and *in situ* hybridization histochemistry. Note the high density of both receptors in the hippocampus and that, as compared to GR, MR show a more selective pattern of expression in other brain areas. *(Unpublished drawing by O.F.X. Almeida, G. Condé & P. von Rosenstiel).*

Corticosterone and cortisol are the prototypic endogenous ligands of the GR, although they actually show a higher affinity for the MR (Reul et al 2000). This is interesting, because the prototypic MR agonist, aldosterone, is not produced in sufficiently high concentrations to reach the brain. Therefore, central MR and GR are both occupied by corticosterone (or cortisol in humans). Under basal conditions, glucocorticoid (GC) secretion exhibits a 24-hour circadian profile, with peak of secretion occuring during the early morning in humans and early evening in rodents. During the circadian trough, the endogenous hormone occupies more than 90% of MR and only 10% of GR. However, during stress and/or circadian peak of GC secretion, MRs are saturated and GR become increasingly occupied (Reul and de Kloet 1985). The study of the specific biological actions mediated by MR and GR is facilitated by the availability of synthetic agonists and antagonists of the respective receptors. Dexamethasone (DEX) and RU28362 may be considered highly selective GR agonist, while RU38486 (mifepristone) is one of the best GR antagonists.

Both, GR and MR are ligand-induced transcription factors belonging to the steroid/thyroid receptor superfamily (Kalman and Spencer 2002). Like other members of the same superfamily of intracellular receptors, these receptors are modular proteins with distinct functional and structural domains (for details, see Box 1.2). Upon hormone (corticosteroid) binding, the receptor-ligand complex translocates from cytoplasm to nucleus where it regulates the transcription of many genes (see Fig 1.15).

Box 1.2 Domain structure and action of the steroid/thyroid receptor superfamily



The receptor consists of a DNA-binding domain (DBD), a ligand-binding domain (LBD), nuclear localization signal (NLS) and two transcription-activation functions (AF1 and AF2). The DBD includes two DNA-binding zinc fingers, which participate in receptor dimerization, nuclear translocation, and transactivation (AF2). The LBD is composed of α-helices and β-sheets that form a hydrophobic pocket and is also crucial for receptor dimerization, heat shock protein association and hormone-dependent transactivation. The N-terminal part contains the AF1 trans-activation domain and interacts with components of the basal transcription machinery and/or with other transcription factors. When inactive (ligand-free), these receptors reside predominantly in the cytoplasm, being tethered by chaperones, e.g. heat shock proteins hsp40, hsp70 and hsp90) and co-chaperons (e.g. p23 and p60). The chaperone molecules are believed to maintain the receptor in a high affinity ligand-receptive state. Ligand binding results in hyperphosphorylation and translocation of the receptor to the nucleus where it initiates gene transcription after binding to specific DNA sequences (glucocorticoid response elements, GREs) and/or a variety of other proteins (e.g. sequence-specific DNA-binding transcription factors, transcriptional coactivators/corepressors, and integrators of transcription (for review see Glass and Rosenfeld 2000) (*unpublished drawing by O.F.X. Almeida).*

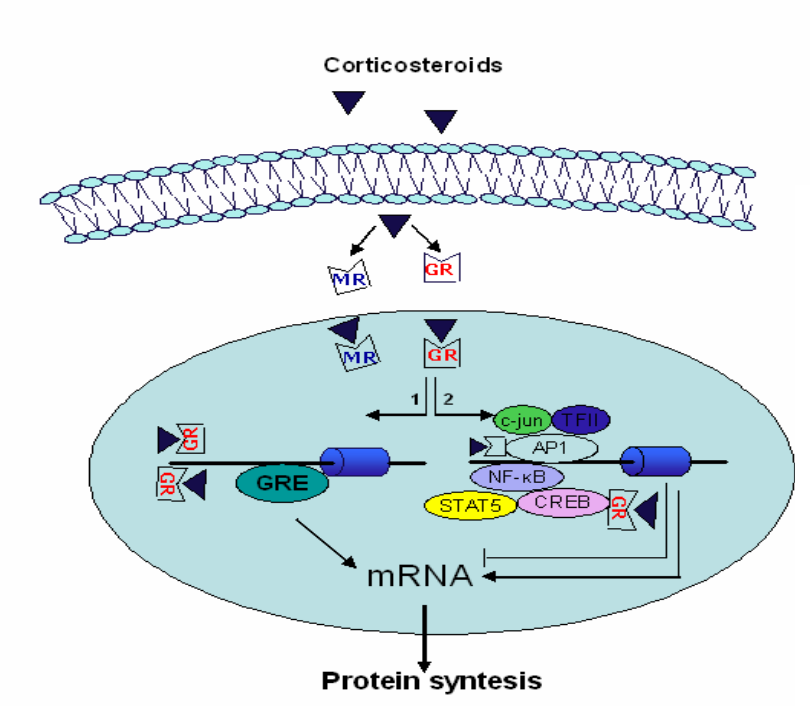


Fig. 1.15. Nuclear receptor-mediated mechanisms of corticosteroid action. Corticosteroids arriving via the plasma membrane bind to, and activate, cytoplasmic MR and GR which are subsequently translocated to the nucleus where they exert their transcriptional effects through one of two mechanisms: (**a**) ligand-activated MR or GR bind to specific DNA sequences GRE) in the promoter region of target genes; or (**b**) the activated receptor interacts with other transcription factors, such as c-jun, activator protein 1 (AP1), nuclear factor-κB (NF-κB), the TFIID (transcription factor IID) complex, signal transducer and activator of transcription 5 (STAT5), or the cAMP response element binding protein (CREB).

1.4.3.3 Role of corticosteroids in the brain: The high concentrations of MR and GR in the hippocampus suggest that these receptors may have an important role in the development, structural integrity and function of this brain region. The hippocampus, more correctly referred to as the 'hippocampal formation,' is a morphologically well-defined structure folded into the interior of the cerebral cortex. It comprises the Ammon's horn or hippocampus proper (CA3, CA2, and CA1 pyramidal cell regions) and the dentate gyrus, with the entorhinal cortex providing the major source of cortical input via the so-called perforant pathway to the granule cells of the dentate gyrus; the perforant pathway also innervates distal dendrites of the CA3 pyramidal cells which, in turn, project to the CA1 region via the Schaffer collaterals.

As already mentioned, the hippocampus is crucial in memory storage and retrieval. Under normal conditions, when MR are predominantly occupied, long-term potentiation (LTP, the electrophysiological correlate of memory formation), is facilitated in the hippocampus; in contrast, when GR are occupied, hippocampal LTP is inhibited and long-term depression (LTD) is induced (Joels 2001). While MR influences interpretation of environmental information and selection of the appropriate behavioral response to cope with the challenge, GR participate in the consolidation of newly acquired

information (for review see Conrad et al 1999). In fact, there appears to be an "inverted U-shaped relationship" between the occupation of brain receptors for corticosteroids and memory performance (Roozendaal et al 2000). Importantly, reversal of hypercortisolemic states has been shown to be followed by recovery of hippocampal atrophy and an improvement of cognitive functioning (Sousa et al 2000; Starkman et al 2003; Sousa and Almeida 2002). Studies in rats and monkeys have shown that repeated stress or excessive exposure to high glucocorticoid levels causes dendritic atrophy, synaptic loss and, in extreme cases, cell death among certain hippocampal neuronal populations (Conrad et al 1999; Sousa and Almeida 2002). In humans, a correlation between cortisol levels, impaired cognition and reduced hippocampal volumes has been described, with the impairments of cognition being reversible upon reductions in cortisol secretion (Lupien et al 1998, Starkman et al 1999). It is also interesting to note that glucocorticoid actions are not limited to the hippocampus. For example, stress enhances dopaminergic activity and increases extracellular glutamate levels (Arnsten and Goldman-Rakic 1998) in the prefrontal cortex, an area recently shown to suffer neuronal loss following GR activation (Cerqueira et al 2005a and b). The latter study also showed this treatment to compromise behavioral flexibility and working memory; the effects of MR activation on these parameters were markedly less pronounced.

In contrast to their deleterious effects, depletion of corticosteroids by adrenalectomy (ADX) results in massive apoptosis in the dentate gyrus (Sloviter et al 1993), demonstrating that low, MR-activating, corticosteroid levels are essential to maintain the structural integrity of this brain region, and ascribing a neuroprotective role to this receptor (Almeida et al 2000; Sousa and Almeida 2002, Crochemore et al 2005). The latter view is supported by studies showing that MR agonists can attenuate GR-mediated cell death (Hassan et al 1996; Crochemore et al 2005). Interestingly, in light of the above-mentioned glucocorticoid-induced changes in prefrontal cortical structure and function, ADX did not have any significant effect on this brain area (Cerqueira et al 2005).

The mechanisms through which glucocorticoids affect the structural integrity of the hippocampus remain enigmatic, but seem to hinge on the maintenance of a

certain balance between MR and GR occupancy (see Almeida et al 2000; Sousa and Almeida 2002) with manifestations on the regulation of the proto-oncogene and cell cycle-related protein p53 and pro- and anti-apoptotic members of the BCl-2 family (Crochemore et al 2002; Almeida et al 2000). In addition, it is important to note that glucocorticoids appear to render neurons more vulnerable to a variety of neuro-endangering stimuli, including glutamate (Armanini et al 1990), free radicals and Aβ (Behl 1998).

Stress and corticosteroids also influence hippocampal structure, and possibly function, at another level. They significantly reduce the number of newly generated neurons (neurogenesis) in the dentate gyrus (Gould et al 1999; Wong and Herbert 2005) and modulate synaptic spine density in the CA1 region (Shors 2004), while depletion of corticosteroids by adrenalectomy (ADX) represents a potent stimulus for neurogenesis (Fischer et al 2002). The anti-proliferative effects of elevated glucocorticoids can be attenuated by drugs that reduce excitatory amino acid transmission or enhance serotonin transmission indicating cross-talk between corticosteroids and neurotransmitter systems in the determination of neuronal cell fate (Sherwood et al 1999). Nevertheless, the significance of these alterations in neurogenesis for cognition and other behavioral and physiological functions remains unclear (for review see Shors 2004).

1.4.3.4. HPA axis and AD brain pathology: The above brief review of evidence linking glucocorticoids with impaired cognition clearly forms a good basis for trying to evaluate the role of glucocorticoids in the etiology and progress of AD. Clinical studies indicate that many aged subjects have a compromised ability to cope with the allostatic load induced by stress, and are therefore potentially at risk for suffering from the undesired effects of excess exposure to glucocorticoids as previously mentioned. Inappropriate responses to stress are seen in the form of exaggerated and prolonged cortisol secretion (Starkman et al 1999; Jerningan et al 2001), indicating impaired negative feedback at the central neural mechanisms that regulate the activity of the HPA axis (Nasman et al 1995). This is also reflected in a sub-population of subjects that show elevated basal levels of cortisol secretion and a flattening of the amplitude of the daily rhythm of cortisol production (Van Cauter et al

1996; Giubilei et al 2001). In fact, elevated cortisol levels have been reported in AD/SDAT patients (Umegaki et al 2000; Rasmuson et al 2001, 2002; Elgh et al 2006). These observations strengthen the hypothesis that glucocorticoids may have a role in the disease especially in light of the demonstration of reduced glucocorticoid negative feedback inhibition in AD/SDAT subjects (Ferrier et al 1988) and reports that the severity of behavioral problems in patients correlates positively with cortisol levels (Lawlor et al 1992).

At least in aged animals there is a marked down-regulation of MR and GR (Hassan et al 1999), findings, which might explain trends towards higher basal and stress-induced corticosterone secretion (Reul et al 1991). This downregulation of feedback efficacy means that a vicious circle is set in place insofar that the individual shows an increasingly poor capacity to shut off its stress response over time (Goul and Tanapat 1999). Under these conditions, corticosteroid target cells and tissues are exposed to higher concentrations of the steroid for longer durations and the potential damage can be appreciated when one considers previous studies showing that aged animals are more susceptible to cell death when exposed to glucocorticoids (Hassan et al 1996). However, the fact that decreases in brain volume and weight (accompanied by increases in ventricular volume and other cerebrospinal fluid spaces) (Anderton 2002) suggests that increased HPA axis activity is not the sole cause of brain atrophy (cf. Lupien et al 1998), i.e. a certain amount of atrophy occurs during the normal course of aging.

Lastly, it is interesting to remark on the fact that at least two transgenic mouse strains display elevated corticosterone secretion (Touma et al 2004; Green et al 2006) although it is not clear whether (i) the hypercorticalism is causal to the deposits of Aβ and the neuritic pathology and cognitive impairments seen in this model, or (ii) the increased adrenal activity was a result of hippocampal damage or a consequence of the genetic manipulation itself. Significantly, in light of the previously mentioned preponderance of women to be at greater risk for developing AD, the males of this transgenic mouse strain display adrenocortical hyperactivity much earlier than their female siblings (Touma et al 2004).

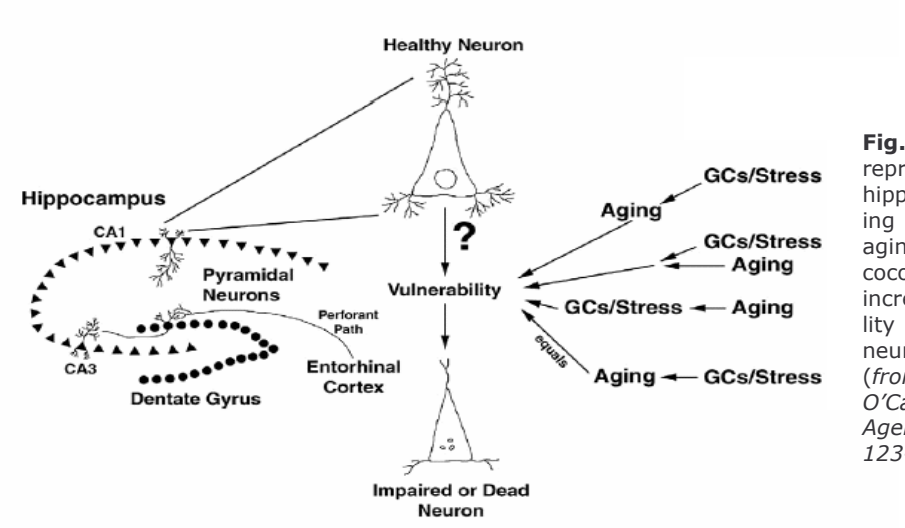


Fig. 1.16. Schematic representation of the hippocampus, illustrating the ways in which aging, stress and glucocorticoids interact to increase the vulnerability of hippocampal neurons. (*from Miller and O'Callaghan (2005) Ageing Res. Rev., 4: 123-140).*

The above summary makes a strong case for investigating the role of, and mechanisms through which, corticosteroids can influence AD-related brain pathology. The foundations for investigating this were previously laid down in the 'Glucocorticoid cascade hypothesis' by Sapolsky & McEwen (1986) which proposed a causal relationship between hippocampal degeneration and cognitive deficits with impairments in the feedback regulation of the HPA axis (see Fig. 1.16). Extending that model, the scheme shown in Fig. 1.17 hypothesizes that cumulative stressful events over the lifetime increase neuronal atrophy and eventual death, resulting in progressive impairments of cognitive functions.

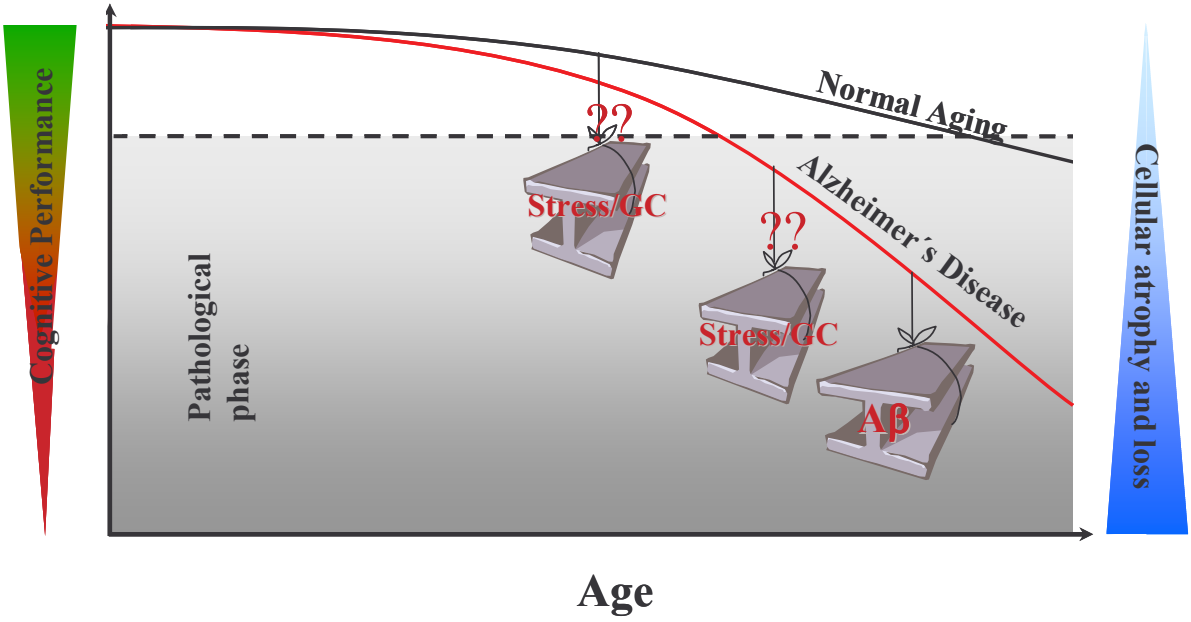


Fig. 1.17. Hypothetical representation of stress/ glucocorticoid influence on cellular atrophy and cognitive performance in normal aging and AD (by I. Sotiropoulos and O.F.X. Almeida).

Aims of thesis

The overall aim of this work was to systematically examine the involvement of stress and/or glucocorticoids (GC) in the initiation of Alzheimer disease-like pathology in laboratory rodents and cellular models, with a view to understanding etiopathogenic mechanisms of the disease and gaining insights into eventual preventative and therapeutic strategies. Specifically, these studies addressed the following questions:

- What are the morphological correlates of stress/GC in the hippocampus and prefrontal cortex, two areas that are strongly involved in the control of cognition and known to be subject to neurodegeneration in AD? *(Chapter 2)*
- Do stress and GC influence the biochemistry of the two main proteins implicated in AD pathology, namely amyloid precursor protein (metabolism) and tau (abnormal hyperphosphorylation)? *(Chapters 2, 3 and 4)*
- What might be the cellular mechanisms that underlie the hypothesized stress/GC-induced alterations in Alzheimer disease-related proteins? *(Chapters 3, 4 and 5)*
- Are the behavioral impairments seen after stress/GC paralleled by any of the aforementioned pathological cascades and biochemical parameters? *(Chapters 3 and 4)*
- Since mood disorders are strongly correlated with the risk of developing Alzheimer's disease, might similar or common pathways account for stress influences on cognition and mood? *(Chapters 3, 4 and 6)*

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Chapter 2

MATERIALS AND METHODS

Materials and Methods

1. Experiments to assess corticosteroid-induced changes in brain morphology and neurochemistry

1.1. Animals and treatments

Male Wistar rats (Charles River, Sulzfeld, Germany), were used in this study. All treatments and in vivo examinations were performed in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and local regulations on animal welfare. Animals were housed 5–6 per cage under standard environmental conditions (temperature 22 ^{o}C; relative humidity 70%; 12 h light: 12 h dark cycle [lights on at 6 a.m.]; ad libitum access to food and drinking solution). Treatments were initiated when the animals were 8 weeks of age and were continued over a period of 11 weeks; body weights were recorded twice weekly. The experiment included the following treatment groups:

(i) Controls (CON) were sham-adrenalectomized rats maintained on tap water (n = 5).

(ii) Adrenalectomized (ADX) rats (hypocortisolism) were prepared surgically under halothane anesthesia and maintained on 0.9% saline as drinking solution (n = 5).

(iii) Corticosterone-replaced ADX (ADX + CORT) animals (normo-cortisolism) were ADX as above and received a drinking solution consisting of 7.5 µg/ml corticosterone (CORT) in 0.9% saline (n = 6). CORT (Sigma, Deisenhofen, Germany) was initially dissolved in 2-hydroxy-b-cyclodextrin (Sigma). Pilot experiments showed that this dose did not activate high-affinity glucocorticoid receptors.

(iv) Dexamethasone-replaced ADX (ADX + DEX) rats (hypercortisolism) were ADX as described above and received the prototypic glucocorticoid receptor agonist, dexamethasone (DEX), at a dose of 0.25 µg/ml in 0.9% saline (n = 7). Soluble DEX (Fortecortin^{e}) was obtained from Merck (Darmstadt, Germany).

1.2. Magnetic resonance imaging and proton spectroscopy protocols

Animals were transferred to the magnetic resonance imaging (MRI) facility of the Max Planck Institute of Psychiatry (Munich, Germany) several hours before scanning to allow adaptation. Animals were anesthetized with halothane, orally intubated and mechanically ventilated at a rate of 50 breaths/min with 1.7% isoflurane in 40% O_2/60% N_2. Ventilation was controlled using a Datex AS/3 anesthesia monitor (Datex, Finland). Animals were placed in a custom-built holder with head fixation, an integrated surface head coil (courtesy of M. Neumeir) and a heating pad. Body temperature was monitored with a rectal probe and maintained at 38.0 ± 0.5 ^{o}C. A fibre-optic pulse oximeter (Nonin, USA) was affixed to the left hindpaw to measure arterial O_2 saturation and heart rate throughout the experiment. Throughout the scanning period the following physiological parameters were monitored: oxygen, partial pressure of carbon dioxide, concentration of inhalative anesthetic, airway pressure, arterial oxygen saturation, heart rate, and body temperature.

Three mutually orthogonal (coronal, axial, and sagittal) pilot images were used for reproducible anatomical orientation of 20 coronal rapid-acquisition relaxation-enhancement (RARE) images (TR=4000 ms, TE=19.4 ms, TE_{eff} = 43.8 msec, RARE factor four, six averages, spatial resolution 0.068 x 0.068 x 0.75 mm^3, 0.75 mm slice thickness with 0.1 mm gap, field-of-view 3.5 x 3.5 cm^2, matrix 512 x 384, 39 min 19 sec scan duration) on a 7 Tesla scanner (Bruker Avance Biospec 70/30, Karlsruhe, Germany), which were positioned perpendicular to a line connecting the superior end of the olfactory bulb with the superior end of the cerebellum according to Wolf *et al* (2002a*). The most rostral slices started at the most posterior point of the olfactory bulb as the anterior landmark.

For proton magnetic resonance spectroscopy (^{1}H-MRS) of the left hippocampus we used a point-resolved spectroscopy sequence (PRESS, TR=5000 ms, TE=17 msec, NA=256, voxel size 3 x 2 x 2 mm^3, 21 min 20 sec scan duration) (Fig. 1 and 2).

*Wolf et al (2002) *Brain Res Protoc* 10: 41-46.

1.3. Image and data analysis

MR volumetry: For volumetric analysis right and left hippocampi as well as brain volume (BV) were outlined manually on the coronal slices by one rater (MS) blinded to group status using the Paravision ROITool software (Bruker, Karlsruhe, Germany) and a standard rat brain atlas (Paxinos and Watson, 1998*) for reference. Hippocampus was present on six consecutive slices, from approximately –2.12 mm (mean –2.19 mm) from Bregma rostrally to approximately –7.04 (mean –6.65 mm) from Bregma caudally, similar to Wolf *et al* (2002). Brain volume was measured on 14 to 16 consecutive slices, from approximately 4.20 mm (mean 4.22 mm) from Bregma rostrally to approximately –8.8 mm (mean –8.63 mm) from Bregma caudally, excluding the olfactory bulb and the cerebellum (Kalisch, et al 2006**). The most rostral slice was defined by the prefrontal cortex having a stake larger or equal to 50% of this slice. This included a caudal part of the olfactory bulb. Multiple features defined the caudal boundary: the central nucleus of inferior colliculus, the caudal end of the aqueduct, which was the last slice anterior to the cerebellum. No attempt was made to exclude ventricular, CSF volume or the sinus. Volumes were calculated by multiplying the number of pixels with the pixel size accounting for the interslice gap in all but the last slice and are given in mm^3. To correct for variations in head and brain size, hippocampal volumes were normalized to BV. To assess the intra-rater variability the hippocampal outlining of all animals was repeated after a period of one month by the same rater (MIS).

Proton spectroscopy: Spectra were obtained in 21 rats (ADX, n=5; ADX+CORT, n=6; ADX+DEX, n=6; CON, n=4). The metabolite concentrations of N-acetyl aspartate (NAA), total creatine (Cr), choline-containing compounds (Cho), *myo*-inositol (mI), glutamate (Glu), glutamine, glucose and taurine were estimated using the automated fitting program LCModel (Provencher, 1993), and expressed as ratios to brain water. We only included metabolites with an estimated standard deviation of less than 30%, i.e. NAA, Cho, Cr, mI and Glu.

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*Paxinos G, Watson C (1998): *The rat brain in stereotaxic coordinates*. Academic Press: San Diego.

**Kalisch et al (2006) *Neuropsychopharmacology,* 31: 925-932.

1.4. Post-mortem procedures

Animals were sacrificed by decapitation immediately after removal from the scanner, while they were still anesthetized. Trunk blood was collected for subsequent analysis of serum corticosterone levels by radioimmunoassay (Corticosterone RIA kit, ICN Biochemicals, Costa Mesa, CA). Thymi were excised and maintained on saline-soaked filter papers until weighing.

1.5. Volumetry

The ACC was outlined according to the landmarks defined by Wolf et al., 2002b* (see also Fig. 3.1.1 in Results chapter). Briefly, a line connecting the extreme most dorso-lateral point of the corpus callosum was connected to the most dorsal and medial intra-hemispheric point of the cortex; this line was continued by the inter-hemispheric line until the intersection of the corpus callosum with the midline and then turned laterally following the corpus callosum to its most dorso-lateral point. The entire cortex within the limits previously defined was measured, starting at the closure of genu of the corpus callosum and terminating at the rostral limit of the hippocampus (see Fig. 3.1.2 in Results Chapter); the chosen profiles for measurement necessarily excluded some of the ACC as its limits are poorly defined in MRI, thus potentially compromising the precision of the volume estimates. Since the retrosplenial granular cortex represents the caudal continuation of the ACC, this region of the brain was measured from the rostral limit of the hippocampus until the slice prior to the opening of the corpus callosum (see Fig. 3.1.2 in Results Chapter), using the procedures described above.

The right and left anterior cingulate and retrosplenial areas were manually delineated by one rater (JJC), blinded to treatment status, in consecutive coronal slices containing these regions using StereoInvestigator[e] (MicroBrightField, Williston, VT) software. Intra-rater reliability coefficient was 0.96. Volumes were calculated by multiplying the area by the inter-slice distance. Determination of hemispheric volume (excluding the olfactory bulb,

*Wolf et al (2002) *Neuroendocrinology* 75: 241-249.

cerebellum and brain stem) was also performed and cingulate cortex and hippocampal volumes were normalized according to this value.

1.6. Histological procedures and estimations

A subset (n = 4) of animals from each experimental group was used for histological analysis. The left hemispheres were removed and immediately immersed in isopentane followed by liquid nitrogen. Consecutive 30 lm coronal cryosections were stained with Giemsa and coverslipped. Multiple identical linear measurements were taken from fresh sections and in slides following processing; importantly, measures in all three dimensions (along the x, y and z axis) were obtained. The determination of the shrinkage factor (SFv) resulted from the calculation of tissue retraction in every dimension; this procedure was performed in all experimental groups to determine differential tissue shrinkage factors.

The volume of the left ACC and hippocampus was estimated on the basis of the Cavalieri's principle. Briefly, every 4^{th} section was used for the estimates; the cross-sectional area of the ACC was estimated by point counting (final magnification x 112) using a test point system in which the interpoint distance, at the tissue level, was 150 lm. The volume of the left ACC and hippocampus was then calculated from the number of points that fell within the area of interest and the distance between the systematically sampled sections.

1.7. Western Blot analysis

Levels of APP and C99, total and phosphorylated Tau were monitored by Western blot analysis (see 2.4. for details).

1.8. Statistical analysis

Effects of treatment on body weight and thymus weight were examined by one-way analysis of variance (ANOVA) with Bonferroni correction. Post-hoc

linear polynomial contrast tests were applied to test whether means differed significantly from each other (pair-wise comparisons). Correlation between volumetric determinations in MRI and histological sections was determined by Pearson's correlation test and Bland– Altman agreement analysis (Altman and Bland, 1983). Differences were considered to be significant if $p < 0.05$. Group differences in normalized hippocampal volumes and metabolites were tested using multivariate analysis of variance (MANOVA) followed by post hoc Bonferroni and post hoc least square difference (LSD) testing due to the small sample size.

2. APP misprocessing and aberrant tau phosphorylation as a cellular response linking chronic stress with AD

2.1. Animal procedures

Male Wistar rats, aged 14 months (Charles River, Barcelona, Spain) were used; all treatments and in vivo examinations were performed in accordance with the European Communities' Council Directives of 24 November 1986 (86/609/EEC) and local regulations on animal welfare. Animals were housed 4-5 per cage under standard environmental conditions (temperature 22^{o}C; relative humidity 70%; 12 h light: 12 h dark cycle [lights on at 6 a.m.]; ad libitum access to food and drinking solution). One sub-group of rats was submitted to a chronic, unpredictable stress paradigm (Sousa et al., 2000*) for 1 month. Animals were then subjected to a first behavioral testing (see below) before receiving a brain infusion cannula (Alza Corp, Mountain View, CA) implant in the left lateral ventricle (i.c.v; stereotaxic coordinates: AP, −1.0 mm; DV, −2.5 mm; and ML, +1.5 mm (right) with bregma as a reference, under pentobarbital (50 mg/kg) anesthesia. Polyethylene tubing was used to connect the cannulae to miniosmotic pumps (Alzet 2002, Alza), placed s.c. The minipumps were filled with either freshly solubilized amyloid beta$_{1-40}$ (4.2 nmole Aβ_{1-40}/200 µl) in sterile distilled

*Sousa et al (2000) Neuroscience 97:253-266.

water, or vehicle, and were left in place for 14 d. The $A\beta_{1-40}$ was obtained from American Peptide Co Inc (Sunnyvale, CA). Subgroups of minipump-implanted animals concomitantly received daily s.c. injections (14 d) of the glucocorticoid (GC) dexamethasone (300 µg/ml/kg BW), delivered in an oily suspension (depot) (1:10 Fortecortin™ [Merck, Darmstadt, Germany] in sesame oil [Sigma, St. Louis, MI]). The following experimental groups, comprising 6-8 rats each, were used: (i) vehicle-infused (CON), (ii) vehicle-infused + GC, (iii) $A\beta_{1-40}$-infused, (iv) $A\beta_{1-40}$-infused + GC; (v) stressed, vehicle-infused; (vi) stressed, $A\beta_{1-40}$-infused; (vii) stressed, $A\beta_{1-40}$-infused + GC. Following these treatments, animals were subjected to a second behavioral test before decapitation. After sacrifice, minipumps were removed and checked for patency and functionality.

Three biometric parameters, daytime serum corticosterone (CORT) levels (measured with a radioimmunoassay kit from ICN, Costa Mesa, CA), body weight changes, and thymus weights at autopsy, served to verify the efficacy of the stress paradigm: Stressed rats showed significant elevations in daytime serum CORT levels (CON: 54.7 $\pm$ 4.8 *vs.* stressed: 384.6 $\pm$ 65.9 ng/ml; $p = 0.016$), a net loss of body weight (-12.1 $\pm$ 2.1 g *vs.* a net gain of 6.1 $\pm$ 1.3 g in CON rats; $p \leq 0.0001$), and thymic gland atrophy (stressed: 4.3 $\pm$ 0.4 *vs.* CON: 7.6 $\pm$ 0.9 mg/kg BW; $p = 0.007$). These parameters did not differ significantly between CON and stressed animals after superimposition of the $A\beta_{1-40}$ infusions. Exposure to GC resulted in similar changes to those observed in the stressed groups ($p = 0.025$).

2.2. Behavioral tests

Tests of spatial memory and reversal memory were conducted in a circular black tank (170 cm diameter) filled to a depth of 31 cm with opaque water (at 22°C) and placed in a dimly lit room with extrinsic clues. The tank was divided into imaginary quadrants and had a black platform (12 cm diameter, 30 cm height) placed in one of them. Data were collected using a video tracking system (Viewpoint, Champagne-au-Mont-d'Or, France). Animals were tested for spatial

reference memory over 4 consecutive days (4 trials per day), as previously described (Cerqueira et al., 2005*).

Reversal learning was assessed after the reference memory procedure (on the fifth day) using the maze set-up described above. For this, the escape platform was re-positioned in the opposite quadrant; rats were tested in a four-trial paradigm (1 trial per day), and distance and time spent swimming in each quadrant were recorded (Cerqueira et al., 2005*).

Emotional behavior was analyzed in the open-field test and in the elevated plus maze (EPM), which allow inferences of locomotor, exploratory and anxiety behavior (Sousa et al., 2006**). The open-field consists of an arena (43.2 x 43.2 cm; transparent acrylic walls and white floor; purchased from MedAssociates Inc., St. Albans, VT). Rat's instant position was monitored on-line over a period of 5 min with the aid of two 16-beam infra-red arrays. Total distances travelled were used as indicators of locomotor activity. Time and distances in the central and pre-defined peripheral areas were recorded and used to calculate the ratio of time spent in the central quadrant of the platform, and the distance travelled in this central area (vs. rest of the open field platform). The number and duration of rearings were also recorded.

Anxiety behavior was evaluated using the elevated plus maze (MedAssociates Inc., St. Albans, VT) which consisted of a black polypropylene plus-shaped platform elevated 72.4 cm above the floor; the maze consisted of two open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm). All assessments were carried out under white light and animals were placed in the maze for a total period of 5 min and the time spent in the open *vs.* closed arms was recorded online. Data were processed to yield the ratio of time spent in the open arms versus total time.

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*Cerqueira et al (2005) J Neurosci 25:7792-7800.
**Sousa et al (2006) Genes Brain Behav 5 Suppl 2:5-24.

2.3. Tissue collection

Animals were killed by decapitation 1 day after the last behavioral test. Brains were excised immediately. One half of each brain was fixed by immersion in 4% paraformaldehyde for 2 days and embedded in paraffin for subsequent immunohistochemical analysis. The PFC, entorhinal cortex and hippocampus were dissected (on ice) from the other half of the brain, snap-frozen in liquid nitrogen, and stored at –80^{o}C until Western blot analysis.

2.4. Western Blot analysis

Immunoblotting was used to assess levels of total tau and hyperphosphorylated tau, APP and its cleavage products as well as a panel of kinases implicated in tau hyperphosphorylation and acetylated tubulin (Ac-Tub); reduced levels of Ac-Tub indicate dysregulated microtubule stability (Cho and Johnson, 2004*). Unless specifically stated, all reagents were purchased from Sigma. Frozen brain areas were homogenized in lysis buffer (100 mM Tris-HCl, pH 8, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 250 mM NaCl, 1% NP40, protease and phosphatase inhibitors using a *Dounce* glass homogenizer. After centrifugation (14,000 *g*, 15 min, 4^{o}C) and determination of protein concentrations (Lowry method), lysates were stored at –80^{o}C.

Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after dilution in Laemmli buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.002% bromophenol blue), and heat denaturation (5 min, 95^{o}C). Samples (30-40 µg protein) were loaded onto 8% or 10% SDS-PAGE gels and electrophoresed in 25 mM Tris, 192 mM glycine and 0.2% SDS (100 V, 2 h). High and low molecular weight markers (Biorad, Hercules, CA) were run in parallel. Electrophoresed samples were subsequently semi-dry transferred onto nitrocellulose membranes (Protran BA 85, Schleicher & Schuell, Dassel, Germany). Equality of protein transfer was verified using Ponceau-S Red stain. Blots were then washed in 15 nM Tris-HCl, 200 mM NaCl and 0.1% Tween-20 (pH 7.5; TBST), blocked in TBST

*Cho JH, Johnson GV (2004) J Neurochem 88:349-358.

containing 5% non-fat milk, before incubation with primary antibodies of interest (1h at RT, or overnight at 4°C). Besides immunoblotting with a panel of phosphorylation-dependent antibodies, blots were also incubated with antibodies directed against total tau (tau-5, BD Biosciences, San Jose, CA) and active and/or total forms of various tau-related kinases (ERK1/2, pERK1/2, JNK, pJNK, cdk5, p35, GSK3, pGSK3, and CaMKII). Microtubule stability was monitored by measuring levels of acetylated α-tubulin (Sigma; 1:2000). Additionally, levels BACE and Nicastrin, enzymes that are involved in APP processing towards the amyloidogenic pathway, were monitored (see Table 1). For normalization purposes, blots were also probed with anti-β-actin (Chemicon, Temecula, CA) or anti-tubulin (Sigma). Characteristics and working dilutions of antibodies against the various kinases and forms of tau are listed in Table 1. After incubation, blots were rinsed extensively in TBS-T before incubation with an appropriate dilution of secondary antibody (in blocking buffer; 1 h, RT), and washed in TBST. Chemiluminescence was used for detection (ECL, Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Films were scanned and quantified (after checking for linearity) using TINA 3.0 Bioimaging software (Raytest, Straubenhardt, Germany). To distinguish between mature and immature isoforms of APP, tissue lysates were digested with endoglycosidase F (Roche, Mannheim, Germany), according to the manufacturer's instructions.

2.5. Immunoprecipitation of C99

Tissue lysates were immunoprecipitated with APP369 antibody in a mixture of Protein A and G beads (Roche, Mannheim, Germany), according to the manufacturer's instructions, before analysis by electrophoresis on Tris-Tricine gradient gels (KMF Laborchemie, Lohmar, Germany) and immunoblotting with an APP antibody (OPA1-01132; Affinity Bioreagents, Golden, CO).

2.6. In situ hybridization

For in situ detection of APP mRNA levels, paraffin sections (12 μm) were deparaffinized (see above), and fixed in 4% *p*-formaldehyde in PBS containing 0.1% diethylpyrocarbonate (DEPC; Sigma). After washing in PBS-DPEC, sections

were acetylated (0.1 M triethanolamine, 0.25% acetic anhydride in DPEC), delipidated in chloroform (5 min), dehydrated through a graded series of ethanol and air-dried. A 40-mer oligonucleotide probe (GCTG GCTG CCGT CGTG GGAA CTCG GACT ACCT CCTC CACA) was used to hybridize APP695-KPI. Slides were first hybridized with (10^6 cpm/slide) of antisense or sense (control) ^{35}S-dATP labeled-oligoprobe, dissolved in hybridization buffer. Labeled probe was placed over the sections and slides were incubated overnight in a humidified oven at 42°C. Subsequently, stringency washes were performed in 1x saline sodium citrate (SSC) at 55°C (15 min). Slides were next dehydrated by brief sequential immersion 1x SSC, H_2O, 65% ethanol, and 95% ethanol, before air drying, mounting on filter paper, and dipped in photographic emulsion (1:1 Kodak NTB2 in distilled water), and exposed for 1 month. Sections were developed, counterstained with toluidine blue and viewed under bright-field or polarized light to view cells and silver grains, respectively. Different regions of interest were examined, using a CCTV camera to acquire images. Hybridization signal was semi-quantitatively scored by two independent observers on a scale of 0.5 to 5.

2.7. Immunohistochemistry

The right hemisphere of each brain (the left one was used for biochemical measurements described above) was placed in 4% paraformaldehyde (PFA) immediately after sacrifice and immersion-fixed for 48 h before embedding in paraffin. Sections (8 µm thick) were cut and antigen retrieval was achieved by treating sections with a sodium citrate solution in a microwave oven for 30 min. Sections were then washed in TBS and endogenous peroxides were neutralized with H_2O_2 (3%) before incubation (30 min) in an appropriate non-immune serum (1:10 in TBS, pH 7.6). Slides were incubated (overnight, RT) with primary antibodies of interest (see Table 1), before washing in TBS and incubation with biotinylated secondary antibody (BioGenex Detection Kit, San Ramon, CA) at RT for 30 min. After rinsing 3 times in TBS, slides were incubated (30 min, RT) with a horseradish-peroxidase complex (ABC substitute by BioGenex, CA, USA). Immunostained structures were visualized with diaminobenzidine (1 mg/ml in 0.01% H_2O_2) by incubating (5 min) in the dark. Chromogenic development was

monitored using a microscope. Negative controls were performed by omitting the primary antibody (no staining was seen in any sections). The degree of antibody staining was scored by visual examination on a scale of 0.5 to 5, by two independent scientists who were blind to the treatments.

2.8. Statistical analysis

Results are expressed as group means ± SEM. Except for the data from reference memory task performance which was analyzed by applying repeated-measures ANOVA on the average results (across the 4 d) of each trial, all other data were evaluated for their statistical significance by using 1-ANOVA and appropriate post hoc pairwise tests. Statistical analyses were conducted using SPSS (Chicago, IL) and SigmaStat (Systat, Erkrath, Germany) software packages; differences were considered to be significant if $p < 0.05$.

3. *In vitro* analysis of glucocorticoid regulation of amyloid precursor protein (APP) and tau.

3.1. Cell lines

PC12 cells stably transfected with either a human tau (0N3R isoform) transgene ($PC12_{htau}$) or an empty vector ($PC12_{vect}$), and wild-type PC12 cells (all cell lines kindly provided by Dr. R. Brandt; Maas et al., 2000*; Fath et al., 2002**), were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Eggenstein, Germany), supplemented with 0.75% fetal bovine serum and 0.75% horse serum (both from Invitrogen) and maintained at 37°C in a humidified incubator under 5% CO_2; $PC12_{htau}$ and $PC12_{vect}$ cells were additionally supplemented with geneticin (250 µg/ml; Invitrogen). Cells were differentiated with 7S nerve growth factor (NGF; Invitrogen) and were maintained in NGF thereafter. Differentiated

*Fath et al (2002) *J Neurosci* 22, 9733-41.
**Maas et al (2000) *J Biol Chem* 275, 15733-15740.

wild-type and transfected PC12 cells did not differ morphologically, and showed the expected characteristics of differentiated neuronal cells.

For comparison purposes, a human neuroblastoma cell line SH-SY5Y (derived from the SK-N-SH line; European Collection of Cell Cultures, Salisbury, UK) was also used. SH-SY5Y cells, cultured in DMEM supplemented with 1% fetal bovine serum (Invitrogen), (37^{o}C under 5% CO_2), were used after differentiation with 10^{-5} M retinoic acid (RA; Sigma, St. Louis, MO).

3.2. Drugs

Treatments were initiated after cells were cultured for 6-7 d. The synthetic glucocorticoid dexamethasone (DEX; Fortecotrin®, Merck, Darmstadt, Germany) was applied for 24-48 h at a concentration of 10^{-6}M. The glucocorticoid receptor (GR) antagonist mifepristone (RU 38486), provided by the National Hormone & Peptide Program (NIDDK) (Torrance, CA), was used at a dose of 10^{-5} M). Daunorubicin (Sigma) was obtained as an HCl salt. Synthetic peptide $A\beta_{25-35}$ (human; American Peptide Company, Sunnyvale, CA), was used dissolved in distilled water and used at concentrations ranging between 0.1 and 5 μM. $A\beta_{25-35}$ were dissolved in distilled water. β-secretase activity was blocked with BACE inhibitor IV (IC50=15nM; Calbiochem, San Diego, CA). The following kinase inhibitors were used: indirubin-3'-monoaxime from Alexis Biochemicals (Lausen, Switzerland), used at 1-30 μM ($IC50_{GSK3\beta}$ = 22 nM; $IC50_{cdk5}$ = 100 nM); indirubin-5-sulfonate from Alexis, used at 1-50 μM ($IC50_{GSK3\beta}$ = 80 nM; $IC50_{cdk5}$ = 7 nM); Alsterpaullone from Sigma ($IC50_{GSK3\alpha/\beta}$ = 4 nM; $IC50_{cdk5}$ = 40 nM); PNU 112455A (N^4-(6-Aminopyrimidin-4-yl)-sulfanilamide, HCl) from Calbiochem, used at 1-100 nM (Ki_{cdk5} = 2 μM); the GSK3α/β inhibitor (3-(3-Carboxy-4-chloroanilino)-4-(3-nitrophenyl) maleimide) from Calbiochem, used at 1-30 μM ($IC50_{GSK3\alpha/\beta}$ = 26 nM; $IC50_{GSK3\beta}$ = 5 μM) and Ca^{+2}/Calmodulin–dependent protein kinase II (CaMK II) inhibitor 281-309 from Calbiochem, used at 1-30 μM (IC50 = 80nM).

3.3. RNA extraction and RT-PCR

Relative mRNA levels of human and rat tau, APP and of the house-keeping gene cyclophilin were measured using reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, total RNA, free from chromosomal DNA contamination, was isolated from cells using the *RNAeasy® Mini Kit* (Qiagen, Hilden, Germany). The RNA was subsequently heat denatured and reverse transcribed with Qiagen's *Omniscript® Reverse Transcriptase Kit* for first-strand cDNA synthesis. The reverse transcription reaction mixture (20 µl), consisting of 2 µg RNA in 0.5 mM of each dNTP, 1 µM oligo$(dT)_{15}$ (Promega, Madison, WI), 10 U RNase inhibitor (Sigma), 1xRT buffer and 4 U of Omniscript® reverse transcriptase, was incubated for 60 min at 37°C. For the PCR reaction, a 5 µg aliquot of reverse transcribed cDNA was used in a 45 µl reaction mixture containing 3′ and 5′ primers (each at 0.4 µM; see below for sequences), 200 µM of each dNTP, 1 U Taq polymerase (Sigma), and 1xPCR buffer (Qiagen). PCR amplification was performed over 28 (human and rat tau), 30 (APP) or 22 (cyclophilin) cycles, under the following conditions: denaturation 40 s at 94° C; annealing 1 min at 58° C (human tau), 60° C (rat tau), 56 ° C (APP) or 56° C (cyclophilin); primer extension 2 min at 72° C; the linear range of each assay was determined in pilot optimization experiments. PCR products were electrophoresed on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide. The amplified products had sizes of approximately 565 bp (human tau), 278 bp (rat tau), 562 bp (APP_{770}), 505 bp (APP_{751}), 337bp (APP_{695}) and 325bp (cyclophilin). Control amplifications (omission of either reverse transcriptase or cDNA) were run in each assay to discount amplification of genomic or contaminating DNA. Gene-specific primer pairs (MWG Biotech, Ebersberg, Germany) were designed on the basis of published rat and human cDNA.

human tau: 5′-CGA AGT GAT GGA AGA TCA CG-3′ (sense)
5′-CAT TCT TCA GGT CTG GCA TG- 3′ (antisense)

rat tau: 5′-GGT GAG GGA TGG GGG TGG TA-3′ (sense)
5′-GTG ACT GGT TCT CGT GGT A-3′ (antisense)

APP: 5′-GAG CGG ACA CAG ACTATG CTG A-3′ (sense)
5′-GAC ATT CTC TCT CGG TGC TTG-3′ (antisense)

Cyclophilin: 5′-CTC CTT TGA GCT GTT TGC AG (sense)
5′-CAC CAC ATG CTT GCC ATC C-3′ (antisense)

3.4. Transient transfection with *htau*

The human tau gene (0N3R) framed in a pEGFP-C1 host vector (Clontech Mountain View, CA), was kindly provided by Dr. R. Brandt; Maas et al., 2000*). PC12 cells were grown in 6-well plates (40,000 cells/well), differentiated with NGF (see above), and were transiently transfected after 6 d. Cells were grown in 6-well plates and transfected with 2 µg of cDNA/well using Lipofectamine 2000® (Invitrogen), according to manufacturer's instructions; both the cDNA and transfecting agent were diluted in Optimem medium (Invitrogen). The transfection efficiency was 5-10% and cells were used 12 h later. Cells transfected with an empty vector did not differ from those transfected with *htau*, as judged by morphology and survival rates.

3.5. Immunoprecipitation

Cell lysates were immunoprecipitated with APP369 antibody (kindly provided by Dr. Sam Gandy, Philadelphia, PA) in a mixture of Protein A and G beads (Roche, Mannheim, Germany), according to the manufacturer's instructions, before analysis by electrophoresis on Tris-Tricine gradient gels (KMF Laborchemie, Lohmar, Germany) and immunoblotting with an APP antibody (OPA1-01132; Affinity Bioreagents, Golden, CO).

3.6. Western blotting

Unless specifically stated, all reagents were purchased from Sigma. Cells were washed 2 times in 1xPBS (on ice) and scraped off dishes, with a rubber policeman, in the presence of lysis buffer (see above). Cell lysates were kept on ice (10 min.) before sonification on ice, centrifugation (14,000 *g*, 15 min, 4° C) and storage at –80° C after determination of protein concentrations (Lowry method). Ice-thawed lysates were subsequently diluted in Laemmli buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, 2% β-mercaptoethanol,

*Maas et al (2000) *J Biol Chem* 275, 15733-15740.

and 0.002% bromophenol blue), heat denatured (5 min, 95° C) and loaded (30-40 µg protein) onto 8-10% SDS-PAGE gels, before electrophoresis in 25 mM Tris, 192 mM glycine and 0.2% SDS. High and low molecular weight markers (Biorad, Hercules, CA) were run in parallel. Electrophoresed samples were subsequently semi-dry transferred onto nitrocellulose membranes (Protran BA 85, Schleicher & Schuell, Dassel, Germany). Equality of protein transfer was verified using Ponceau-S Red stain. Blots were washed in 15 nM Tris-HCl, 200mM NaCl and 0.1% Tween-20 (pH 7.5; TBST), blocked in TBST containing 5% non-fat milk, before incubation with primary antibodies of interest (1h at RT, or overnight at 4° C). The characteristics and dilutions at which antibodies against the various proteins and peptides of interest were used are listed in Table 2. For normalization purposes, blots were also probed with anti-β-actin (Chemicon, Temecula, CA) or anti-tubulin (Sigma). After incubation, blots were rinsed extensively in TBS-T before incubation with an appropriately diluted secondary antibody (in blocking buffer; 1 h, RT), and washed in TBST. Chemiluminescence was used for detection (ECL, Amersham Biosciences, Little Chalfont, UK), following the manufacturer's instructions. Films (ECL Hyperfilm, Amersham) were scanned and quantified (after checking for linearity) using TINA 3.0 Bioimaging software (Raytest, Straubenhardt, Germany). Alkaline phosphatase pre-treatment was employed in control experiments. Briefly, 25 µg of protein extract in lysis buffer (100 mM Tris-HCl, 10% glycerol, 1mM EDTA, 5 mM $MgCl_2$, 250 mM NaCl, 1% NP40, protease inhibitors and phosphate inhibitors) were treated with 15 U of calf intestine alkaline phosphatase (Roche, Mannheim, Germany) at 37^{o} C for 24 h.

For analysis of secreted APP (sAPP), cell culture supernatants were concentrated by centrifugation (3,000 *g*, 30 min), using Vivaspin concentrators (Vivascience, Goettingen, Germany) and analyzed by Western blotting.

3.7. Assay of tau protein stability

NGF-differentiated PC12 cells were cultured (24 h) in methionine/cysteine-free medium, supplemented with [^{35}S]-methionine/cysteine (185 µCi/ml; Amersham). Cells were then washed, exposed to test drug (DEX) in normal, non-radioactive medium, and lysed (see above) at different time points after drug exposure. Tau proteins were immunoprecipitated with a mixture of A- and G-agarose beads (Roche) using Tau-5 antibody (see Table 2). Immunoprecipitates were analyzed by SDS-gel electrophoresis, Coomassie-stained and dried on filter paper under vacuum. Radioactivity in the dried gels was evaluated using a Fuji Bio-image 3000 system and AIC 7770 analysis software (Adaptec, Milpitas, CA).

3.8. Confocal microscopy

Unless specifically stated, all reagents were purchased from Sigma. Cells were cultured on coated glass coverslips (0.8 mg/ml gelatin and 0.1 mg/ml poly-D-lysine). After washing with PBS, cells were fixed (20 min) in 4% paraformaldehyde/PBS containing 4% sucrose, incubated (20 min) in 0.1 mM glycine/PBS, and permeabilized (5 min) in 0.2% TritonX-100/PBS. Immunostaining was performed in PBS containing 5% normal serum or 1% BSA in PBS-0.1% Tween, to which 0.02% sodium azide was added. After overnight incubation (4^{o} C) with primary antibodies (Table 2), cells were washed and incubated with either anti-mouse or anti-rabbit biotinylated second antibody (RT, 1 h); they were then thoroughly washed before incubation with fluorescence-conjugated avidin-streptavidin (RT, 30 min). After coverslipping under DABCO antifade mounting medium, cells were exmined by scanning laser confocal microscopy (Zeiss LSM). When double immunostaining was performed, the second primary antibody and subsequent procedures were done after completing staining of the first antigen of interest.

3.9. Cell survival assay

Cell viability was assessed using MTT cytotoxicity assays. Cells were grown and differentiated at an initial density of 700 cells per well (96-well dishes) for 6 days. Cells were then treated with MTT (Sigma; dissolved to a final concentration

of 455 µg/ml in 0.9% NaCl) and incubated for 4 h (37^{o} C, 5% CO_2). The formazon product resulting from MTT cleavage was solubilized by the addition of 0.1 ml 0.04 N HCl in isopropanol, and incubation (RT) until disappearance of formazon crystals. The O.D. were then measured on a Dynatech (MR 630) plate reader (570 nm; reference wavelength of 630 nm). Relative Viability (%) was determined by comparison against O.D. values obtained for control (no drug treatment) cells. Sextuplet measurements were obtained for each treatment condition.

3.10. Statistical Analysis

Numerical data are expressed as group means ± SEM. All data sets were subjected to 1-way ANOVA, before application of appropriate *post hoc* pair-wise comparisons (SigmaStat, Systat, San Jose, CA). Differences were considered to be significant if $P \leq 0.05$.

Table 1. Antibodies used in *in vivo* studies

Antibody	Antigen	Source	Dilution
CP-13	phospho-Ser202 tau	Dr. P. Davies (Bronx, NY)	1:30 (W.B.) 1:50 (IHC)
CP-9	Phospho-Thr231 tau	Dr. P. Davies (Bronx, NY)	1:30 (W.B.) 1:50 (IHC)
12E8	phospho-Ser262/ Ser356 tau	Dr. P. Seubert (South San Francisco, CA)	1:100 (IHC)
PHF1	phospho-Ser396/ Ser404 tau	Dr. P. Davies (Bronx, NY)	1:30 (W.B.) 1:50 (IHC)
PG5	phospho-Ser409 tau	Dr. P. Davies (Bronx, NY)	1:30 (W.B.) 1:50 (IHC)
tau-5	(phosphorylation-independent tau)	BD Biosciences, San Jose, CA	1:1000 (W.B.) 1: 200 (IHC)
ERK1/2	Total ERK1/2	Cell Signaling, Danvers, MA	1:2500
active ERK1/2	phospho-Thr202/204 ERK1/2	Cell Signaling, Danvers, MA	1:2500
JNK	Total JNK	Cell Signaling, Danvers, MA	1:1000
active JNK	phospho-Thr183/Tyr185 JNK	Cell Signaling, Danvers, MA	1:1000
cdk5	Total cdk5	Upstate, Lake Placid, NY	1:1000
p35	Total p35	Santa Cruz, Santa Cruz, CA	1:600
GSK3	Total GSK3	Santa Cruz, Santa Cruz, CA	1:2000
active GSK3	phospho-Tyr216/279 GSK3	Biosource, Camarillo, CA	1:2000
CaMKII	Total CaMKII	Cell Signaling, Danvers, MA	1:1000
APP369	APP	Dr Sam Gandy (Thomas Jefferson University, USA)	1:5000
BACE-1	BACE-1	ProSci Inc, Poway, CA	1:500
Nicastrin	Nicastrin	Sigma	1:5000

Table 2. Antibodies used in *in vitro* studies

Antibody	Source	Dilution
AT8 (phospho-Ser202/205 tau)	Innogenetics	1:15 (W.B.) 1: 50 (ICC)
CP-9 (phospho-Thr231 tau)	Dr P. Davies (Albert Einstein College, USA)	1:30 (W.B.) 1: 50 (ICC)
PHF1 (phospho-Ser396/404 tau)	Dr P. Davies (Albert Einstein College, USA)	1:30 (W.B.) 1: 50 (ICC)
PG5 (phospho-Ser409 tau)	Dr P. Davies (Albert Einstein College, USA)	1:30 (W.B.) 1: 50 (ICC)
MC1 (conformational-dependent tau Ab)	Dr P. Davies (Albert Einstein College, USA)	1: 50 (ICC)
Alz50 (conformational-dependent tau Ab)	Dr P. Davies (Albert Einstein College, USA)	1: 50 (ICC)
Tau-5 (Total tau Ab)	BD Biosciences	1:1000 (W.B.)
APP 369 APP C-terminal	Dr S Gandy (Thomas Jefferson University, USA)	1:5000 (W.B.) 1:100 (IF)
OPA1-01132 Total APP	Affinity Bioreagents, Golden, CO	1:1000 (W.B.)
22C11 APP N-terminal	Chemicon, Temecula, CA	1:1000 (W.B.)
BACE BACE -1	ProSci Incorporated, Poway, CA	1:500 (W.B.)
Nicastrin	Sigma	1:5000 (W.B.)
Cdk5 Total cdk5	Upstate, Lake Placid, NY	1:1000 (W.B.)
cdk5 active phospho-cdk5	Santa Cruz, Santa Cruz, CA	1:600 (W.B.)
GSK3 Total GSK3	Santa Cruz, Santa Cruz, CA	1:2000 (W.B.)
GSK3 active phospho-Tyr216/279 GSK3	Biosource, Camarillo, CA	1:2000 (W.B.)
ERK1/2 Total ERK1/2	Cell Signaling, Danvers, MA	1:2500 (W.B.)
p-ERK1/2 (active) phospho-Thr202/204 ERK1/2	Cell Signaling, Danvers, MA	1:2500 (W.B.)
CaMK II Total CaMKII	Cell Signaling, Danvers, MA	1:1000 (W.B.)
Actin Total actin	Chemicon, Temecula, CA	1:2000 (W.B.)
Tubulin Total tubulin	Sigma	1:2000 (W.B.)

Chapter 3

RESULTS

Results

3.1. Assessment of corticosteroid-induced changes in brain morphology and neurochemistry

Background

Besides the major histopathological findings of AD that include senile plaques and neurofibrillary tangles together with loss of cortical neurons and synapses, there is no established non-invasive test to aid pre-symptomatic diagnosis. Most exciting among the current attempts in this direction is the detection of senile plaques by magnetic resonance imaging (MRI) and/or single photon emission computed tomography (SPECT). Neuroimaging can also provide volumetric (MRI) and functional data (functional MRI) on the hippocampus and cortex with a high degree of anatomical resolution. In fact, numerous MRI studies have shown atrophy of hippocampal and cortical areas in brain of AD patients as well as in diseases characterized by disturbances in glucocorticoids (e.g. Cushing and Addison's syndromes, and depression). Furthermore, it is well documented that imbalances in corticosteroid levels induce structural changes (e.g. volume loss) in hippocampus and cortical areas such as prefrontal cortex while glucocorticoid actions extend to neuronal atrophy and synaptic loss or, in extreme cases, cell loss. Besides of cell and synaptic loss, AD patients exhibit disturbances of corticosteroid secretion mechanisms; this raised the possibility that there might be a interrelation between corticosteroid status, brain area volume AD-like histopathology, namely, tau hyperphosphorylation and changes in APP levels and processing. To this end, three different chronic alterations of rat adrenocortical status were chosen: hypocortisolism (induced by adrenalectomy, ADX), normocortisolism (ADX with low-dose corticosterone) and hypercortisolism (ADX with high-dose dexamethasone supplementation).

3.1.1. Biological efficacy of hormone manipulations

The efficacy of the various hormonal manipulations was proven by their effects on body weight as judged by ANOVA ($F = 99.7$; $p < 0.001$).

Compared to the CON and ADX + CORT-treated groups, ADX resulted in a significant decrease in body weight over the experimental period ($p < 0.005$). ADX + DEX-treated animals showed an even greater loss of body weight as compared to CON ($p < 0.001$) and other treatment groups (vs. ADX and vs. ADX + CORT $p< 0.001$) (Fig. 3.1.3(a)).

ANOVA on ranks showed that all treatments resulted in significant reductions of daytime plasma CORT levels ($F = 13.8$; $p = 0.003$) (Fig. 3.1.3(b)). ADX and ADX + DEX animals had undetectable levels of CORT. Pair-wise analysis revealed that CORT levels in ADX + CORT animals were significantly higher than in both ADX ($p = 0.019$) and ADX + DEX-treated rats ($p = 0.03$) and significantly lower than those found in CON animals ($p = 0.005$). The elevated CORT levels in adrenal-intact animals reflect the stressful nature of the MRI procedure inasmuch as blood samples were collected immediately after MRI acquisition.

As expected, thymus weight proved to be a better indicator of the corticosteroid status during the entire experimental period. ANOVA revealed a significant effect of treatment on thymus weight at the time of autopsy ($F = 6.0$; $p < 0.05$). As compared to controls, the thymus to body weight ratio was significantly increased in ADX animals ($p = 0.04$), and this ratio was significantly decreased in the ADX + DEX group of animals ($p = 0.002$). Attesting to the fact that the CORT-replacement paradigm did not involve occupation of glucocorticoid receptors, ADX + CORT-treated rats did not show any significant reduction in their thymus to body weight ratios (Fig. 3.1.3(c)).

3.1.2. MR volumetry

Hemispheric volumes Although there was a slight increase in the volume of both left and right hemispheres in ADX animals and a reduced volume in ADX + DEX-treated animals, ANOVA failed to reveal significant differences ($F = 3.1$; $p = 0.54$ for right and $F = 2.7$; $p = 0.74$ for left hemispheric volumes)

in this parameter (Fig. 3.1.4(a)).

Anterior cingulate cortex and retrosplenial cortex volumes - ANOVA indicated a significant effect of treatment on the absolute volume of the left ACC ($F = 5.8$; $p < 0.005$). Post-hoc tests revealed a significant reduction on the left ACC in ADX + DEX-treated animals when compared to CON ($p = 0.008$), ADX ($p = 0.006$) and ADX + CORT ($p = 0.04$)-replaced groups. On the contralateral side, there was a similar trend but ANOVA failed to reveal a significant effect of treatment ($F = 3.0$; $p = 0.06$) on the volume of the ACC (Fig. 3.1.4(b)).

Normalized values (anterior cingulate/hemispheric volume) were also significantly affected by treatment on the left hemisphere ($F = 3.4$; $p < 0.05$) but not on the right ($F = 1.3$; $p = 0.32$). Comparisons among groups revealed that ADX + DEX-treated animals have a significant reduction of the normalized left ACC volume when compared to controls ($p = 0.02$) and ADX ($p = 0.04$) rats (Fig. 3.1.4(c)). None of the treatments caused significant volumetric alterations in the left or right RSC ($F = 0.49$; $p = 0.69$ for right side and $F = 0.12$; $p = 0.95$ for left side) (Fig. 3.1.4(d)). Normalized values (retrosplenial/hemispheric volume) also failed to reveal significant differences on both hemispheres ($F = 0.68$; $p = 0.58$ for right and $F = 0.46$; $p = 0.72$ for left side) (Fig. 3.1.4(e)).

In order to test for the occurrence of a shift on the demarcation between the two regions (since we were using external landmarks), we computed and analysed the combined RSA and ACC volumes. ANOVA of these combined volumes revealed a significant effect of treatment on the left hemisphere ($F = 4.26$; $p = 0.02$), ADX + DEX-treated animals having a statistically significant smaller volume (10.2 ± 0.30 mm^3) than both CON (11.6 ± 0.31 mm^3; $p = 0.006$) and ADX (11.4 ± 0.19 mm^3; $p = 0.014$) but not ADX + CORT–treated (10.9 ± 0.3 mm^3; $p = 0.084$) rats. On the right side there was a similar trend (CON 12.0 ± 0.48 mm^3; ADX 11.7 ± 0.30 mm^3; ADX + CORT 11.1 ± 0.36 mm^3; ADX + DEX 10.5 ± 0.33 mm^3) but ANOVA failed to reveal

a significant effect of treatment ($F = 3.198$; $p = 0.050$).

To further elucidate the observed volume reductions, the number of slices measured and a per slice average volume were computed for each region (data shown on Table 3.1.1). On the number of slices ANOVA failed to reveal significant differences between groups both for ACC ($F = 1.100$; $p = 0.376$) and for RSC ($F = 0.305$; $p = 0.822$). According to the whole volume results, analysis of per slice volumes indicated a significant effect of treatment on the left ACC ($F = 3.401$; $p = 0.042$), but not on the right ACC ($F = 1.678$; $p = 0.209$), the left RSC ($F = 0.228$; $p = 0.875$) or the right RSC ($F = 0.093$; $p = 0.963$). Post-hoc analysis confirmed a significant volume reduction on the left ACC in ADX + DEX-treated rats as compared to CON ($p = 0.02$) and ADX ($p = 0.015$) but not ADX + CORT-treated ($p = 0.106$) rats.

Hippocampal volume - Normalized hippocampal (HV) and brain volumes (BV) differed significantly between groups (Wilks' Lambda=0.287, $p<0.05$), due to altered BV ($F(3,19)=6.341$, $p<0.05$) and significant effects from normalized right HV (RHV, $F(3,19)=4.196$, $p<0.05$) and left HV (LHV, $F(3,19)=4.552$, $p<0.05$). LSD post hoc testing revealed that normalized RHV and LHV were significantly reduced in the ADX group compared to all other groups. The reduced RHV and LHV in ADX vs. ADX+DEX ($p<0.05$ each), and the reduced LHV in ADX vs. CON ($p<0.05$) survived Bonferroni correction with a trend of reduced RHV and LHV in ADX vs. ADX+CORT ($p=0.085$ and $p=0.072$) and a trend of reduced RHV in ADX vs. CON rats ($p=0.065$). No difference emerged between ADX+CORT and controls. In addition, the BV in the ADX+DEX group was significantly smaller compared to all other groups ($p<0.05$) which survived Bonferroni correction *vs.* the groups ADX and ADX+CORT ($p<0.05$). All groups showed consistently larger right than left HV ($p<0.05$) (Table 3.1.2).

3.1.3. Proton spectroscopy

The statistical analysis of the hippocampal metabolites was led by the hypothesis of adrenalectomy-induced NAA loss, and a further NAA loss in conjunction with elevated glutamate in the dexamethasone treated group (ADX+DEX). Based on

this hypothesis, we first compared these two metabolites between ADX vs. CON, ADX+DEX vs. CON and ADX+DEX vs. ADX groups by using MANOVA with post hoc LSD testing. The groups differed in the metabolite profiles (Wilks' Lambda=0.164, $F(15,36.289)=2.236$, $p<0.05$), mainly due to significant changes in glutamate (Glu) ($F(3,17)=4.284$), myo-inositol (mI) ($F(3,17)=5.813$) and choline-containing compounds (Cho) ($F(3,17)=3.382$). LSD post hoc testing was focused on Glu and NAA according to our hypothesis. ADX+DEX rats demonstrated significantly elevated Glu compared with all other groups and significantly reduced NAA compared with ADX and a trend reduction compared with controls ($p=0.064$). There were no significant alterations between ADX and controls (Table 3.1.3).

The exploratory comparison of the further metabolic profiles between ADX+DEX, ADX and controls was done with LSD and after Bonferroni correction of multiple testing. A significant increase of mI was found in ADX+DEX vs. ADX (also after Bonferroni correction). LSD tests furthermore revealed reduction of mI in ADX vs. CON ($p<0.05$, non significant after Bonferroni correction), and elevation of Cho in ADX vs. ADX+DEX ($p<0.05$, non significant after Bonferroni correction) and a borderline elevation of Cho in ADX vs. CON (LSD, $p=0.089$) (Table 3.1.3).

3.1.4. Histology and its correlation with MR volumetry

The shrinkage factor was 1.08, 1.17, 1.07 and 1.04, respectively, for controls, ADX, ADX + CORT and ADX + DEX treatment groups. The slight variations found in ADX and ADX + DEX-treated groups are likely to reflect the expected reduced water content in brain tissue of experimental groups (Fig. 3.1.5). Although ANOVA on the histology data did not reach significance ($F = 2.83$; $p = 0.08$), a strong correlation was found between the histological and MRI estimations of volumes of the left ACC ($r = 0.91$; $p < 0.001$) (Fig. 3.1.6). The mean difference between the two methods of estimation was ~0.28 ± 0.56 mm^3 (Bland–Altman analysis of agreement). Similarly, ANOVA testing revealed no significant effects in LHV obtained by histology and normalized to MRI-derived BV ($F(3,18)=1.241$, $p=0.324$). In terms of absolute

volumes, there was overall a trend effect ($F(3,18)=2.634$, $p=0.081$) with a significantly reduced LHV in ADX+DEX rats compared to ADX+CORT ($p<0.05$) and CON rats ($p<0.05$) with LSD post hoc testing.

When accounting for the shrinkage factors, there was a moderate but significant correlation between MRI-based volume calculations and stereological histology-based volume estimations of the absolute LHV (Pearson's correlation coefficient, $r = 0.50$ $p<0.05$). Intra-rater variability for MR hippocampal volumetry was 0.97 for the right, 0.98 for the left, and 0.99 for the total hippocampal volume (Pearson's correlation coefficient, $p<0.05$).

3.1.5. Tau phosphorylation pattern and APP processing

Both, amyloid precursor protein (APP) metabolism and microtubule-associated protein tau phosphorylation, two highly regulated cellular processes, are intrinsically linked to neuronal function and survival. Since the MRI data showed that alterations of the glucocorticoid *milieu* result in neuronal dysfunction and atrophy in the rat hippocampus and PFC, it was of interest to monitor the effect of manipulations of the corticosteroid environment on total levels of tau and APP as well as on APP misprocessing and aberrant phosphorylation of tau. Figures 3.1.9 and 3.1.10 show recently-obtained data that extend the aforementioned volumetric and functional MRI data.

As shown in Fig 3.1.9, administration of either CORT or DEX to ADX animals stimulates APP production (3.1.9A) and increases levels of BACE-1, the enzyme that cleaves APP (3.1.9C) in the prefrontal cortex (PFC). Both GC agonists also acted similarly insofar that they decreased C-99 levels, the product of γ-secretase cleavage. These results confirm other findings in this thesis (Chapter 3.2.6) that show that GC stimulate the amyloidogenic pathway of APP processing. The data from ADX animals are more difficult to interpret. Like GC, ADX resulted in an upregulation of APP and BACE, but not nicastrin, resulting in an exaggerated accumulation of C99. It should be noted, however, that the C-99 data come from pooled immunoprecipitates since the tissue lysates were insufficient for individual

analysis; therefore these data should be treated as preliminary.

