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AMYLOID PRECURSOR PROTEIN:

CELLULAR STUDIES AND ANIMAL MODELS

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I'm just trying to find a decent melody

A song that I can sing in my own company

Bono and The Edge

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by a number of neuropathological features, including extracellular deposits composed primarily of amyloid-β (Aβ) peptides. Aβ is derived from proteolysis of the amyloid precursor protein (APP) by consecutive action of β- and γ-secretases. APP is a ubiquitously expressed type I transmembrane protein with a large N-terminal extracellular domain, a single transmembrane span and a short C-terminal cytoplasmic tail. Its physiological function is still unknown, but the existing data suggest that APP may function by linking extracellular cues, such as ligand- or substratum-binding, to intracellular signalling pathways. Because the natural ligand of APP is unknown, several studies have used antibodies against different epitopes in APP extracellular domain to mimic the ligand-receptor interaction. In our study (*paper I*), using the antibody approach, we examined the role of APP in activation of gene transcription, and found that antibody-bound APP upregulates expression of ornithine decarboxylase (ODC), which is the initial and rate-controlling enzyme in polyamine biosynthesis. The induced ODC expression was rapid and biphasic, resembling growth-factor stimulated signalling events. This APP signalling did not require γ-secretase cleavage, as it was independent of the presence of presenilin-1 and -2. In ***paper II***, we investigated the localisation and levels of ODC protein in AD brain. Specifically, we examined the ODC immunoreactivity in three different brain regions; in hippocampus, frontal cortex and cerebellum; taken from control, possible and definite AD cases. Qualitative and quantitative analyses of these results demonstrated that ODC translocates in AD, from the nuclear compartment towards the cytoplasmic. Western blotting of frontal cortex homogenates showed that the levels of ODC in AD are increased. Both the translocation and change in ODC levels occur early in the disease process, i.e. in possible AD.

Animal models of AD are extremely valuable for the discovery and development of new treatments. Most of these models have been generated in mice. However, for decades the rat has been the preferred model for pharmacological and behavioural studies. In ***paper III and IV***, we report the establishment and characterization of transgenic rats expressing human APP695 with the Swedish double mutation (tgAPPswe). These rats were generated by pronuclear injection, using a construct that contained human APPswe cDNA driven by the ubiquitin promoter (***paper III***). The highest expression of the transgene was observed in cortex, hippocampus and cerebellum. Immunohistochemical examination of brain tissue revealed extracellular Aβ42 staining, either as cerebrovascular deposits or very rare diffuse plaques in the deep layers of the cortex, but the amyloid pathology was limited and occurred at a high age, above 15 months (***paper III***). Western blot analysis of hippocampal and cortical brain homogenates showed hyperphosphorylated tau, but no neuronal and synaptic loss (***paper IV***). We further characterised the tgAPPswe rats using behavioural tests, finding that these rats were more active (in the open-field) and showed impaired acquisition of learning (in the Morris water maze) when compared to the controls (***paper IV***). We also investigated the hippocampus and lateral ventricles of transgenic rats by *in vivo* MRI at the age of 16 months (***paper IV***). Both visual examination of the MR images and quantitative determination of the areas of these two structures suggest hippocampal atrophy and enlargement of lateral ventricles in transgenic rats compared to age-matched controls. We believe that the tgAPPswe rats represent a unique model of early AD.

LIST OF PUBLICATIONS

I. **Tatjana Nilsson**, Katarzyna Malkiewicz, Maria Gabrielsson, Ronnie Folkesson, Bengt Winblad, and Eirikur Benedikz

Antibody-bound amyloid precursor protein upregulates ornithine decarboxylase expression

Biochemical and Biophysical Research Communications (2006) **341**, 1294-1299

II. **Tatjana Nilsson**, Nenad Bogdanovic, Inga Volkman, Bengt Winblad, Ronnie Folkesson, and Eirikur Benedikz

Altered subcellular localization of ornithine decarboxylase in Alzheimer's disease brain

Biochemical and Biophysical Research Communications (2006) **344**, 640-646

III. Ronnie Folkesson, Katarzyna Malkiewicz, **Tatjana Nilsson**, Ewa Kloskowska, Elena Popova, Nenad Bogdanovic, Inga Volkman, Teit Johansen, Ursula Ganten, Detlev Ganten, Bengt Winblad, Michael Bader, and Eirikur Benedikz

Amyloid precursor protein transgenic rat model of Alzheimer's disease

Manuscript (2006)

IV. **Tatjana Nilsson**, Ewa Kloskowska, Johanna Öberg, Shunwei Zhu, Katarzyna Malkiewicz, Bengt Winblad, Ronnie Folkesson, Christian Spenger, Abdul Mohammed, and Eirikur Benedikz

Behavioural and brain changes in transgenic rats expressing human amyloid precursor protein with the Swedish mutation

Manuscript (2006)

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid-β peptide
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
APH-1	Anterior pharynx defective-1
APLP1	APP-like protein 1
APLP2	APP-like protein 2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APP-BP1	APP binding protein 1
APPswe	Mutant APP carrying the Swedish mutation
BACE 1	β-site APP cleaving enzyme 1
CDK 5	Cyclin-dependent kinase-5
FAD	Familial AD
GSK-3β	Glycogen synthase kinase-3β
HEK293	Human embryonic kidney 293
JIP-1	c-Jun NH2-terminal kinase-interacting protein-1
KPI	Kunitz-type protease inhibitor
NFT	Neurofibrillary tangles
ODC	Ornithine decarboxylase
PDGF	Platelet-derived growth factor
PEN-2	Presenilin enhancer-2
PI3-kinase	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PP-1	Protein phosphatase-1
PP-2A	Protein phosphatase-2A
PS1	Presenilin 1
PS2	Presenilin 2
siRNA	Small interfering RNA
tgAPPswe	Transgenic for APPswe
UbC	Ubiquitin

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 General introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, the fourth most common cause of death in western industrialised nations and, according to the World Health Organization, one of the major contributors to the global burden of disease. Currently over 24 million people worldwide are estimated to be demented and, with the expected increase of the elderly population over coming decades, this will rise to 81 million by 2040 (Miller et al., 2006). Thus AD is going to become an even bigger health problem, for which there is no definitive treatment or cure at the present time.

AD is a chronic progressive disease clinically characterised by memory loss and deficits in one or more of the following cognitive domains: aphasia (language disturbance), agnosia (failure to recognize people or objects in presence of intact sensory function), apraxia (inability to perform motor acts in presence of intact motor system), or executive function (plan, organize, sequence actions, or form abstractions). In AD patients these deficits are severe enough to interfere with daily life or work, and represent a significant decline from an earlier level of function (Brown et al., 2005).

The main neuropathological hallmarks of AD are intracellular neurofibrillary tangles (NFT) and extracellular amyloid-β (Aβ) deposits, found as senile plaques in the brain parenchyma and vascular amyloid in the walls of meningeal and cerebral blood vessels, the latter called cerebral amyloid angiopathy (Beher and Graham, 2005; Goedert et al., 1991).

1.1.2 Amyloid precursor protein: a key molecule in the pathogenesis of Alzheimer's disease

Despite a multitude of studies, the pathogenesis of AD is still unclear in many respects. Research conducted in the mid-1980s, showing that senile plaques in AD brain tissue are composed primarily of the sticky Aβ, later led to the proposal of the 'amyloid cascade hypothesis'. According to this hypothesis, the accumulation of Aβ is the primary event that leads to all subsequent events in the pathology of AD (Verdile et al.,

2004; Zimmermann et al., 2005). On the other hand, there is also evidence suggesting that Aβ deposition may be a consequence, rather than a causative factor. This has resulted in alternative hypotheses to explain the mechanism of the disease, such as abnormal tau phosphorylation and subsequent NFT formation (Verdile et al., 2004), cholinergic dysfunction (Oddo and LaFerla, 2006), oxidative stress (Harman, 2006), metal ion dysregulation (Maynard et al, 2005), inflammation (Sastre et al., 2006), vesicular transport deficits (Suzuki et al., 2006), and cell cycle abnormalities (Neve and McPhie, 2006). All these pathological events are associated with AD and most likely play a role in the disease process, but the production and cerebral deposition of Aβ is still considered, by most scientists, crucial to development of this disease.

The first gene identified in association with inherited susceptibility to AD was the amyloid precursor protein (APP) gene on chromosome 21. Initial studies of familial AD focused on this chromosome for two reasons: people with Down's syndrome, who have an extra copy of chromosome 21, invariably develop AD pathology by age 40; and Aβ peptide had been shown to be derived from APP (Goate, 2006; Spires and Hyman, 2005; Tanzi and Bertram, 2005). To date, a number of familial genetic mutations that result in early-onset AD have been found. In these families, mutations in either APP or presenilins, which are involved in APP processing, have in common altered Aβ production (Xie and Tanzi, 2006; Tanzi and Bertram, 2005).

