Department of Neurobiology, Caring Sciences and Society
Karolinska Institutet, Stockholm, Sweden

In vitro modelling of tau phosphorylating kinases: emphasis on Cdk5

Anne Jämsä

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Det krävs ett helt nytt sätt att tänka för att lösa de problem vi skapat med det gamla sättet att tänka.

Albert Einstein

ABSTRACT

The main hallmarks of Alzheimer's disease (AD) are extracellular deposits of beta-amyloid (Aβ) and intracellular neurofibrillary tangles (NFT) composed of highly phosphorylated tau protein. Abnormal hyperphosphorylation of tau is the most deleterious step in NFT formation making the use of kinase inhibitors an attractive treatment possibility in AD. To enable development and screening of selective kinase inhibitors, well-characterized cellular assays are essential. In this thesis, different cell culture systems were investigated as in vitro models for tau phosphorylating kinases, with an emphasis on cyclin-dependent kinase-5 (Cdk5).

In paper I, differentiated SH-SY5Y cell line was investigated as a model for tau phosphorylation. Sequential differentiation of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor induced a prominent increase in the content and the phosphorylation state of tau. Of the investigated kinases, glycogen synthase kinase 3β (Gsk3β) contributed most to tau phosphorylation whereas Cdk5 made a minor contribution. Lithium, a GSK3β-inhibitor, reproducibly inhibited tau phosphorylation in a wide concentration range indicating that this model can be used to screen for GSK3β inhibitors.

In paper II, differentiated SH-SY5Y cells were treated with neurotoxic stimuli or transfected with p25 in order to activate Cdk5. Glutamate increased Cdk5 and p35 protein levels thereby elevating Cdk5 activity and tau phosphorylation. When p25 was transfected to the cells, increased tau phosphorylation was achieved but could not be reduced with the Cdk5 inhibitor Roscovitine. This is possibly through activation of ERK1/2, another tau phosphorylating kinase, detected in Roscovitine treated cells. An additional finding of this study was degradation of p25 via proteasome in cells treated with Cdk5 inhibitors.

In paper III, investigation of Aβ treated hippocampal organotypic cultures revealed increased tau phosphorylation at the Ser396 epitope probably through activation of Gsk3β whereas Cdk5 involvement was not detected.

In paper IV, alternative Cdk5 substrates were looked for in Cdk5/p25 transfected HEK293 cells. A non-muscle myosin heavy chain, type B (NMHC-B), was identified as a novel Cdk5 substrate. Only Cdk5 phosphorylates NMHC-B in HEK293 cells and its phosphorylation was concentration-dependently inhibited with Cdk5 inhibitors. A new screening system for Cdk5 inhibitors was established using NMHC-B phosphorylation as a read-out.

Many kinases, some with reciprocal interactions, are involved in tau phosphorylation in differentiated SH-SY5Y cells complicating its use as a model for Cdk5 mediated tau phosphorylation. The assay with NMHC-B phosphorylation as a read-out in Cdk5/p25 transfected HEK293 cells is, however, very specific and sensitive and allows validation of compounds designed to inhibit Cdk5.

LIST OF PUBLICATIONS

I. Jämsä A, Hasslund K, Cowburn RF, Bäckström A and Vasänge M
The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau phosphorylation
Biochem. Biophys. Res. Commun. (2004) 319: 993–1000

II. Jämsä A, Bäckström A, Gustafsson E, Dehvari N, Hiller G, Cowburn RF and Vasänge M
Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells
Biochem. Biophys. Res. Commun. (2006) 345: 324-331

III. Johansson S*, **Jämsä A***, Vasänge M, Winblad B, Luthman J and Cowburn RF
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Neuroreport (2006) 17:907-911

IV. Jämsä A*, Agerman K*, Radesäter A-C, Ottervald J, Malmström J, Hiller G, Liu G and Vasänge M
Identification of non-muscle myosin heavy chain as a substrate for Cdk5 and tool for drug screening
Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