Manipulation of the GC milieu was found to also alter the pattern of tau phosphorylation (Fig. 3.1.10). Adrenalectomy (ADX) and ADX+DEX-treated animals showed increased tau phosphorylation, as measured by the PHF-1 tau antibody which recognizes phosphorylated tau at Ser396/404. In contrast, ADX animals receiving corticosterone (CORT) showed decreased amounts of tau phosphorylation.

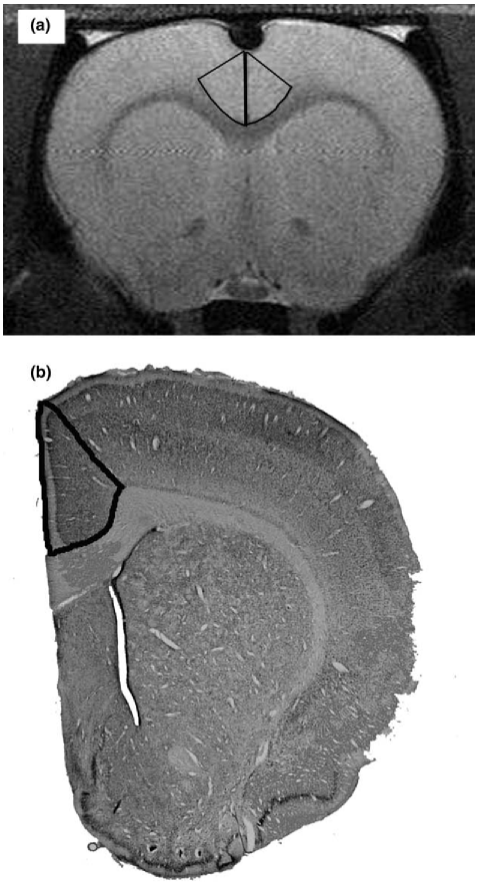


Fig. 3.1.1. Landmarks used to delineate the anterior cingulate cortex in MRI scans (a) and histological sections stained with Giemsa (b) are shown. To estimate cross-sectional areas of regions of interest, a systematic set of points was randomly overlaid on an image of the scan/section and the points hitting the area under study were counted (see Section 2)

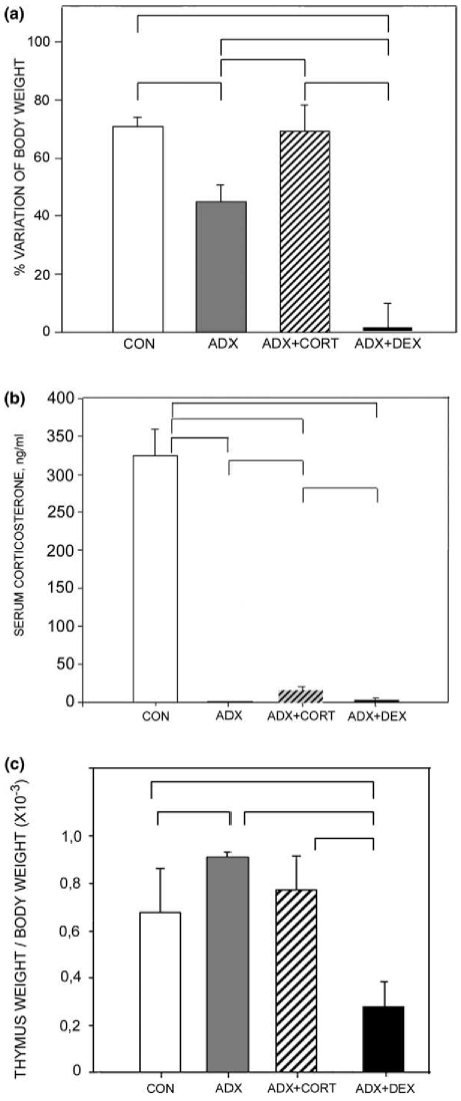


Fig. 3.1.3. Effects of hormonal manipulations on body weight (a), serum corticosterone level (b) and thymus/body weight (c). CON controls; ADX adrenalectomized rats; ADX + CORT adrenalectomized rats with corticosterone replacement; ADX + DEX adrenalectomized rats with dexamethasone treatment. Lines indicate significant differences ($p < 0.05$) between experimental groups: (A) CON vs. ADX $p < 0.005$; CON vs. ADX + DEX $p < 0.001$; ADX vs. ADX + CORT $p < 0.005$; ADX vs. ADX + DEX $p < 0.001$; CORT vs. ADX + DEX $p = 0.001$; (B) CON vs. ADX $p = 0.005$; CON vs. ADX + CORT $p < 0.01$; CON vs. ADX + DEX $p < 0.01$; ADX + CORT vs. ADX $p < 0.02$; ADX + CORT vs. ADX + DEX $p < 0.05$; (C) CON vs. ADX $p < 0.05$; CON vs. ADX + DEX $p = 0.002$; ADX vs. ADX + DEX $p < 0.001$; ADX + CORT vs. ADX + DEX $p < 0.001$.

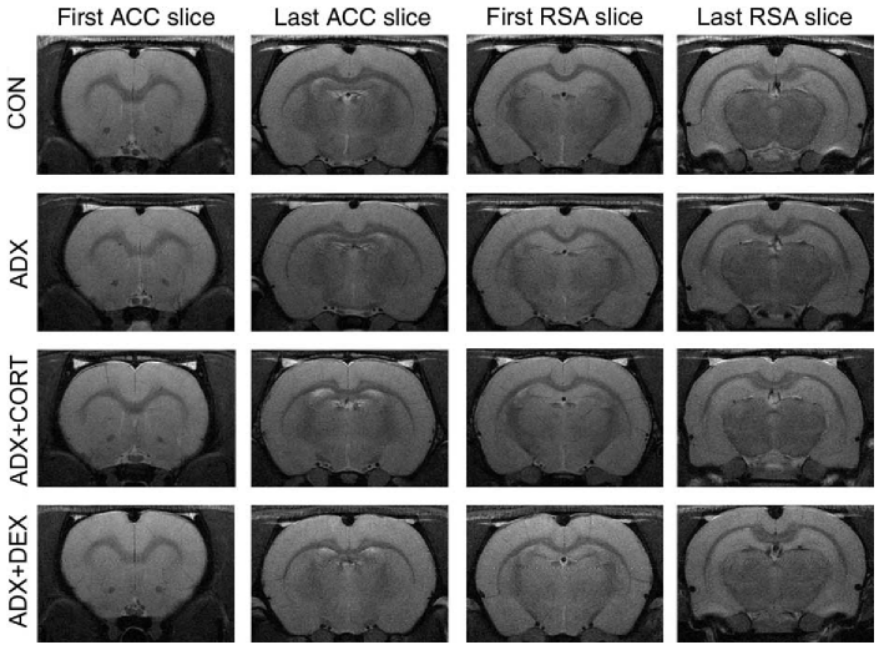


Fig. 3.1.2. Examples of the first (rostral) and last (caudal) MR slices used for ACC and RSC volume estimations in one animal of each treatment group. ACC was measured from the closure of the genu of the corpus callosum (first slice where it was closed) to the rostral limit of the hippocampus (last slice before its appearance). RSC was measured from the rostral limit of the hippocampus (first slice where it was present) to the caudal opening of the corpus callosum (last slice before it was opened). See Section 2. CON controls; ADX adrenalectomized rats; ADX + CORT adrenalectomized rats with corticosterone replacement; ADX + DEX adrenalectomized rats with dexamethasone treatment

Table 3.1.1
Detailed analysis of data generated through MR images

	CON	ADX	ADX + CORT	ADX + DEX
ACC slice number	5.25 ± 0.25	5.25 ± 0.25	5.17 ± 0.17	4.86 ± 0.14
RSC slice number	3.75 ± 0.25	3.75 ± 0.25	3.50 ± 0.22	3.71 ± 0.18
Left ACC per slice volume[a]	1.51 ± 0.060	1.52 ± 0.065	1.45 ± 0.036	1.35 ± 0.033
Right ACC per slice volume	1.55 ± 0.078	1.52 ± 0.045	1.46 ± 0.024	1.41 ± 0.044
Left RSC per slice volume	1.03 ± 0.018	0.99 ± 0.021	1.00 ± 0.029	0.98 ± 0.068
Right RSC per slice volume	0.98 ± 0.035	0.92 ± 0.031	0.98 ± 0.030	0.98 ± 0.075

CON, controls; ADX adrenalectomized rats; ADX + CORT adrenalectomized rats with corticosterone replacement; ADX + DEX adrenalectomized rat with dexamethasone treatment. Mean number of MRI slices used for ACC and RSC volume estimations and average per slice volume for each region studied.

a Left ACC per slice volume: CON vs. ADX + DEX $p = 0.020$; ADX vs. ADX + DEX $p = 0.015$.

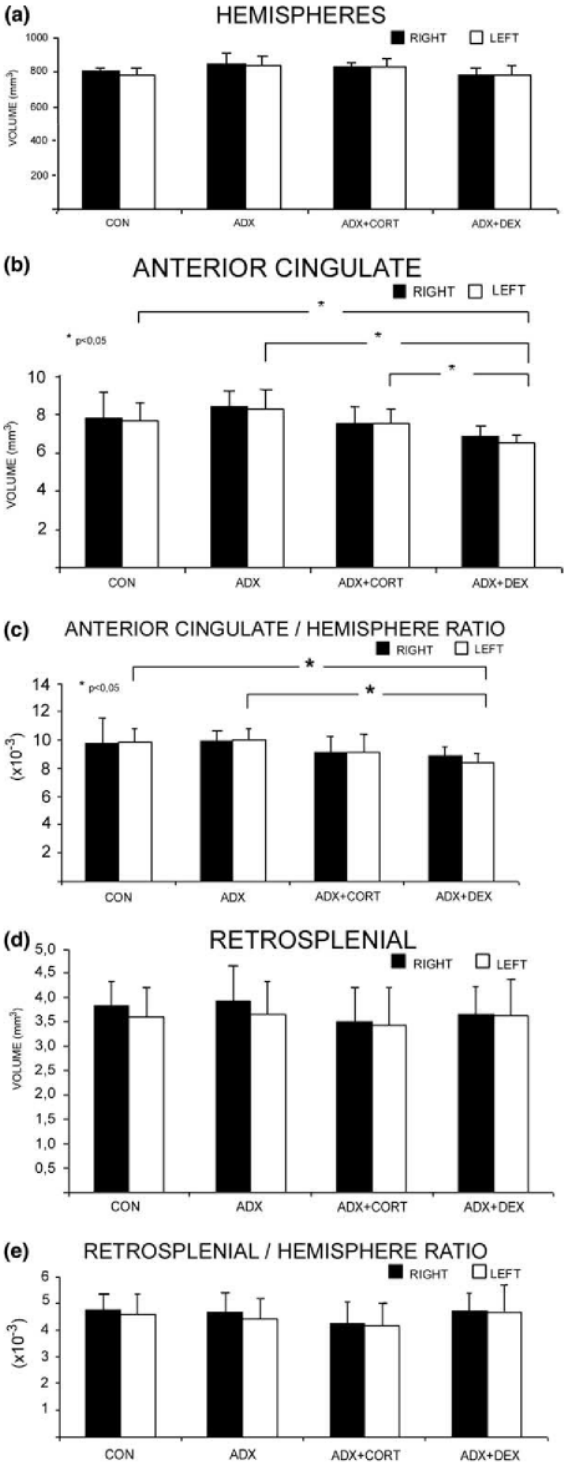


Fig. 3.1.4. Volumetric determinations derived from MRI images. CON, controls; ADX adrenalectomized rats; ADX + CORT adrenalectomized rats with corticosterone replacement; ADX + DEX adrenalectomized rats with dexamethasone treatment. (a) Hemispheric volumes. (b) Cingulate cortex volume: CON vs. ADX + DEX p = 0.008; ADX vs. ADX + DEX p = 0.006; ADX + CORT vs. ADX + DEX p = 0.04. (c) Cingulate cortex volume expressed as a function of hemispheric volumes: CON vs. ADX + DEX p = 0.02; ADX vs. ADX + DEX p = 0.04. (d) Retrosplenial cortex volume. (e) Retrosplenial cortex volume expressed as a function of hemispheric volumes.

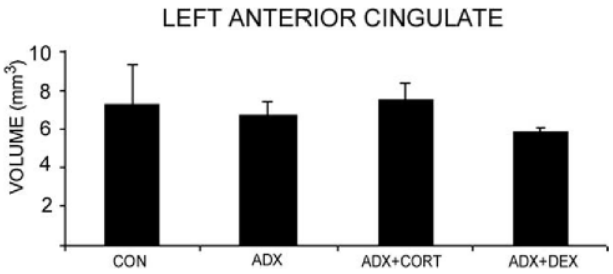


Fig. 3.1.5. Volumetric determinations of the left anterior cingulate cortex in histological sections. CON, controls; ADX adrenalectomized rats; ADX + CORT adrenalectomized rats with corticosterone replacement; ADX + DEX adrenalectomized rat with dexamethasone treatment.

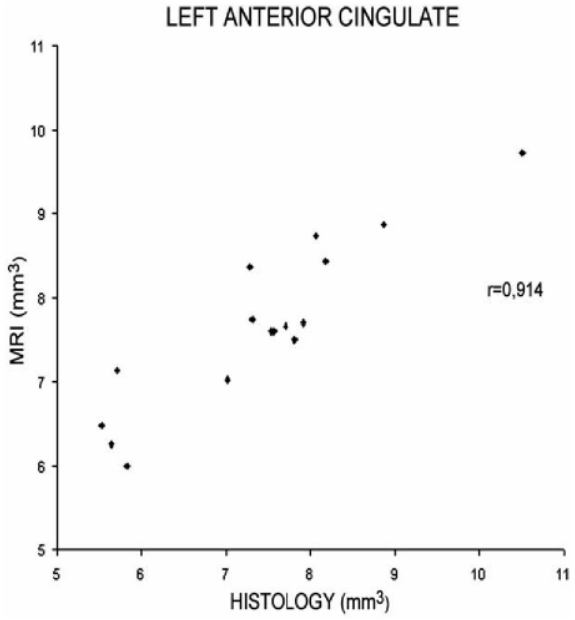


Fig. 3.1.6. Correlation between volumetric determinations from MRI images and histological analysis. For each animal, the volume estimated from histological sections is plotted on the x-axis and the volumes estimated from MRI against the y-axis; r represents Pearson~s coefficient of correlation.

Table 3.1.2: Volumetric and histological measures in rats after alteration of the corticosteroid status.

Measure	CON (n=5)	ADX (n=5)	ADX+CORT (n=6)	ADX+DEX (n=7)
Volumetric measures				
BV [mm^3]	1537.81±33.74	1582.57±87.25	1591.51±49.22	1454.16±69.55*
LHV [mm^3]	47.79±2.38	41.95±2.18	48.69±2.37	44.76±2.45
RHV [mm^3]	47.92±1.52	45.25±2.16	49.32±2.82	45.61±2.91
LHV/BV	0.0311±0.0021	0.0283±0.0017*	0.0306±0.0011	0.0308±0.0005
RHV/BV	0.0312±0.0014	0.0286±0.0018*	0.0310±0.0015	0.0314±0.0011
Histological measures				
LHV [mm^3]	49.73±2.95	45.14±5.43	49.50±6.70	42.17±5.88*

CON: controls, ADX: adrenalectomized rats, ADX+CORT: adrenalectomized rats with corticosterone replacement, ADX+DEX: adrenalectomized rats with dexamethasone treatment, BV: brain volume, RHV: right hippocampal volume, LHV: left hippocampal volume. Values are given as means ± SD. MANOVA (*p<0.05, **p<0.01, ***p<0.001). Significant differences between experimental groups: BV: ADX+DEX *vs.* ADX p=0.003, ADX+DEX *vs.* ADX+CORT p=0.001, ADX+DEX *vs.* CON p=0.036; RHV/BV: ADX *vs.* ADX+CORT p=0.014, ADX *vs.* ADX+DEX p=0.004, ADX *vs.* CON p=0.011; LHV/BV: ADX *vs.* ADX+CORT p=0.012, ADX *vs.* ADX+DEX p=0.006, ADX *vs.* CON p=0.004. LHV (histology, ANOVA): ADX+DEX *vs.* ADX+CORT p=0.029, ADX+DEX *vs.* CON p=0.032.

Table 3.1.3: Absolute hippocampal metabolite concentrations in rats after alteration of the corticosteroid status.

	CON (n=4)		ADX (n=5)		ADX+CORT (n=6)		ADX+DEX (n=6)	
	AbsConc	EQ	AbsConc	EQ	AbsConc	EQ	AbsConc	EQ
NAA	2760±325	10.5±4.4	2802±126	8.8±0.8	2591±313	11.5±3.0	2423±248*	10.2±0.8
Cr	2888±259	9.8±0.5	2734±243	9.4±1.3	2640±292	10.5±2.8	2760±279	8.8±1.0
Cho	1012±103	16.5±3.0	1224±302*	12.8±3.0	935±71	18.7±4.8	919±141	15.3±2.9
mI	1455±111	21.0±1.6	1220±137*	23.4±3.6	1350±120	22.3±3.7	1547±159*	17.0±4.5
Glu	2378±134	16.0±2.8	2400±200	14.6±2.5	2378±174	15.7±1.9	2728±247*	11.7±0.8

AbsConc: absolute metabolite concentration (means ± SD), EQ: LCModel percent estimation quality (EQ) standard deviation (means ± SD), NAA: N-acetyl aspartate, Cho: choline-containing compounds, Cr: total creatine, mI: *myo*-inositol, Glu: glutamate. Hippocampal metabolites: MANOVA (*p<0.05). Significant differences between experimental groups: Glu: ADX+DEX *vs.* ADX p=0.014, ADX+DEX *vs.* ADX+CORT p=0.007, ADX+DEX *vs.* CON p=0.014; mI: ADX+DEX *vs.* ADX p=0.001, ADX+DEX *vs.* ADX+CORT p=0.022, ADX *vs.* CON p=0.019, Cho: ADX *vs.* ADX+CORT p=0.014, ADX *vs.* ADX+DEX p=0.011, NAA: ADX+DEX *vs.* ADX p=0.030.

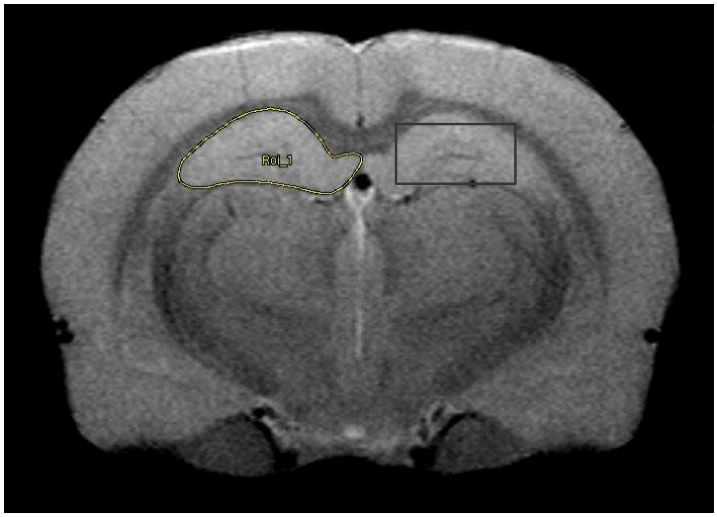


Fig. 3.1.7: Example of image analysis by manual outlining of the (right) hippocampus (bright line) at approximately -3.14 mm from Bregma using FUNtool software (Bruker, Karlsruhe, Germany).

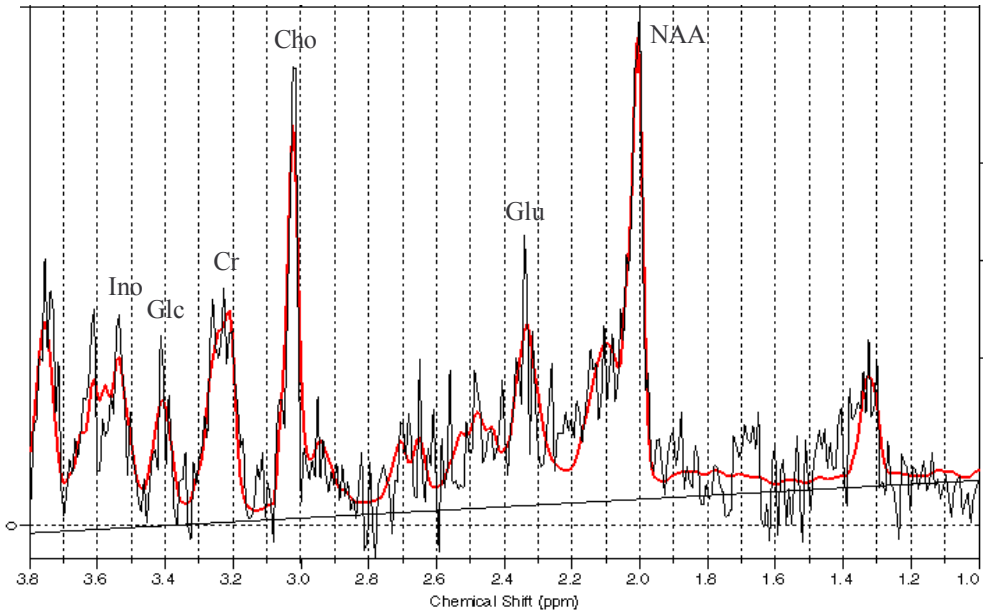


Fig. 3.1.8: Example of proton spectroscopy of the left hippocampus (position of voxel as shown in Fig. 1) of an ADX+DEX rat (a) *vs.* an ADX+CORT rat (b). Cho: choline and choline-containing molecules (3.2 ppm), Cr: creatine (3.0 ppm), mI: *myo*-inositol (3.5 ppm), NAA: N-acetyl-aspartate (2.0 ppm), Glu: glutamate (2.5 ppm, arrow).

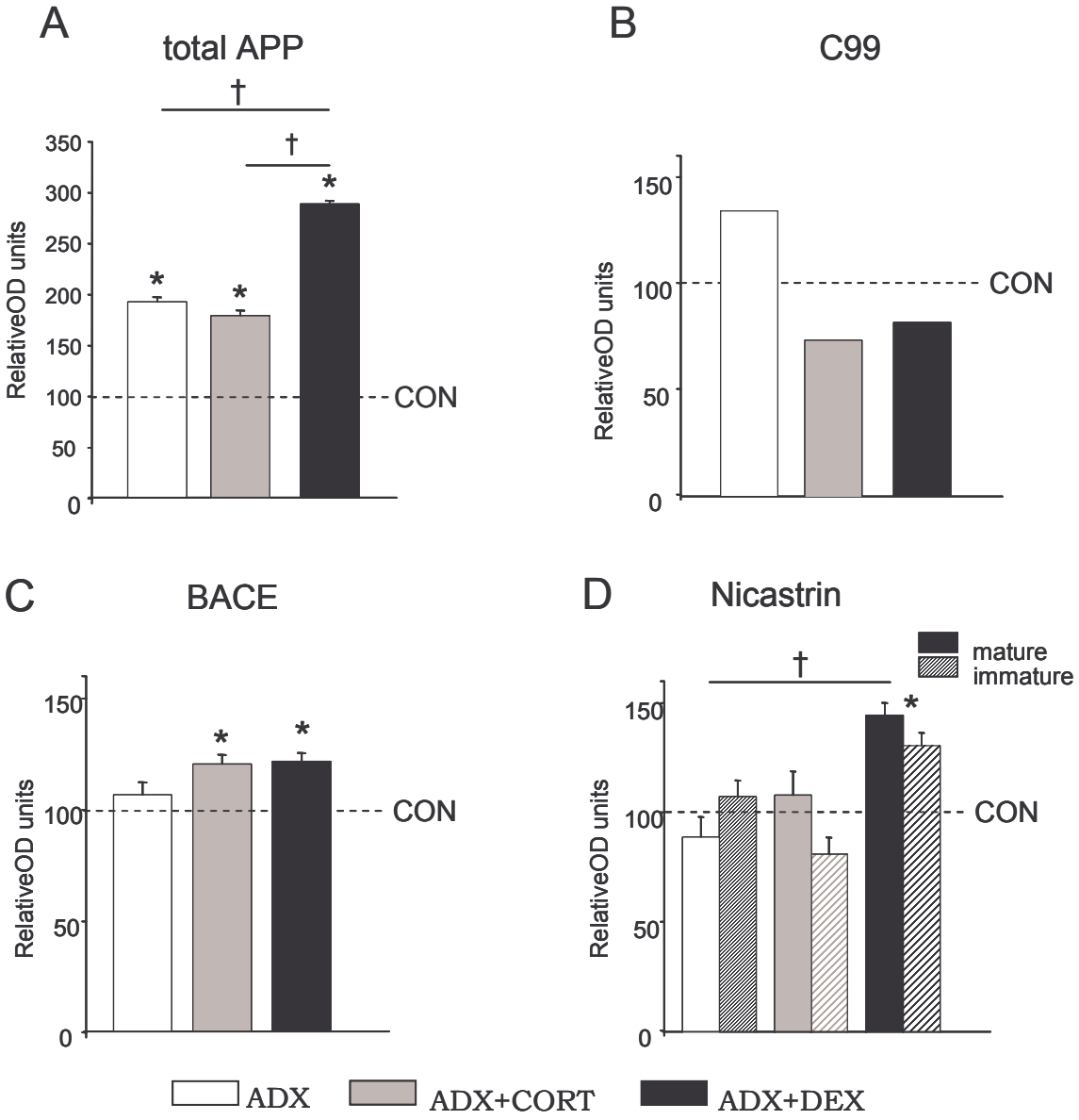


Fig 3.1.9. Alterations in APP metabolism in the rat prefrontal cortex (PFC) following manipulation of the glucocorticoid (GC) milieu by adrenalectomy (ADX), ADX+corticosterone (CORT) or ADX+dexamethasone (DEX).

CORT is the natural GC in rodents; DEX is a synthetic GC receptor (GR) agonist. Both CORT and DEX in ADX animals stimulate APP production (**A**) as well as BACE (**C**) and nicastrin levels (**D**). Again, both GC agonists acted similarly by decreasing C-99 amount possibly cleaved by γ-secretase. Together with the BACE and nicastrin data, these preliminary findings on C-99, confirm other findings in this thesis that suggest that GC stimulate the amyloidogenic pathway of APP processing. Like GC, ADX resulted in an upregulation of APP and BACE, but not nicastrin, resulting in an exaggerated accumulation of C99. Each data set, except for that for the C-99 fragment, are means ± SEM from 6-7 animals each. In the case of the C-99 data, the results shown come from pooled immunopreciptates since the tissue lysates were insufficient for individual analysis.

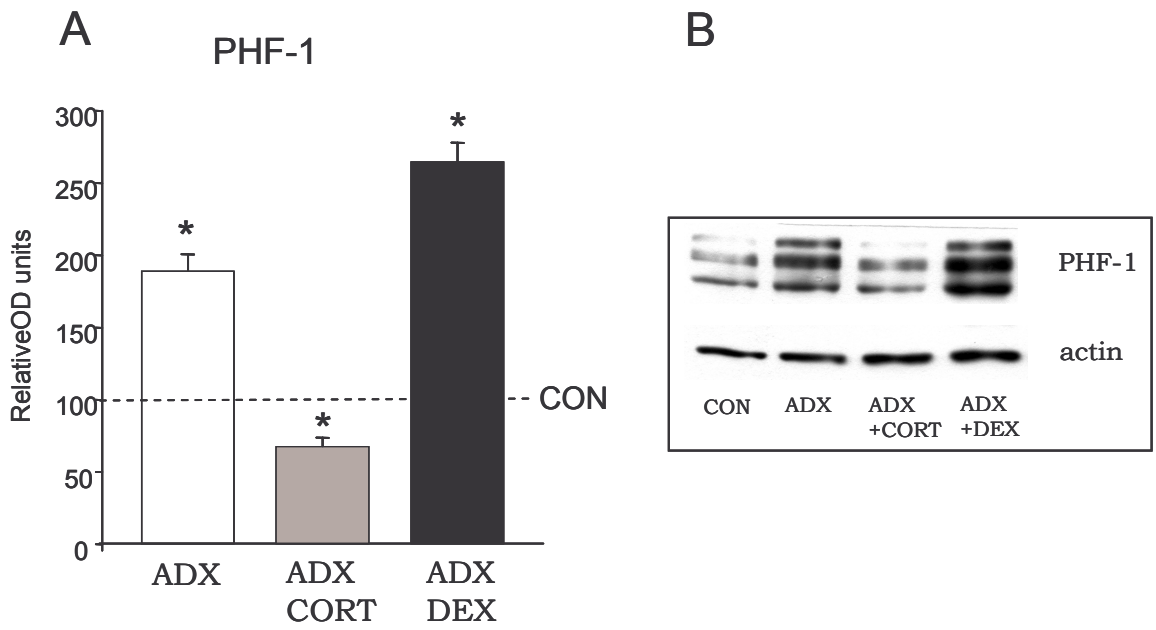


Fig 3.1.10. Manipulation of the glucocorticoid (GC) milieu alters tau phosphorylation pattern.
(A) Adrenalectomized (ADX) as well as ADX animals treated with dexamethasone (DEX) show increased tau phosphorylation as measured by PHF-1 tau antibody (recognizes phosphorylated tau at Ser396/404). In contrast, ADX animals receiving corticosterone (CORT) show the opposite effect. Data are normalized against actin levels and each data set are means $\pm$ SEM from 6-7 animals each. **(B)** Representative blot from Western blot analysis.

3.2. APP misprocessing and aberrant tau phosphorylation as a cellular response linking chronic stress with AD

Background

Memory deficits, strongly correlated with atrophy in the hippocampus, entorhinal cortex and prefrontal cortex, are the principal manifestation of AD (see Chapter 1.1, 1.2 and 1.4.3.4). Similar patterns of dendritic atrophy and synaptic loss are observed in humans and animals displaying impaired cognition after exposure to elevated glucocorticoid (GC) levels and/or stressors (see Chapter 1.4.3.3). While AD patients frequently display hypercortisolism (see Chapter 1.4.3.4), the involvement of GC in the etiopathogenesis and course of the disease is not known. Having shown in the previous experiment (see Chapter 3.1.5) that elevated GC levels induce neuronal dysfunction and brain atrophy, and affect tau phosphorylation and APP processing, it was of interest to investigate whether elevated GC levels and stress can trigger tau and cytoskeletal pathology, thus influencing the initiation and/or maintenance of AD pathology.

3.2.1. Chronic stress and GC induce hyperphosphorylation of tau

Western blot analysis of the hippocampus (Fig. 3.2.1*a*) and PFC (Fig. 3.2.1*b*) revealed that exposure to chronic unpredictable stress results in significantly increased levels of phosphorylated tau (pSer202, pThr231, and pSer396/404) ($p \leq 0.05$ in all cases); pSer262/356 tau is not detectable by Western blotting. In addition, exposure to stress produced increased levels of pSer409 tau in the hippocampus ($p \leq 0.05$); this phospho-epitope was undetectable in the PFC. Treatment with GC also resulted in increased expression of pSer202 and pSer396/404 tau ($p < 0.05$) in the hippocampus and of the pSer231 epitope in the PFC ($p < 0.05$).

The above analyses, showing that stress and GC treatment induce tau hyperphosphorylation, were confirmed by immunohistochemistry. Stress was associated with increased staining intensity of tau phosphorylation-dependent antibodies such as CP13 (recognizes pSer202) and 12E8 (pSer262/Ser356)

within the subiculum of the hippocampal formation (Fig. 3.2.1*c*) and PFC (Fig. 3.2.1*d*). Most of the 12E8 and CP13 immunoreactivity was observable in the soma of pyramidal neurons, as indicated in the corresponding micrographs. All the tau phosphorylation sites recognized by these antibodies have been identified in paired helical filaments-tau proteins in AD brains.

A number of kinases, in particular MAP kinases, cdk5 and GSK3β, have been implicated in hyperphosphorylation of tau in AD (see Chapter 1.3.2.4 and Box 1.1) as well as in other forms of neuronal dysfunction and degeneration. This prompted our investigation of whether any of these kinases are concomitantly activated by stress or GC. Results of our Western blot analysis in hippocampus and prefrontal cortex are shown in Fig. 3.2.1*e* and *f*. Interestingly, not all kinases examined were activated by stress. Stressed rats showed significantly increased levels ($p \leq 0.05$) of active ERK1/2 and JNK in the hippocampus and PFC (Fig. 3.2.1*e*), whereas GC treatment resulted in significantly elevated levels ($p \leq 0.05$) of only active ERK1/2 in these areas (Fig. 3.2.1*f*). Active GSK3β levels were increased in the hippocampus, but not PFC, of GC-treated rats ($p \leq 0.05$; Fig. 3.2.1*f*); there were no significant changes in levels of this form of the protein after exposure to stress (Fig. 3.2.1*e*). Although neither stress nor GC induced changes in the levels of cdk5, levels of its activator p35 were significantly elevated in the hippocampus and PFC of stressed animals ($p \leq 0.05$; Fig. 3.2.1*e*). Both stress and GC treatment led to significant increases in CaMKII levels in the hippocampus ($p \leq 0.05$; Fig. 3.2.1*e*, *f*), although these treatments did not influence expression of this kinase in the PFC.

3.2.2. Involvement of glucocorticoids

Previous studies have shown that GC can render neurons more vulnerable to the neurotoxic effects of Aβ and Aβ can trigger tau neuropathology (see Chapter 1.3.1.3 and 1.4.3.3). Accordingly, we next investigated whether GC interplays with Aβ to influence tau biochemistry. Chronic i.c.v. infusions of Aβ stimulated the expression of a number of tau phospho-epitopes in both the hippocampus and PFC ($p \leq 0.05$; Fig. 3.2.2a, b). Previously, GC treatment alone was also seen

to increase tau phosphorylation (Fig. 3.2.1a and b); now, as shown in Fig. 3.2.2a and b, GC significantly potentiated Aβ-induced increases of pSer202 tau in the hippocampus and PFC, and of pSer396/404 and pSer409 tau in the hippocampus ($p \leq 0.05$).

The above results, showing that GC can exacerbate the effects of Aβ, were supported by immunohistochemical analysis of the hippocampus and PFC of unstressed animals. As compared to control animals, those treated with Aβ displayed higher levels of immunoreactive pSer202 tau (detected with CP13 antibody) within the somata of subicular and PFC neurons; when GC and Aβ were administered concomitantly, there was a further increase in immunoreactive signal (Fig. 3.2.2*c*). Immunohistochemistry also revealed an intensification of pSer262/356 tau (12E8) immunoreactive signal in the subiculum and PFC after combined treatment with Aβ and GC (Fig. 3.2.2*d*). Another phosphorylation-independent antibody, PHF-1 (against pSer396/404 tau) was more strongly stained in the hippocampal mossy fiber network of GC+Aβ-treated animals than in that of unstressed animals receiving Aβ only (Fig. 3.2.2*e*).