1.1.3 Tangles and plaques

NFT are composed primarily of abnormally hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986). Tau is an axonal protein that binds to microtubules and promotes their assembly and stability. This is dependent on the phosphorylation state of tau, controlled by the balance between the activities of kinases (for example, GSK-3β and CDK 5) and phosphatases (for example, PP-1 and PP-2A) (Iqbal et al., 2005). When perturbation of these enzymes results in hyperphosphorylated tau, a pathological sequence of events takes place; sequestration of normal tau, disassembly of microtubules, impaired axonal transport, and compromised neuronal and synaptic function. In addition, hyperphosphorylated tau tends to aggregate into insoluble fibrils to form NFT and by that further endangers neuronal function (Blennow et al., 2006; Iqbal et al., 2005).

Plaques are not uniform in nature, and have been divided into two types: diffuse and neuritic. In diffuse plaques, the Aβ peptides are not in the β-pleated sheet conformation typical to amyloids of all compositions. Therefore, these plaques are also called 'pre-amyloid'. Neuritic plaques, also known as 'classical', have on the other hand a central amyloid core surrounded by a halo of dystrophic neurites (Morris and Nagy, 2004). Both NFTs and Aβ plaques can be seen in the cerebral cortex of cognitively normal aged individuals; but it is their number and widespread distribution, and the occurrence of neuritic plaques that distinguish the pathology of AD from the cognitive normal individuals (Boche et al., 2006).

Initially thought to be an abnormal peptide, Aβ is produced constitutively during normal cell metabolism. Brain Aβ is degraded by enzymes, such as insulin-degrading enzyme, neprilysin and endothelin-converting enzyme (Carson and Turner, 2002). Aβ is also cleared from the brain in a process balanced by the efflux and influx of this peptide across the blood-brain barrier. The Aβ efflux is mediated by low-density lipoprotein receptor-related protein 1, and the influx, by the receptor for advanced glycation end products (Blennow et al., 2006; Tanzi et al., 2004). Thus, under normal conditions, the steady-state level of Aβ is maintained by the balance of its synthesis and clearance. However, in AD, these processes appear to be perturbed. In familial AD (FAD; see 1.1.5 Genetics), the synthesis of Aβ is altered. Normally, there are two major forms of this peptide: the shorter Aβ40 accounting for ~ 90% of all Aβ released from cells; and the longer Aβ42 accounting for only ~ 10% (Verdile et al., 2004; Morgan et al., 2004). The majority of FAD mutations cause an increase of Aβ42 levels, increasing the Aβ42 to Aβ40 ratio (Sankaranarayanan, 2006; Xie and Tanzi, 2006; Beher and Graham, 2005). Aβ42 is more hydrophobic and more prone to aggregation than Aβ40. Interestingly, two very recent papers (Bentahir et al., 2006; Theuns et al., 2006) have reported that not only the increase in Aβ42- but also a reduction in Aβ40-production could be responsible for increase of the ratio. In sporadic AD, on the other hand, changes in production of Aβ have not been observed, and the onset of these forms of AD may be attributed to impaired clearance of the peptides (Higuchi et al., 2005).

1.1.4 Plaques versus tangles

The existence of two distinct pathological lesions in AD brains has led to a long-lasting debate over which is responsible for initiating the disease. NFT formation correlates better with cognitive decline (Turner et al., 2003), and therefore this lesion is believed to play a primary role in the disease process. On the other hand, there is also evidence suggesting that Aβ pathology occurs earlier than NFT and is upstream in the disease pathway (Corder et al., 2004). This has been studied experimentally in mice expressing mutant tau protein. These mice developed NFT pathology, but showed aggravation of this pathology after being injected intracerebrally with Aβ42 (Gotz et al., 2001). Double transgenic mice, expressing mutant tau and APP, displayed enhanced neurofibrillary degeneration, whereas amyloid plaques were similar in number and distribution to those of comparably aged single mutant APP mice (Lewis et al., 2001). Triple transgenic mice carrying mutations in tau, APP and PS genes, confirmed that Aβ deposits precedes the neuronal accumulation of hyperphosphorylated tau (Oddo et al., 2006) and showed that administration of Aβ specific antibodies reduced not only the Aβ pathology but also early forms of tau pathology. Thus, tau pathology is likely to be downstream of Aβ in the AD pathway.

1.1.5 Genetics

Genes play an essential role influencing the onset and modifying the progression of AD. Specifically, inheritance of AD shows an age-related dichotomy in which rare but highly penetrant mutations transmitted in an autosomal dominant manner are responsible for early (≤ 60 years) onset FAD, while common polymorphisms with low penetrance, such as apolipoprotein E (APOE) ε4 allele, appear to have their greatest effect on the more frequent, later onset forms of the disease (Bertram and Tanzi, 2004).

Mutations in three genes, APP on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1, are responsible for up to 50 % of early onset cases of FAD (Xie and Tanzi, 2006). Presenilins form the active site of the γ-secretase complex involved in the production of Aβ (see 1.2.2 Processing). To date, 18 different pathogenic mutations have been described in the APP gene, and all of these are missense mutations lying within or close to the domain encoding the Aβ peptide. 10 mutations are identified in the PS2 gene, but the vast majority of FAD mutations (over

100) reside in the PS1 gene (Xie and Tanzi, 2006). As discussed above, the FAD mutations result in increased Aβ42 to Aβ40 ratio.

Inheritance of the APOE ε4 allele is a major risk factor for late onset AD. The APOE gene on chromosome 19 exists in three isoforms: ε2, ε3, and ε4. In the normal population, the ε3 allele is the most common followed by the ε4 allele (Sjogren et al., 2006). The presence of the ε4 allele is neither necessary nor sufficient to actually cause the disease. Rather, it appears to be a genetic risk-modifier or susceptibility factor, that predominantly acts through decreasing the age of onset in a dose-dependent manner (Bertram and Tanzi, 2004; Tanzi and Bertram, 2005). The ε4-allele increases the risk of the disease by three times in heterozygotes and by 15 times in homozygotes, and each allele copy lowers the age of onset by almost 10 years (Sjogren et al., 2006; Blennow et al., 2006; Bertram and Tanzi, 2004; Tanzi and Bertram, 2005). The mechanism behind the contribution of ε4 allele to the increased risk of AD is currently unknown. However, the link between the protein encoded by ε4 allele and AD is probably related to the ability of this protein to interact with the Aβ peptide and influence its clearance, aggregation, or conformation (Holtzman, 2004).

1.2 AMYLOID PRECURSOR PROTEIN

1.2.1 Expression

APP is expressed in all mammalian cells and shows high degree of evolutionary conservation. It belongs to a small family of proteins that also includes APP-like proteins 1 and 2 (APLP1 and APLP2). APP is a type I transmembrane protein; it exists in at least three isoforms that arise as a result of variable exon splicing of a single gene. APP770 and APP751, containing a Kunitz-type protease inhibitor (KPI) domain, are predominantly expressed in peripheral tissues. The shorter APP695 has no KPI domain and represents the main isoform found in brain tissue (Morris and Nagy, 2004).

1.2.2 Processing

APP can be processed by one of two pathways: the amyloidogenic that leads to Aβ production and involves the β- and γ-secretases; and the nonamyloidogenic that involves the α- and γ-secretases (Figure 1). In the amyloidogenic pathway, β-secretase cleaves APP at the N-terminal of the Aβ domain, releasing the soluble sAPPβ fragment

(Russo et al., 2005). The residual membrane attached C-terminal fragment C99 (99 amino acid residues) is subsequently cleaved by γ-secretase. Interestingly, proteolytic processing by this enzyme seems to involve a dual cleavage; one occurring approximately in the middle of the transmembrane domain of the substrate (γ-cleavage), in this case cleavage of C99 to release the Aβ peptide. The other cleavage event, ε-cleavage, takes place close to the cytoplasmic surface of the membrane and liberates the cytoplasmic APP intracellular domain (AICD) (Vardy et al., 2005; Beher and Graham, 2005; Zimmermann et al., 2005). The sequence of these proteolytic actions is unknown, although it seems that ε-cleavage may be the initial event (Reinhard et al., 2005; Beher and Graham, 2005).
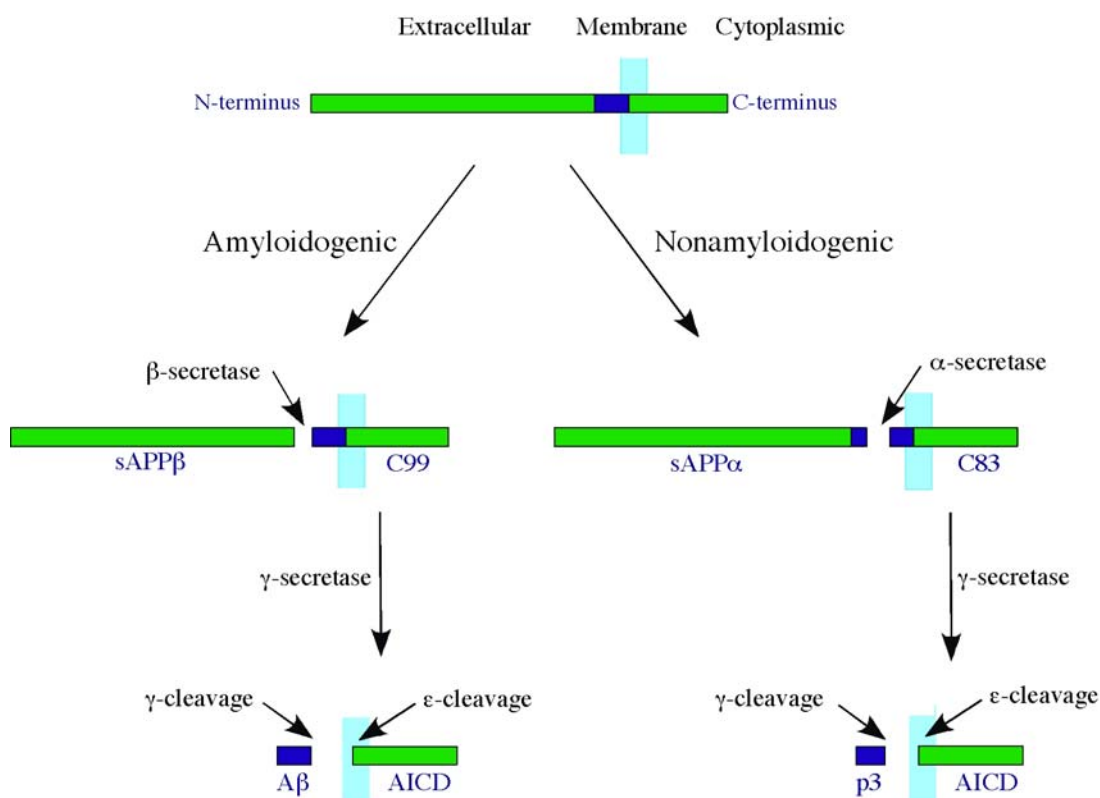