Aβ	amyloid-beta peptide
ACh	acetylcholine
AChE	acetylcholine esterase
AchE-I	acetylcholine esterase inhibitor
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
AICD	APP intracellular domain
APP	amyloid precursor protein
BACE1	β-site APP cleaving enzyme 1
BDNF	brain-derived neurotrophic factor
Cdk	cyclin-dependent kinase
Cdk5	cyclin-dependent kinase-5
CNS	central nervous system
ERK	extracellular signal-regulated kinase
FAD	familial AD
GSI	γ-secretase inhibitor
Gsk-3β	glycogen synthase kinase-3β
JNK	c-Jun N-terminal protein kinase
LTP	long-term potentiation
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MBDs	microtubule binding repeat domains
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NMHC-B	non-muscle myosin heavy chain, type B
NGF	nerve growth factor
PDPK	proline-directed protein kinase
PHF	paired helical filaments
PP-1	protein phosphatase-1
PP-2A	protein phosphatase-2A
SF	straight filaments

INTRODUCTION

General introduction to Alzheimer´s disease

About 24 million people worldwide are estimated to suffer from dementia, the most common cause being Alzheimer's disease (AD) (Qiu et al., 2007). With an increasing age of the population, the number of affected people will increase making AD a growing health problem throughout the world (Johnson et al., 2000).

AD is a progressive neurodegenerative disorder characterized by a gradual decline of intellectual capacity. The symptoms include memory loss and deficits in one or more of the cognitive domains: aphasia, agnosia, apraxia or executive function (Brown et al., 2005). The disease progression leads to a significant decline in patients ability to manage daily life and finally to total dependence of caregivers. The duration of disease varies from 5 to 15 years. The cause of death is usually associated with secondary diseases such as cardiac infarct, pneumonia or other infections.

AD can be classified into familial (FAD) and sporadic forms, which are separated according to the presence or absence of other demented individuals in the family or in rare cases identification of a specific gene mutation. The FAD-linked mutations are located in β-amyloid precursor protein (APP), presenilin-1 or presenilin-2 genes (Campion et al., 1999). FAD cases are usually associated with early-onset i.e. the disease manifests before 65 years of age, whereas sporadic forms are mostly late-onset with disease debut occurring after age of 65 years. Sporadic late-onset forms of AD account for over 90% of all cases. Both forms of the disease have similar histopathological changes suggesting common etiology and pathophysiology.

The macroscopic changes seen in AD brain include shrinkage of gyri, widening of sulci and enlargement of vesicles. AD is further characterized by synaptic and neuronal loss, the cholinergic neurons being especially vulnerable (Davies and Maloney 1976). The most affected brain regions are hippocampus, entorhinal cortex, amygdala, neocortex and certain basal forebrain nuclei (Braak and Braak 1994). The main microscopic neuropathological hallmarks of AD are the deposits of beta-amyloid (Aβ) as extracellular plaques or in the cerebrovasculature (Glenner and Wong 1984) and intracellular aggregations of highly phosphorylated tau protein in neurofibrillary tangles (NFT) (Grundke-Iqbal et al., 1986) (Figure 1).

Tau protein and neurofibrillary tangles

In the human brain, tau proteins constitute a family of six isoforms ranging from 352 to 441 residues with a molecular weight of 45-65 kDa (Bueé et al., 2000). Alternative splicing of a single gene produces these different isoforms, which vary in having 3 or 4 microtubule binding repeat domains (MBDs) in the C-terminal half of the tau and in the number and size of the N-terminal inserts (Billingsley and Kincaid, 1997). All six isoforms are present in adult brain whereas only the shortest isoform is expressed in fetal brain (Kosik et al., 1989). Tau proteins belong to the microtubule-associated protein (MAP) family (Weingarten et al., 1975). Tau proteins are predominantly found in central nervous system (CNS) where they mainly localize in the axonal compartment of neurons (Binder et al., 1985).