In addition to measuring tau phosphorylation pattern, we also monitored the expression profiles of a series of kinases in the PFC and hippocampus by immunoblotting. Aβ treatment produced major increases in activated ERK1/2 levels in the hippocampus and PFC ($p \leq 0.05$; Fig. 3.2.2*f* and *g*). The Aβ infusion paradigm also produced variable increases in the levels of pGSK3β (hippocampus: 15%; PFC: 27%), cdk5 (hippocampus: 8%; PFC: 50%) and its activating partner p35 (hippocampus: 19%; PFC: 7%) and CaMKII (hippocampus: 38%; PFC: no change). When animals were given the combined treatment of Aβ+GC, a striking accentuation of the pERK1/2 and pJNK response was observed in the hippocampus ($p \leq 0.05$; Fig. 3.2.2*f*); this treatment regimen produced only minor changes in kinase expression in the PFC (Fig. 3.2.2*g*). The lesser kinase changes in the PFC after Aβ+GC administration are commensurate with the fewer number of tau epitopes that were hyperphosphorylated in this area (cf. Fig. 3.2.2*b*).

3.2.3. Previous stressful experience increases sensitivity to GC and Aβ

Having shown that stress and Aβ alone, as well as Aβ together with GC, can induce tau hyperphosphorylation, we next sought to investigate the effect of Aβ and Aβ+GC on tau phosphorylation in previously stressed animals.

As shown in Fig. 3.2.3*a* and *b*, the combined treatment of Aβ+GC in stressed rats resulted in increased levels of several tau phospho-epitopes in the hippocampus and PFC, as compared to treatment with Aβ alone. Specifically, GC significantly potentiated the stimulatory effects of Aβ on the levels of pSer202 tau (hippocampus and PFC), pThr231 tau (PFC only), pSer396/404 tau (hippocampus and PFC) and pSer409 tau (hippocampus only). Additionally, the entorhinal cortex, an area that displays neuropathological lesions in AD (see Chapter 1.2), also showed signs of altered expression of phospho-tau epitopes in stressed rats that were subsequently treated with Aβ+GC. The data for the entorhinal cortex were obtained from tissue pools from 4 animals. Western blot analysis indicated that, as compared to mono-therapy with Aβ, the combinatorial treatment regimen results in markedly higher levels of pSer396/404 tau and pSer409 tau (Fig. 3.2.3*c*). The above described changes in levels of the various forms of phosphorylated tau in the hippocampus and PFC were paralleled by increased levels of various tau-related kinases, represented by pERK1/2 and pJNK in Fig. 3.2.3d and e. Levels of pGSK3β, p35 and CaMKII levels were also significantly elevated in the hippocampus after Aβ treatment ($p < 0.05$), with a further significant rise in CaMKII levels being observed in rats exposed to Aβ+GC ($p < 0.05$). In the PFC, Aβ resulted in significant increases in pGSK3β and cdk5 levels ($p < 0.05$), with a further increase occurring in rats given Aβ+GC ($p < 0.05$).

Furthermore, as compared to non-stressed animals receiving Aβ+GC, rats treated with Aβ+GC subsequent to stress exposure showed greater staining intensities with the antibody PHF-1, which labels pSer396/404 tau (cf. Figs. 3.2.3*f* and 2*e*). Interestingly, PHF-1 immunoreactivity became evident in the somata of hippocampal neurons in the animals subjected to the stress+Aβ+GC paradigm (Fig. 3.2.3*h*), contrasting with the result obtained in non-stressed

animals that received Aβ+GC; in the latter, PHF-1 immunoreactivity was largely confined to the mossy fibers (cf. Fig. 3.2.2*e*). In the PFC, there was a clear increase in the intensity of PHF-1 labeling in the stress+Aβ+GC group *vs.* non-stressed+Aβ+GC group. As shown in Fig. 3.2.3*g* and *h*, this labeling was exclusively found in neuronal perikarya.

3.2.4. Tau accumulation and interference with microtubule assembly dynamics after exposure to stress and/or GC

Besides tau hyperphosphorylation, another neuropathological characteristic of AD and other tauopathies is the relocation of tau from axons to perikarya, and its accumulation at the latter site (see Chapter 1.3.2). Based on this, we next monitored tau distribution by immunohistochemistry with the pan-tau antibody, Tau-5 (Fig. 3.2.4). This analysis revealed an obvious accumulation of Tau in the perikarya of PFC and hippocampal CA3 neurons when Aβ was co-administered with DEX; notably, these changes are much more striking in the PFC of stressed *vs.* unstressed animals (semi-quantification results in lower panel of Fig. 3.2.4).

Cytoskeletal disruption associated with abnormally hyperphosphorylated tau protein is emerging as an important mechanism in Alzheimer's disease (AD) (see Chapter 1.3.2.5). Here, we monitored a post-translational modification of α-tubulin (acetylation), decreases of which correlate with dysregulation of microtubule dynamics and stability and associate with tau neuropathology (see Chapter 1.3.2). Immunoblotting showed that chronic stress significantly reduces Ac-Tub levels (14%; $p \leq 0.05$). Whereas Aβ alone did not influence Ac-Tub levels, GC+Aβ led to a 17% decrease (17%) in this parameter ($p \leq 0.05$). The same pattern of changes was observed after Aβ+GC administration to previously stressed animals (16%; $p \leq 0.05$). Together, with the earlier-shown data on tau hyperphosphorylation (pThr231 and pSer262/356 tau), these findings suggest that stress and Aβ+GC contribute to microtubule dysregulation and cytoskeletal disturbances.

3.2.5. Synaptic dysregulation by stress and GC

Synaptic plasticity provides a cellular basis for cognitive functions. Soluble Aβ induces synaptic dysfunction and loss, effects which correlate better with cognitive impairment in AD than Aβ deposits (see Chapter 1.3.1.3). Previous studies showed that stress and GC, acting through glucocorticoid receptors (GR), result in dendritic atrophy and neuronal loss in the hippocampus and PFC (see Chapter 1.4.3.3). Since other results in this chapter demonstrate that GC treatment increases abnormal tau hyperphosphorylation and accumulation, as well as cytoskeletal disturbances, pilot studies (Fig 3.2.6) were carried out to correlate the observed alterations in tau biochemistry with synaptic changes. To this end, immunocytochemistry using presynaptic and postsynaptic markers (synapsin I and PSD-95, respectively), was employed on sections from rats treated as indicated. As shown in Fig 3.2.5, Aβ decreases pre- and post-synaptic sites in both the PFC (cingulate cortex) and hippocampus (subiculum); GC were found to potentiate the actions of Aβ, being more obvious for the reduction in synapsin I staining; stress was also found to lead to a reduction in the expression of both markers.

3.2.6. Chronic stress and GC drive APP processing along the amyloidogenic pathway

Exposure to stress resulted in a slight increase in the expression levels of *APP* mRNA (Table 3.2.1). While analysis of hippocampus (Fig. 3.2.6A) and PFC (Fig. 3.2.6B) revealed that stress did not influence total levels of APP (immature and mature forms), levels of the C99 fragment of APP were significantly increased ($P \leq 0.05$) in tissues from stress-treated rats. Consistent with this last observation, levels of BACE-1 were also slightly, but significantly, elevated after exposure to stress ($P \leq 0.05$). Interestingly, the levels of immature and mature nicastrin were selectively increased in the hippocampus and PFC of rats that had been exposed to stress ($P \leq 0.05$). Lastly, it is of interest to note that the above stress-induced magnitude of changes in C99 and nicastrin were generally greater in the hippocampus than in the PFC. The increased stress-induced vulnerability of the hippocampus *vs.* PFC is consistent with the fact that signs of AD neuropathology

occur earlier in the hippocampus than in the PFC (see Chapter 1.2).

Since GC secretion is an important physiological response to stress, we next considered the possibility that GC may be responsible for mediating the above-reported effects of stress on APP metabolism. To examine this possibility, rats were given daily injections of the synthetic glucocorticoid receptor agonist, dexamethasone. As shown in Table 3.2.1 and Fig 3.2.10, GC treatment resulted in an increase in *APP* mRNA levels and significant increases in the hippocampal (Fig. 3.2.7A and B) levels of APP. Furthermore, both brain areas also showed significantly increased levels of C99 ($P \leq 0.05$). BACE-1 expression was, however, only significantly elevated in the hippocampus ($P \leq 0,05$; Fig. 3.2.7A), indicating tissue-selectivity. In contrast to stress, GC failed to alter nicastrin levels in both the hippocampus and PFC (Fig. 3.2.7A and B), suggesting that signals, other than GC, are responsible for mediating the above-reported ability of stress to increase nicastrin levels.

3.2.7. Exogenous Aβ triggers APP misprocessing

As Aβ is known to induce AD pathology and its infusion is widely used as a model of AD (see Chapter 1.3.1.3), we here attempted to parallel Aβ and stress/GC effects by giving middle-aged rats constant infusions of Aβ into one lateral ventricle. Furthermore, in light of studies suggesting that Aβ can stimulate its own production in vitro(see Chapter 1.3.1.3), it was also of interest to investigate how exogenous Aβ influences APP metabolism in vivo. Aβ treatment resulted in an increase in *APP* mRNA expression (Table 3.2.1) and significant reductions in the levels of immature and mature APP in the hippocampus (Fig. 3.2.8A) and PFC (Fig. 3.2.8B). This may be explained by the finding that Aβ induced major increases in C99 production in both regions ($P \leq 0.05$, but greater in the hippocampus than in the PFC), with parallel significant increases in the levels of BACE-1 and nicastrin ($P \leq 0.05$). These findings demonstrate that Aβ treatment triggers APP mis-processing into C99 (through BACE-1) which has the potential to be cleaved into amyloid peptides (through γ-secretase cleavage). The close

resemblance of the effects of Aβ and stress on APP processing suggest that stressful events have the potential to trigger pathological mechanisms that result in APP mis-processing.

3.2.8. Stress history exacerbates APP misprocessing

Using transgenic mouse models of AD, Jeong et al. (2006) recently showed that chronic stress potentiates Aβ deposition and induces cognitive deficits, and Green et al (2006) showed that elevated GC exacerbate amyloidogenesis. On the other hand, the organism experiences stressful events throughout its lifetime, and aged individuals tend to show exaggerated GC secretory responses to stress (see Chapter 1.4.3.3). In light of these considerations and in an attempt to mimic life events, we here examined the sequential effects of stress and GC on AD-like pathology by simultaneously treating previously-stressed and non-stressed rats with Aβ (i.c.v. infusions) and/or GC (s.c. injections). Here, stress did not influence the effects of subsequently-applied Aβ on APP metabolism (data not shown), but stress accentuated the amyloidogenic effects of Aβ+GC in rats that had been previously exposed to stress. Specifically, as shown in Fig. 3.2.9, concomitant treatment with Aβ and GC triggered APP mis-processing in both hippocampus and PFC in non-stressed rats by elevating C99 levels ($P \leq 0.05$), an effect that was significantly exacerbated in previously-stressed animals. Comparison of BACE-1 levels in Aβ+GC-treated non-stressed and stressed rats revealed increases that were only significant in the PFC (Fig. 3.2.9B; $P \leq 0.05$). However, nicastrin levels in both the hippocampus and PFC were significantly elevated by Aβ+GC in stressed *vs.* non-stressed rats (Fig. 3.2.9, $P \leq 0.05$), indicating the greater likelihood of C99 processing along the amyloidogenic pathway. Thus, a history of stress increases vulnerability to subsequent exposures to GC and Aβ.

Jeong et al (2006) FASEB J. 20: 729-731.
Green et al (2006) J Neurosci. 26: 9047-9056.

3.2.9. Behavioral performance

Since impairments in learning and memory are the primary behavioral manifestations of AD, we considered it important to evaluate the extent to which the various treatments used in the present study influenced cognitive performance. Specifically, we examined (i) hippocampus-dependent spatial learning/memory, and (ii) PFC-dependent reverse learning. Consistent with our previous findings (see Chapter 1.4.3.3), both chronic stress and GC exposure impaired reference memory ($p<0.05$) and reversal learning ($p < 0.05$) (data not shown). Additionally, as shown in Fig. 3.2.11*a*, i.c.v. infusion with Aβ resulted in impairment of spatial reference memory ($p < 0.001$); this effect was aggravated in terms of latency times ($p < 0.05$; data not shown) in animals that received concomitant treatment with GC, although the between-group differences did not reach statistical significance in terms of path-length. The cognitive impairments following simultaneous treatment with Aβ and GC were even more pronounced in animals that had been previously exposed to chronic stress (stress *vs.* stress+Aβ, $p < 0.05$; stress *vs*. stress+Aβ+GC, $p < 0.0001$; Fig. 3.2.11*b*).

The results of the reversal learning test are shown in Fig. 3.2.11*c* and 3.2.11*d*. Treatment with Aβ impaired behavioral flexibility ($p < 0.001$; Fig. 3.2.11*c*); this effect was again to some extent potentiated by Aβ and GC (Fig. 3.2.11*c*). Interestingly, further impairments were observed when Aβ and GC were given to previously stressed animals ($p < 0.05$; Fig. 3.2.11*d*), although the administration of Aβ alone to previously-stressed animals did not add to the effects of stress on this behavioral parameter. These cognitive deficits correlate closely with the finding that the stress+Aβ+GC paradigm has a greater influence on tau hyperphosphorylation and APP misprocessing than the Aβ+GC paradigm has (cf. Fig 3.2.3, Fig 3.2.9 and Fig. 3.2.11).

In light of studies suggesting an interaction between emotion and cognition (and evidence that AD patients show hyperanxiety (Dolan, 2002; Phelps, 2006), we assessed the emotional state of the variously-treated groups of animals. In accordance, we found that all experimental procedures produced signs of

increased anxiety as determined by % of time spent in open arm of the elevated plus maze than in controls ($p \leq 0.001$), and animals given Aβ also displayed increased signs of anxiety when compared both to their controls or stress counterparts ($p \leq 0.01$). Unexpectedly, there were no differences between non-stressed and previously stressed animals that received the combined treatment of Aβ and GC. A similar profile was detected in the analysis of % of distance travelled in the center vs periphery of the open-field, which is also an estimator of anxiety-like behavior. Importantly, this anxious phenotype cannot be attributed to locomotor deficits as none of the experimental procedures produced differences in total distances travelled in the open-field, nor in the number of close arm entries in the EPM. Interestingly, there were impairments in exploratory behavior as assessed by rearing activity. Briefly, stress- and CG-treated rats had smaller number and duration of rearings ($p \leq 0.042$ and $p \leq 0.047$ for number and $p \leq 0.042$ and $p \leq 0.015$ for duration of rearings) than controls. Aβ-treated rats also displayed decreased exploratory behavior when compared to controls ($p \leq 0.05$) (Table 3.2.2)

-Dolan RJ. Science 2002; 298: 1191-1194
-Phelps EA. Annu Rev Psychol. 2006, 57: 27-53

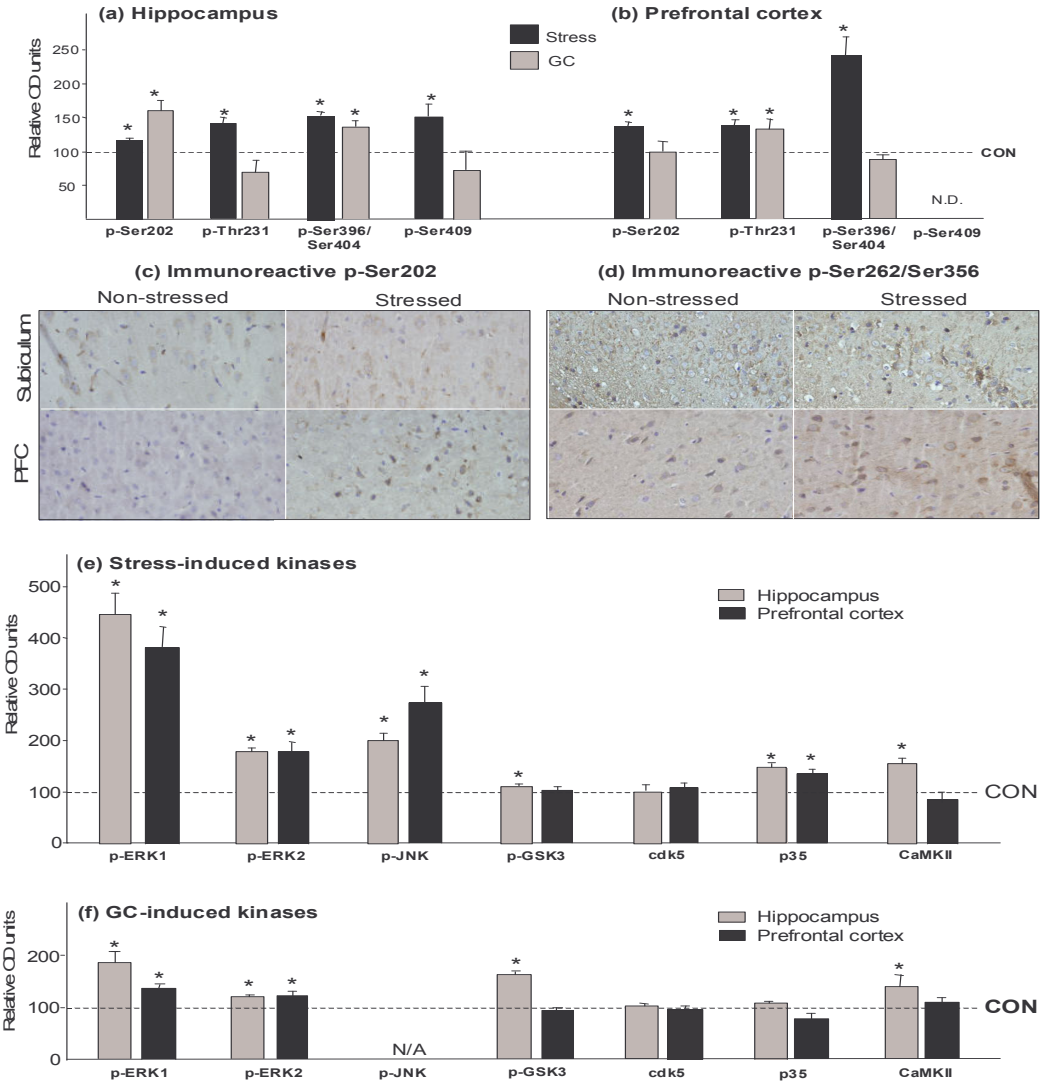


Figure 3.2.1. Stress and GC induce tau hyperphosphorylation in the hippocampus and PFC. Western immunoblotting and immunocytochemistry were used to analyze the phosphorylation status of tau in the hippocampus and PFC of chronically stressed or GC-treated rats (treatment details summarized in legend to Fig. 1). Upper panels show changes in hippocampal (*a*) and PFC (*b*) expression of a panel of antibodies that recognize various phospho-tau epitopes of relevance to AD pathology. The data shown have been normalized against total tau levels. Immunohistochemical staining with CP-13 (pSer202 tau) or 12E8 (pSer262/356 tau) antibodies in the subiculum (hippocampus) and PFC is shown in panels (*c*) and (*d*), respectively. Note that stressed animals showed greater intensity of staining (both antibodies) and that the immunoreactive signal is confined to the somata of pyramidal neurons. Both stress (*e*) and GC (*f*) induced the expression of a number of kinases in the hippocampus and PFC. In the case of data for pERK1/2 and pJNK, relative optical density (OD) values were normalized with respect to total ERK1/2 and JNK levels, respectively. In addition, all kinase data were normalized against total actin levels in the respective individual tissue extracts. All numerical data shown (panels *a, b, e* and *f*) represent mean $\pm$ SEM values, and are depicted with respect to data obtained in control tissues (CON, dotted line), set at 100%. * indicates significant differences from CON values ($p \leq 0.05$). Bars in photomicrographs represent 50 μm.

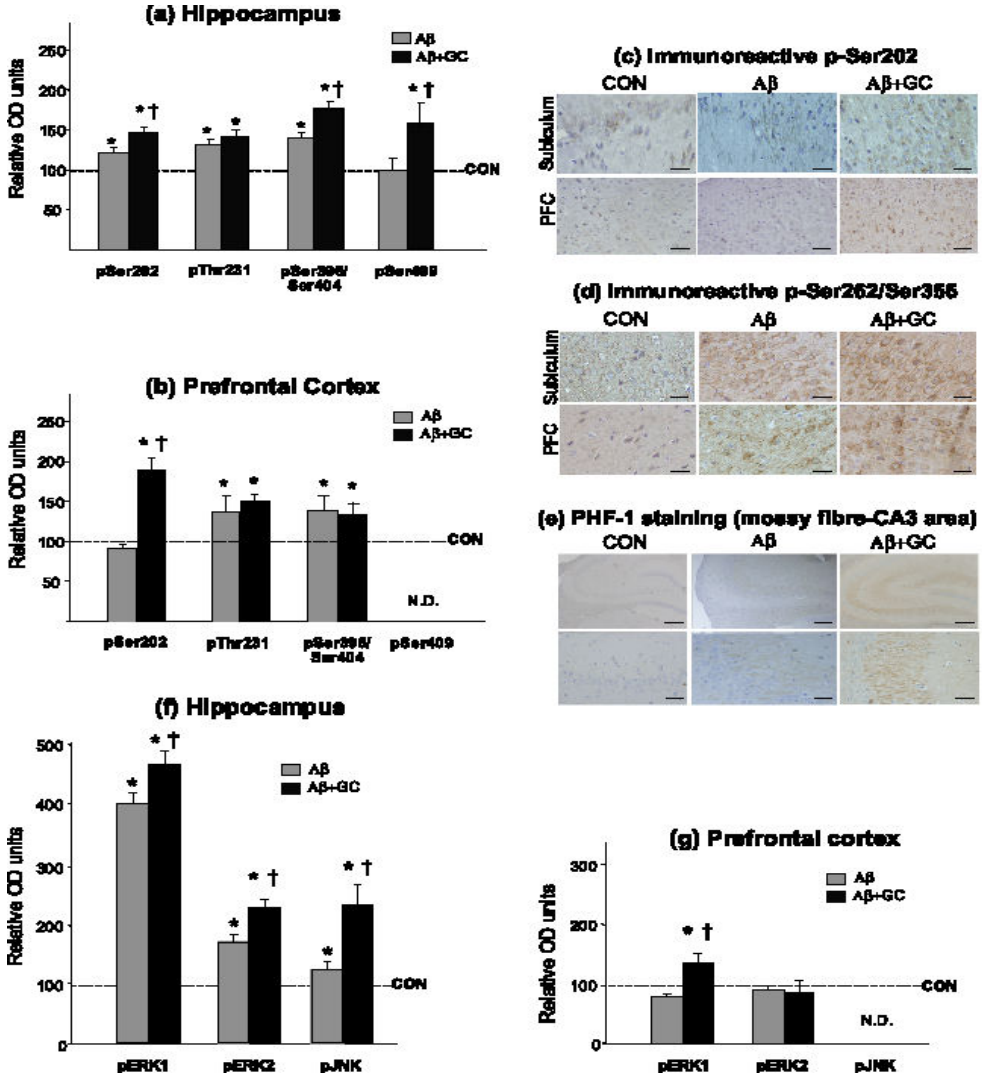


Figure 3.2.2. GC potentiate Aβ-induced increases in tau phosphorylation levels. Concomitant administration of Aβ and GC resulted in increases in the levels of several phospho-tau isoforms in the hippocampus (*a*) and PFC (*b*) of non-stressed rats, as measured by Western blotting; these increases were significantly greater than after treatment with Aβ alone ($p \leq 0.05$). Note the differential responses of the hippocampus and PFC in terms of number of phospho-tau epitopes that were affected by the combined Aβ+GC treatment. Immunocytochemical analysis, which focused on the PFC and hippocampus (subiculum and/or mossy fiber-CA3 area) confirmed the immunoblotting results. Panels *c and d* show staining of the hippocampus and PFC with the antibodies CP-13 (pSer202 tau) (*c*), 12E8 (pSer262/356) (*d*), while panel *e* illustrates PHF-1 (pSer396/404) in the mossy fiber system (*e*) in non-stressed rats; comparisons between Aβ and Aβ+GC treatments are shown. Note that while CP-13 and 12E8 immunoreactivity was localized to neuronal perikarya, that of PHF-1 was predominant in the mossy fiber system. The alterations in expression levels of the various phospho-tau epitopes shown in panels *a* and *b*, were accompanied by significant changes ($p \leq 0.05$) in the expression of several tau-related kinases, represented by the data for pERK1/2 and pJNK levels in the hippocampus (*f*) and PFC (*h*). All numerical data shown (panels *a, b, f* and *g*) represent mean $\pm$ SEM values, and are depicted with respect to data obtained in control tissues (CON, dotted line), set at 100%. Procedures for semi-quantitative analysis of Western blots are described in the legend to Fig. 2. * indicates significant differences from CON values ($p \leq 0.05$); † denotes significant difference ($p \leq 0.05$) between corresponding pairs of values for each treatment group (Aβ *vs*. Aβ+GC). Scale bars in panels *c* and *d* represent 50 μm. Scale bar in the *upper* and *lower rows* of micrographs in *e* represent 200 μm and 50μm., respectively.

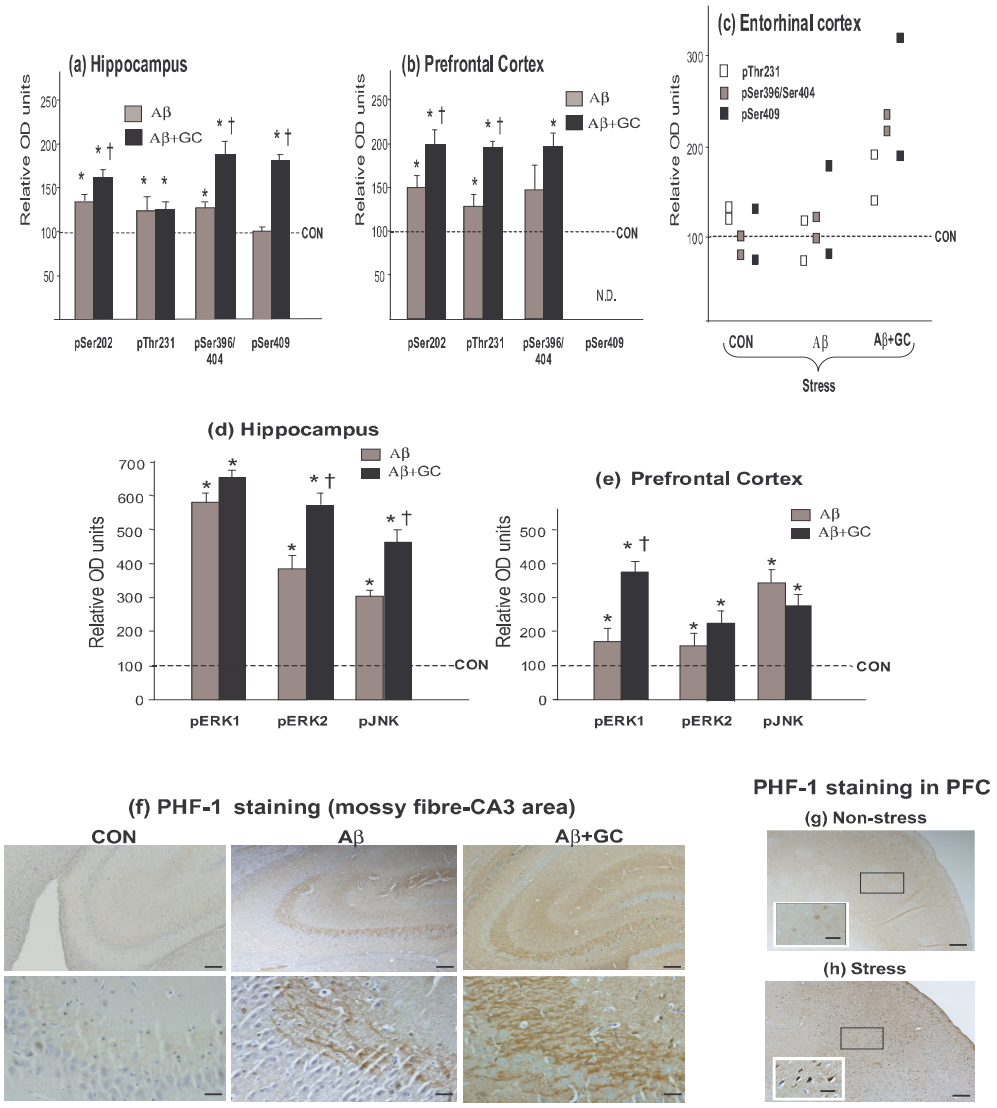
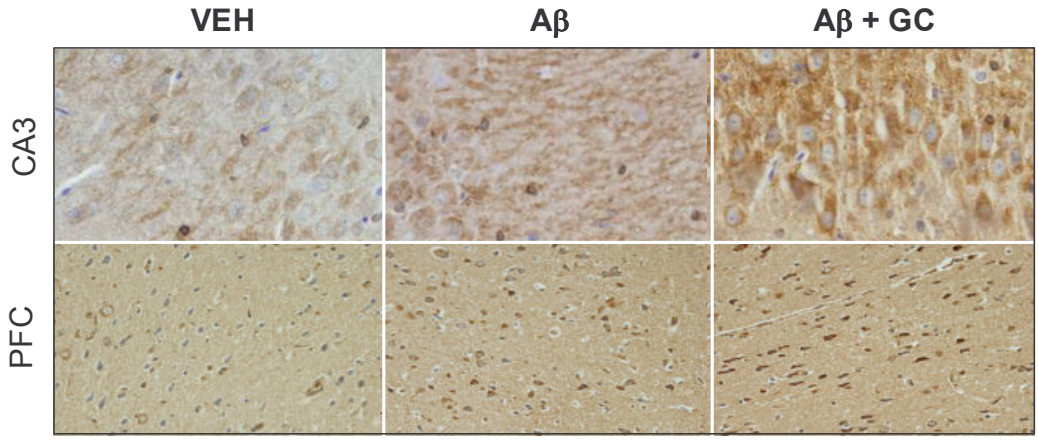


Figure 3.2.3. Previous stressful experience increases sensitivity to Aβ+GC.
Rats that had experienced chronic, unpredictable stress (1 month) responded to co-treatment with GC and Aβ with increases in tau phosphorylation (*a, b*) and tau-related kinases (*c, d*) to levels greater than those found after application of Aβ alone. As compared to the responses of non-stressed rats (cf. Fig. 3 *a, b, f and g*), exposure of previously stressed rats to Aβ+GC responded with more pronounced increases in hyperphosphorylated tau (*a, b*) and tau-related kinases (represented by pERK1/2 and pJNK in *d, e*) in the hippocampus and PFC. Levels of three phospho-tau isoforms measured in pooled extracts from the entorhinal cortex are also shown (*c*). Comparison of the low- and high-magnification images in (*f*) illustrate enhanced PHF-1 immunoreactivity in the mossy fiber network of the hippocampus of stressed rats receiving Aβ or Aβ+GC (Aβ+GC > Aβ > vehicle). Panel (*h*) shows that PHF-1 immunoreactivity was strongly stained in the perikarya of PFC neurons of stressed+Aβ+GC-treated rats (micrograph of the PFC region from a non-stressed rat that had been treated with Aβ+GC is shown for comparison, *g*). The insets in panels *h* and *g* are high-power magnifications of the rectangular areas marked in the respective low-magnification micrographs. All numerical data shown (panels *a-e*) represent mean ± SEM values, and are depicted with respect to data obtained in control tissues (CON, dotted line), set at 100%. Analysis of Western blots was done as described in the legend to Fig. 2. * indicates significant differences from CON values ($p \leq 0.05$); † denotes significant difference ($p \leq 0.05$) between corresponding pairs of values for each treatment group (stress+Aβ *vs*. stress+Aβ+GC). Scale bars in *f (upper* row*)* represent 200 μm; in *f (lower row)*, bars represent 50 μm. Bars in *g* represent 200 μm; the bars in the insets in *g* represent 50 μm.



Total Tau staining

Treatment		CA3	Prefrontal Cortex
CON		+ +	+
Aβ		+ + +	+
Aβ + GC		+ + + +	+ +
Stress	VEH	++	+ +
	Aβ	+ +	+ +
	Aβ + GC	+ + + + +	+ + + + +

Figure 3.2.4. Previous stress exacerbates GC- and Aβ-induced accumulation of tau in hippocampal CA3 and PFC neurons. Tau was stained using phosphorylation-independent pan-tau antiserum (Tau-5). Micrographs (upper panel) show Tau-5 immunoreactivity in hippocampal (CA3) and PFC regions; semi-quantitative scores of intensity of immunoreactive tau signal (*see Materials and Methods*) in the hippocampus and PFC of variously treated non-stressed and stressed rats are tabulated (lower panel).

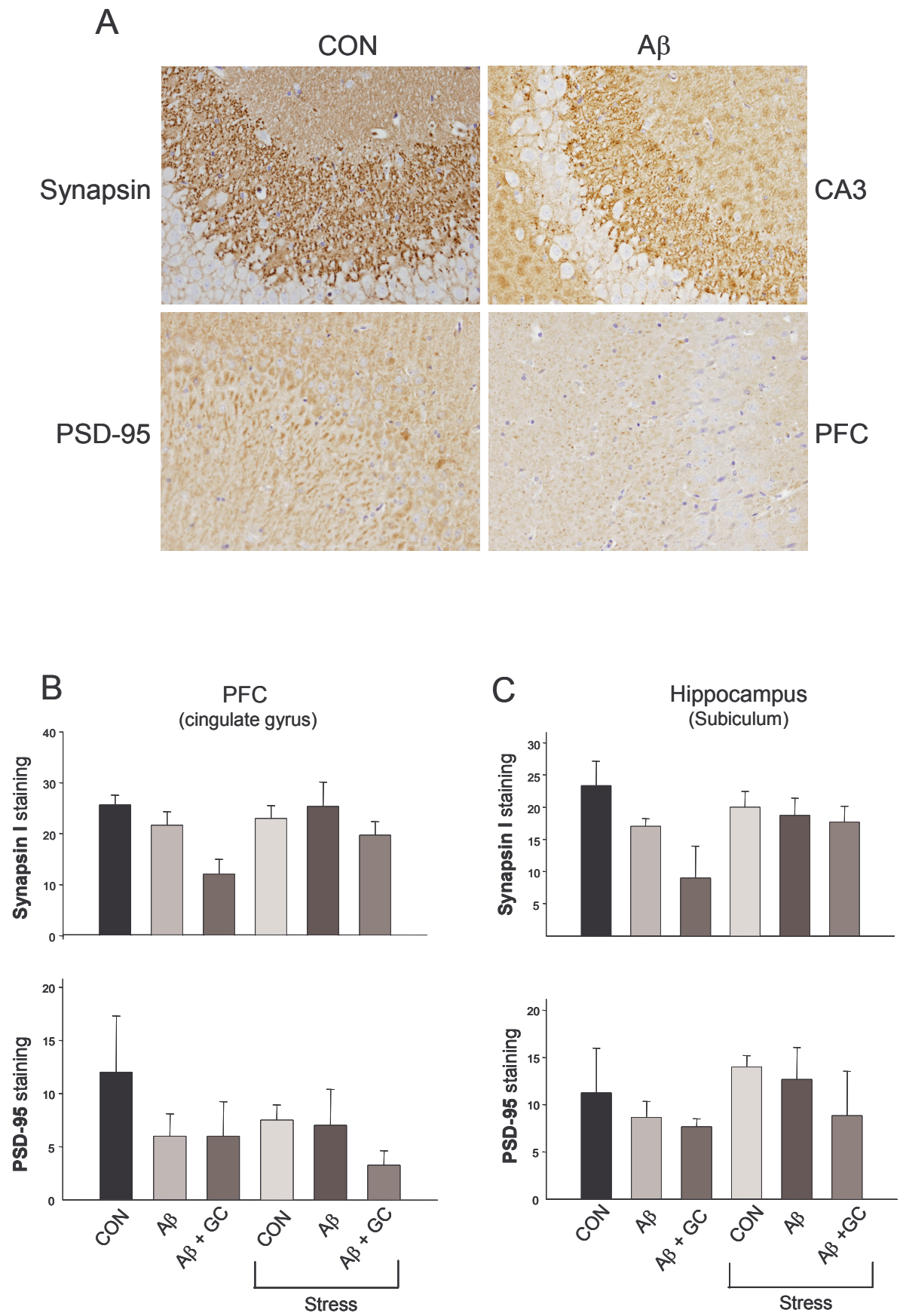


Figure 3.2.5. Immunocytochemical micrographs (A) using presynaptic and postsynaptic markers (synapsin I and PSD-95, respectively) in both the PFC (cingulate cortex) and hippocampus (subiculum); semi-quantitative scores of intensity of immunoreactive synapsin and PSD-95 signal (B and C).