Figure 1. Schematic illustration of the amyloidogenic and nonamyloidogenic APP processing pathways.

In the nonamyloidogenic pathway, α-secretase cleaves the APP within the Aβ domain, releasing the soluble sAPPα fragment. The remaining membrane-bound C83 undergoes subsequent cleavage, probably first at the 'ε-site', liberating the AICD followed by γ-

cleavage to yield the short p3 peptide (Beher and Graham, 2005; Reinhard et al., 2005; Zimmermann et al., 2005).

α-secretase activity is mediated by two proteases, ADAM 10 and ADAM 17 (also known as tumour necrosis factor-α converting enzyme or TACE), which are members of the ADAM (a disintegrin and metalloprotease) family. Most β-secretase activity originates from an aspartic protease called β-site APP cleaving enzyme 1 (BACE 1). γ-secretase is a protease complex, consisting of four components: presenilin, nicastrin, PEN-2, and APH-1, with presenilin constituting the active site (Vardy et al., 2005; Verdile et al., 2004; Zimmermann et al., 2005).

1.2.3 Domain structure

APP is a membrane protein with a large N-terminal extracellular domain, a single transmembrane span and a short C-terminal cytoplasmic tail. Most of the extracellular domain has been structurally characterised and can be regarded as a sequence of several individual domains or 'folding units' (Figure 2). Even though a complete crystal structure of APP is required to see to what extent the conformation of individual folding units is conserved in the context of the whole protein (Reinhard et al., 2005), knowledge about these structures helps to understand the function of this protein. For example, the most N-terminal domain, growth factor-like domain (GFLD), contains a hydrophobic surface patch that is conserved within the whole APP family.
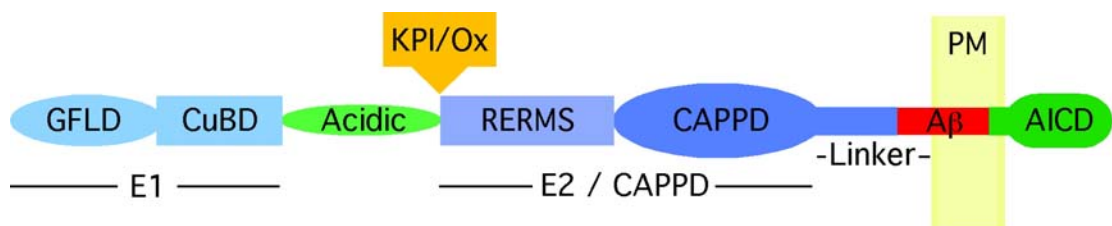
Figure 2. Domain organisation of APP. The N-terminal E1 consists of the growth factor-like domain (GFLD) and the copper-binding domain (CuBD). The acidic region links E1 to E2, the latter also called central APP domain (CAPPD). This is followed by the linker, the transmembrane domain and the APP intracellular domain (AICD). Aβ is shown in red. The KPI domain, which is present in APP751 and APP770, and the Ox2 sequence, which is present in APP770, are shown above their insertion site.

Considering that such patches are known to be the key players in the protein-protein interactions, it is suggested that this hydrophobic surface may contribute to the binding of APP to its extracellular partners, ligand or substratum, or it may provide an APP-APP dimerisation interface (Rossjohn et al., 1999; Reinhard et al., 2005). Moreover, structural similarities with cysteine-rich growth factors suggest that GFLD itself may be responsible for the growth factor-like properties of APP. This domain has also been shown to contain one of the APP-heparin binding sites (Rossjohn et al., 1999; Reinhard et al., 2005).

The following copper-binding domain (CuBD) contains a copper binding site that favours Cu(I) ions (Barnham et al., 2003). It is believed that the CuBD binds first to Cu(II) and then facilitates the reduction of these ions to Cu(I). This domain has structural homology to copper chaperones, and is proposed to act also as a Cu(I) binding neuronal metallochaperone. The suggested function is in accordance with the findings that APP knockout mice have elevated copper levels in the cerebral cortex, whereas APP overexpressing transgenic mice have reduced brain copper levels. Importantly, copper binding to APP was shown to greatly reduce $A\beta$ production in vitro (Barnham et al., 2003).

The subsequent acidic region and the membrane-neighbouring linker region are not believed to participate in any standard secondary structures and thus are 'flexible linkers' that connect the individual folding units (Reinhard et al., 2005). The acidic region is followed by the E2 domain, also called central APP domain (CAPPD). This domain comprises additional heparin-binding sites, but also the well-studied RERMS sequence, implicated in memory and cognition (Turner et al., 2003), and growth-promoting properties of APP (Reinhard et al., 2005).

To approximate the physiological structure of $A\beta$ and avoid its aggregation, most of the structural studies on this peptide have been performed in membrane-mimicking environments. Surprisingly, $A\beta40$ and 42 peptides appear to consist of two α-helices linked via a 'kink' or a loop. In amyloid fibrils, however, the $A\beta$ peptide shows a wide content of β-sheet, but the triggers of the conformational switch from α-helical to β-sheet structures are still not fully understood (Reinhard et al., 2005; Morgan et al., 2004).

Structural analysis of AICD shows that this domain adopts no stable conformation in solution. Several nascent secondary structures are observed over a broad pH range, but no tertiary contacts, which may describe an early state in the protein folding process. Interaction with another protein could promote the further protein folding of AICD, and stabilize the resulting structure as well. Considering that AICD interacts with several proteins (for example, the members of the X11 and Fe65 families), the structure of AICD could be different depending on the binding partner (Reinhard et al., 2005; Ramelot et al., 2000).

1.2.4 Intracellular binding partners

A number of proteins that interact with the short cytoplasmic APP domain have been described to date, including Go, APP binding protein 1 (APP-BP1), members of the X11 and Fe65 families, and c-Jun NH2-terminal kinase-interacting protein-1 (JIP-1) (Russo et al., 1998; Miller et al., 2006). Go binds to amino acid residues 657-676 (numbering for the APP695 isoform); no data are available on the APP cytoplasmic residues involved in APP-BP1 interaction (Russo et al., 1998); but most of the other binding partners interact with the YENPTY motif (residues 682-687), which is completely conserved from *C.elegans* to humans (Reinhard et al., 2005; Miller et al., 2006).

The heterotrimeric G protein Go was shown to be activated by APP when antibodies to the APP extracellular domain were employed to mimic the binding of the unidentified ligand. These data were the first to support the receptor function of APP, anticipated from the structure. Following research suggested that Go-mediated APP signalling causes apoptosis in neurones. Transfection of neurones with APP695 containing FAD linked substitutions at valine 642, all resulted in constitutive activation of Go and subsequent apoptosis. Together, these findings suggest that Go may function as a transducer of APP-induced cell death signals (Neve and McPhie, 2006; Turner et al., 2003).

APP-BP1 is a cell cycle protein, overexpression of which in dividing cells drives the cell cycle through the S-M checkpoint. In primary neurones, on the other hand, the overexpression causes DNA synthesis and apoptosis (Chen et al., 2000; Chen et al., 2003). It has been proposed that interaction of APP-BP1 with APP is important for

normal brain function, but regulation of this interaction may be perturbed in AD, resulting in cell cycle regulatory failure and consequent apoptosis in neurones.