Highly phosphorylated tau polymerises to paired helical filaments (PHFs) and to straight filaments (SF). PHFs are the major components of NFTs, one of the pathological hallmarks in AD brain (Grundke-Iqbal et al., 1986, Kosik et al., 1986). Normal tau binds to microtubules in a phosphorylation dependent manner stabilizing microtubules and thereby modulating axonal morphology (Xie et al., 1998). When tau becomes hyperphosphorylated, a pathological sequence of events takes place: dissociation of tau from microtubules and microtubule disassembly, abnormal accumulation of tau in the somatodendritic compartment, impaired axonal transport, loss of synapses and finally death of neurons (Alonso et al., 1997, Lau L et al., 2002, Iqbal and Grundke-Iqbal 2005). Hyperphosphorylation of tau reduces its degradation rate further promoting accumulation of tau in the cells (Khatoon et al., 1992).

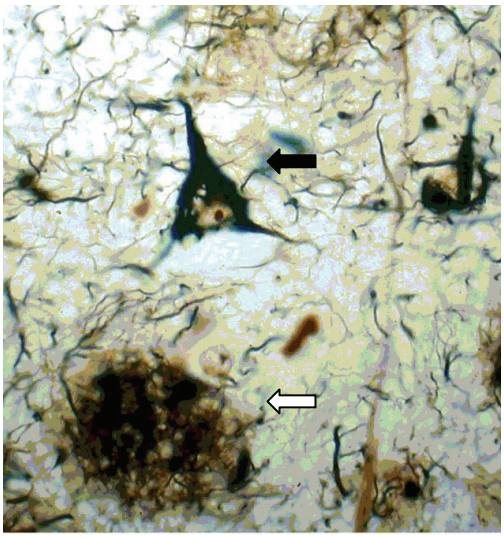

Figure 1. The main pathological hallmarks of AD; intracellular neurofibillary tangles (black arrow) and extracellular amyloid plaques (white arrow), Bielschowsky silver staining (Courtesy of Dr Nenad Bogdanovic).

Most of the phosphorylation sites in the tau protein are located on serine-proline (Ser-Pro) and threonine-proline (Thr-Pro) motives (Bueé et al., 2000). Some of these sites are phosphorylated only in fetal brain tissue and mitotic cells. However, some sites occurring in fetal brain are phosphorylated also in AD brain whereas some sites, such as Thr212/Ser214 (recognized by the AT-100 antibody) are believed to be AD specific (Imahori and Uchida 1997). Some tau phosphorylation sites have been shown to correlate with the severity of AD pathology. For instance Ser202/Thr205 (AT8) and Ser396/Ser404 (PHF-1) epitopes show intense staining in advanced AD cases (Augustinack et al., 2002). Figure 2 shows the major tau phosphorylation sites in AD.

Tau phosphorylation

The phosphorylation state of tau is controlled by a balance between activities of different kinases and phosphatases (Iqbal et al., 2005). Tau protein serves as a substrate to many kinases that can be divided into proline-directed protein kinases (PDPKs) and non-PDPKs (Morishima-Kawashima et al., 1995). PDPKs mainly modify Ser-Pro and Thr-Pro motifs in the flanking domains whereas tau phosphorylation by non-PDPKs mainly occurs at the MBDs where Ser or Thr is not followed by a Pro. The PDPKs consist of cyclin dependent kinase-5 (Cdk5), glycogen synthase kinase-3α/β (GSK-3α/β) and mitogen-activated protein kinase (MAPK) family: extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal protein kinase (JNK). Deregulation of all PDPKs is reported to occur in AD although Cdk5 and Gsk-3β are suggested to be the central players in tau hyperphosphorylation (Iqbal and Grundke-Iqbal 2007). The protein phosphatases mainly responsible for dephosphorylation of tau are PP-1 and PP-2A, activities of which are also compromised in AD brain (Gong et al., 1995).