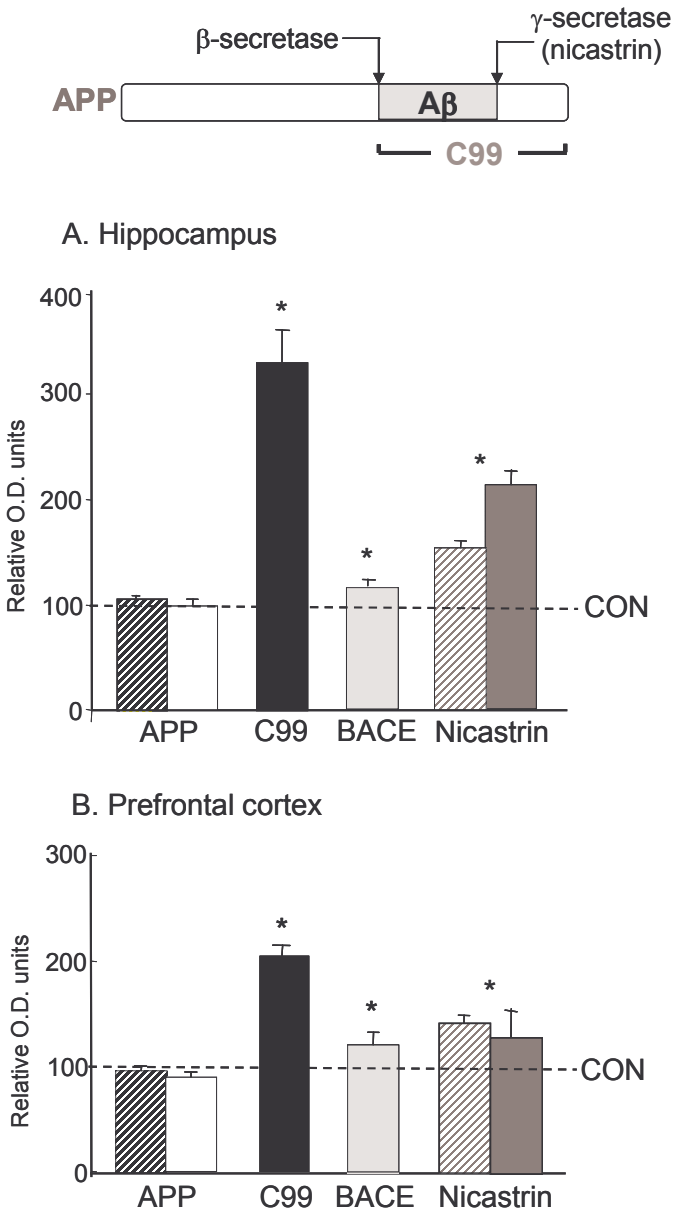


Figure 3.2.6. Stress drives amyloid precursor protein (APP) metabolism towards amyloidogenesis. The upper panel is a simplified schematic representation of how APP is sequentially metabolized by β-secretase (BACE-1) and γ-secretase into C99 and amyloid peptide(s) (Aβ). To facilitate interpretation of results, it should be reiterated that the generation of amyloid peptides results from the sequential post-translational processing of APP, starting with its cleavage by BACE-1 into C99; C99 is subsequently cleaved by γ-secretase into neurotoxic Aβ (see *Fig. 1*). Although, much prominence is given to Aβ in the pathogenesis of AD, it should be noted that C99 has the potential to induce neuronal apoptosis and cognitive impairment. Note that nicastrin is a major and essential component of the γ-secretase complex. Hippocampal (*A*) and prefrontal cortex (PFC) (*B*) tissues from chronically stressed middle-aged rats were analyzed for their contents of APP, C99 (carboxy-terminal fragment of APP), BACE-1 and nicastrin, by immunopreciptation and Western blotting. Stressors were applied over a period of 14 days. Compared to the PFC, the hippocampus showed greater C99 responses to stress. In both brain areas, nicastrin was upregulated by stress, indicating the potential for C99 to be further processed to amyloid peptide. Data shown are from semi-quantitative assessment (optical densities) of immunoreactive bands, normalized against actin; data point represents the mean $\pm$ SEM from 6-8 rats. Asterisks indicate $P \leq 0.05$ *vs.* control values.

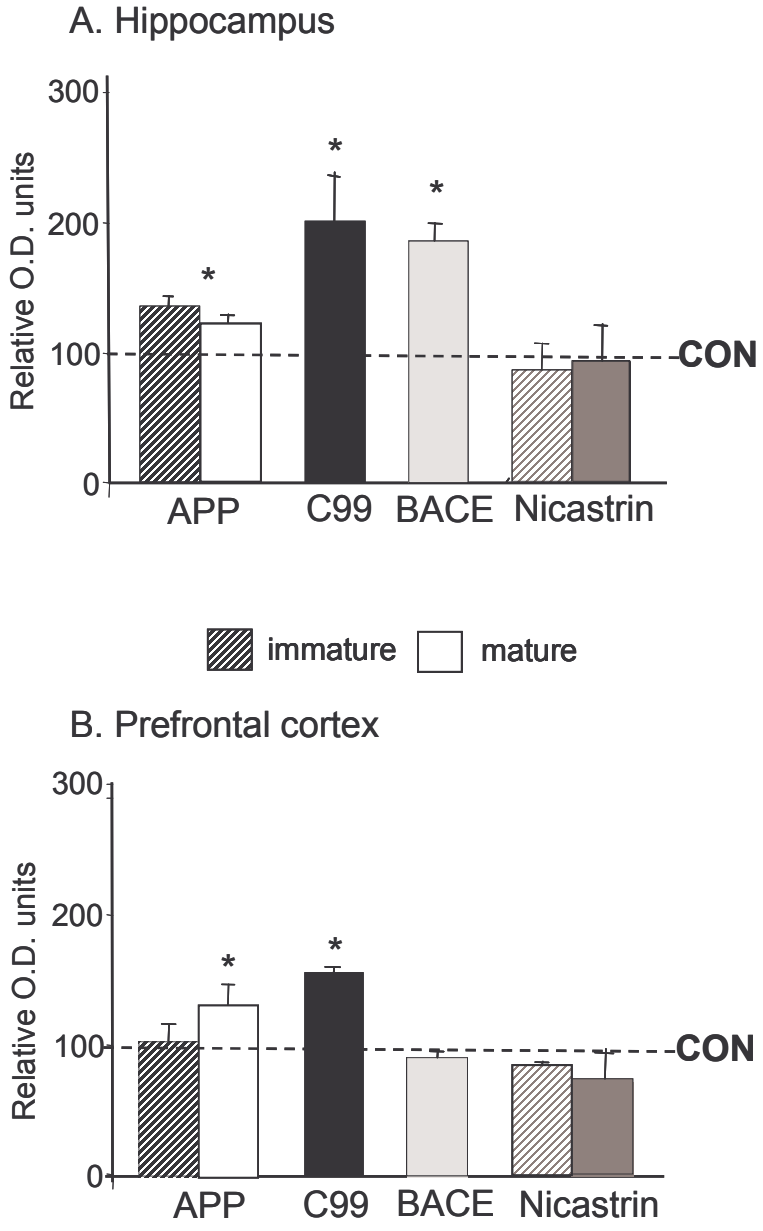


Figure 3.2.7. Glucocorticoids stimulate C99 production without upregulating nicastrin levels. Shown are the changes in APP, C99, BACE-1 and nicastrin expression in the hippocampus (*A*) and PFC (*B*) of middle-aged rats that had been treated with the synthetic glucocorticoid (GC), dexamethasone for 14 days. Note that nicastrin levels were not increased in either brain area; thus the high levels of C99 produced in response to the GC are unlikely to have been processed into amyloid peptides. Means $\pm$ SEM are depicted (n = 6-8 rats). The data emerged from semi-quantitative assessment (optical densities) of immunoreactive bands, normalized against actin. Asterisks indicate $P \leq 0.05$ *vs.* control values.

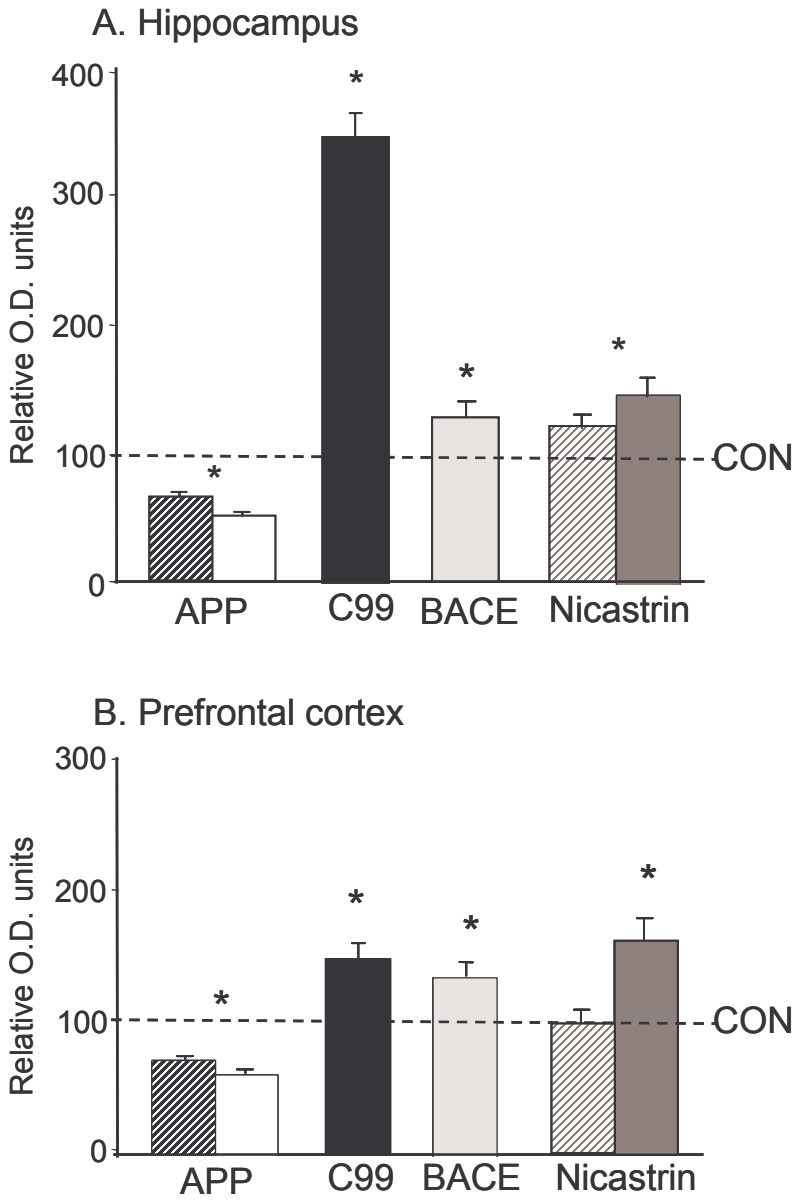


Figure 3.2.8. Aβ stimulates amyloidogenesis. Aβ administered by chronic, constant-rate infusion into one lateral ventricle resulted in significant increases in C99 production, and concomitantly in nicastrin levels in the hippocampus (*A*) and PFC (*B*), suggesting the potential for further processing of C99 to amyloid peptides(s). The treatment resulted in significant reductions in APP levels, most likely due to its rapid processing into C99. Means $\pm$ SEM (n = 6-8 rats) are shown. Data represent optical densities of immunoreactive bands, normalized against actin. Asterisks indicate $P \leq 0.05$ *vs.* control values.

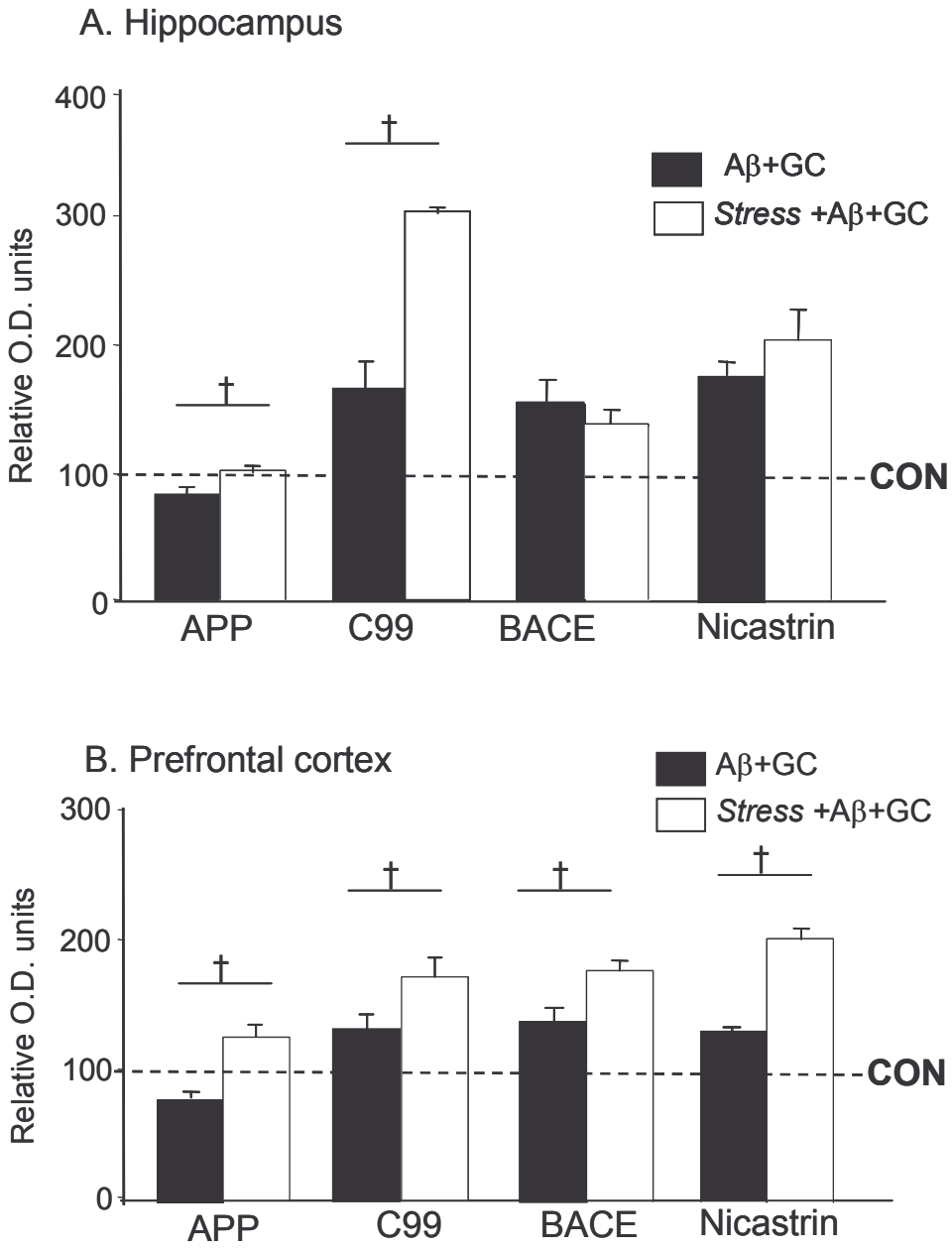


Figure 3.2.9. Stress history exacerbates the amyloidogenic effects of Aβ+GC treatment. Rats that had been exposed to a chronic unpredictable stress paradigm for 1 month previously, responded to the co-administration of Aβ+GC (14 days) with markedly increased levels of C99, BACE-1 and nicastrin in the hippocampus (*A*) and PFC (*B*). Note that while only the hippocampus showed significant responses in terms of BACE-1, C99 and nicastrin levels when non-stressed rats were given Aβ+GC, both the hippocampus and PFC showed significant changes in the parameters when the same treatments were given to previously-stressed rats. Means $\pm$ SEM are depicted (n = 6-8 rats). The data shown are optical density readings from immunoreactive bands, normalized against actin. Asterisks indicate $P \leq 0.05$ *vs.* control values.

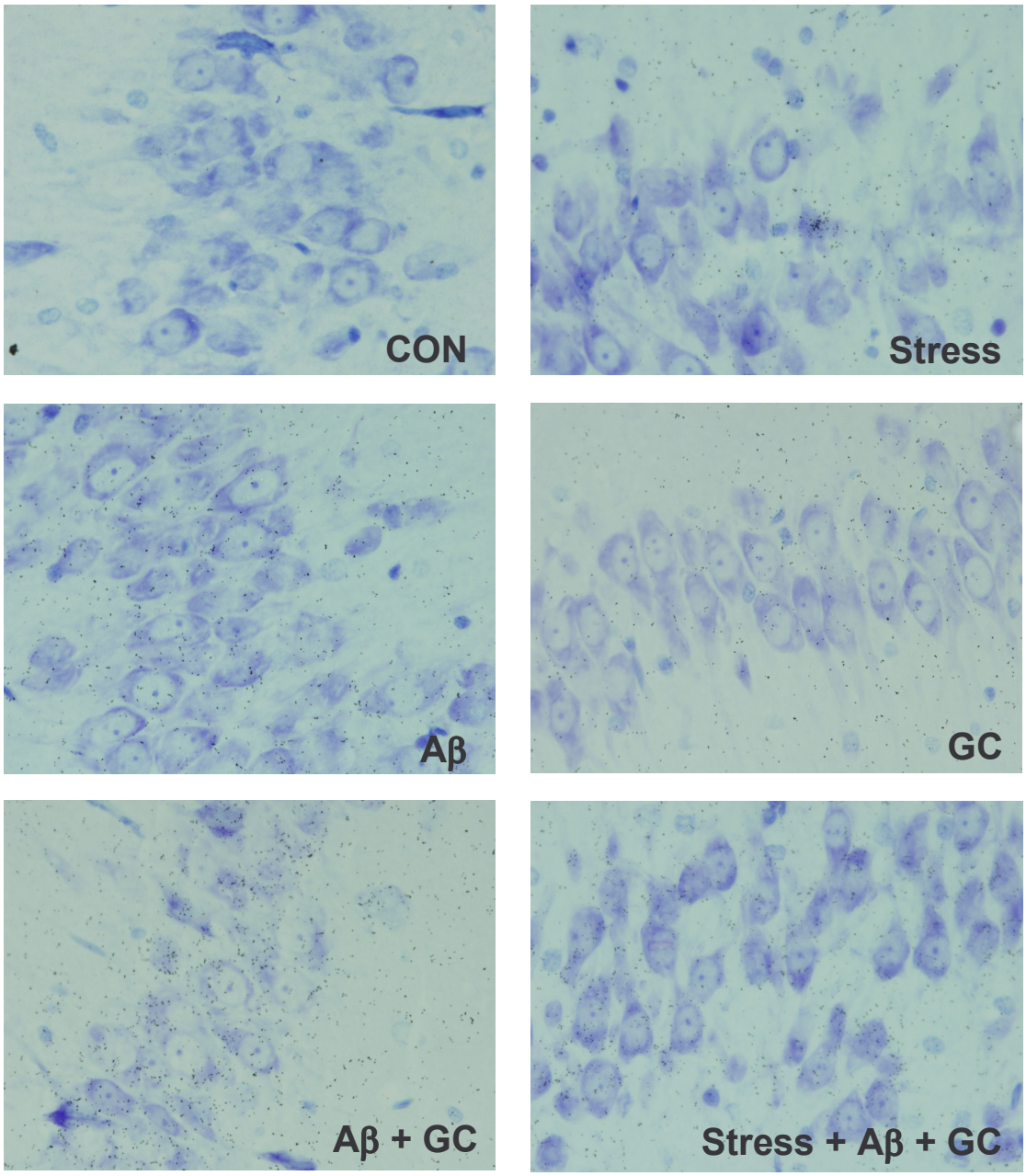


Figure 3.2.10. Representative images from preliminary *in situ* hybridization histochemistry assays (after photo-emulsion dipping) for *APP* mRNA in the CA1 region of the hippocampus from variously-treated groups of rats.
Note that stress and GC increased *APP* mRNA levels compared to controls; Aβ produced a stronger increase in this parameter and this effect was further accentuated in GC-treated rats. Table 1 shows semi-quantitative scores of silver grain signal intensity (n = 3 rats/group).

Table 3.2.1. Semi-quantitative evaluation of silver grains representing *APP* mRNA in the CA1 and CA3 subfields of the hippocampus and the prefrontal cortex (PFC) of rats exposed to various treatments involving exposure to stress, Aβ, GC or Aβ+GC, or a combination thereof.
In situ hybridization histochemistry was performed as described in *Methods*, using a 40-mer oligo-probe designed to recognize APP695-KPI. Representative hybridization images from the CA1 region are shown in Fig. 3.2.10.

Treatment	Hippocampus	
	CA1	**CA3**
Control	+	+
Stress	++	++
GC	+/++	+/++
Aβ	++++	++++
Aβ + GC	+++++	++++
Stress + Aβ + GC	+++	+++

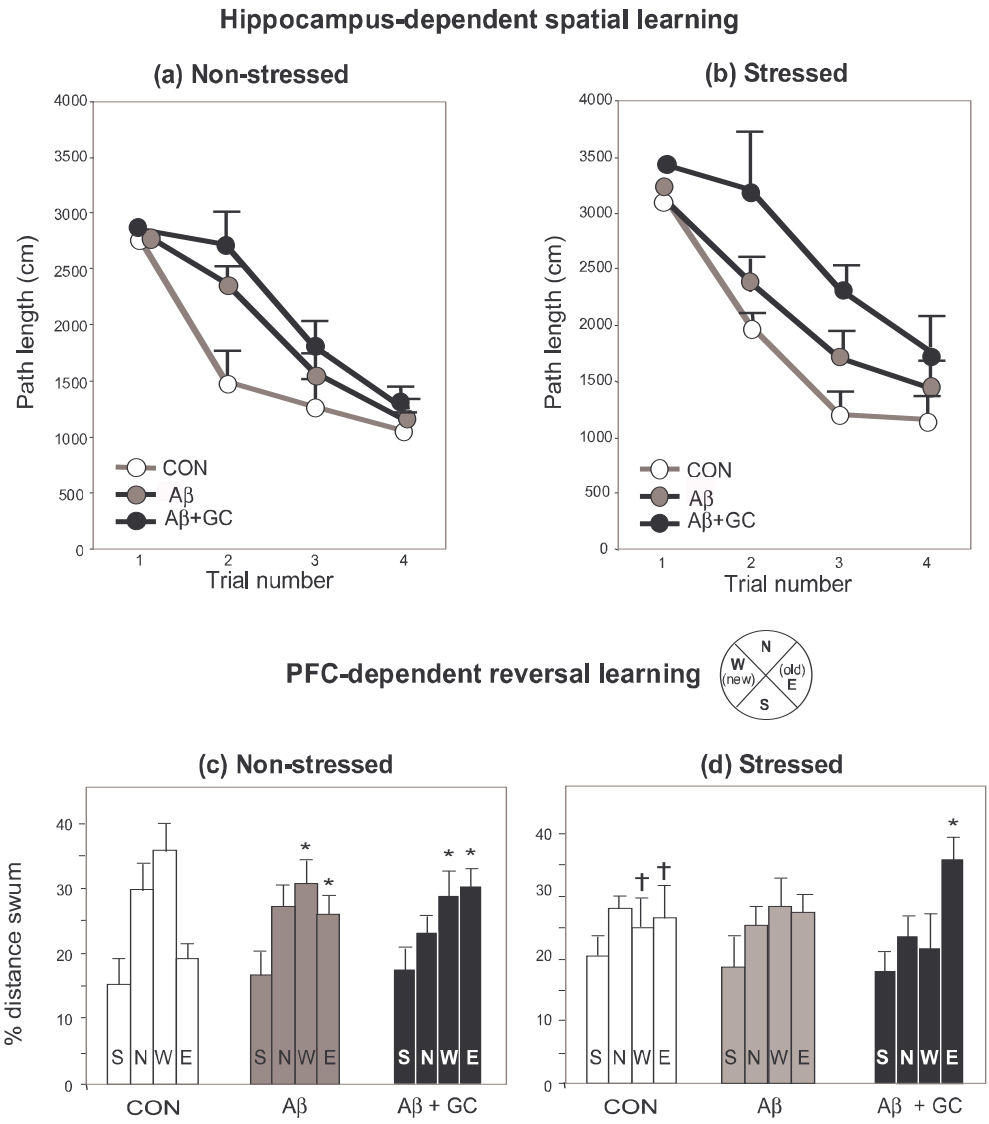


Figure 3.2.11

Stress exacerbates the cognition-impairing actions of Aβ and GC. Two forms of cognitive behavior were monitored, namely, hippocampus-dependent spatial reference memory (*a, b*) and PFC-dependent reversal learning. Behavioral tests were conducted in non-stressed (*a, c*) and stressed (*b, d*) rats. Chronic, unpredictable stress was delivered for 1 month before sub-groups of animals were treated with either vehicle, Aβ or Aβ+GC. $A\beta_{1\text{-}40}$ was chronically infused (0.59 µl/h) into the lateral ventricle at a dose of 0.3 nmol/d; dexamethasone (synthetic GC) was given as a daily depot (s.c.) injection at a dose of 300 µg/kg. In non-stressed animals, spatial reference memory was significantly impaired after Aβ treatment ($p < 0.001$) and concomitant GC administration resulted in slight worsening of the deficit (*a*). Treatment with Aβ alone impaired spatial memory in previously stressed rats ($p < 0.05$), and this deficit was further amplified in stressed rats that received combined treatments of Aβ and GC ($p < 0.0001$) (*b*). As compared to their non-stressed counterparts, stressed rats showed significant deficits in the reversal learning task ($p < 0.05$) (cf. *c* and *d*). Treatment of non-stressed rats with Aβ significantly reduced performance in the reversal learning test ($p < 0.05$), an effect that was not changed in GC+Aβ-treated animals (*c*). Although reversal learning was not significantly disrupted after administration of Aβ alone to previously-stressed animals, the behavior was significantly impoverished in stressed animals that were subsequently given the combined Aβ+GC regimen ($p < 0.05$) (*d*). * indicates significant differences from CON values; † denotes significant difference between stressed and non-stressed animals.

Table 2.

Tests of exploratory and emotional behavior and spatial memory.
Data shown are means ± SEM (n = 6-7 rats per treatment group).

	CON	Stress	DEX	Aβ	Aβ DEX	Stress Aβ	Stress Aβ DEX
Locomotor behavior – open field							
% distance (cm) in central area	26.6 ± 9.8	22.5 ± 3.7	17.7 ± 5.6	19.1 ± 4.5	11.9 ± 2.5	20.5 ± 2.1	14.0 ± 6.5
No. of rearing/extensions	21.0 ± 4.0	12.1 ± 2.0	10.0 ± 1.5	9.5 ± 2.6	9.8 ± 1.4	7.7 ± 1.7	7.2 ± 2.3
Anxiety level – Elevated plus maze							
% Time in open arms	26.1 ± 6.7	14.9 ± 3.3	8.8 ± 2.8	8.3 ± 2.0	4.4 ± 3.0	10.0 ± 1.9	11.2 ± 2.0

3.3. *In vitro* analysis of glucocorticoid regulation of amyloid precursor protein (APP) and tau.

Background

Alzheimer's disease (AD) pathology is believed to result from (i) the mis-processing of amyloid precursor protein (APP) into a 99-amino acid C-terminal fragment (C99) and amyloid β (Aβ) and (ii) the abnormal hyperphosphorylation of the cytoskeletal protein tau. The current consensus in the field is that APP mis-processing precedes tau hyperphosphorylation, even though both events can independently lead to neurodegeneration and behavioral disruption (see Chapter 1.3). Although laboratory species produce both APP and tau, they do not normally develop AD neuropathology unless engineered to express human forms of APP and tau. This raises the possibility that some unique property(ies) of the human versions of tau and/or APP may be responsible for the disease development. Recent studies in transgenic mouse models, expressing mutated human *APP* and/or *tau*, described the ability of elevated glucocorticoid (GC) levels to induce signs of AD-like pathology (Jeong et al., 2006; Green et al., 2006). In an initial attempt to understand the mechanisms that render neurons sensitive to GC-induced AD-like pathology, we here employed a rat neuronal cell line (PC12) that had been stably transfected with a *human tau* (*htau*) gene.

3.3.1. Human tau renders PC12 cells more vulnerable to various insults

Accumulating evidence suggests that tau participates in neurodegenerative processes (see Chapter 1.3.2); the paucity of data showing a similar role for this protein in animal models led us to examine whether the human form of the protein confers vulnerability to neuroendangering insults. In this experiment, differentiated PC12 (rat pheochromocytoma) cells stably transfected with human tau ($PC12_{htau}$) were monitored for their sensitivity to a variety of insults. Under basal conditions, PC12 wild-type ($PC12_{wt}$), PC12 cells transfected with an empty vector and $PC12_{htau}$ cells did not differ in survivability (Fig. 3.3.1). In contrast, the

Jeong et al (2006) FASEB J. 20: 729-731.
Green et al (2006) J Neurosci. 26: 9047-9056.

apoptosis-inducing drug daunorubicin triggered significantly more concentration-dependent (0.1-20 μM) cell death in the $PC12_{htau}$ than in PC12 cells ($P \leq 0.05$; Fig. 3.3.1A). Another known cell death-inducing stimulus, NGF withdrawal also led to significantly poorer survival rates in the $PC12_{htau}$ cells ($P \leq 0.05$; Fig. 3.3.1B). Likewise, exposure to 1 μM of Aβ peptide, a neurotoxin that is strongly associated with AD neuropathology, resulted in significantly reduced viability of $PC12_{htau}$ *vs.* PC12 cells ($P \leq 0.05$; Fig. 3.3.1C). Finally, treatment with the synthetic glucocorticoid (GC) dexamethasone (10^{-6} M), a known pro-apoptotic stimulus for neurons (see Chapter 1.4.3.3) led to higher levels of cell death $PC12_{htau}$ *vs.* PC12 cells ($P \leq 0.05$; Fig. 3.3.1D). This set of data demonstrates that the presence of human tau can render a rat neuronal cell line sensitive to the cell death-inducing actions of a variety of stimuli.

3.3.2. GC induce tau hyperphosphorylation in $PC12_{htau}$ cells

Aberrant tau hyperphosphorylation, an event that can lead to cytoskeletal disruption, synaptic loss and eventually neuronal death, is considered to be key pathogenic factor in AD (see Chapter 1.3.2). Accordingly, we next examined the influence of GC in inducing tau hyperphosphorylation. Using a panel of phosphorylation-dependent tau antibodies (see Chapter 2, Table 2) in Western blot analyses, we observed that exposure to GC for 48 h resulted in marked increases of tau phosphorylation ($P \leq 0.01$; Fig. 3.3.2A) in $PC12_{htau}$, but not PC12, cells; no significant effects were observed after 24 h of GC exposure. Interestingly, the GC effects did not extend to all potential phosphorylation sites but only to selected ones, namely, those recognized by the AT8, CP9 and PHF-1 antibodies, but not PG5; all of these antibodies' corresponding epitopes show increased phosphorylation in AD brains. Confocal microscopy, using phosphorylation-dependent antibodies AT8 and PHF-1 confirmed the results obtained by Western blotting (Fig. 3.3.2B). In addition, increased levels of immunoreactivity with the conformation-dependent antisera MC1 and Alz50 were also observed in GC-treated $PC12_{htau}$ cells (Fig. 23.3.B); both MC1 and Alz50 recognize aberrant tau forms found in AD brains. Similar effects (not shown) were observed in SHSY5Y human neuroblastoma cells expressing the same

human tau isoform as PC12$_{htau}$ cells; this finding supports the view that it is the human version of tau protein that confers GC sensitivity. That all of these actions of GC are mediated by glucocorticoid receptors (GR) was demonstrated by the fact that GC-induced tau hyperphosphorylation in PC12$_{htau}$ was blocked by concomitant treatment with mifepristone (RU 38486), as shown in Fig. 3.3.2C.

Tau is known to be a substrate of several protein kinases (see Chapter 1.3.2.4 and Box 1.1), the most prominent ones in the context of AD being cyclin-dependent kinase 5 (cdk5), glycogen synthase kinase 3 (GSK3), extracellular signal-related kinases ERK1/2, and calcium/calmodulin protein kinase II (CaMKII). It was therefore of interest to determine the total and active levels of these kinases in GC-treated PC12$_{htau}$ cells. As shown in Fig. 3.3.3A, GC application resulted in significant ($P \leq 0.05$) increases in phospho-cdk5 and phospho-GSK3α and β, as well as phospho-ERK1/2; GC treatment did not affect CaMKII levels.

The above findings were verified by concomitantly treating PC12$_{htau}$ cells with GC and selective kinase inhibitors. The GC-induced increases in tau phosphorylation, as measured with PHF-1 and CP9 tau antibodies, were significantly reduced by both the GSK3β inhibitor [3-(3-Carboxy-4-chloroanillino)-4-(3-nitrophenyl) maleimide] ($P \leq 0.05$; Fig. 3.3.3B) and the cdk5 inhibitor (PNU 112455A) in a dose-dependent fashion ($P \leq 0.05$; Fig. 3.3.3C); interestingly, the effects of the GSK3β inhibitor were more pronounced on the phosphorylation of epitopes recognized by PHF-1 than by CP9, probably reflecting the fact that tau can be phosphorylated in a sequential manner at multiple sites, depending on the particular kinases that are activated and other prevailing conditions (see Chapter 1.3.2.4). These findings were confirmed by experiments in which the less selective kinase inhibitors indirubin-3-monoaxine and indirubin-5-sulfonic acid were used (data not shown). Consistent with our observations that GC did not influence CaMKII levels (Fig. 3.3.3A), the CaMKII inhibitor 281-309 was without effect. Thus, the actions of GC on tau hyperphosphorylation appear to be primarily mediated by GSK3β and cdk5.

3.3.3. Role of GC in tau synthesis and stability

Disruption of the normal phosphorylation pattern of tau results in tau malfunction and redistribution of tau into the somatodendritic compartment (see Chapter 1.3.2). Here, using PC12 cells that were transiently transfected with *EGFP-htau*, we demonstrated that GC treatment leads to an accumulation of htau in the somata (Fig. 3.3.4A).