When bound to AICD, Fe65 has been shown to recruit one more protein, the histone acetyl transferase Tip 60, and as a tripartite complex function in gene transactivation (Cao and Sudhof, 2001). The genes regulated by Fe65 signalling are not properly known, but reported targets include proteins that are associated with AD pathogenesis, such as APP itself, BACE1, GSK-3β, neprilysin, and Tip60 (Miller et al., 2006). Interestingly, targeted deletion of Fe65 and Fe65 Like 1, another member of the Fe65 family, produce phenotypes similar to those observed in APP/APLP1/APLP2 triple knockout mice (see 1.2.5 Function), further supporting that APP is a component of signal transduction pathways (Suzuki et al., 2006).

In neurones, two X11 proteins are found, X11α and X11β, and they appear to regulate APP metabolism. Recent studies have shown that APP-X11 complexes interact with a type I membrane protein named Alcadein, and the resulting tripartite complexes are metabolically stable. On the other hand, naked APP is quickly processed (Suzuki et al., 2006). X11 transgenic mice, when crossed with mice expressing APP with the Swedish mutation (see 1.2.6 Transgenic animals), yield offspring showing reduced Aβ40 and Aβ42 levels, and a reduction in the number of amyloid plaques when compared with single transgenic mice expressing only the mutant APP (Miller et al., 2006; Suzuki et al., 2006).

JIP-1 mediates the binding of APP to the kinesin I light chain, and is thought to be the most important adaptor protein in APP vesicular transport (Suzuki et al., 2006).

The interaction of APP with some of the intracellular partners appears to be influenced by its phosphorylation state. Namely, the recently identified APP binding proteins Shc A and C, and Grb2, that are thought to activate MAPK pathways (Russo et al., 2005), require the specific phosphorylation of tyrosine 682 of the YENPTY motif (Xie and Tanzi, 2006; Russo et al., 2005). The Fe65, X11, and JIP-1 proteins, on the other hand, interact with this motif but in a phosphorylation-independent manner (Suzuki et al., 2006). However, another phosphorylation event in the nearly situated VTPEER, i.e. the phosphorylation of threonine 668, was shown to influence the interactions of APP with

Fe65 and JIP-1. For example, it promotes the interaction with JIP-1 protein (Muresan and Muresan, 2005), but suppresses the binding of Fe65 by inducing a conformational change in the APP cytoplasmic domain (Suzuki et al., 2006). At the same time, this phosphorylation has little or no impact on the interaction of APP with X11 (Reinhard et al., 2005). All these data suggest that phosphorylation events most likely control the functions of APP by regulating its affinity for distinct binding partners.
.

1.2.5 Function

The physiological function of APP is still unknown. APP is a 'resource' rich protein: two proteolytic processing pathways, generating at least five different fragments; a large extracellular sequence, consisting of several individual domains with different properties; AICD, interacting with several proteins and probably adopting several different conformations. The extensive research performed on APP to date has given a broad and relatively diverse list of potential functions, for example: regulator of neurite outgrowth, dendritic arborisation, synaptogenesis and synaptic plasticity; adhesion cell-cell and cell-substratum molecule; surface receptor and activator of gene transcription; axonal transport cargo receptor; and regulator of cell survival (Turner et al., 2003; Reinhard et al., 2005).

Biological models deficient for APP have also provided some insights into the function of this protein. APP knockout mice are viable and initially appeared normal, probably due to the functional compensation by APLP1 and APLP2. Triple knockout mice deficient for both APP and APLPs, however, die prematurely. At the morphological level, these mice show a high incidence of cortical dysplasias, characterised by a fragmented basal lamina and 'overmigration' of neurones, which emphasises the importance of the APP family in neuronal cell adhesion and migration (Reinhard et al., 2005; Herms et al., 2004).

Additional research is required to better understand the role of this enigmatic protein in the whole organism and in particular in the brain. Nevertheless, the existing data suggest a model in which APP may function at both sides of the plasma membrane, linking extracellular cues, such as ligand- or substratum-binding, to intracellular signalling pathways (Reinhard et al., 2005).

1.2.6 Transgenic animals

Numerous laboratories have used transgenic approaches to examine the *in vivo* consequences of expressing the mutant APP. Mice are the most commonly used AD model organisms. Since people with Down's syndrome develop neuropathological characteristics of AD early in life and they have an extra copy of the APP gene, many groups worked under the theory that overexpression of wild type (wt) human APP might result in amyloid pathology similar to that seen in AD (Spires and Hyman, 2005). For example, in 1991, the NSEAPP mice expressing wt human APP751 under control of the neuron specific enolase (NSE) promoter were generated. Even though diffuse forms of amyloid plaques increasing in number with age were observed in these mice, mature dense core plaques were rarely seen (Sankaranarayanan, 2006). To date, many mice models carrying single or multiple mutations in APP and/or presenilin have been created, but none of them recapitulate completely the AD process (Sankaranarayanan, 2006; Spires and Hyman, 2005; McGowan et al., 2006). All of the single APP transgenic (see Box 1) and APP/presenilin double transgenic mice models have in common the presence of amyloid deposits that progress with age and the absence of NFT. Expression of AD-linked presenilin variants alone on the other hand is not sufficient to induce amyloid pathology in mice. Synaptic and neuronal loss, which is another prominent feature of AD neuropathology, differs considerably between various APP and APP/PS models. Behavioural testing has also exposed varying degree of memory deficits (Sankaranarayanan, 2006; Spires and Hyman, 2005, McGowan et al., 2006).

Inability to produce a mouse model that recapitulates all the aspects of AD probably reflects the limitations of using a rodent system to reproduce a human disease that lasts several decades and involves higher cognitive function (McGowan et al., 2006). However, compared to mice, rats are considered the animal of choice in memory and neurobehavioural testing. Its size, pharmacokinetics and accessibility for brain imaging are additional advantages of this model.

To date, only three APP or APP/PS1 transgenic rat models have been reported. In 2004, one group described a transgenic rat model that expresses human APP carrying the Swedish K670N/M671L mutation (APPswe) under the control of the platelet derived growth factor (PDGF) promoter (Ruiz-Opazo et al., 2004). These rats exhibit: low-levels of APPswe expression, and no extracellular Aβ deposits up to 18 months of age.

Unexpectedly, the cognitive performance of these rats was significantly better than age-matched 6- and 12-months old controls.

Box 1. APP transgenic mouse models (reviewed in McGowan et al., 2006; Sankaranarayanan, 2006)

PDAPP: First mutant APP transgenic model with robust plaque pathology. Mice express human APP cDNA with the Indiana V717F mutation, under control of the platelet derived growth factor (PDGF) promoter. Plaque pathology begins between 6-8 months in homozygous animals. There is a synapse loss but no evident cell loss. PDAPP mice show memory impairments that increase with age. This model has been used widely in vaccination therapy strategies.

Tg2576: Mice express APP695 with the Swedish K670N/M671L mutation under control of the hamster prion promoter. Plaque pathology is observed from 9 months of age. These mice have age-related memory deficits, but no synaptic or cell loss. It is one of the most widely used transgenic models.

APP23: Mice express APP751 with the Swedish mutation, under control of the mouse Thy-1 promoter. These mice develop both amyloid plaques and cerebral amyloid angiopathy starting at around 6 months of age. Neuronal loss was detected in the hippocampus, but no in the cortex. APP23 mice develop memory deficits.

APP/Ld/2: Mice carry the London V717I mutation in APP695, under control of the Thy-1 promoter. Plaque pathology starts at 12 months of age. Behavioural deficits from 3 months.

TgCRND8: Mice express multiple APP mutations (Swedish plus Indiana) in APP695, under control of the hamster prion promoter. Memory deficits coincide with rapid extracellular plaque development at ∼ 3 months of age. The cognitive deficits can be reversed by Aβ vaccination therapy.

J20: Mice express both the Swedish and Indiana mutations in APP-751, under control of the PDGF promoter. Age-dependent increase in Aβ42 production, decrease in synaptophysin immunoreactivity proportional to Aβ42 levels, and amyloid deposition at 5-7 months of age were important findings in these mice.

APP (Swedish & London): Mice express both the Swedish and London mutation in APP751 under control of the murine Thy-1 promoter. Plaque pathology develops after 3-5 months of age. No cognitive deficits are observed in these mice.

The same year, a series of papers on a double-transgenic APP/PS1 rat model were published (Vercauteren et al., 2004; Lopez et al., 2004, Echeverria et al., 2004a and 2004b). These rats express APP containing the Swedish and Indiana mutations and a FAD-linked PS1 mutation. No extracellular amyloid was seen in these animals up to 24 months of age. The main pathological feature, visible after 6 months, was the accumulation of Aβ intracellularly in neurones of the hippocampus and cortex. The reported memory impairment investigated in the water maze was mild, but significant. At the Society for Neuroscience meeting in 2003, a multi-transgenic rat model was reported (Flood et al., 2003), expressing two mutated APP constructs and one mutated PS1. According to the abstract, triple transgenic homozygous animals had onset of amyloid deposition at the age of 7 months and the deposits appeared similar to that seen in AD mice models. No behavioural studies were reported and unfortunately this work has still not been published.