Cyclin-dependent kinase-5

Regulation of Cdk5

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family of Ser/Thr kinases (Meyerson et al., 1992). Unlike other Cdk's, Cdk5 is not involved in cell cycle control. The activity of Cdk5 is regulated by its binding to neuron-specific activator proteins p35 and p39 (Tsai et al., 1994, Tang et al., 1995). Binding to activator proteins is the only absolute

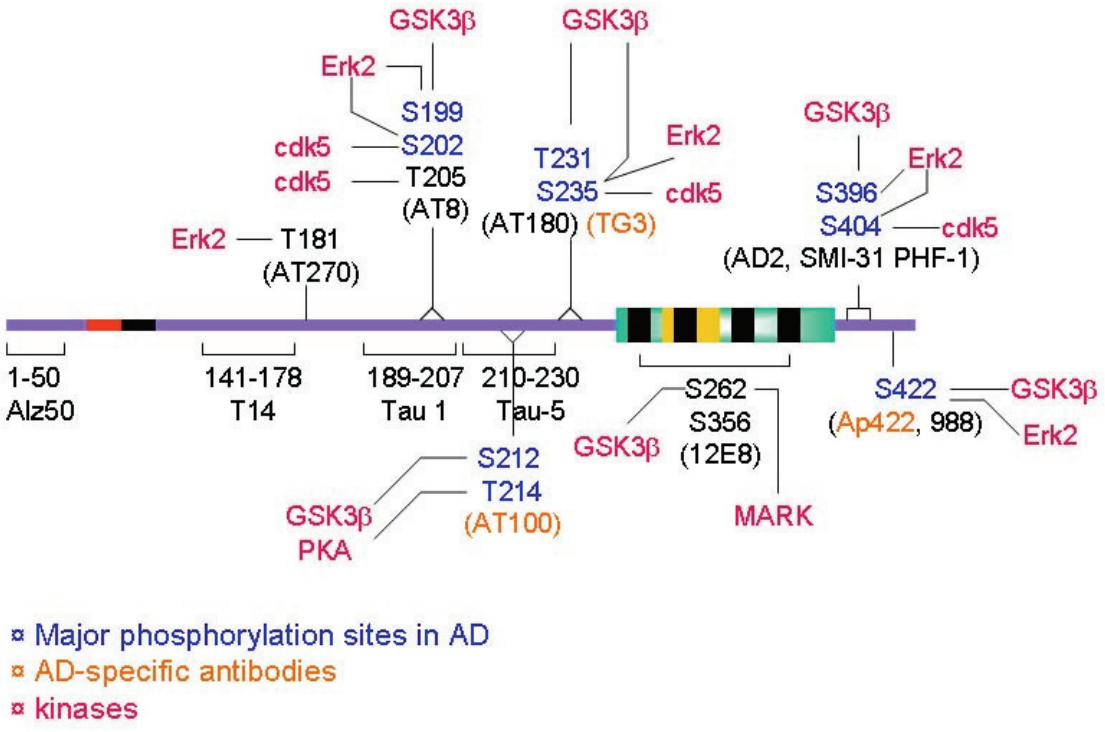

Figure 2. Schematic presentation of the major tau phosphorylation sites in AD (Courtesy of Dr Ratan Bhat).

requirement for Cdk5 activity. Further stimulation can, however, be achieved by phosphorylation at Tyr15 by Src-related tyrosine kinases (Zukerberg et al., 2000).

Although Cdk5 is expressed in most tissues, its enzymatic activity is predominantly found in the CNS. This is due to the restricted expression of its activator proteins in post-mitotic neurons (Zheng et al., 1998). The spatial and temporal expression of p35 and p39 in the brain seems to be complementary (Zheng et al., 1998) and they localize to overlapping but distinct subcellular compartments in growth cones, synapses, detergent-insoluble cytoskeleton and membrane fractions (Dhavan and Tsai 2001). p35 and p39 have an amino-terminal myristoylation signal motif that targets these proteins to cell membranes and dictates thus the subcellular distribution of Cdk5 (Patrick et al., 1999). In vitro studies indicate similar substrate specificity for Cdk5/p35 and Cdk5/p39 although p39 binds to Cdk5 with lower affinity than p35. p39 can compensate for some but not all functions of p35 in vivo (Ko et al., 2001).