Since GC bind to GR and activate transcription, we were prompted to examine whether the GC-triggered increases in tau within neuronal perikarya might have resulted from GC effects on tau synthesis and metabolism. As shown in Fig. 3.3.4B, GC did not upregulate steady-state levels of tau mRNA, as measured by RT-PCR, although there was a significant increase in the expression levels of tau protein within the same time period (Fig. 3.3.4B). Additionally, inhibition of *de novo* synthesis by cyclohexamide did not alter tau levels after GC exposure. Given the observation that GC treatment results in an accumulation of tau (Fig. 3.3.4A and B) and the fact that hyperphosphorylation impedes tau degradation (see Chapter 1.3.2), we next monitored the stability (turnover) of tau protein in a pulse-chase experiment. The results depicted in Fig. 3.3.4C show that GC treatment leads to a significant reduction in the rate at which ^{35}S-labeled tau protein is degraded, explaining the increased levels of tau after GC treatment.

3.3.4. Glucocorticoids differentially influence production and processing of APP in PC12$_{wt}$ and PC12$_{htau}$ cells

Recent studies in transgenic mice have suggested that GC may induce tau pathology by stimulating the production of Aβ (Jeong et al 2006; Green et al 2006 - see above). Accordingly, it was of interest to investigate the influence of GC on APP synthesis and processing in PC12$_{htau}$ cells. All of the effects reported for APP below were observed within 24h of GC application; in contrast, GC effects on tau hyperphosphorylation (see above) were first detectable only after 48h.

Western blot analysis (Fig. 3.3.5A) revealed that application of GC (10^{-6} M dexamethasone) causes a significant increase in APP levels within 24h of

application to PC12 cells ($P \leq 0.05$). In contrast, the same treatment in $PC12_{htau}$ cells results in significantly reduced levels of APP ($P \leq 0.05$). These differential effects of GC treatment were confirmed by confocal analysis (Fig. 3.3.5B). In a further step of analysis, we used reverse-transcriptase PCR (RT-PCR) to study the influence of GC on the synthesis of the mRNAs encoding APP (APP_{770}, APP_{751} and APP_{695}); as shown in Fig. 3.3.5C, three major bands (562 bp, 505 bp and 337 bp, corresponding to the respective PCR products) were detected. Following GC treatment, the mRNA levels of all three *APP* isoforms were upregulated in $PC12_{wt}$ but down-regulated in $PC12_{htau}$ cells ($P \leq 0.05$; Fig. 3.3.5C). The GC-induced effects on APP mRNA and protein levels were blocked in the presence of the glucocorticoid receptor (GR) antagonist mifepristone (10^{-5} M; data not shown), indicating mediation by GR.

Next, the effects of GC on APP processing were examined. The analysis focused on the amyloidogenic pathway and involved measurements of the C99 fragment as well as of the two proteolytic enzymes, BACE and nicastrin, through whose actions Aβ can be derived from C99 (Fig 3.3.6A). Treatment with GC resulted in a highly significant increase ($P \leq 0.001$) of C99 levels in $PC12_{htau}$ cells (Fig. 3.3.6C), but not PC12 cells (Fig. 6B). Moreover, $PC12_{htau}$, but not PC12, responded to GC application with significantly increased ($P \leq 0.05$) levels of BACE and nicastrin ($P \leq 0.05$) (Fig. 3.3.6B and C). The concomitant increases in BACE, C99 and nicastrin levels following GC treatment strongly imply APP processing through the amyloidogenic pathway increased in $PC12_{htau}$.

In a next step, it was sought to investigate the role of GC in the non-amyloidogenic processing of APP. To this end, we concomitantly applied GC and a specific BACE inhibitor to preclude the production of sAPPβ; the inhibitor was used at a dose optimized in pilot experiments. The results of this experiment are shown in Fig. 3.3.6D, E. Both, PC12 and $PC12_{htau}$ cells responded to the BACE inhibition with increased production of sAPPα ($P \leq 0.05$). Since sAPPα levels did not differ between cells treated with the BACE inhibitor only *vs.* those treated with the BACE inhibitor + GC, it may be inferred that GC do not trigger non-

amyloidogenic sAPPα.

3.3.5. GC exacerbation of Aβ-induced neurotoxicity in PC12$_{htau}$ involves tau hyperphosphorylation

As shown in Fig. 3.3.1, GC can accentuate cell death in PC12$_{htau}$ cells and previous studies showed that GC potentiate the actions of various neuroendangering agents, including Aβ (see Chapter 1.4.3.3). In addition, recent studies described the ability of stress/GC to amplify the incidence of pathological signs in transgenic mouse models of AD (see above). In light of these observations it was therefore of interest to analyze whether PC12$_{htau}$ cells show increased vulnerability to the combined treatment of GC + Aβ and whether abnormal tau hyperphosphorylation might contribute to this process. As reported above (Fig. 3.3.1D), GC treatment resulted in significantly more neuronal cell death ($P \leq 0.05$) as well as tau hyperphosphorylation (Fig. 3.3.2A). In addition, as shown in Fig. 3.3.7A, GC exposure was found to potentiate the neurotoxic effects of Aβ, an effect that was more pronounced when Aβ was applied at 0.1 µM (Fig. 3.3.7A). In parallel, GC potentiated the ability of the higher dose of Aβ (1 µM) to induce tau hyperphosphorylation, as detected by CP9 and PHF-1 antibodies (Fig. 3.3.7B); this finding indicates that tau hyperphosphorylation is unlikely to be the sole trigger of cell death induced by low-dose Aβ (Fig. 3.3.7A).

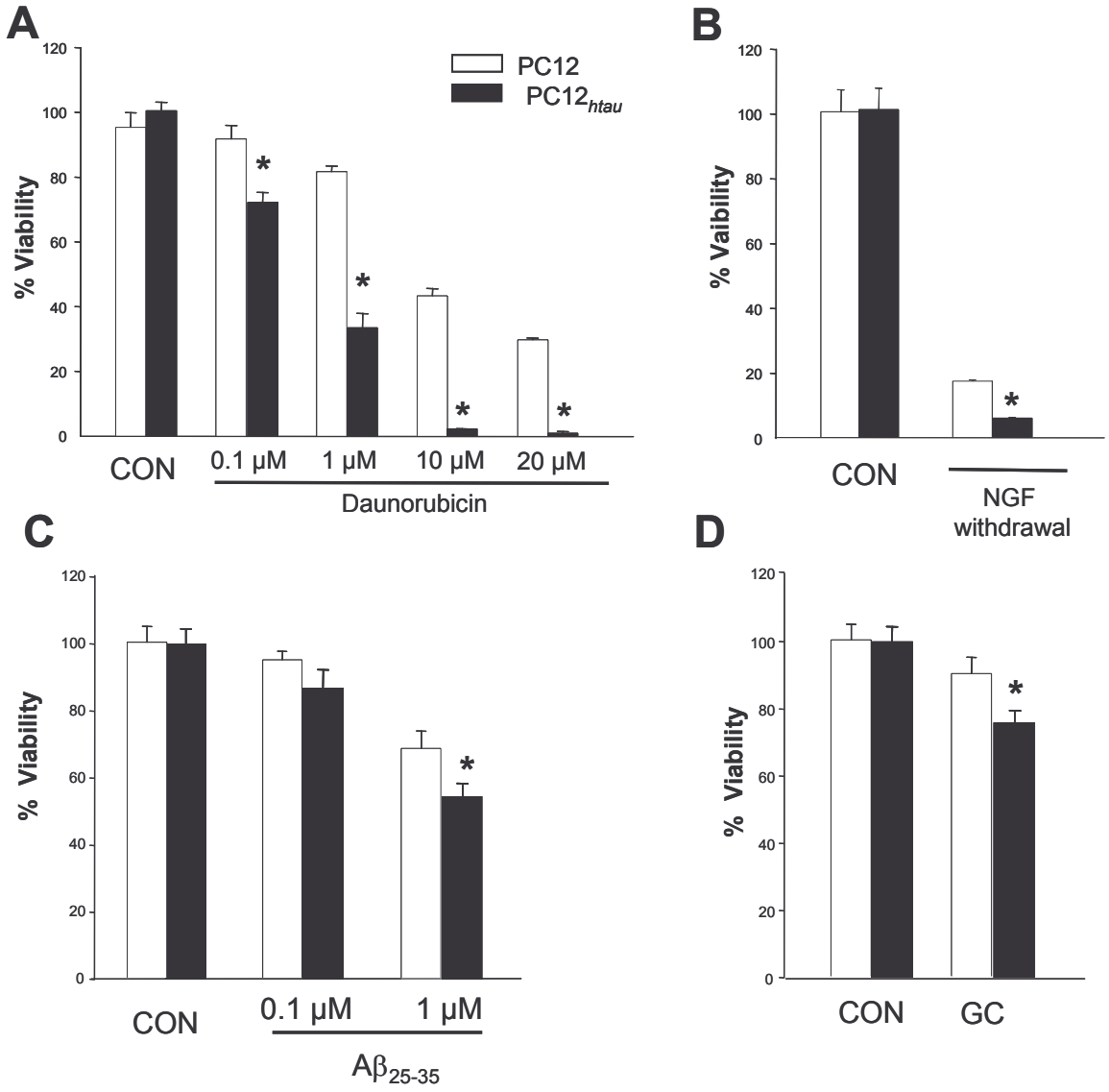


Figure 3.3.1.
PC12$_{htau}$ cells are more sensitive to multiple insults. As compared to normal PC12 cells, PC12$_{htau}$ cells showed reduced resistance to the cell death-inducing actions of (**A**) daunorubicin, (**B**) NGF-withdrawal, (**C**) Aβ (1 μM), and (**D**) GC (dexamethasone, 10^{-6} M), as measured by MTT assays. All treatments were applied for 48 h. Numerical data shown represent mean ± SEM values (n = 6-8 individual measurements). Asterisks indicate significant differences between the two cell lines ($P \leq 0.05$).

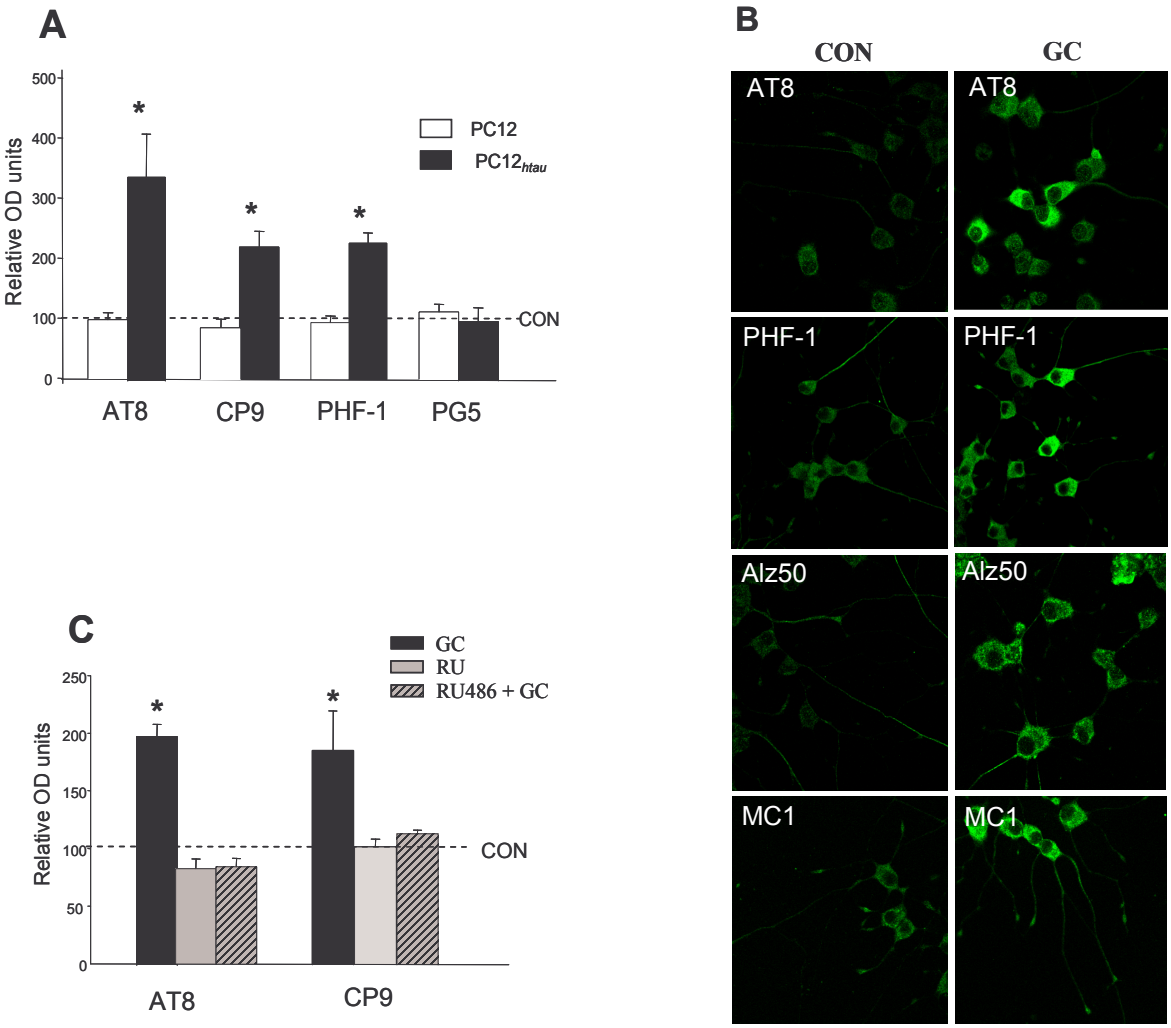
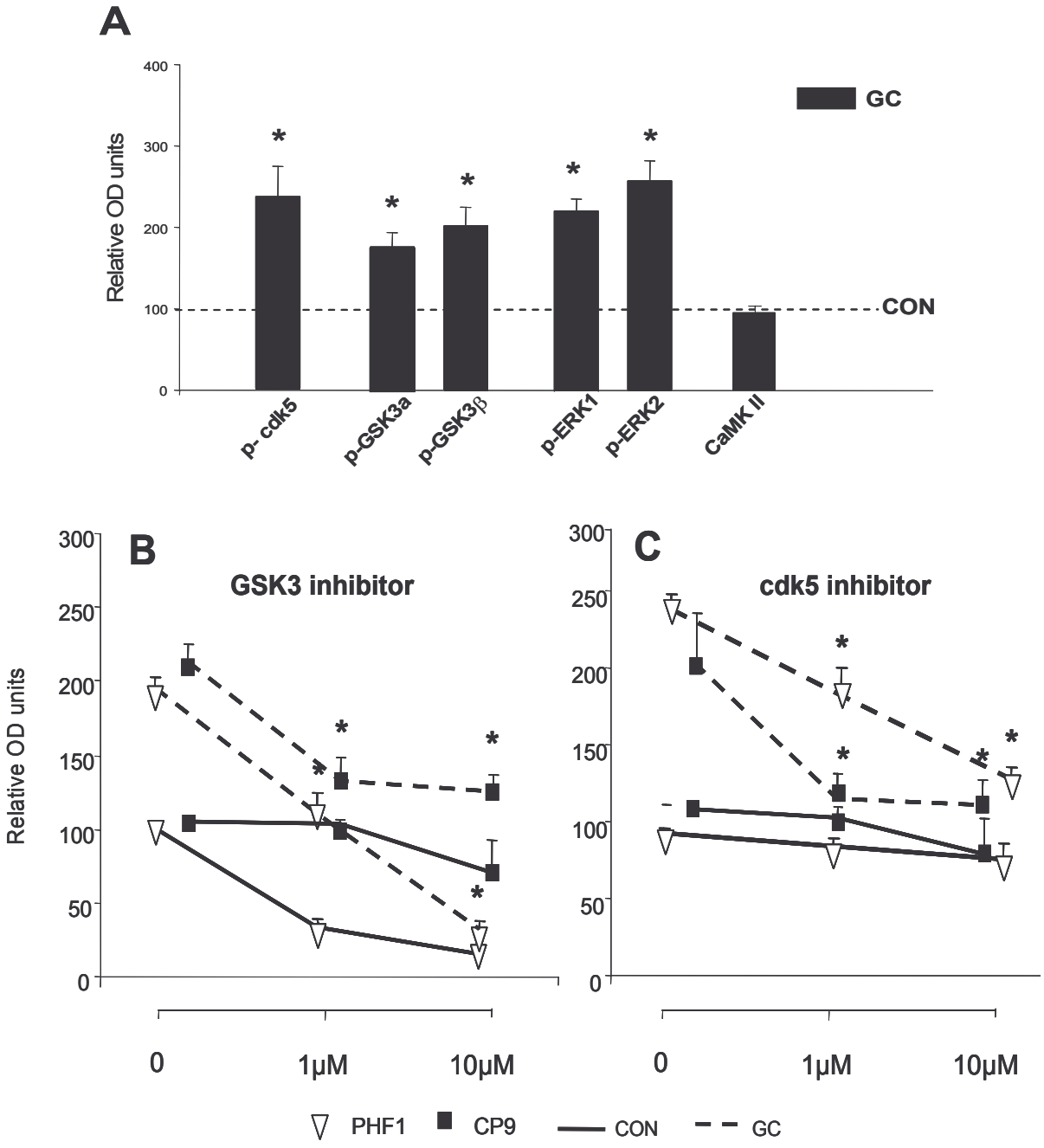


Figure 3.3.2.
GC induce tau hyperphosphorylation in PC12$_{htau}$ cells only. Results shown are from Western blotting and confocal microscopy experiments. Immunoblotting data have been normalized against total tau levels. (**A**) shows alterations in the expression levels of different phosphorylation-dependent tau epitopes after treatment of PC12$_{htau}$, but not PC12, cells. (**B**) Staining (immunofluorescence) of control (CON) and GC-treated (dexamethasone, 10^{-6} M, 48 h) PC12$_{htau}$ cells with various phosphorylation-dependent (AT8; PHF-1) and conformation-dependent (Alz50; MC1), confirming data in (**A**); magnification X63. (**C**) The GC actions were mediated by the GR, since GC-induced changes in tau phosphorylation were abolished in the presence of the GR antagonist mifepristone (RU38486). All numerical data shown represent mean $\pm$ SEM values (6-8 individual measurements per data point); they are depicted with respect to data obtained in control cells (CON, set at 100%, dotted line). Asterisks indicate significant differences from CON values ($P \leq 0.05$).



Fgure 3.3.3.
Kinases involved in GC-induced tau hyperphosphorylation. (**A**) Treatment with GC (dexamethasone, 10^{-6} M, 48 h) resulted in increased levels of active (phosphorylated) forms of GSK3β, cdk5 and ERK1/2; there were no changes in the levels of total CaMKII. (**B, C**) Specific inhibitors of GSK3β ((3-(3-Carboxy-4-chloroanilino)-4-(3-nitrophenyl) maleimide)) and cdk5 (PNU 112455A) dose-dependently attenuated the GC-induced tau hyperphosphorylation, as shown by immunoblotting with PHF-1 and CP9 antibodies. Semi-quantitative results (means $\pm$ SEM; each data point represents 6-8 individual measurements) from evaluation of Western blots are shown as optical densities (O.D). In the case of the data for p-GSK3β, p-cdk5 and p-ERK1/2 O.D. values were normalized with respect to total levels of the corresponding kinase. Asterisks denote significant differences between GC-treated and control (CON) cells ($P \leq 0.05$).

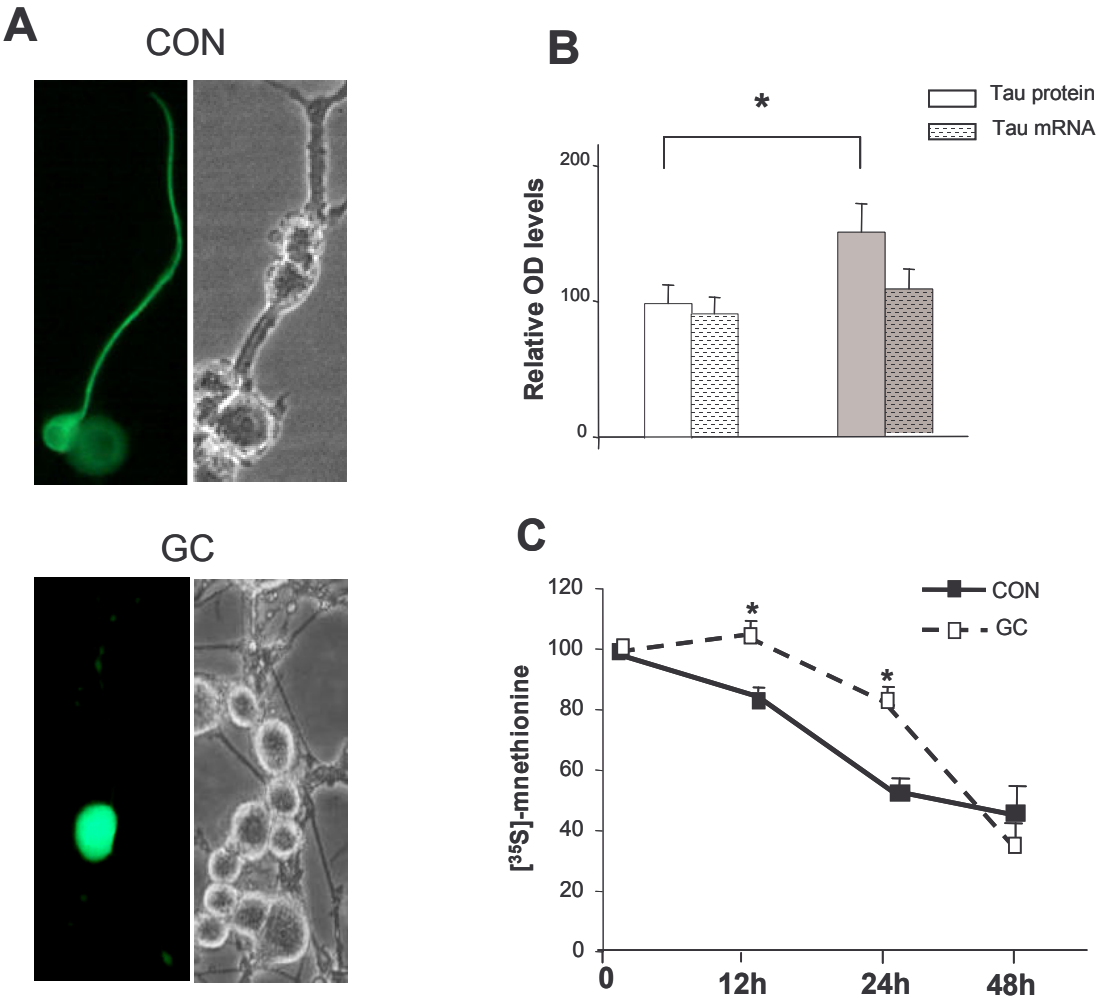


Figure 3.3.4.
GC induce tau accumulation and alter tau turn-over. Panel **A** shows confocal (*left*) and phase-contrast (*right*) images of $PC12_{htau}$ under basal conditions (CON) or after exposure to GC (dexamethasone, 10^{-6} M, 48 h). Note that the GC-treated cells show accumulation of htau-EGFP in the neuronal perikaryon; in contrast the fusion protein is diffusely distributed within the neurites and perikarya of untreated cells; magnification X40. Panels (**B** and **C**) show that significantly higher levels of tau protein are found in cells exposed to GC *vs*. untreated $PC12_{htau}$ cells, but that GC treatment does not influence *tau* mRNA expression. Panel (**C**) shows the results of a pulse-chase experiment to monitor the stability of tau in untreated and GC-treated $PC12_{htau}$ cells. Numerical values shown represent means $\pm$ SEM (n = 6-8 individual measurements in the case of **B**, and n = 4 in the case of **C**). Asterisks indicate significant differences between GC-treated and control cells ($P \leq 0.05$).

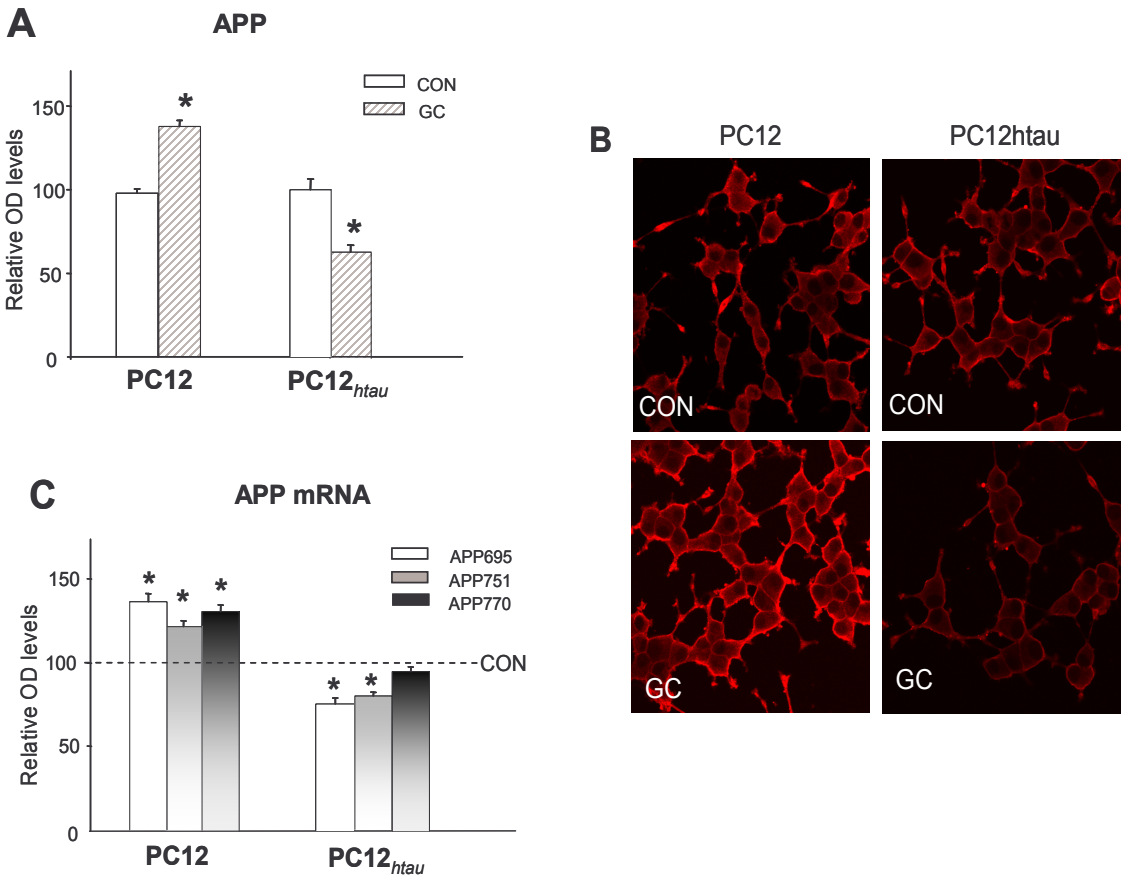


Figure 3.3.5.
Differential effects of GC on APP synthesis and levels. (**A**) Western blotting revealed that APP levels in normal PC12 and PC12$_{htau}$ cells are, respectively, up- and down-regulated after treatment with GC (dexamethasone, 10^{-6} M, 48 h). (**B**) Confocal images confirm the results derived by immunoblotting in panel **A**. Panel **C** compares *APP* mRNA levels, encoding the three isoforms of APP (APP695, APP751 and APP770) in PC12 and PC12$_{htau}$ cells; steady-state mRNA levels were determined by RT-PCR. All numerical data shown represent mean $\pm$ SEM (n = 6-8); data in panel **A** were normalized respect to values obtained in control cells (CON, set at 100%, shown as dotted line). Asterisks denote significant differences from CON values ($P \leq 0.05$).

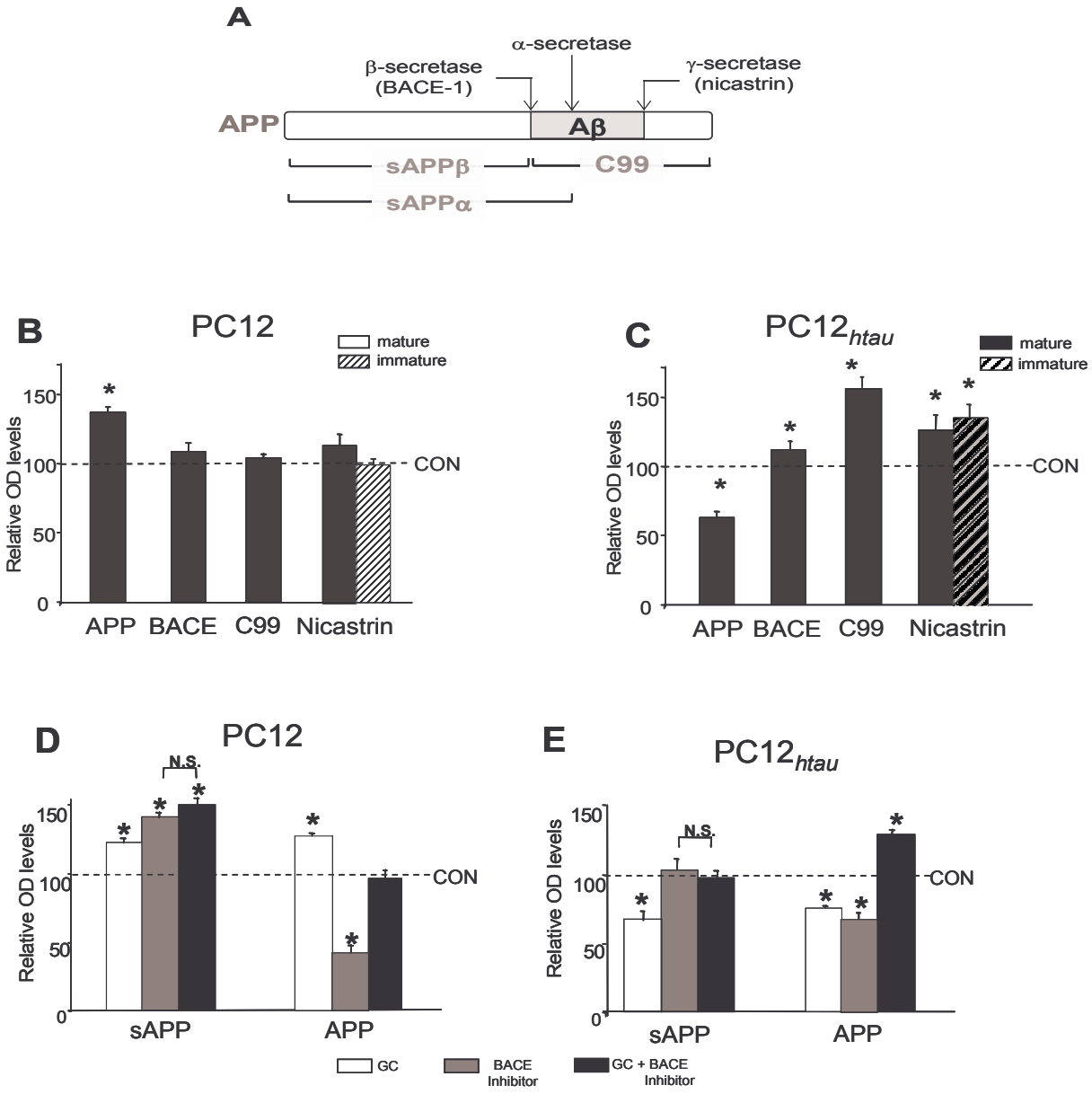


Figure 3.3.6.
GC treatment leads to APP mis-processing in PC12$_{htau}$ cells. (**A**) Schematic representation of APP processing along the non-amyloidogenic and amyloidogenic paths. (**B and C**) In PC12$_{htau}$ cells, but not in normal PC12 cells, GC treatment (dexamethasone, 10^{-6} M, 48 h) is accompanied by increased levels of β-secretase (BACE) and C99 from which Aβ can be eventually cleaved. GC exposure also increases levels of nicastrin, an essential component of the γ-secretase complex that is responsible for cleaving Aβ out of C99. Generation of C99 and Aβ results from mis-processing of APP via the so-called amyloidogenic pathway. (**D**) Levels of secreted APP (sAPP, representing sAPPα) are increased after exposing cells to a specific BACE inhibitor, indicating that GC do not influence the non-amyloidogenic pathway. Results shown in **B**, **C** and **D** derive from semi-quantitative (mean ± SEM values are given; n = 6-8) Western blot assays after normalization with respect to data obtained in control cells (CON, set at 100%, dotted line). Asterisks indicate significant differences from CON values ($P \leq 0.05$).

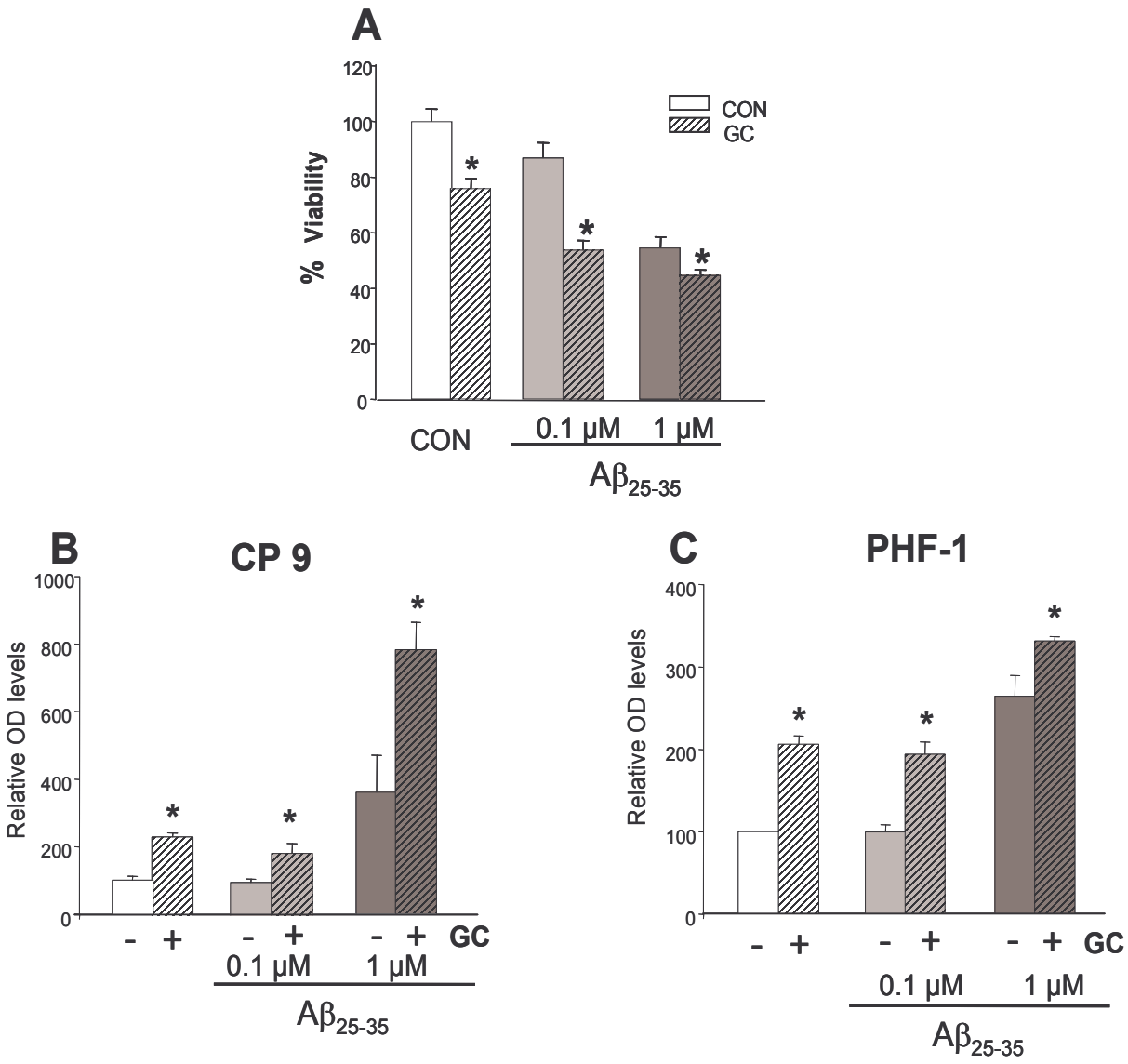


Figure 3.3.7.