2   AIMS OF THE STUDY

APP is a key molecule in AD pathogenesis – understanding the role of this protein will be a large step forward in understanding the pathogenesis of the disease. The general aim of the work presented here was to better understand the function of this intriguing protein. Cell cultures were utilised to study the function of 'normal' APP, specifically to examine the putative receptor and activator of gene transcription functions. Finding that APP upregulates ODC expression created the next aim, i.e. to gain an insight into the role of ODC in AD by examining its localisation and levels in AD and control brains. To study the consequences of expression of 'mutant' APP, we established transgenic rats carrying APP with the Swedish mutation. Specifically, the aims of the animal studies were to characterise these rats by examining the expression of the transgene, neuropathology, behavioural deficits, and MRI changes in hippocampus and lateral ventricles, and by that investigate whether these rats can be used as a model of AD.

3 RESULTS AND DISCUSSION

3.1 ANTIBODY-BOUND AMYLOID PRECURSOR PROTEIN UPREGULATES ORNITHINE DECARBOXYLASE EXPRESSION

APP appears to function as a cell surface receptor involved in regulation of gene transcription (Cao and Sudhof, 2001; Baek et al, 2002). Because the natural ligand of APP remains unknown, several studies have used antibodies against different epitopes in the extracellular domain of this protein to mimic ligand-receptor interaction (Kawasumi et al, 2002; Mbebi et al, 2005). In this study, using the antibody approach, we examined the role of APP in activation of gene transcription.

For this purpose we used AP-180, an antibody raised against residues 46-61 in the N-terminal domain of APP. This affinity purified antibody, generated in our laboratory, specifically stains APP on Western blots similarly to commercially available APP antibodies. The effects of AP-180 treatments on mRNA expression were investigated in HEK293 cells using Human signal transduction pathway finder gene arrays (GEArray Q Series; Super Array, Inc.). Among the 96 genes included, the largest change was observed for ornithine decarboxylase (ODC), the initial and rate-controlling enzyme in polyamine biosynthesis (Schipper and Verhofstad, 2002). To further examine these results, we analysed the levels of ODC protein in AP-180 treated cells by Western blotting, which showed an even larger upregulation of this protein in the treated cells (Figure 3). Basal levels of ODC, on the other hand, were difficult to detect by Western blotting and could only be seen when the gels were loaded with very high amounts of the total protein.
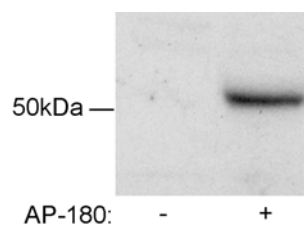

Figure 3. Treatment of HEK293 cells with AP-180 antibody induces ODC expression.

We confirmed our findings by showing that ODC protein is also upregulated in human neuroblastoma SH-SY5Y cells and primary neurones, cortical and hippocampal, when treated with AP-180. These results were also supported by the findings that a commercially available Anti-APP (Upstate), an antibody raised against the same amino acids' sequence as AP-180, also induces ODC expression. On the other hand, MAB348 (clone 22C11 formerly sold by Roche), the first antibody used in APP ligand-mimicking experiments in which it has shown to be neurotoxic, did not influence ODC expression, evaluated both by microarrays and Western blotting. We also tested MAB348 for neuronal toxicity without success. We have since learned that others that have published on neurotoxicity of antibody clone 22C11 have been unable to reproduce those results with recent batches of MAB348 [www.alzforum.org/ des_antibodies.asp (posted 21 January 2005)].

To show that ODC upregulation is induced by the AP-180 antibody, we ran a number of different control experiments. For example, we preincubated the antibody with either 10 or 100 µg/ml of the synthetic APP_{46-61} peptide containing the epitope for AP-180, before treating the cells. Higher peptide amounts led to lower ODC levels, proving that AP-180 is the trigger for ODC upregulation.

To demonstrate that the AP-180 triggered signalling event is mediated by APP, we investigated whether the amount of cellular APP influences the expression of ODC. We first compared APP overexpressing and control cells, and conclude that the more APP in the cells, the more ODC expressed. We also performed siRNA experiments to knockdown the endogenous APP in the cells, and then treated these cells with AP-180. The APP suppression by siRNA significantly reduced the antibody induced ODC expression, and showed again that upregulation of this protein, in our study, is mediated by APP.

The ODC expression is a rapid event, namely AP-180 treatment for only 30 min results in a large accumulation of ODC protein. In the continuous presence of AP-180 during a 24 h – period, the expression pattern of ODC is biphasic. This is an interesting finding as many signalling systems (e.g. Ras, PI3-kinase, PKC) in cells treated continuously with growth factors show two-waves of activity during a cell-cycle period (Jones and Kazlauskas, 2001). Since alteration in ODC activity seems to be accompanied by roughly equivalent changes in the amount of this enzyme (Isomaa et al., 1983; Laitinen

et al., 1984), the biphasic upregulation of ODC in our experiments may signify biphasic enzyme activity.

It has been suggested that APP signalling occurs by a mechanism similar to that of the Notch receptor, in which the intracellular tail released by γ-secretase translocates to the nucleus and activates transcription (Cao and Sudhof, 2001, Hass and Yankner, 2005). We investigated whether AICD is involved in the AP-180 induced signalling pathway by use of a cell-based quantitative reporter gene assay that monitors the γ-secretase cleavage of APP (Karlstrom et al., 2002). Unexpectedly, this assay showed that antibody binding reduced the processing of APP. This is in accordance with a very recently described APP signalling mechanism that does not require γ-secretase cleavage, but involves recruitment of Tip 60 by holo-APP to the membrane (Hass and Yankner, 2005). We also examined whether AP-180 triggered ODC expression is dependent on presenilin by use of BD8 cells that are deficient for both PS1 and 2. We found that absence of the presenilin did not block the induced ODC expression, demonstrating that APP can signal to the nucleus in presenilin-independent mode of action.

3.2   ALTERED SUBCELLULAR LOCALISATION OF ORNITHINE DECARBOXYLASE IN ALZHEIMER'S DISEASE BRAIN

In *paper I* we showed that antibody-bound APP upregulates expression of ODC. The precise role of ODC in central nervous system is unknown. However, this enzyme is very sensitive to a variety of harmful stimuli: physical, thermal, chemical and metabolic stress - all induce increase in ODC activity (Kauppinen and Alhonen, 1995; Johnson, 1998; Bernstein and Muller, 1999; Seiler, 2000). It is still debated whether this upregulation is neuroprotective or contributes to neuropathology, but a growing number of studies support the view that increased ODC activity and polyamine levels are part of a reparative response to support regeneration of damaged neurones (Frolich et al., 1998; Khan et al., 1992; Ha et al., 1998; Lukkarinen et al., 1999; Lukkarinen et al., 1998; Reeben et al., 1996). There are only a few reports on ODC in AD and they suggest increase of its activity (Morrison et al., 1998) and elevated levels (Bernstein and Muller, 1995) in the cortex of AD patients. The latter was determined by immunohistochemical examination of primary cortical areas in definite AD cases. We

performed Western blotting on frontal cortex homogenates from both possible and definite AD brains. Our results support the increase of ODC levels, but also suggest that this increase occurs earlier in the disease, i.e. in possible AD.

Insight into the cellular and subcellular localisation of ODC might provide valuable information on its role in AD. Since there is a characteristic, hierarchical pattern of distribution and progression of pathology in this disease, we examined ODC immunoreactivity in three different brain regions; hippocampus, a region with early and the most severe changes in the disease; frontal cortex that is affected in the later stages; and cerebellum where neurofibrillary pathology and neuritic plaques are not found. Specifically, ODC immunostaining was analysed in the anterior hippocampus, frontal associative cortex [Brodmann Area (BA) 9/46], and anterior lobulus of neocerebellum, taken from control, possible and definite AD brains.

In the control cases, ODC was predominantly localised in the nuclear compartments of the cells. The most pronounced ODC immunoreactivity was found in pyramidal cells of hippocampus and frontal cortex, whereas in cerebellum, only a minor portion of Purkinje cells were stained. No ODC immunoreactivity was seen in glial cells.

In the possible AD brains, ODC staining was shifted to the cytoplasm but still found in the nucleus. In hippocampus, pyramidal cells of CA1 were the most immunoreactive; and in cortex, pyramidal cells in layers III and V. Double staining with ODC- and AT8-antibodies, the latter recognizing abnormally phosphorylated tau protein (tau-P) and hence tangles, was also performed. This showed colocalisation of the two proteins in the cytoplasmic compartments of hippocampal and cortical pyramidal cells. ODC seems to appear in the cytoplasm before tangle formations, i.e. in hippocampus this protein was seen in pyramidal cells without any obvious pathology as well as in cells stained for tau-P. In frontal cortex, the pyramidal cells of both layers III and V were stained for ODC, whereas tau-P staining began to appear only in cells of layer V, which is hierarchically the first to show tangle pathology (Bussiere et al., 2003a; Bussiere et al., 2003b). It should also be noted that in this stage of AD, the pyramidal cells of the hippocampus qualitatively exhibit much more intensive cytoplasmic staining for ODC than cortical pyramidal cells. This may reflect the neuropathological staging where the hippocampus is affected prior to cortical regions. In cerebellum of possible AD brains,

the number of ODC stained Purkinje cells increased significantly and the staining was found in both the cytoplasm and nucleus.