p35 and p39 are short-lived proteins (Patrick et al., 1998). The protein amount of p35 and p39 primarily determines the Cdk5 activity in neurons, while the amount of p35 and p39 is determined by their synthesis and degradation. The expression of p35 is induced by

extracellular stimuli: neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Tokuoka et al. 2000, Harada et al., 2001) or extracellular matrix molecules such as laminin (Paglini et al., 1998). Phosphorylation of p35 by Cdk5 is a signal for ubiquitination and degradation via the ubiquitin/proteasome system. Inhibition of Cdk5 activity increases the stability of p35 several fold and p35 mutant proteins that lack Cdk5 phosphorylation sites have a longer half-life (Patrick et al., 1998). Membrane interaction is reported to be a novel mechanism to regulate Cdk5 kinase activity, membrane-bound Cdk5/p35 being inactive and soluble Cdk5/p35 the active complex (Zhu et al., 2005). Membrane association in turn is dependent on the phosphorylation state of p35 (Sato et al., 2007).

Cdk5 activator proteins p35 and p39 can be cleaved to smaller fragments, p25 and p29, by a calcium activated protease calpain (Kusakawa et al., 2000, Lee et al., 2000). Due to loss of myristoylation site, p25 and p29 preferentially locate to the cytosol and gain access to alternative substrates resulting in a qualitative change in Cdk5 function (Patrick et al., 1999, Kusakawa et al., 2000, Lee et al., 2000). Calpain cleavage causes also a quantitative change in Cdk5 activity since p25 activates Cdk5 more efficiently than p35 (Patrick et al., 1999, Amin et al., 2002). Moreover, p25 and p29 have longer half-lifes than uncleaved proteins (Patrick et al., 1999). Cdk5 induced phosphorylation of p35 reduces calpain mediated p35 cleavage suggesting possible autoregulation (Saito et al., 2003). Cleavage of Cdk5 activator proteins is often detected in neurotoxic conditions, especially when prominent calcium influx is involved (Kusakawa et al., 2000, Lee et al., 2000).

Physiological function of Cdk5

Cdk5 is involved in regulation of diverse cellular processes. The best-known role for Cdk5 is probably in development of the CNS. Mice that are deficient of Cdk5 show disruptions in neuronal layering of many brain structures including the cerebral cortex, hippocampus and cerebellum indicating neuronal migration defects (Ohshima et al., 1996, Gilmore et al. 1998). In addition, the laminar organization of neurons is inverted in the cerebral cortex. Mice lacking p35 show similar inverted cortical layering than Cdk5 -/- mice, but have only mild disruptions in the hippocampus and have a fairly normal cerebellum (Chae et al., 1997, Kwon et al., 1998). p35-mutant mice have defects in the fasciculation of several prominent axon tracts indicating a role for Cdk5 in axon guidance and targeting (Kwon et al.,1999). p39-deficient mice show no noticeable deficits whereas the phenotype of the p35/p39 double mutant is indistinguishable from that of Cdk5-/- mice (Ko et al., 2001). In

addition to migration and axon guidance, Cdk5 also regulates dendrite development and dendritic spine morphogenesis (Cheung and Ip, 2007). The exact molecular mechanisms and the target proteins involved in these developmental events in the nervous system are still inadequately known but might be through regulation of the cytoskeleton, cellular adhesion or the response to guidance cues by Cdk5 (Gupta and Tsai 2003).

Numerous substrate proteins have been identified for Cdk5. Cdk5 phosphorylates cytoskeletal components such as neurofilament and microtubule-associated proteins tau and MAP1B (Grant et al., 2001, Baumann et al., 1993, Paglini et al., 1998). Cdk5 also phosphorylates PAK1 kinase, which is implicated in actin polymerization (Nikolic et al., 1998). Cdk5 has been linked to axonal transport by the identification of Nudel as a Cdk5 substrate (Niethammer et al., 2000). The role of Cdk5 in adhesion is mediated by its phosphorylation of β-catenin and regulation of cadherin-catenin complexes (Kesavapany et al., 2001). Cdk5 phosphorylates APP and thereby affects its processing (Iijima et al., 2000).