Synergistic action of GC and Aβ on tau hyperphosphorylation and cell death induction. (**A**) Treatment of $PC12_{htau}$ cells with GC (dexamethasone, 10^{-6} M, 48 h) resulted in a potentiation of the neurotoxic actions of Aβ, as measured by MTT cell survival assays. (**B, C**) The ability of GC to increase tau hyperphosphorylation was significantly potentiated when $PC12_{htau}$ cells were co-treated with Aβ (1 μM), as shown in the semi-quantitative data obtained after immunoblotting with PHF-1 and CP9 antibodies. Data shown are means ± SEM (n = 6-8 individual measurements); asterisks represent $P \leq 0.05$.

Chapter 6

General Discussion

General Discussion

Living organisms continuously face demands imposed by changes in their external environment and need to maintain the integrity of their *milieu interior* in order to survive; they achieve this through homeostatic mechanisms. The term 'stress' refers generally to such external demands; depending on their quality and intensity, stressors challenge, and may disrupt, homeostasis. The organism attempts to maintain 'stability through change' (also referred to as 'allostasis') by mounting the so-called 'stress response,' a process involving the activation of the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoids (GC). The stress response (and adaptations to it) is governed by neural circuits in the brain, including the hippocampus and prefrontal cortex (PFC). Both these regions are involved in the regulation of the HPA axis and are targets of GC actions; they are also regions with important roles in cognitive functions such as learning and memory.

Many studies have associated elevated GC levels, such as those found after stress, to impair cognitive performance (Lupien et al 1998; Starkman et al 1999; Sousa and Almeida 2002; Cerqueira et al 2005a, b; Radley et al 2006). These impairments may involve GC-induced dendritic atrophy and synaptic loss or, in extreme cases, neuronal death through the apoptotic mechanisms (Almeida et al 2000; Crochemore et al 2002). Similar neurodegenerative changes are observed in the hippocampus and PFC of Alzheimer's disease (AD) patients, with synaptic losses correlating especially strongly with memory deficits (Lue et al 1999; Mucke et al 2000), the principal manifestation of AD (Chetelat and Baron 2003; Teipel et al 2005). AD patients frequently display hypercortisolism and dysregulation of the homeostatic mechanisms controlling the HPA axis (Hatzinger et al 1995; Hartmann et al 1997; Umegaki et al 2000; Rasmuson et al 2001; Elgh et al 2006; Miller et al 1998), suggesting a causal link between adrenocortical function and AD.

Although stress and GC have been implicated in the initiation and progress of AD pathology, the role and mechanisms through which GC may influence its etiopathogenesis have not been systematically investigated. However, given the prominent role ascribed to amyloid β (Aβ) and hyperphosphorylation of the microtubule-associated protein tau in the neuropathological and behavioral symptoms of AD, consideration of these molecules seems to be a good starting point. Aβ and its immediate precursor, C99, are derived from the cleavage of a much larger precursor, amyloid precursor protein (APP); both peptides have neurotoxic and cognition-impairing properties. In its soluble form, Aβ is clearly neurotoxic (Roselli et al 2005), although much attention has been given to its deposition as insoluble senile plaques. Interestingly, although a major neuropathological feature of the AD brain, senile plaque density correlates poorly with the degree of cognitive impairment. In contrast, the behavioral deficits of AD match better with the amount of abnormally hyperphosphorylated tau; the latter leads to microtubule disassembly, and formation of neurofibrillary tangles which ultimately result in neuronal atrophy and death. Neuropathological studies suggest that abnormalities in tau represent an early sign of AD and reflect the degree of cognitive impairment.

The clinical and histopathological description of '*dementia praecox*' by Alois Alzheimer in 1906 (the disease was later named after him) (Möller and Graeber 1998; http://www.whonamedit.com/doctor.cfm/177.html), stimulated researchers in the last decade or so to exploit the possibility to create mutant mouse and cellular models of the disease (Brandt et al 2005; McGowan et al 2006). These approaches involved the over-expression of human genes, in particular mutant forms of *APP*, *APOE* and *tau*, but also of genes involved in the pathological processing of APP and tau (e.g. the presenilins, BACE, GSK3β, and cdk5). It should be noted that these efforts were made despite the fact that only some 7% of AD cases (so-called familial AD, FAD) have a genetic basis.

Mice carrying *tau* mutations show many of the neuropathological features that are seen in AD. In contrast, *APP* transgenic mouse lines display altered APP

processing and age-dependent amyloid deposition and memory deficits, but neither tau pathology nor widespread neuronal loss. On the other hand, transgenic animals carrying both *APP* and *tau* mutations show AD-related neuropathology and behavioral impairments, giving strength to the hypothesis that APP and/or Aβ can directly influence and trigger tau pathology (Lewis et al 2001; Oddo et al 2003). Nevertheless, the picture remains somewhat confusing since turning off *tau* in triple transgenic mice improves cognitive performance but not tau pathology (Santacruz et al 2003), and immunization against Aβ partly reverses memory deficits but not amyloid pathology (Dodart et al 2002; Cleary et al 2005). Apart from hinting at the potential reversibility of the pathological effects of Aβ and tau, these findings indicate that amyloid deposition and tau aggregation may be less important than soluble Aβ and its subsequent tau hyperphosphorylation during early disease stages. This view is consistent with human studies showing that soluble Aβ levels correlate better with cognitive impairment (Lue et al 1999).

Infusion of soluble Aβ has been used by some investigators to create alternative, more natural, rodent models of AD. The basis for this approach is that soluble Aβ will induce synaptic dysfunction and degeneration and trigger cell signaling cascades, even before the formation of Aβ aggregates and plaques (for review see Stephan and Phillips 2005). Normal rodents that receive exogenous Aβ have been found to present several neuropathological characteristics and behavioral features of AD, providing a valuable experimental model for studying specific pathogenic processes and for monitoring the impact of environmental factors (e.g. stress). Importantly, such a model is not confounded by genetic manipulations that can potentially mask or exaggerate the influence of other factors or pathways. The latter is particularly important given the role of several AD-related proteins in normal cell function as well as in the regulation of cerebral development (Wines-Samuelson and Shen 2005).

As the aim of this work was to investigate the role of stress in the initiation and maintenance of AD pathology, exogenous Aβ infusion was chosen to mimic one

aspect of the disease. It was decided to infuse $A\beta_{1-40}$ since this peptide aggregates slowly as compared to $A\beta_{1-42}$, allowing analysis of the effects of soluble and diffusible Aβ. The regional pattern of Aβ-induced pathology critically depends on a number of factors (e.g. diffusion, penetration, binding characteristics and metabolism of Aβ) governing the effective intra- and extra-cellular concentrations of Aβ. Accordingly, it should be noted that this paradigm does not completely recapitulate the anatomical distribution of changes associated with natural AD. It is therefore of interest to note that, in this study (see Chapter 3.2.), pathobiochemical changes occurred in the hippocampus, PFC and entorhinal cortex, three areas that are most severely affected in AD. Also, it is important to note that such a paradigm is unlikely to capture all the changes and symptoms found in the human disease; the disease manifests itself over several decades, and its main feature, memory impairment, probably only becomes evident long after the first pathobiochemical changes were initiated.

Another point to be considered is that of species-specificity in the probability of developing tau and APP pathology (Geula et al 1998). For example, as opposed to higher primates, aged rodents exhibit very little or no Aβ-related neurodegenerative features (e.g. cortical neuronal loss, abnormal tau hyperphosphorylation and microglia proliferation). Results presented in Chapter 3.3. of this thesis suggest that species-specific differences in tau protein, including susceptibility to hyperphosphorylation at specific sites (see Busciglio et al 1995; Le et al 1997; Shea et al 1997), may account for differential vulnerability to the neurotoxic actions of Aβ. This view receives support form other studies in cell lines and non-vertebrate models of AD that show the paramount role of human tau in causing disturbances of cellular homeostasis (Wittmann et al 2001; Jackson et al 2002; Stamer et al 2002). Lastly, human tau seems to determine tau pathology in a dose-related manner (for review, see Brandt et al 2005) and interestingly, tau depletion was shown to abrogate Aβ-induced neurodegeneration (Rapoport et al 2002). These various observations indicate that the availability of human tau may play a decisive role in setting a cell's vulnerability to insults such as Aβ or GC.

Whereas animal models allow testing of hypotheses in physiological settings, they are limited in that one cannot analyze cellular or molecular mechanisms conveniently. For this reason, this study also included work on the rat-derived PC12 neural cell line. After differentiation, this cell line has been widely used for studies on the biology of neuronal development and disease, including AD. Besides using differentiated wild-type PC12 cells, we also had the opportunity to use PC12 cells that had been engineered to stably express human tau protein ($PC12_{htau}$ cells) (Maas et al 2000; Fath et al 2002) (see Chapter 3.3. of this dissertation). The manipulated cells allowed us to demonstrate that human tau renders rat neurons sensitive to Aβ or GC, seen as mis-processing of APP and aberrant hyperphosphorylation of tau, two features central to the etiology of AD.

As already mentioned, GC are thought to interfere with cognitive performance by inducing neuronal atrophy and loss in the hippocampus and PFC. Hippocampal and cortical volume loss and metabolic dysfunction can also serve as surrogate markers of AD. The Results section of this thesis (Chapter 3.1.) describes studies involving surgical and pharmacological manipulation of the endogenous corticosteroid *milieu* of rats before performance of brain magnetic resonance imaging (MRI) and functional magnetic resonance spectrometry (MRS), (and eventually morphometry by conventional histological methods). The imaging studies, performed at 7 Tesla resolution, allowed validation of the suitability of such methods, with a view to eventually using them for longitudinal monitoring of brain changes in rodents. The results of this first study showed that corticosteroid removal by adrenalectomy (ADX) causes selective neuronal atrophy (reduced volume) of the hippocampus, but not of the PFC [anterior cingulate cortex (ACC) and retrosplenial cortex (RSC)]. On the other hand, exposing rats to supraphysiological doses of GC levels (as might be found during stress response) led to reduced volumes of both the hippocampus and PFC, accompanied by marked changes in hippocampal metabolism; the latter implies impaired neuronal function. Besides opening the way for the design of new studies, the results of this work point to the specific sensitivities of different neuronal populations on alterations in the GC environment. Specifically, the

volumetric differences found in the ACC of dexamethasone-treated animals most likely depend on the relative abundance of the two corticosteroid receptors in this region: while the ACC expresses a high level of glucocorticoid receptors, its complement of mineralocorticoid receptors is low (Chao et al 1989). It therefore appears that the relative abundance of each corticosteroid receptor subtype, rather than its absence per se, may render the ACC vulnerable to the excess of circulating corticosteroids. Interestingly, although the RSC shows a pattern of corticosteroid receptor distribution similar to that found in the ACC, dexamethasone-induced hypercortisolism did not produce marked changes in RSC volume. This result resembles the observation that pyramidal neurons in the hippocampus are not subject to ADX, corticosteroid and stress-induced cell death despite their expression of both mineralocorticoid and glucocorticoid receptors (Hassan et al 1999; Sloviter et al 1989). Since virtually all neurons express corticosteroid receptors, the present differential responses of the ACC and RSC (as well as of the various hippocampal cell types) raise the interesting question of what might be the unique properties of particular cell types (e.g., anterior cingulate cells) that render them more vulnerable to corticosteroids. Additionally, in this study, animals were subjected to ADX of elevated GC for just 2 weeks. Thus, the significant changes detected by brain imaging demonstrate that GC can act relatively fast to re-organize neural substrates that are important for cognition, and which are affected during early stages of AD (Braak and Braak, 1997).

In studying the role of stress and GC in triggering AD-like pathology, a considerable portion of this thesis (see Chapter 3.2. and 3.3, and parts of 3.1.) focused on APP misprocessing and tau hyperphosphorylation, as these processes are widely accepted to be the primary cause of neuronal dysfunction and death in brain areas (e.g. hippocampus and PFC) involved in the regulation of memory and other cognitive functions. In this respect it should be mentioned that both brain areas are well endowed with glucocorticoid receptors (GR), and as already stated, respond to stress and GC with dendritic atrophy and synaptic loss. Pilot

studies (Chapter 3.1.) showed that manipulations of adrenocortical function result in altered levels of APP, findings that are in agreement with previously-published work (Islam et al 1998; Budas et al 1999). In a further step, the pro-amyloidogenic role of GC was revealed in terms of GC-stimulated increases in C99 and BACE and nicastrin, two enzymes that are critically involved in the generation of Aβ. In contrast, hypocortisolism, induced by ADX, led to increased production of C99, but these changes were not accompanied by increased levels of nicastrin, precluding the production of amyloid. It should be noted that, here and elsewhere in this thesis, technical limitations precluded direct measurements of Aβ; accordingly, concomitant increases in C99 and nicastrin levels were interpreted as being suggestive of the potential for increased Aβ production.

The pilot results reported in Chapter 3.1. also showed that ADX results in tau hyperphosphorylation; GC replacement therapy, with the physiological hormone corticosterone (normocortisolism), restores the usual tau phosphorylation pattern, and on the other hand, supplementation of ADX animals with a synthetic GC, dexamethasone (the prototypic GR agonist), also results in tau hyperphosphorylation. These results may be explained by the fact that whereas high GC levels activate GR, lower levels only activate mineralocorticoid receptors (MR) (Reul et al 2000). Work from this laboratory has previously demonstrated duality of GC actions, mediated by MR and GR, on neuronal survival, atrophy and death (Sousa et al 1999; Almeida et al 2000; Crochemore et al 2005). Although the studies reported in Chapter 3 involved the use of the pure GR ligand, dexamethasone, the previous work on MR suggests that important new insights may be gained from future studies on the role of MR activation in the regulation of APP and tau metabolism; based on the existing knowledge, one might expect to uncover mechanisms through which selective GC ligands might afford neuroprotection in the context of AD.

Synaptic plasticity provides a cellular basis for cognitive functions such as learning and memory. Cytoskeletal proteins and the kinases that govern their phosphorylation status, and therefore ability to maintain the integrity of

microtubules, seem to play a critical role in synaptic plasticity and maintenance of neuritic structures (Manji et al 2001; Sweatt 2004; Johnson and Stoothoff 2004; Chen and Manji, 2006). In an extension of the pilot data presented in Chapter 3.1, those described in Chapter 3.2 demonstrate that stress/GC have profound effects on tau biochemistry in middle-aged rats. Both treatments induce aberrant hyperphosphorylation of tau (at AD-relevant sites), increase tau accumulation and diminish microtubule stability. Specifically it is shown, for the first time, that stress/GC treatment can influence the biological activity of the cytoskeletal protein tau by inducing its hyperphosphorylation; at least two of the phospho-tau sites (Thr231 and Ser262) altered here have been strongly correlated with the reduced microtubule binding capacity of tau and, subsequently, cytoskeletal disturbances (Sengupta et al 1998). Additionally, levels of pThr231 correlate with rates of hippocampal atrophy in AD patients (Hampel et al 2005) and pSer262 levels are strongly associated with cytoskeletal pathology in AD (Lauckner et al 2003). The changes in tau hyperphosphorylation occurred in tandem with increases in the steady-state levels of a number of kinases implicated in tau pathology and were accompanied by decreased levels of Ac-Tub, suggestive of disruption of microtubule stability (cf. Hempen and Brion, 1996; Cho and Johnson, 2004). These changes are reminiscent of those seen in the AD brain and are thought to contribute to severe cytoskeletal disturbances and eventually neuronal death. Interestingly, stress/GC induce the above changes in the hippocampus and PFC, areas that are strongly implicated in learning and memory; in the present study, parallel measurements of PFC- and hippocampus-dependent behaviors (reversal learning and spatial memory, respectively) revealed that the extent of stress/C-induced cognitive impairment correlated with the degree of tau hyperphosphorylation.

Other data presented in this dissertation (Chapter 3.2) show that GC can exacerbate tau pathology and cognitive deficits in rats infused with Aβ peptide. Specifically, concomitant exposure to GC and Aβ produced increases in aberrant tau hyperphosphorylation and accumulation, as well as in levels of several active kinases of tau, accompanied by markedly impaired spatial reference memory and

reversal learning. Notably, reciprocal regulatory relationships between cognition and emotion are being increasingly recognized (Dolan, 2002; Ochsner and Gross, 2005; Phelps, 2006), but such interactions have not attracted much attention in the AD field. In light of reports that AD patients show signs of hyper-anxiety (Grossberg, 2003) and the implication of stress and GC in disorders of anxiety (Tatsch et al 2006) and cognition (Starkman, 2001; Lupien et al 2005), we here evaluated emotional behavior in rats exposed to stress, GC or Aβ regimens. All three treatments were associated with elevated emotionality (decreased locomotion in the central area of an open field) and anxiety (less time spent in open arms of a plus maze). Together with the clinical observations referred to above, these observations suggest that emotional behavior may be an important aspect to be considered in understanding the neurobiological basis of cognitive impairment in AD. Together, these findings offer novel insights into the underlying mechanism(s) of stress- or GC-evoked neuronal impairment and atrophy (dendritic atrophy and synaptic loss) as well as cognitive deficits.

The mechanism through which hyperphosphorylated tau leads to neuropathology involves axonal-to-soma relocation and accumulation of tau, disruption of axonal transport, and eventual cytoskeletal destabilization (Johnson and Stoothoff, 2004). Most of the tau epitopes monitored in this study are known to be abnormally phosphorylated in AD (Augustinack et al 2002; Lauckner et al 2003; Hampel et al 2005). Inspection of the data for individual treatments and brain areas reveals, however, that not all phospho-epitopes were equally affected. This may be due to the fact that tau hyperphosphorylation occurs in a sequential and graded manner (Morishima-Kawashima et al 1995). On the other hand, several studies ascribe functional significance to hyperphosphorylation at distinct sites. For example, pSer262 (recognized by antibody 12E8) and pThr231 (recognized by antibody CP9) are more closely associated with cytoskeleton disruption than other phospho-tau isoforms (Seubert et al 1995; Sengupta et al 1998; Lauckner et al 2003). In the present study, both pSer262 and pThr231 were detectable in hippocampal and PFC neurons (stronger signal in stressed *vs.* unstressed rats receiving Aß+GC >> Aß or GC), changes that were accompanied by signs of

reduced microtubule stability. It should be noted that increased levels of pSer262 are strongly associated with microtubular disruption (Lauckner et al 2003) and synaptic loss (Callahan et al 2002). Since stress is known to lead to loss of synapses in the hippocampus (Sousa et al 2000; Sandi 2004) and synaptic input to the PFC (Cerqueira et al 2006), pilot data from this study showing that stress and GC trigger a significant loss of pre- and post-synaptic markers, support our suggestion that tau-related cytoskeletal disturbances which lead to synaptic degeneration can underlie the stress-induced cognitive deficits offering novel information and perspectives for further investigation.

The current consensus is that APP mis-processing may precede the manifestation of defects in tau phosphorylation. Results presented in Chapter 3.2. cannot fully support this view, but do not refute it either. Specifically, in this study, we found that stress and GC upregulate APP mRNA expression, but not protein levels in the hippocampus and PFC. These observations suggest increased metabolism of APP. Consistent with this interpretation are our findings that both treatments resulted in increased BACE-1 levels as well as C99. The upregulation of BACE-1 by stress is most likely mediated by GC since Sambamurti et al (2004) described a glucocorticoid response element (GRE) in the promoter region of the BACE gene. BACE-1 is essential for C99 production, and previous studies have shown that even slight increases in this enzyme can result in the generation of high levels of amyloidogenic products such as Aβ (Haass 2004; Johnston et al 2005; Li et al 2006). Our results therefore strongly suggest that stress and GC activate the amyloidogenic pathway. However, although both GC and stress stimulate APP processing to C99, the two treatments seem to differ in one important respect: stress resulted in significant increases in the levels of nicastrin in both the hippocampus and PFC; in contrast, GC actions 'stop' at the pre-amyloid (C99) stage. Nicastrin is an essential component of the γ-secretase complex (Herreman et al 2003); by recognizing and presenting substrate (C99) to the presenilins (also part of the γ-secretase complex), nicastrin is crucial for the generation of Aβ from C99 (Shah et al 2005). Thus, besides GC, stressful stimuli most likely recruit other, as yet unknown, mechanisms that allow it to carry C99 metabolism

through to Aβ production. On the other hand, the possibility that GC can also induce amyloidogenesis, by acting on other members of the γ-secretase complex, cannot be excluded. Nevertheless, it should be emphasized that both Aβ and C99 can induce neuronal damage and behavioral deficits; moreover, as was indicated earlier, stress-induced effects on C99 production are likely to depend on GC mediation.

In fact, the differential temporal effects of GC on APP mis-processing and tau hyperphosphorylation seen in $PC12_{htau}$ cells (Chapter 3.3.) do go along with this proposed sequence of events - GC rapidly induce the production of BACE, C99 and nicastrin earlier than they alter the phosphorylation status of tau. Returning to the suggestion that APP mis-processing occurs prior to tau hyperphosphorylation, it should be noted that Aβ is known to activate a number of tau kinases, making the suggested temporal cascade plausible. Additionally, using i.c.v. Aβ infusion in middle-aged rats, we observed that Aβ upregulates *APP* mRNA levels, but reduces APP steady-state protein levels; at the same time, the treatment resulted in increased tissue levels of C99 and nicastrin. Thus, as was the case with stress and GC, Aβ appears to accelerate APP metabolism, increasing the potential for amyloid peptide generation. This finding suggests that Aβ may mediate the well-known cognition-impairing effects of stress and GC (Lupien et al 2005) and, at the same time, supports the view that stress and GC may contribute to the etiopathogenesis of AD.

A starting hypothesis for these studies held that interactions between endogenous and exogenous factors (e.g. age, mutations and Aβ production on the one hand, and stress on the other) may determine the onset and progress of AD. The organism experiences intermittent stressors throughout life while its endogenous production of Aβ increases with age (Braak and Braak, 1997). Recent studies in two different transgenic animal models of AD which exhibit enhanced Aβ production reported that chronic stress potentiates Aβ deposition and induces cognitive deficits (Joeng et al 2006) and that elevated GC exacerbate amyloidogenesis (Green et al 2006). In Chapter 3.2., we attempted to mimic life

events by exposing rats to a chronic unpredictable stress paradigm before subsequent treatment with GC and/or central infusions of Aβ. The results clearly demonstrate that stress history exacerbates the APP mis-processing effects of Aβ and GC and, strikingly, also activate the amyloidogenic pathway in the PFC, an area that had proven rather resilient to the individual stimuli. Additionally, previous stress also exacerbated the GC-potentiating role of Aβ-induced tau hyperphos-phorylation; this effect was more robust in the PFC and entorhinal cortex, two areas that mildly responded to individual treatments. These findings indicate a type of 'build-up' or cumulative phenomenon whereby stressful stimuli trigger APP- and tau-related pathological mechanisms, producing greater deterioration of cognition than that induced by the individual treatments. Briefly, stress history and previous exposure to elevated GC can markedly worsen the effects of subsequent exposures to stress/GC and Aβ on AD-like biochemical and behavioral pathology. Although further increases in anxiety could not be discerned in rats given the combinatorial treatment ('floor' effects of stress, Aβ and GC themselves), exposure to stress before treatment with Aβ and GC produced greater deterioration of spatial memory than that induced by the individual treatments. Together, this set of experiments shows that stress history and previous exposure to elevated GC can markedly worsen the effects of subsequent exposures to stress/GC and Aβ on AD-like biochemical and behavioral pathology. Accordingly, stress would seem to be an important precipitating and exacerbating factor in AD. Given this, strategies to reduce the impact of stress might be helpful not only in preventing AD, but also for impeding the rate of progress during early phases.

In attempt to initiate a mechanistic analysis of the observations in animals, as mentioned already, some of the work reported in this thesis was carried out *in vitro*, using a differentiated neural cell line (PC-12) (Chapter 3.3.). Those studies showed that GC treatment induces tau hyperphosphorylation through the mediation of GSK3β, cdk5 and ERK1/2; previously, all of these kinases have been causally implicated in AD tau-related neurodegeneration. Interestingly, the increases in tau phosphorylation were accompanied by reduced tau degradation, suggesting that tau hyperphosphorylation affects the turnover of tau, resulting in

tau redistribution and accumulation in neuronal somata vs. neurites; these features are also observed in AD-affected brains. Lastly, it is important to note again that the pathobiochemical changes seen after GC (and Aβ, below) treatment of rat PC12 cells seemed to depend strongly on the presence of human tau. This finding may seem to be at odds with our observations of AD-like pathology in normal rats (see Chapter 3.2.), and a suitable explanation for the differences between cells and intact rodents is not readily available. Uncovering the underlying reasons will be a challenge that must be taken on in the future.

Since Aβ is known to induce tau-related neuronal dysfunction and death, the present observations (Chapter 3.3.) that the changes in tau were apparently triggered by GC-evoked misprocessing of amyloid precursor protein (APP) favoring the APP amyloidogenic pathway and that GC do not seem to drive the non-amyloidogenic pathway are interesting. These GC-induced alterations in APP and tau may be responsible for rendering cells more vulnerable to subsequent insults, including Aβ itself. Indeed, other results presented in Chapter 3.3. showed that pre-treatment with GC increases neuronal vulnerability to the neurotoxic actions of Aβ, seen as potentiated tau hyperphosphorylation and cell death. It is interesting to note that such a 'build-up' phenomenon was also observed in the *in vivo* studies reported in Chapter 3.2. where previously stressed rats treated with Aβ+GC showed enhanced APP mis-processing and tau pathology and cognitive decline when compared to non-stressed animals receiving the same treatment. The latter results strongly indicate that a history of stress/GC exposure may sensitize individuals to the tau pathology-inducing effects of Aβ and GC. As concluded for Chapter 3.2., if these findings are extrapolated to humans, they suggest the potential usefulness of stress/GC reduction in the clinical management of AD and other stress-related disorders of cognition. Additionally, our preclinical observations reiterate the need for judicial use of GC therapy in older subjects, especially in light of the apparently poor efficacy of GC (cf. certain non-steroidal anti-inflammatory drugs, NSAIDS) to slow the progression of AD (cf. in t' Veld et al 2001; Aisen 2002).

In conclusion, the work presented in this thesis reports a novel cellular response to stress and GC that involves APP misprocessing, resulting in generation of C99 and Aβ. Both C99 and Aβ can have deleterious effects on neuronal function and survival as well as on cognition. Further, it is proposed that the activation of kinases (e.g. GSK3β, cdk5 and ERK1/2) by Aβ may be responsible for triggering tau pathology, namely, tau hyperphosphorylation and accumulation, microtubule instability, and eventually synaptic loss and neuronal death (see model in Fig. 4.1). Besides their immediate relevance to AD, these findings provide new insights that may help understand the interaction between GC, neuronal cytoskeletal dynamics and synaptic loss, especially in the context of stress-related cognitive disorders such as major depression which are also characterized by hypersecretion of GC and neuronal remodelling and atrophy. These studies also warrant a closer examination of the suitability of the various cellular and animal models available for studying the causes and progress of AD, as well as for developing disease prevention and reversal strategies. In particular, they raise questions with respect to the risk afforded by human forms of APP and tau, the two proteins most commonly implicated in AD.

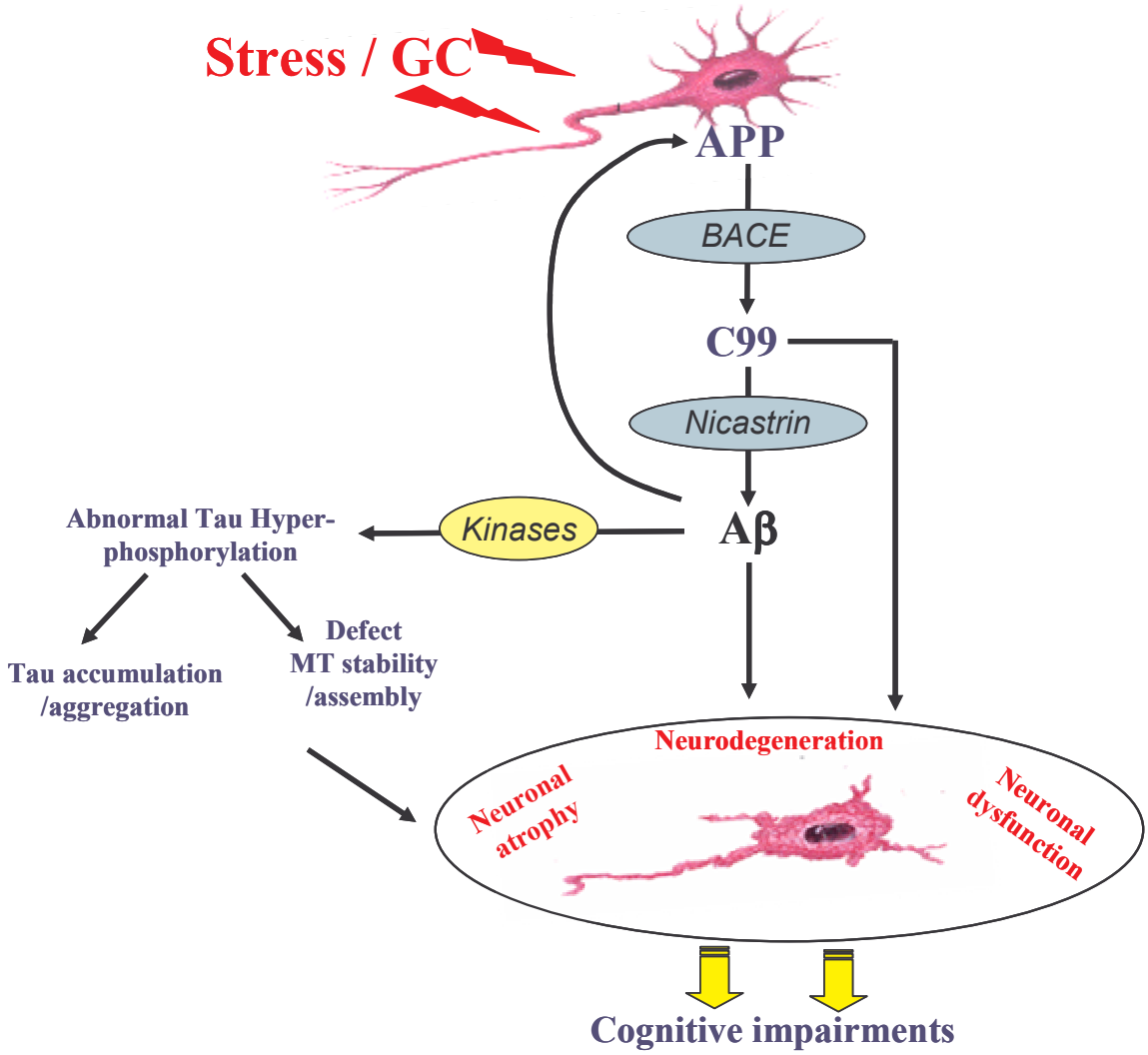


Fig. 4.1. Schematic representation summarizing the role of stress/GC in APP misprocessing and tau protein based on results described in this dissertation. It is proposed that stress/elevated GC primarily act to induce amyloidogenic mis-processing (stimulation of BACE, C99 production, and nicastrin). Besides inducing neurodegenerative and behavior-impairing effects of its own, Aβ, in turn, stimulates several tau kinases which lead to tau hyperphosphorylation of specific sites, culminating in cytoskeletal disturbances and neuronal death. Note that C99 also shares some of the deleterious properties of Aβ (drawn by Sotiropoulos I. and Almeida O.F.X.).

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ANNEX

- **Invited review**
  Catania C, Sotiropoulos I, Breen K, Almeida OFX (2006) A steroid hormone-Alzheimer's disease connection? Upsides-Downsides. In: Molecular Bases of Neurodegeneration. Eds. I. Di Liegro & G. Savettieri. Research Signpost, Kerala, pp 21-42

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2 A steroid hormone-Alzheimer's disease connection? Upsides, downsides

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Abstract

Alzheimer 's disease (AD) is the fourth leading cause of death in people over 65 years of age, while dementia of Alzheimer 's type is the commonest form of dementia. Clinical studies suggest that steroid hormones may be involved in the neuropathology of AD, a neurodegenerative disorder that results in the loss of neurons that contribute to memory processes. A large body of evidence shows that estrogen, a female sex steroid, can protect against neuronal death, and several studies suggest that estrogens, whose

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secretion declines with increasing age, may improve cognition in humans and animals; however, the beneficial role of estrogens is currently strongly debated. Hypersecretion of the stress hormone, cortisol, from the adrenal glands is associated with cognitive impairments, and hypercortisolism is observed in a subgroup of AD patients. While these observations indicate a role for adrenocorticosteroids (glucocorticoids) in the etiology and progression of AD, little is known about the key mechanisms involved. This review will discuss the current state of knowledge regarding how steroids are implicated in the onset and progress of AD; it also outlines some of the underlying pathological mechanisms that might be targeted in attempts to prevent or slow progression of the disease.

Introduction

Alzheimer's disease (AD) is a progressive age-related disorder, affecting 7-10% of the population aged over 65 years [1]. The disease, named after Alois Alzheimer, is the most common form of senile dementia, clinically characterized by progressive loss of cognitive abilities which first become manifest around 65-70 years of age. Most forms of AD are sporadic. However, genetic inheritance is responsible for some 7% of cases, referred to as familial Alzheimer's disease (FAD).

Senile dementia of the Alzheimer type (SDAT) is the diagnostic term reserved for patients suspected to be suffering from AD. Diagnosis of SDAT is based on neurological examination and the exclusion of other causes of dementia. Definitive diagnosis of AD itself can only be made *post mortem* and is based on neuropathological lesions in the neocortex, hippocampus, and basal forebrain cholinergic system. The lesions are caused by the extracellular accumulation of *senile plaques,* derived from amyloid precursor protein (APP), and intracellular *neurofibrillary tangles* (NFT) which are thought to result from abnormal phosphorylation of the cytoskeletal protein tau [2, 3, 4]. With time, these lesions lead to neuronal dystrophy, reduced synaptic input and cell death within neural circuits responsible for attention and memory.