In the definite AD brains, ODC immunoreactivity was preferentially found in the cytoplasmic compartments of pyramidal (hippocampal and cortical) and Purkinje cells. In double stained specimens, ODC was found in almost all AT8 positive pyramidal cells. However, the more affected the neurones are and therefore more heavily stained by AT8 antibody, the less staining for ODC they display as evaluated by qualitative analysis. This phenomenon could be seen in both cortex and hippocampus.

In frontal cortex of definite AD brains, besides the neuronal loss and huge amount of silver-stained tangles and neuritic plaques, there was also a population of ODC-positive astrocytic cells, found especially around neuritic plaques. These findings are in agreement with the earlier reports, showing that ODC is generally localized in neurones, but strong stimulation (e.g. kindling, epileptic seizures, and lesions) leads to long lasting expression of ODC immunoreactivity in astroglia (Bernstein and Muller, 1999; Bernstein and Muller, 1995). In hippocampus and cerebellum of definite AD, staining of astrocytic cells was not observed. These regional differences probably reflect the fact that fewer neuritic plaques are found in hippocampus and none in cerebellum; therefore very few or no active astrocytes are found in those regions, respectively.

We also performed a quantitative analysis of ODC immunoreactivity, confirming the qualitative results. Thus, these results together suggest an early shift of ODC protein in AD from the nuclear compartments toward the cytoplasmic. Interestingly, ODC *(paper I)* is not the only protein linked to APP that is shifted to the cytoplasm in AD. NEDD8, an ubiquitin-like protein activated by APP-BP1, is also translocated towards the cytoplasm in hippocampal neurones in AD brains (Chen et al., 2003).

3.3 AMYLOID PRECURSOR PROTEIN TRANSGENIC RAT MODEL OF ALZHEIMER'S DISEASE

AD animal models are extremely valuable for the discovery and development of new treatments. Most of the transgenic models of AD have been generated in mice, and these recapitulate some, but not all, of the neuropathological features of this human disease (Folkesson et al., 2002; Spires and Hyman, 2005). However, for decades the rat has been the preferred model for pharmacological and behavioural studies (Wells and Carter, 2001; Cenci et al., 2002). Rat models of AD should enable a more sophisticated characterization of behavioural phenotype, and perhaps afford better resolution of the relationship between pathology and behaviour, or treatment effects on behaviour (Higgins and Jacobsen, 2003). Here we report the establishment and characterization of transgenic rats expressing human APP695 with the Swedish double mutation (tgAPPswe).

The tgAPPswe rats were generated by pronuclear injection, using a construct that contained human APPswe cDNA driven by the ubiquitin (UbC) promoter. PCR analysis of the genomic DNA, extracted from the tail, identified five transgenic lines that show presence of the UbC promoter and human APPswe transgene. Of these, one turned out to be infertile, most likely due to the insertion site of the transgene, another did not show germline transmission of the construct, and the remaining three lines were investigated further. RT-PCR analysis of brain samples revealed the expression of APPswe mRNA in two of these lines. This was confirmed by Western blotting, which also showed that the two rat-lines, named tg6601 and tg6590, express APPswe protein in the brain but at different levels (Figure 4).
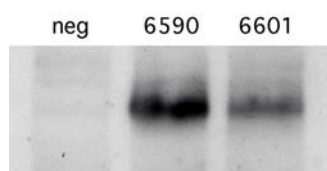

Figure 4. Analysis of transgene expression. Western blot of brain homogenates from transgenic rats using human APP specific antibody 6E10, showing different expression levels in the two lines.

Previous experience with APP transgenic mice suggests that high expression levels of APP are required for full fledge plaque formation. Western blotting analysis of APP in brain homogenates from tgAPPswe and control animals demonstrated that this protein is overexpressed, even in heterozygous transgenic animals. Interestingly, despite using the UbC promoter, that might be expected to give uniform APPswe expression in different brain regions, we find that the levels of the transgene vary considerably, being the highest in cortex, hippocampus and cerebellum (Figure 5).
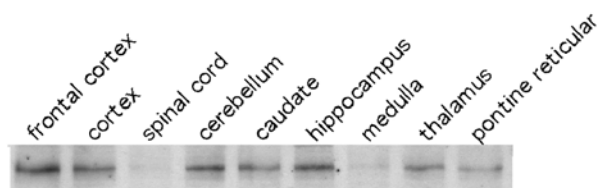

Figure 5. A representative Western blot showing human APP expression in different regions of the brain from a transgenic rat.

APP can be cleaved by either α- or β-secretase, resulting in a membrane bound C-terminal APP fragment (CTF) of 12 or 14 kDa respectively. We analysed this processing event in different brain regions from the tgAPPswe rats. Using a C-terminal specific antibody that recognises both the α- and β-cleaved CTFs, it was shown that the caudate-putamen displays the highest levels of α-cleaved and the hippocampus the highest levels of β-cleaved fragments. The latter was confirmed by another antibody that recognizes only the β-cleaved CTF, suggesting that the hippocampus has the highest levels of Aβ production. It has been reported that the activity of β-secretase increases with age in humans (Fukumoto et al., 2004). To examine whether age had an effect on APP processing in tgAPPswe rats, we determined the amount of CTFs in cortical and hippocampal samples from animals of different ages, finding that the levels of both α- and β-cleaved fragments increase with age.

tgAPPswe animals have been analysed with regard to pathology. In younger animals, the most predominant immunohistochemical feature in the brain is staining of the APPswe in neurones. At about the age of 15 months, homozygous animals begin to exhibit early stages of amyloid deposition either as cerebrovascular deposits or very rare diffuse plaques in the deep layers of the cortex. Both types of deposits are

immunoreactive for Aβ42 but only the cerebrovascular deposits show some Aβ40 immunoreactivity. Considering that APP with the Swedish mutation was used for establishment of the tgAPPswe rats, accumulation of Aβ in the cerebral blood vessels is to be expected, as it is common in patients carrying this mutation.

3.4 BEHAVIOURAL AND BRAIN CHANGES IN TRANSGENIC RATS EXPRESSING HUMAN APP WITH THE SWEDISH MUTATION

In *paper* III we characterized the expression pattern of human mutant APP in the tgAPPswe rats. Here we further characterise these rats using behavioural tests, magnetic resonance imaging (MRI) and Western blotting.

We first investigated the behavioural phenotype in these rats. In the open-field at the age of 8 months, the male tgAPPswe rats were more active than the controls. The tgAPPswe rats displayed higher motor activity as measured by: frequency of entering both the corner and central zones; total duration of movement in the corner zones; higher raring frequency than the control rats. The female tgAPPswe rats, on the other hand, did not differ significantly from the controls in the open-field. At the present time we do not have the explanation for this difference. However, gender has shown to be of importance in some of the AD-mouse models (Kobayashi and Chen, 2005).

At the age of 14 months, male tgAPPswe rats demonstrated impaired acquisition of learning in the Morris water maze when compared with the controls. Testing was performed in 4 consecutive days. Analysis of the latency showed a significant difference on days 3 and 4 (Figure 6). On each day of the experiment, control rats showed significant improvement in the time needed to find the hidden platform ($p < 0.002$). On the other hand, there was substantial heterogeneity in the transgenic rats that on average demonstrated some improvement over the 4 days, but it was not statistically significant ($p < 0.303$). Expressing the data as a number of failures to reach the platform within the 65 sec trail time also showed significant differences on days 3 and 4. By day 3, all of the control animals found the platform within the 65 sec time period.
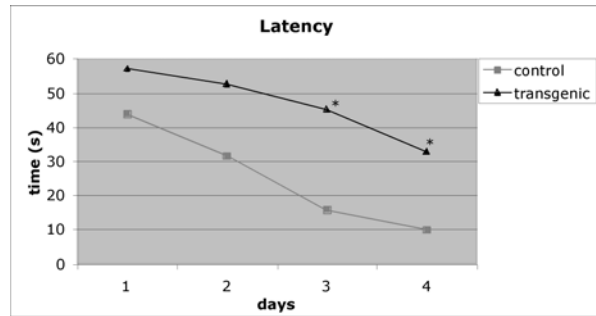
Figure 6. Impaired acquisition of place learning in the Morris water maze at the age of 14 months. Figure shows the average latency to find the platform over 4 days. * p<0.05

Considering the atrophy of the hippocampus and dilatation of the lateral ventricles in patients with AD (Morris and Nagy, 2004), we investigated these two structures in tgAPPswe rats by *in vivo* MRI. Most of the animals tested in the Morris water maze (one transgenic and one control were lost) were at the age of 16 months subjected to MRI. Visual examination of the MR images suggests hippocampal atrophy and enlargement of lateral ventricles in tgAPPswe rats, when compared to age-matched controls (Figure 7).
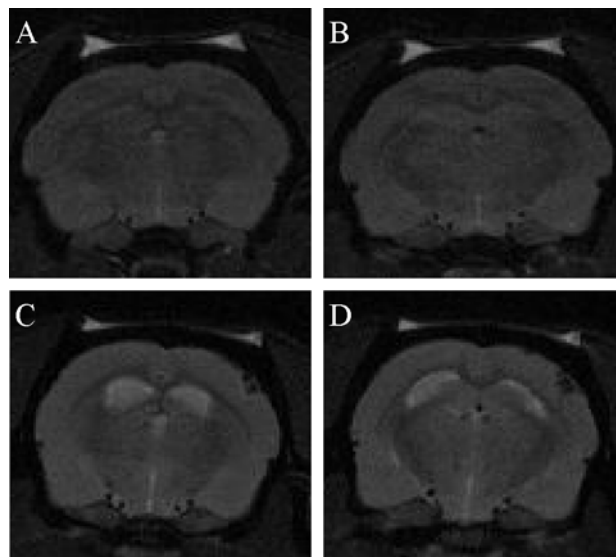

Figure 7. Representative magnetic resonance images of coronal sections (1 mm thick) from a control (A – B) and transgenic rat carrying the Swedish mutation (C – D). The coordinates for the pictures are as follows: 2.30 mm (A, C) and 3.30 mm (B, D) posterior to Bregma.