In this brief review, we will summarize some of the recent advances in our knowledge about the pathobiochemistry of the disease. In addition, we will present an appraisal of what is currently known about likely risk and/or potentiating factors with a particular focus on the role of genetic mutations, sex hormones, and stress. The latter two are particularly interesting since prominent changes are observed in the secretory profiles of the adrenocortical and gonadal steroid hormones as the organism ages, and as already indicated above, aging is the principal risk factor for developing AD [5]. Steroid hormones produced in the periphery provide a means for easy communication between the periphery and brain, and understanding their role in the disease

will cast light on the interplay between peripheral physiology and brain function and pathology while, at the same time, providing preventative and therapeutic hints. Insights into the pathobiochemistry of AD are essential for identifying cellular and biochemical targets for treatments aimed to alleviate symptoms and/or forestall disease progression. Such studies have already led to the development of promising 'biomarker assays' for the early differential diagnosis of SDAT *versus* other forms of cognitive impairment, as will be discussed towards the end of this chapter.

1. Alzheimer's disease

1.1. Molecular pathobiochemistry of AD

Senile plaques and amyloid precursor protein (APP)

Senile plaques - also known as *amyloid plaques* – which represent one of the neuropathological hallmarks of AD, consist of a central extracellular core of aggregated amyloid â-peptide surrounded by distended abnormal neurites. While this central core contains numerous proteins, a 39-43 amino acid peptide, known as amyloid â-peptide (Aâ) is the principal component relevant to AD pathology. Aâ is derived from the proteolytic processing of *amyloidprecursor protein (APP)*. It is important to note that there exist two amyloid precursor-like proteins (APLP1 and 2), which have extracellular and intracellular domains similar to those found in APP, but which lack the 42-amino acid Aâ region.

APP is a type-I trans-membrane sialo-glycoprotein. It has a single membrane-spanning domain, a large extracellular glycosylated N-terminus and a shorter cytoplasmic C-terminus. APP can be found in many membranous structures, including the endoplasmic reticulum, Golgi, and cell membrane [for review, see 6]. The *APP* gene has 18 exons and, through alternative splicing of exons 7, 8 and 15, can give rise to at least 8 APP protein isoforms. Neurons always express the product of exon 15 of the *APP* gene. This product shows greater propensity to form amyloid aggregates, i.e. it is more amyloidogenic than other isoforms, thus increasing the chances of plaque formation on, or in, the vicinity of neurons. There are 3 neuronal isoforms of APP (APP_{695}, APP_{751} and $_{APP770,}$ consisting of 695, 751 and 770 amino acid residues, respectively); both $_{APP751}$ and $_{APP770}$ contain the 56-amino acid Kunitz protease inhibitor (KPI) domain, which is encoded by exon 7 [see reviews, 7,8].

APP undergoes proteolytic cleavage in two different pathways. One is termed 'amyloidogenic pathway' as it results in the generation of the Aâ peptide; the other is referred to as 'anti-amyloidogenic pathway' since it does not result in the production of Aâ (see Fig. 1). The amyloidogenic pathway is initiated by APP cleavage through BACE-1 (â-secretase, a type-I membrane-bound aspartyl protease preferentially located in the Golgi apparatus and endosomes)

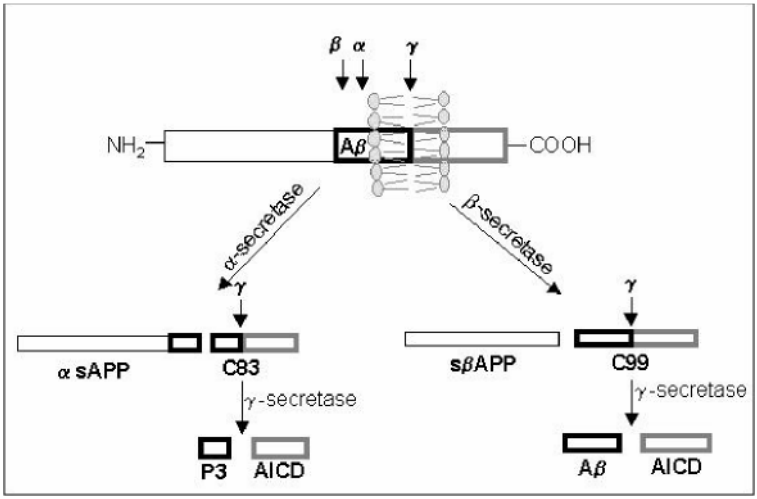

Figure 1. APP-processing pathways. Cleavage by the membrane-associated metalloprotease á-secretase enzymes occurs within the Aâ domain (between residues 16 and 17), thereby preventing the formation of Aâ, and instead resulting in the release of the large soluble extracellular N-terminal portion of APP (APPsα) and a C-terminal fragment consisting of 83 residues (C83). C83 can undergo further processing by ã-secretase to release a peptide called p3. Aâ is generated through 2 sequential endoproteolytic steps involving distinct enzymatic activities: â- and ã-secretase. â-secretase, also known as BACE1, cleaves APP at the N-terminal region of the Aâ sequence. Cleavage by â-secretase generates a slightly shorter soluble N-terminus (sAPPâ) and the amyloidogenic C-terminal fragment (C99). The cleavage of C99 by ã-secretase liberates the C-terminal 50 residues of APP, the APP intracellular domain (AICD) and Aâ.

at the N-terminus of the Aâ-containing domain. This cleavage generates soluble sAPPâ and a C-terminal fragment (C99); the latter undergoes a second cleavage by ã-secretase within the transmembrane domain of APP. ã-secretase is a heteromeric protein complex consisting of presenilin, nicastrin, PEN-2, and APH-1. In contrast, the anti-amyloidogenic pathway starts with APP cleavage by á-secretase, a member of the ADAM (a disintegrin and metalloprotease) family of proteases. á-secretase cuts within the Aâ domain, thus precluding the generation of the neurotoxic Aâ peptide. The residual C-terminal fragment undergoes ã-cleavage to yield the p3 peptide.

The functions of APP are still poorly understood. *In vitro* studies suggest that secreted APP can function in an autocrine manner to stimulate cell proliferation and adhesion or to augment the neurite-promoting actions of

nerve growth factor. Other studies have implied APP in signal transduction and, in association with other proteins, in the regulation of transcription [9]. Aâ can have multiple adverse effects on the functions and integrity of both pre- and post-synaptic terminals. These include the induction of oxidative stress, impairment of calcium homeostasis and perturbation of mitochondrial and endoplasmic reticulum functions. It appears that Aâ is most toxic when it is in the form of soluble oligomers [10], and synapses seem to be particularly susceptible to aggregating forms of Aâ.

Tau and neurofibrillary tangles (NFTs)

The major component of *neurofibrillary tangles* (NFT), the other characteristic feature of AD, was shown by immuno- and protein chemistry to be tau, a cytoskeletal protein [11]. NFT are in fact made up of highly insoluble *paired helical filaments* (PHF) that appear as left-handed double helices and *straight filaments* (SF) in which tau is abnormally hyperphosphorylated. Besides NFT, intraneuronal neurofibrillary lesions observed in AD brain include (i) *neuropil threads* (NT) in distal dendrites, and (ii) *neuritic plaques* (NP) which are argyrophilic dystrophic neurites often associated with extracellular amyloid plaques. Both, NFT and NT can apparently develop in the absence of amyloid deposits, although they co-exist in the final stages of disease. NP generally develop later and show an irregular distribution.

Tau proteins are soluble low M_r microtubule (MT)-associated proteins which are predominantly expressed in axons; astrocytes and oligodendrocytes express these proteins at very low levels [12]. The adult human brain expresses 6 isoforms of tau (352 to 441 amino acid residues) which are all encoded by a single gene consisting of 16 axons; the isoforms result from alternative mRNA splicing of exons 2, 3 and 10. The C-terminal segment of the molecule contains 3-4 tandem repeat sequences (31 or 32 amino acids each, encoded by exons 9, 10, 11, 12) which make up the MT-binding domain; tau binding to MT promotes polymerization and provides stability and orientation to the MT [for review, see 13]. During fetal development, tau is alternatively spliced to yield only the shortest isoform.

Tau protein is subject to several types of post-translational modifications, namely, glycosylation, ubiquitination, glycation and phosphorylation; the latter has received most attention. In its longest form (441 amino acids), tau bears 79 putative sites (serine and threonine residues) at which phosphorylation can occur (see Fig. 2), of which approximately 30 are not associated with neuropathology [14]. The degree of tau phosphorylation gradually declines with age [15, 16].

At least 25 sites have been identified in PHF-tau purified from AD brains [17] at which tau can be hyperphosphorylated; this results in reduced affinity of tau for MT, leading to disruption of the neuronal cytoskeleton and the

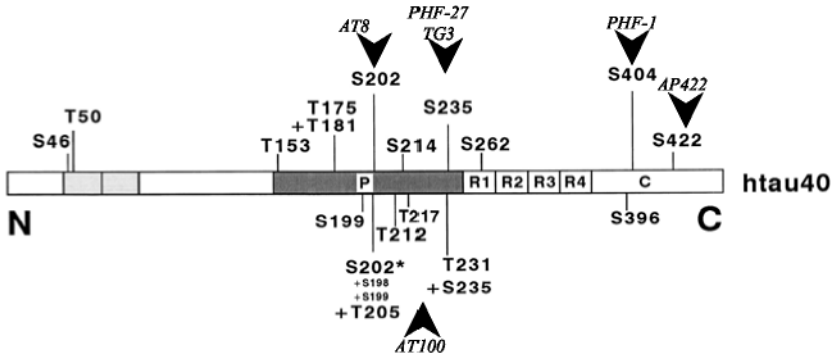

Figure 2. Schematic drawing of Tau protein showing threonine (T) or serine (S) residues that can be phosphorylated; some antibodies which recognize specific phospho-tau epitopes are shown in italics.

accumulation of hyperphosphorylated tau in the form of PHF in neuronal perikarya and their dendritic processes, thus compromising dendritic plasticity and axonal transport [18]. In contrast, dephosphorylation of tau promotes rapid and extensive MT polymerization [19, 20]. PHF can be formed from all 6 isoforms of tau, with the C-terminal portion which contains the MT-binding domain forming the core of the detergent-insoluble PHF [21].

Tau phosphorylation is mediated by either (i) proline-directed kinases such as glycogen synthetase kinase 3â (GSK-3ß or tau protein kinase I), cyclin-dependent kinase 5 (cdk5 or tau protein kinase II), the mitogen-activated kinases (MAP) p38 and JNK, and several other stress kinases (e.g. cdc2), or (ii) non-proline-directed kinases such as protein kinase A (PKA), protein kinase C (PKC), calmodulin kinase II (CamKII) and microtubule affinityregulating (MARK) kinase [22]. Ultimately, however, the phosphorylation state of tau is balanced by the prevailing activities of different kinases and phosphatases (e.g. protein phosphatase I and II [PPI and PPII] PP2B and PP5 [for review see 23].

1.2. Genetics of AD

Some 90% of AD cases occur sporadically. The remaining (FAD) cases, which show early onset and are autosomal dominant, have been attributed to polymorphic mutations in 3 genes, namely *APP* (mapped to chromosome 21), *Presenilin 1 (PSEN1,* on chromosome 14) and *Presenilin 2 (PSEN2,* on chromosome 1) [24]. The products of the *PSEN1* and *PSEN2* genes seem to form the catalytic core of the ã–secretase complex. To date, 12 AD-associated

mutations have been found in the *APP* gene, 69 in *PSEN1,* and 5 in *PSEN2 [25]*. Interestingly, all of these mutations result in increased production of Aâ peptide fragments.

Late-onset non-familial (sporadic) AD has been associated with a polymorphic allele of the *apolipoprotein E (APOE)* gene, located on chromosome 19. The mutation å4 has been shown to increase the risk of developing AD in a gene-dosage-dependent manner. However, < 50% of non-familial AD cases are burdened with the ApoEå4 allele [26]. In contrast to the mutations in APP, *PSEN1* and *PSEN2, APOå4* reduces Aâ production, but promotes Aâ deposition and plaque formation [27]. Meanwhile, some 30 other candidate genes have been suggested to contribute to the risk of developing sporadic AD [28].

In 1998, mutations were found in the *tau* gene in several frontotemporal dementia- and Parkinson's disease-afflicted families; these patients exhibited extensive neuronal loss and tau deposition [29], implying that mutations of tau alone are sufficient to cause both neuronal loss and tau deposition. The tau mutations were mapped to chromosome 17 (FTDP-17).

1.3. Neuropathological features

The progressive build-up of Aâ-containing plaques and NFT over years (even decades) eventually leads to lesions that involve neuronal dystrophy and death and loss of synaptic connections. Attention- and memory-relevant neuronal circuits in the neocortex, hippocampus, and basal forebrain cholinergic system seem particularly vulnerable to these insults. *Post mortem* observations suggest that even as these lesions are growing, patients may be largely asymptomatic, i.e. symptoms of cognitive impairment appear at a rather late stage after the onset of disease. However, it remains unclear as to whether a certain threshold needs to be reached before symptoms are observable or whether such a threshold varies between individuals. Interestingly, the distribution pattern (and shape and size of amyloid deposits) varies widely between patients. It is tempting to speculate that individual genetic differences or lifetime experiences or a combination thereof determine these supposed thresholds. In the context of this article, extra-genetic factors such as stress or physiological or pharmacological exposure to adrenal and gonadal steroids could well be important. What seems certain from the literature is that low education status or reduced intellectual activities render some subjects more susceptible to AD.

In contrast to senile plaques, neurofibrillary lesions develop at predictable sites, affecting specific cell types in given cell layers and brain regions. The uniformity of these patterns formed the basis of Braak and Braak's [30] description of the 6 stages of NFT and NT development; these stages,

summarized below, show good correlation with impairment of performance on both memory and mental status tests [31].

- *Stage* I: Projection neurons of the a-layer of the *trans-entorhinal* (TE) region display neurofibrillary changes, notably in the *absence* of amyloid deposits. Patients are asymptomatic and, if at all, show mild changes in the hippocampal CA1 subfield.
- *Stage I:* The TE becomes more severely affected and mild changes occur in the pre-a layer of the entorhinal region. As with Stage I (TE-stage) patients, subjects in this phase of disease development show no signs of cognitive impairment and only minor neurofibrillary disturbances in the CA1 region.
- *StagesI and IV,* also known as the *limbic stages,* are characterized by mild cognitive impairment (MCI). Both pre-a layers of the entorhinal and TE regions are severely lesioned and lesions become more evident in the CA1 area of Stage III patients. Stage III patients also start developing mild lesions in the magnocellular forebrain nuclei, the anterodorsal nucleus of the thalamus and the amygdala. Numerous NFT also occur in the CA1 area at Stage IV.
- *Stages V and VI,* also referred to as *the neocortical stages,* represent fully developed AD. While primary sensory areas are relatively spared, subcortical nuclei show pronounced changes during Stage V. Finally, in Stage VI, the hippocampus is infected with neurofibrillary changes and CA1 neuronal degeneration is rife; all cortical association areas as well as the primary sensory areas and subcortical nuclei are also markedly affected.

It is important to note that a large number of non-demented individuals display plaques and tangles at autopsy. These findings support the view that the pre-clinical phase of AD is protracted, that the appearance of plaques or tangles is a normal accompaniment of aging, and that the magnitude of these lesions must play a decisive role in determining when outwardly clinical signs of AD (amnesic type of memory impairment, deterioration of language, and visuospatial deficits) will become manifested [32].

1.4. Biomarkers and Diagnosis of SDAT/AD

The aforementioned classic clinical features of AD represent a progressive loss of higher level-coordinated daily living activities which, at very late disease stages, can extend to motor and sensory abnormalities, gait disturbances and even seizures. In addition, apathy and relatively severe mood disturbances may develop early in the course of the disease, and psychosis and agitation are characteristic of the middle and later phases. Laboratory tests to

aid definitive diagnosis are highly desirable. In particular, tests that would enable reliable differential diagnosis between AD and other disorders which are accompanied by impaired cognition, e.g. *mild cognitive impairment* (MCI, widely considered to represent a high risk for full-blown AD), major depression and cerebrovascular disease, would facilitate effective clinical and social interventions and benefit the patient while, at the same time, reducing long-term costs by initiating treatment strategies that might help slow progression to severe SDAT.

There are presently no fool-proof non-invasive tests to aid presymptomatic diagnosis, but the problem is receiving much attention. Recently, it has become possible to detect amyloid plaques by magnetic resonance imaging (MRI) and/or single photon emission computed tomography (SPECT) in live subjects [33, 34]. Neuroimaging methods can also provide volumetric and functional data on the hippocampus and entorhinal cortex with a high degree of anatomical specificity. However, while results from such tests can help identify early signs of disease, they need further refinement to allow distinction between SDAT and other disease entities which lead to secondary impairment of cognition (see above) [35].

Significant advances have recently been reported with respect to the detection of AD-related proteins in cerebrospinal fluid (CSF), thanks to the development of antibodies against total tau, different phospho-tau epitopes, and isoforms of Aâ (including the 42-amino acid variant, $A\hat{a}_{42}$). Under normal conditions, detectable amounts of total tau (various fragments, with ranging between 14 and 16 kDa) are secreted to the CSF. The source of these proteins and their relationship to AD progression remains unclear; while some authors report correlations between total tau levels (CSF) and severity of dementia [36], others have not [37]. Further, increased CSF levels of total tau are not unique to AD as they are also elevated in patients with head trauma, Jacob-Creutzfeldt disease [38], normal-pressure hydrocephalus [39], and AIDS [40]. Thus, increased CSF levels of total tau seem to be a general, rather than a specific, biomarker of neuronal cell death and axonal degeneration [41]. Currently, there exist a number of sensitive enzyme-linked immunoassays (ELISA) which can measure total tau levels as well as the protein's different isoforms, cleavage products, and phosphorylated and non-phosphorylated forms of the protein in a highly sensitive manner.

ELISA has been used to detect APP, sAPP, Aâ and sAPPâ in CSF [42]. While CSF $A\beta 40$ levels apparently do not change [43], those of $A\beta 42$ may be decreased by some 40–50% in SDAT patients. The latter reduction was initially thought to be caused by the deposition of $A\beta 42$ in plaques, resulting in lower availability of the peptide for secretion into the CSF. Subsequent studies showed, however, that $A\beta 42$ is also markedly reduced in the CSF of patients

with other conditions where senile plaques do not occur, e.g. Jacob-Creutzfeldt disease [44], amyotrophic lateral sclerosis [45], and multiple system atrophy [46]. These findings raise questions about the relationship between low CSF-Aâ$_{42}$ levels and deposition of $_{A\beta42}$ in plaques. On the other hand, a recent autopsy study found strong correlations between low $_{A\beta42}$ in ventricular CSF and the number of plaques in the neocortex and hippocampus of AD-afflicted subjects [47].

2. Steroid hormones and brain pathology

Steroid hormones are small molecules produced by the adrenals (corticosteroids), the ovary (estrogens and progesterone) and testes (androgens); in addition, the gonads, adrenals and neuroglial cells produce the so-called neuroactive steroids (sometimes also termed 'neurosteroids'). The hydrophobic nature of steroids gives them easy passage to the brain during development and throughout the lifespan. Steroids exert pleiotropic effects on the nervous system, e.g. neurotransmitter synthesis and release, neuroprotection, myelination, control of mood and cognition as well as affiliative behavior and emotion. In addition, they feedback on hippocampal and hypothalamic neurons and cells in the anterior pituitary to regulate their own secretion. Together, these actions help co-ordinate the organism's endocrine and behavioral adaptation to changes in the environment, e.g. stress. Apart from the neurosteroids, which exert their actions by modulating an allosteric site on the $GABA_A$ receptor, all other steroids generally act via intracellular (nuclear) receptors. The latter are transcription factors, i.e. they regulate the expression of specific genes. Specific, high-affinity nuclear receptors for each type of steroid have been localized in various regions of the brain, albeit according to specific distribution patterns which are closely matched with a particular physiological or behavioral function. In recent years, a growing body of evidence supports the view that certain steroids (estrogens, progestins and corticosteroids) can additionally signal by triggering messenger cascades that originate at the plasma membrane (e.g. the MAP kinase pathway).

2.1. Gonadal (sex) steroids and their neuroactive derivatives

The neuroprotective effects of *estrogens* have been documented in many studies in cell culture and whole animal studies. In both, aged animals and different lesion paradigms, treatment with estradiol has been shown to promote neuronal survival and recovery [48, 49]. When applied *in vitro,* estradiol can protect neurons against excitotoxic amino acids, Aâ-induced toxicity, oxidative stress, and glucose deprivation [50, 51]. In addition, estradiol has been shown to decrease Aâ production *in* vitro [52] and in both normal mice

and transgenic models of AD [51]. Further, estrogens stimulate neurogenesis within the adult hippocampal formation [53].

The ability of estrogen to attenuate Aâ production apparently occurs through its ability to stimulate APP trafficking out of the endoplasmatic reticulum and Golgi (the site at which APP is cleaved by â-secretase) [54] and toward the cell surface (where á-secretase activity is located) [55]. One mechanism through which Aâ is thought to induce neuronal atrophy and death is through the generation of oxidative stress. Estradiol and several analogs bearing a phenolic ring structure have been shown to be particularly potent scavengers of reactive oxygen species; by donating a hydrogen atom, estrogens saturate and detoxify free radicals [56]. This and some of the aforementioned neurotropic actions of estrogens appear to occur through non-classical mechanisms, i.e. independently of nuclear estrogen receptors [57].

A large body of evidence accumulated over the last 2 decades has indicated that estrogens can preserve and even restore cognitive functions in older women and reduce a woman's risk of developing SDAT/AD [31, 58]. However, large-scale studies have indicated more recently that, besides increasing the risk of gynecological cancers [59, 60], estrogens might actually be detrimental to cognitive performance insofar that they accelerate cognitive decline, increase risk of dementia and stroke, and lead to hippocampal atrophy [61, 62]. The issue of estrogen supplements for menopausal women (see later in this section) has become a highly-charged topic and remains controversial [63, 64, 65].

Little is known about how *progestins* influence the development of SDAT/AD or whether this class of steroids has any therapeutic potential in preventing or slowing disease progression. However, as will be discussed later, the progestin precursors (pregnenolone and dehydroepiandrosterone and their respective sulfated esters), purportedly have memory-enhancing properties [66].

The significance of *androgens (e.g.* testosterone and dihydrotestosterone) in the context of SDAT/AD is also uncertain. There are reports that circulating testosterone levels are reduced in SDAT/AD subjects as compared to non-demented age-matched subjects [67]. These observations somewhat complement those that testosterone replacement therapy can have beneficial effects on cognitive function [68]. On the other hand, they counter those of a recent study that found testosterone levels to be elevated in SDAT/AD patients, although because of a skew in the respective populations compared, this report deserves cautious interpretation. Care must also be exercised in interpreting studies in which only total testosterone has been studied: testosterone can be 5á-reduced to the pure androgen dihydrotestosterone (DHT) or, in the brain, aromatized to estradiol, thus raising questions about the

nature of the active compound responsible for any beneficial effects observed. This issue is compounded by the recent report that SDAT/AD subjects show higher levels of estradiol and a lower free androgenization index (FAI) as compared to non-affected controls [69].

Besides the few above-mentioned studies in humans, Papasozomenos has addressed whether androgens are implicated in AD. In a rat model of stress (hyperthermia), testosterone was shown to prevent tau hyperphosphorylation by over-activation of GSK-3â (see above); interestingly, this effect was only seen with testosterone and not with its aromatised metabolite, 17â-estradiol [70].

Lastly, there is evidence from studies in cultured neurons that testosterone promotes the processing of APP via the non-amyloidogenic pathway, thereby down-regulating Aâ production [71]. Interestingly, however, the fact that these effects were abolished by an aromatase inhibitor strongly suggests that the anti-amyloidogenic effects of testosterone are in fact mediated by estradiol [72].

The terms *neurosteroid* and *neuroactive steroid* are often used interchangeably. Given that all the major steroids (primarily of peripheral origin) are neuroactive, the term neurosteroid should be reserved for those steroids that are synthesized in the brain independently of peripheral glandular sources. Several of these compounds have been shown to have anxiolytic properties [73] and have been suggested to be neuroprotective. Of particular relevance to this article, are reports that locally-synthesized neurosteroids can help maintain cognitive functions during aging [66]. In line with these observations, SDAT/AD patients tend to have lower brain concentrations of neurosteroids in different brain regions [74]. Interestingly, the neurosteroid *pregnenolone* (PREG) was shown to protect mouse hippocampal cells against Aâ-induced toxicity in a concentration-dependent manner [75], while another study indicated that PREG can alter the dynamics of the neuronal cytoskeleton by directly binding to microtubule-associated protein 2 [76]. Further, sulfated PREG proved to be a potent promnesic (memory enhancer) in rodents [77], probably by modulating NMDA-glutamatergic transmission, adrenergic á-1 receptor activity [78] and/or the central cholinergic system [79]. The latter effects have been ascribed to increased acetylcholine release within the cerebral cortex, amygdala and hippocampus following disinhibition of GABA actions on cholinergic-secreting neurons [80].

Neuroprotective roles have been ascribed to the neuroactive adrenalderived androgens, *dehydroepiandrosterone* (DHEA) and its sulfate (DHEAS). They have been shown to improve learning and memory in various animal models [80], and to attenuate the toxic effects of excitatory amino acids and Aâ [81, 82]; the latter effects are likely to result from the anti-oxidant properties of these steroids [83]. Other neurotropic effects of DHEA(S) have also been observed; these include the stimulation of neuronal differentiation

and survival in culture [84, 85]; the latter actions were ascribed to steroid modulation of NMDA receptor activity [85].

The secretion of DHEA(S) normally declines with age and it has been reported that plasma DHEA/DHEAS levels in male and female SDAT/AD patients are ca. 50% lower than in age- and gender-matched controls [86]. A number of other studies also propose that reduced DHEA production may be related to the pathogenesis of AD [87]. While adrenal production of DHEA(S) declines with age, the glucocorticosteroid (e.g. cortisol) response to stress tends to be exaggerated in at least some older persons, apparently making their neurons more vulnerable to the deleterious effects of glucocorticoids (see Section 2.2). Since many actions of DHEA(S) have been characterized as 'typically anti-glucocorticoid' [88], the reported beneficial effects of DHEA(S) might be explained by their anti-glucocorticoid-like properties. Here, it is also worth mentioning that both the *neurosteroid tetrahydroprogesterone* and the *neuroactive steroid tetrahydro-deoxycorticosterone* display strong anti-glucocorticoid-like actions, while at the same time exerting potent anxiolytic effects [89].

We previously mentioned the subject of *hormone-replacement therapy* (HRT). While no adverse effects of DHEA(S) or any of the other neuroactive/neurosteroid compounds mentioned above have been reported yet, we alluded that the issue of estrogen replacement therapy (ERT) in women, even if used only to primarily alleviate peri- and post-menopausal symptoms, is controversial. Such treatments reportedly preserve or improve cognitive function [90, 91], but the beneficial effects, especially in the context of AD, have been questioned recently [92, 64, 62-93]. As pointed out earlier, proponents of HRT [63, 94, 65] have noted potential sources for the discrepancies between the potent neuroprotective effect of sex steroids in animal models and the negative findings in humans. The role played by progesterone alone (or after its reduction to the neurosteroid tetrahydroprogesterone) has not received much attention despite the fact that decreased capacity of aged nervous tissue to metabolize progesterone may result in reduced 'availability' of neuroprotective steroids [95]. Another issue that is widely ignored is the possibility that steroid signaling may be impaired during advanced age either as a result of reduced receptor numbers or availability of essential receptor coactivators [96, 97] and growth factors [98]. Also to be considered is the fact that specific allelic variants or mutations of gonadal steroid hormone receptors may predispose certain individuals to neurodegenerative disorders; this is well exemplified by the finding that the incidence of a polymorphism in the estrogen receptor alpha gene is higher in women with dementia [99]. It remains to be seen whether this observation is related to the fact that SDAT/AD occurs more commonly in women than in men, even after correcting for the longer lifespan in women [100, 101, 102,

103]. Support for the importance of gender comes from studies in transgenic animals carrying the human APP*swe* and the PS 1-*A246E* mutations [104]. The female transgenics show significantly higher levels of $_{A\beta40}$ and $_{A\beta42}$ as compared to their male counterparts at all ages, indicating that sex itself, or exposure to sex hormones, renders females to a higher amyloid burden. Lastly, studies showing that females are exposed to higher levels of corticosteroids [105] seem to suggest that other hormones might also play a role in the development of SDAT/AD.

2.2. Adrenal (stress) hormones

Corticosteroid secretion (cortisol in humans and other large mammals; corticosterone in rodents) rapidly increases upon the arrival of any noxious stimulus *(stress)* in order to help the organism make physiological and behavioral adjustments that will help it cope with the insult [106]. Primarily, they act to divert energy supply to challenged tissues; among other functions, they control the excitability of neuronal networks that underlie learning and memory processes.

Corticosteroid actions result from the activation of either mineralocorticoid (MR) or glucocorticoid (GR) receptors, both of which act in the nucleus to regulate gene transcription. Whereas GR are expressed ubiquitously throughout the brain (and especially the hippocampus), MR expression is restricted to the hippocampus, hypothalamus and septum [107, 108]; in some cases, MR and GR may be co-localized in individual cells [109]. The glucocorticoids (corticosterone and cortisol) are the prototypic endogenous ligands of the GR, although they actually show a higher affinity for the MR [110]. This is interesting, because the prototypic MR agonist, aldosterone, is not produced in sufficiently high concentrations to reach the brain. Therefore, central MR and GR are both occupied by corticosterone (cortisol). These receptors not only mediate corticosteroid actions in the brain, they also play a crucial role in mediating glucocorticoid negative feedback on the hippocampus and hypothalamus as well as the pituitary, thereby curtailing the endocrine response to stress, thus facilitating the restoration of homeostasis [108, 110]. As will be discussed below, exaggerated hypercorticalism is associated with compromises in neuronal survival.

Corticosteroids have been implicated in mood and cognitive disorders, and their excess secretion has been correlated with hippocampal atrophy [111]. Recent studies have demonstrated them to play a complex role in the acquisition, retrieval and consolidation of new memories [112]. There appears to be an "inverted U-shaped relationship" between the occupation of brain receptors for corticosteroids and memory performance [113], and reversal of hypercortisolemic states has been shown to be followed by recovery of hippocampal atrophy and an improvement of cognitive functioning [114, 115,

116]. The hippocampus is important for certain forms of learning and memory. Under normal conditions, when MR are predominantly occupied, long-term potentiation (LTP), the electrophysiological correlate of memory formation, is facilitated in the hippocampus; in contrast, when GR are occupied, hippocampal LTP is inhibited and long-term depression (LTD) is induced [117]. It is also pertinent to note that MR have been associated with increased ability to interpret environmental information and the selection of the appropriate behavioral responses; although GR apparently interfere with information recall, they appear to be critical in consolidating newly-acquired information [for review see 112].

Numerous studies have shown that high levels of corticosteroids (GR-activating) can alter hippocampal structure by inducing dendritic atrophy and synaptic losses and, in extreme cases, cell death among certain hippocampal neuronal populations [118, 111], and in humans, a correlation between cortisol levels, impaired cognition and reduced hippocampal volumes has been described [119, 120]. These morphological changes are accompanied by marked cognitive impairments, and as mentioned earlier, are reversible after a period of recovery [114, 116]. Both stress- and corticosterone-induced atrophy can be blocked by drugs that reduce excitatory amino acid transmission, extracellular serotonin levels, or by agents that enhance the GABAergic tone; together, they indicate cross-talk between corticosteroids and various neurotransmitter systems in the determination of neuronal cell fate [121]. Besides themselves being able to induce hippocampal cell death [122], glucocorticoids appear to render neurons more vulnerable to a variety of neuro-endangering stimuli, including glutamate [123], free radicals and Aâ [124]. Lastly, there is now clear evidence that occupation of GR results in an inhibition of hippocampal granule cell proliferation; however, the significance of this for cognition and other behavioral and physiological functions remains to be determined [reviews by 125,126].

In contrast to the deleterious effects of corticosteroids (high GR occupation) on hippocampal structure, low levels of corticosteroid receptor occupancy appear to be essential to maintain the structural integrity of this brain region; accordingly, a neuroprotective role has been attributed to the MR [127, 111, 122]. Together with the foregoing information, it seems clear that corticosteroids have binary roles (through the balance of MR *vs.* GR occupation) in the regulation of the structural integrity of the hippocampus; the latter are presumed to be partly responsible for corticosteroid effects on cognitive behavior [117, 113, 119]. These observations form the rationale for the hypothesis that corticosteroids may play a role in the etiology and progress of AD.

There is some evidence that older individuals may have a compromised ability to cope with the allostatic load placed on the organism by stress – allostasis refers to the process of achieving stability through change. The

release of corticosteroids in response to stress is an adaptive process but if there is an over-compensation, damage to various tissues (including the brain) can ensue. Besides a reduced ability to respond appropriately to stress [106, 128], some aged subjects also show elevated basal levels of cortisol secretion and a flattening of the amplitude of the daily rhythm of cortisol production [129]. At least in aged animals, there is a marked down-regulation of MR and GR [97], findings which might explain trends towards higher basal and stressinduced corticosterone secretion [130]. Further, the downregulation of feedback efficacy means that a vicious circle is set in place insofar that the individual shows increasingly poor capacity to shut off its stress response over time [131]. Under these conditions, corticosteroid target cells and tissues are exposed to higher concentrations of the steroid for longer durations. The potential damage can be appreciated when one considers previous studies showing that aged animals are more susceptible to cell death when exposed to glucocorticoids [132].

Clinical studies suggest that elevated cortisol levels, or disturbances in adrenal function, in SDAT/AD patients correlate with hippocampal degeneration and memory impairments [133, 134]. Unfortunately, few researchers have addressed this question in animal models of AD. Although one study of an APP transgenic mouse strain reported elevated corticosterone secretion [135], it is not clear whether the hypercorticalism is causal to the deposits of Aâ, neuritic pathology and cognitive impairments seen in this model, or whether the increased adrenal activity was a consequence of the genetic manipulation, or simply an effect of some other secondary factor; interestingly, adrenocortical hyperactivity occurred much earlier in males than in females.

Recent studies in the authors' laboratory have begun to give credence to the view that corticosteroids might be causal to alterations in the synthesis/processing of APP and the phosphorylation patterns of tau. We have found that glucocorticoids, acting through the GR, can lead to the hyperphosphorylation of tau. The results on GR-regulated APP are more complicated; depending on the particular cell type tested and the amount of tau expressed, glucocorticoids increase or decrease APP synthesis and/or production. Therefore, although these studies are still in progress, their first results support our starting hypothesis - that corticosteroids have a role in the pathobiochemistry of AD.

3. Summary and conclusions

This brief article aims to provide the reader with a broad overview of the clinical symptoms found in AD, as well as current thoughts about pathobiochemical and genetic mechanisms that might contribute to the cause and progress of the disease. In particular, it attempts to provide a picture of

what is known about possible ways in which gonadal and adrenal steroids (as well as neurosteroids) might influence the initiation of AD. Several steroids appear to have the potential to protect neurons from age-related degeneration and dysfunction; since the production of most of these neurotropic steroids declines with age, it is plausible that they play a role in preventing or delaying AD pathology. On the other hand, as the organism ages, it becomes increasingly exposed to glucocorticoids which have the ability to induce dendritic atrophy and neuronal death in brain structures play key roles in cognitive function. As discussed in this review, several issues regarding the role of steroid hormones in AD remain controversial, albeit challenging. Therefore, rather than providing a 'prescription' for how to prevent or treat the disease, we prefer to conclude that further systematic research on the mechanisms through which steroids might contribute to the pathobiochemistry of AD is amply justified.

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