Quantitative determination of the areas of these two structures was performed in two serial coronal sections, one rostral and one 1mm caudal to the first (~ 2.30 mm and 3.30 mm posterior to Bregma respectively for hippocampus, and ~ 1.30 and 2.30 mm posterior to Bregma respectively for lateral ventricles). Even though statistical analysis of the obtained results cannot be performed due to the limited number of animals, calculation of the mean values of the measured areas clearly suggest a tendency to a size difference in hippocampus and lateral ventricles between the two groups. Namely, the decrease in hippocampal size in the tgAPPswe rats is 63% measured in the rostral section and 41% measured in the caudal section, when referred to the controls. The difference in size of lateral ventricles is even more striking, i.e. 320% increase measured in the rostral and 380 % increase in the caudal section, when compared to the control animals. Hippocampal volume reduction has also been described in transgenic mice overexpressing human mutant APP V717F (Redwine et al., 2003). Similar to our results, this volume deficit was shown to occur before any histologically evident formation of Aβ-containing diffuse or compact plaques.

Considering the prominent synaptic and neuronal loss in AD patients, we examined the levels of the synaptic marker synaptophysin and neuronal marker NeuN in hippocampus and cortex of transgenic animals, at the age of 16 to 20 months, using Western blotting. Examination of the levels of synaptophysin suggests no difference between the tgAPPswe and control rats. The same is true for the NeuN. This is maybe due to the limited amyloid accumulation found in the rats at this age. Synaptic and neuronal loss has shown to be very variable in APP and APP/presenilin transgenic mice models, and even missing in some of them (Sankaranarayanan, 2006).

NFT were not successfully modelled in the current APP and APP/PS transgenic animals (Sankaranarayanan, 2006; Spires and Hyman, 2005), but hyperphosphorylation of tau is reported for some of these models (Kobayashi and Chen, 2005; Sankaranarayanan, 2006; Spires and Hyman, 2005). We examined the phosphorylation state of tau in hippocampus and cortex of tgAPPswe animals by Western blotting, using the phospho-specific antibody PHF-1, which detects phosphorylation of serine at amino acids 396 and 404. These immunoblots demonstrated an obvious difference between individual animals, both within transgenic and control groups. However, there is an apparent increase in PHF-1 reactivity in tgAPPswe animals compared to controls. The

blots were stripped and reprobed with tau-5 antibody, indicating similar levels of total tau in the examined regions of transgenic and control animals.

To further characterize the phosphorylation of tau, we used another phosphotau antibody, AT8, which recognises tau phosphorylated on amino acids Ser199, Ser202, and Thr205. However, using this antibody, there was no difference between the transgenic and control animals. Increased tau phosphorylation at PHF-1- but not AT8- recognition sites is consistent with the results found in the double APP/PS rat model (Echeverria et al., 2004a).

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The extensive research on APP to date has provided considerable amount of information about: its structure, binding partners, posttranslational modifications, processing, etc; and even though there are many functions proposed for this protein, its physiological function remains unknown.

In our work, we investigated the 'normal' APP *(paper I)* and 'mutant' APP *(paper III and IV)*. Specifically, in *paper I*, we showed that antibody-bound APP upregulates ODC expression. Since APP is a key molecule in AD pathogenesis, we compared the localization and levels of ODC protein in AD and control cases *(paper II)*. We found that ODC, localised in the nucleus in control brains, is shifted towards the cytoplasm in AD and this occurs early in the disease process. In *paper III* and *IV*, we examined the *in vivo* consequences of expressing mutant APP by establishing transgenic rats carrying the Swedish mutation (tgAPPswe). These rats exhibit limited amyloid accumulation that occurs at a high age, but also behavioural deficits, enlarged lateral ventricles and a smaller hippocampus when compared to the controls.

APP, being the source of Aβ, is intimately connected to AD. Understanding the normal function of APP is essential for understanding AD pathogenesis as dysfunction of this protein most likely contributes to the disease process. Our first study (*paper I*) supports the role of APP in activation of gene transcription, but also shows that APP can signal by a γ-secretase-independent mode of action. Further studies on this signalling pathway will be needed; however, a very recent paper describes a novel APP signalling mechanism that does not require γ-secretase cleavage, but involves recruitment of Tip 60 by holo-APP to the membrane (Hass and Yankner, 2005). Interestingly, Tip 60 has been reported to be upregulated in ODC overexpressing cells (Hobbs et al., 2006).

The findings that APP induces ODC expression (*paper I*) raise one important question: what is the role of ODC in AD? We (*paper II*) and others (Bernstein and Muller, 1995) have reported elevated levels of ODC in AD brains, but we also found that this alteration occurs early in the disease process. Moreover, ODC translocates in AD, from the nuclear compartment towards the cytoplasmic, and this was again observed early in the disease (*paper II*). There is evidence that ODC induction and associated accumulation of brain polyamines is neuroprotective, i.e. transgenic mice

overexpressing ODC exhibit a significantly elevated seizure threshold to chemical and electrical stimuli (Kauppinen and Alhonen, 1995); in these mice expression of neurotrophins is induced (Reeben et al., 1996); polyamines has been shown to function directly as free radical scavengers (Ha et al., 1998) and by that may serve to protect neuronal cells from oxidative stress, etc. The functional significance of ODC compartmentalisation however is unclear, but it may have a reactive and neuroprotective role since it appears prior to the pathological features in AD.

Development of AD animal models has been greatly facilitated by the knowledge of various FAD mutations in APP and PS. There are two main reasons for developing such models: to better understand the pathogenesis of the disease, especially important because the first steps in AD pathogenesis are not accessible in human patients; and to test treatment strategies. However, it has been proved to be very difficult to establish AD models that recapitulate all aspects of the disease. In *paper III and IV*, we characterised our AD model, the tgAPPswe rat, and found that it exhibits limited disease-related pathology. Thus, to shorten the time for development of this pathology, the next steps will be to generate transgenic rats expressing human PS1 with AD associated mutation, and then crossing these animals with our current model.

Despite the limited neuropathology, we believe that the tgAPPswe rats represent a unique model of AD, i.e. they show both behavioural deficits and hippocampal atrophy, and also enlarged lateral ventricles. It has been suggested that hippocampal atrophy can be used as a structural and early MRI marker of AD: first, a large number of studies have shown that MRI measurements of hippocampal atrophy can distinguish AD from cognitively normal elderly people with 80-90 % accuracy (Blennow et al., 2006); second, hippocampal volume has been shown to strongly correlate with Braak neurofibrillary stage; and finally, MRI appears to track even the earliest stages of the disease (Mortimer et al., 2005). The latter even suggests that tgAPPswe rats may represent a model of early AD. However, since AD is a progressive disease, it will be important to investigate how these AD-related MRI and behavioural findings change with age in our rats.

5 MATERIALS AND METHODS

5.1 CELL CULTURES

Human embryonic kidney 293 (HEK293) and SH-SY5Y human neuroblastoma cells were cultured in RPMI 1640 containing 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Blastocyst-derived embryonic stem cells lacking PS1 and PS2 expression, BD8 cells, were cultured in DMEM supplemented with 10% fetal calf serum, 2.4 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, and non-essential amino acids (Donoviel et al., 1999).

Primary cortical and hippocampal cultures were prepared from the cortex and hippocampus respectively of E17 foetal Sprague-Dawley rats. Following dissection and dissociation by mechanical trituration, the cells were seeded on poly-D-lysine coated culture dishes, at a density of 250 cells/mm^2. The cells were kept in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin, for up to 14 days.

5.2 MICROARRAY EXPERIMENTS

Human signal transduction pathway finder gene arrays contain 96 marker genes associated with 18 signal transduction pathways. The assay was performed according to manufacturer's instructions (GEArray Q Series; Super Array, Inc.). In brief, total RNA was isolated from cells using Qiagen RNeasy mini kit. Gene-specific biotin-labelled cDNA probes were generated from 7.5 µg total RNA using gene-specific set of primers for the reverse transcription reaction. The cDNA probes were hybridised with gene specific cDNA fragments spotted on a nylon membrane. The relative expression level of each gene was analysed using ScanAlyse (developed by Michael Eisen at Lawrence Berkeley National Lab) and GEArrayAnalyser.

5.3 APP KNOCKDOWN BY RNA INTERFERENCE

For each transfection sample, we prepared complexes of small interfering RNA (siRNA) duplex oligoribonucleotides directed against APP and Lipofectamine 2000 as follows: 5 µl of 20 µM APP siRNA (with sense sequence 5'-GGGACCAAAACCUGC AUUG-3' and antisense sequence 5'- CAAUGCAGGUUUUGGUCCC-3', synthesised by Ambion) were diluted in 250 µl Opti-MEM I without serum. 10 µl of Lipofectamine

2000 were also diluted in 250 µl Opti-MEM I and incubated for 5 minutes at room temperature. After the 5-min incubation, diluted APP siRNA were combined with diluted Lipofectamine 2000 and this mixture was incubated for 20 minutes at room temperature to allow the siRNA-Lipofectamine 2000 complexes to form. Then, the APP siRNA-Lipofectamine 2000 mixture was added to a 60 mm culture dish with HEK293 cells (that were plated without antibiotics). The cells were then incubated for 48 h.

5.4 WESTERN BLOTTING

Proteins were separated by electrophoresis on polyacrylamide gels and transferred onto nitrocellulose membranes. Blotted membranes were blocked with 5% w/v non-fat dry milk in TBS for 45 min and incubated overnight with indicated primary antibodies. The membranes were washed in TBS-T and incubated with HRP-conjugated secondary antibodies. After washing, antibody binding was visualised using ECL detection system (Amersham).

5.5 IMMUNOHISTOCHEMISTRY

4% formaldehyde-fixed brain material was taken, embedded in paraffin and cut with a microtome to sections of 6-7 µm thickness and mounted onto coated slides. Mounted sections were deparaffinised, rehydrated via a series of graded alcohols, and treated with Dako protein block solution for 0.5-1 h. Sections were incubated with primary antibodies, overnight, and then with biotinylated secondary antibodies for 30 min. Biotin-labelled tissue was further processed using ABC Elite HRP reagents (Vector laboratories) and was developed with a solution of hydrogen peroxide (0.003%) and diaminobenzidine (0.02%). Double staining was performed using ABC-Elite HRP reagents, with final visualisation by SG kit (Vector laboratories). Finally, the sections were dehydrated and mounted in Depex (BDH).

5.6 STEREOLOGICAL ANALYSIS

To estimate the number of neurones in frontal cortex and hippocampus CA1 region as well as Purkinje cells in the cerebellum, an unbiased method based on stereological principle was used (Gundersen, 1986). This method includes counting 100 consecutive random chosen neuronal profiles in a uniform systematic sampling. This procedure ensured the selection of a systematic uniformly random sample of an area of interest in which all neurones had equal probabilities to be presented (Gundersen, 1986; West et

al., 1991). A computer-generated counting frame was superimposed on the screen a CAST – GRID 1.2 software program (Olympus, Denmark). Two sets of motors are connected to the microscope to move the section at known distance in the x- and y-direction. A sampling schema was designed so that 100 consecutive neuronal profiles would be counted under a 100x oil-immersion objective. The area of the counting frames was dimensioned so that two to three neuronal somas would typically be sampled. Counting was performed on computer-governed random-chosen number of position in the determined area selected in those regions. In the frontal cortex selected region contains area which involved apical, lateral and bottom part of the gyrus, vertically cut to the surface but parallel to penetrating arteries. The neurones can be easily differentiated from other cellular profiles regarding the presence of nucleus with a single large nucleolus, no heterochromatin and visible cytoplasm (Berry et al, 2002; Dawson et al., 2003). The neuronal cells were divided in three groups by means of localisation of ODC staining: 1) predominantly nuclear 2) predominantly cytoplasmic and 3) not stained. In the cerebellum two types of Purkinje cells staining patterns were applied: 1) cytoplasmic/nuclear and 2) not stained.

5.7 ESTABLISHMENT OF TRANSGENIC RATS

Human APP695 containing the Swedish mutation (Mullan et al., 1992), was cloned into the pUbi1Z vector. The pUbi1Z vector was generated by replacing the CMV promoter in pcDNA3.1/Zeo with the human ubiquitin (UbC) promoter from pTEJ-8 (Johansen et al., 1990), and also removal of the ampicillin resistance in pcDNA3.1/Zeo.

The construct was sequenced and its integrity verified by transfection into human HEK293 cells followed by Western blotting for APP. The promoter and APP cDNA were excised from the vector and purified. The transgenic rats were generated by pronuclear injection using Hannover Sprague-Dawley rats. Founder animals were identified by PCR using genomic DNA extracted from the tail. Two sets of primers were used, one for amplifying the whole APP coding region using primers APP1, 5'-GCGGCCGCATGCTGCCCGGTTTGGC-3' and APP2, 5'-GGGCCCCTAGTTCTG CATCTGCTC-3' and the second to amplify parts of the promoter and APP using primers pUbC1, 5'-GTTGGCGAGTGTGTTTTGTGAAG-3' and APP3, 5'-AATCGA TGTGGTTCTCTCTGTGGC-3'.

The presence of human APP mRNA was investigated by reverse transcription-coupled PCR (RT-PCR). Total RNA from the cortex of transgenic rats was isolated using RNeasy (Qiagen). The RT-PCR reaction was run using Qiagens OneStep RT-PCR Kit and APP primers (see above).

5.8   SPONTANEOUS OPEN- FIELD TEST

Transgenic rats and control rats at the age of 8 months were used. The apparatus consisted of 4 identical open field arenas, which were located in a dimly lit experimental room. Each arena measured 76.5 x 76.5 x 49 cm and was arranged in a 2 x 2 manner. They were made of wood, painted white and were set up vertically under a digital camera, which was connected to a video recorder and a computer under the control of EthoVision (Nodus, Wageningen, the Netherlands) tracking system. Four rats were tested simultaneously and each rat was placed into the centre of open-field arena. Their behaviours were recorded over a 30-min session.

5.9   WATER-MAZE TESTS

Transgenic and control animals at the age of 14 months were used. Spatial learning and memory was assessed in the Morris water maze. The test is based on the animal's ability to escape the water by climbing on a hidden platform when spatial memory is assessed. The apparatus consists of a pool 140 cm in diameter, filled with tap water at 30 cm height, maintained at $22 \pm 1°$ C. A transparent platform was submerged 0.5 cm below the water surface and the animal was required to locate the submerged platform (10.5 cm diameter) placed at one quadrant of the pool. On day 1 of the experiment, a habituation swim trial of 65 sec with no platform present was given to all the animals. The animals were given 4 trials per day over 4 days. Each trial starts by gently placing the rat on the surface of the water pool facing the wall of the pool and letting it swim for 65 sec to locate the platform. Each starting position was chosen randomly. If an animal failed to find the platform within 65 sec, it was placed on the platform and remained there for 30 s, before being given another trial. On day 5 a probe trial was given in which the platform was removed and the animal allowed to swim for 65 sec before being removed from the pool. The movement of the animals whilst in the pool was video-taped and recorded by a computer tracking system.

5.10 MAGNETIC RESONANCE IMAGING

For MRI studies, transgenic and control rats were imaged at 16 months of age. MRI examinations were performed using a 4.7 T magnet with a horizontal bore (Bruker Biospec Avance 47/40, Bruker, Karlsruhe, Germany) equipped with a 12 cm inner diameter self-shielded gradient system (max. gradient strength 200mTm^{-1}). A commercially available circular resonator (Bruker, Karlsruhe, Germany) with an inner diameter of 72 mm was used for excitation and signal detection. Structural images were obtained producing an axial multi slice package consisting of 21 continuous slices through the brain using spin echo sequences with rapid acquisition with relaxation enhancement (RARE) (Wimmer et al., 1986). The parameters were adjusted as follows: TR 2500 ms, TE 37.4 ms, RARE-factor 8, matrix size 256x256, slice thickness 1 mm, FOV 4 mm and 16 averages.

Rats were anesthetized with 1.5 - 2.0 % isoflurane in air delivered via a mouth piece allowing spontaneous respiration. The rats were then positioned in supine position and the head fixed to an acrylic rig. Body temperature was recorded and maintained at 36 to 37 °C using a MRI-compatible air temperature control system.

Area measurements of hippocampus were performed in two coronal serial sections, centred approximately 2.3 mm and 3.3 mm posterior to Bregma, using Bruker standard software (Paravision 2.1.12b). Hippocampus was distinguished by surrounding structures as follows: corpus callosum clearly demarcated the dorsal border of hippocampus; thalamus which represents the ventral border was discerned by its hypointensity. For measurements of areas of the lateral ventricles, again two serial coronal sections were used, ~1.30 mm and 2.30 mm posterior to Bregma. The identification of lateral ventricles was possible to make because they provide high contrast boundaries towards the surrounding structures.

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