Cdk5 affects synapse formation, synaptic plasticity, learning and memory by phosphorylating numerous synaptic proteins. Cdk5 is involved in regulation of exocytosis of synaptic vesicles through the phosphorylation of synapsin 1, MUNC18 and P/Q subtype voltage-dependent calcium channel (Matsubara et al., 1996, Shuang et al., 1998, Tomizawa et al., 2002). Dynamin 1 and amphiphysin 1, proteins essential for clathrin-mediated endocytosis, are also identified as substrates for Cdk5 (Floyd et al., 2001, Tomizawa et al., 2003). In addition to these presynaptic roles, Cdk5 is also involved in postsynaptic events. Cdk5 regulates neurotransmitter receptor expression (Fu et al., 2001, Xie et al., 2004) and affects clustering of neurotransmitter receptors by phosphorylating PSD-95 (Morabito et al., 2004). Additional ways to control synaptic plasticity exerted by Cdk5 are upregulation of N-methyl-D-aspartate (NMDA) receptor activity by phosphorylating NR2A-subunit (Li et al., 2001) and facilitation of NR2B degradation (Hawasli et al., 2007). Cdk5 also modulates dopaminergic signalling by phosphorylating DARPP32 (Bibb et al., 1999).

Cdk5 in neurodegeneration

Although Cdk5 activity is necessary for development and many physiological functions of the nervous system, deregulated Cdk5 activity is neurotoxic and has been linked to AD and other neurodegenerative disorders such as Parkinson's disease (Smith et al., 2003) and amyotrophic lateral sclerosis (Nguyen et al., 2001). Cdk5 is also implicated in ischemic stroke (Wang et al., 2003) and psychiatric disorders (Sananbenesi et al., 2007).

Deregulation of Cdk5 activity in AD brain is proposed to occur through increased production of p25 causing prolonged activity and altered cellular localization and the substrate specificity of the kinase (Patrick et al., 1999, Tseng et al., 2002). Cdk5/p25 is thought to hyperphosphorylate tau protein in AD brain and thus contribute to the formation of NFT's (Patrick et al., 1999, Lee et al., 1999, Tseng et al., 2002). The published clinical findings on the relevance of this pathological mechanism are somewhat conflicting; both elevated and unchanged p25 levels have been reported in AD brains (Patrick et al., 1999, Takashima et al., 2001, Taniguchi et al., 2001, Tseng et al., 2002, Tandon et al., 2003). This discrepancy can at least partly be accounted for extensive post mortem degradation of this protein (Taniguchi et al., 2001). Also in transgenic mice mimicking the amyloid pathology of AD, both increased and unaltered p25 levels have been reported (Otth et al, 2002, Tandon et al., 2003). Increased Cdk5 enzymatic activity has, however, been documented in AD brain (Patrick et al., 1999, Lee et al., 1999) and Cdk5 has been shown to be associated with NFTs (Yamaguchi et al., 1996, Pei et al., 1998).

Compelling support for a role of Cdk5/p25 in AD pathophysiology has come from the p25 transgenic mice demonstrating both hyperphosphorylation of tau and neurofibrillary pathology derived from endogenous tau (Cruz et al. 2003). Prolonged p25 expression in these mice impaired hippocampal long-term potentiation (LTP) and memory with accompanying synaptic and neuronal loss (Fischer et al., 2005) Remarkably, transient p25 expression enhanced hippocampal LTP, increased dendritic spine density and number of synapses without causing neurodegeneration suggesting that a prolonged expression of p25 may turn a physiological action of Cdk5 into a pathological one. Involvement of Cdk5 in tau pathology was also demonstrated in tangle-forming transgenic mice where an increase in p35 and p25 levels was shown to correlate with the amount of tau in abnormal conformation and in insoluble form (Kelleher et al., 2007).

In vitro studies support the role of p25 in tau hyperphosphorylation and neurodegeneration. Transfection of cell lines or primary neurons with Cdk5/p25 induces tau phosphorylation to greater extent than transfection with Cdk5/p35 complex (Patrick et al., 1999, Hashiguchi et al., 2002, Zheng et al., 2002). Besides increased tau phosphorylation, introduction of p25 to cultured primary neurons causes neurite retraction, microtubule collapse and apoptosis (Patrick et al., 1999, Zheng et al., 2005). Cdk5 has been shown to phosphorylate tau in vitro on sites that are found in PHF-tau (Paudel et al., 1993).

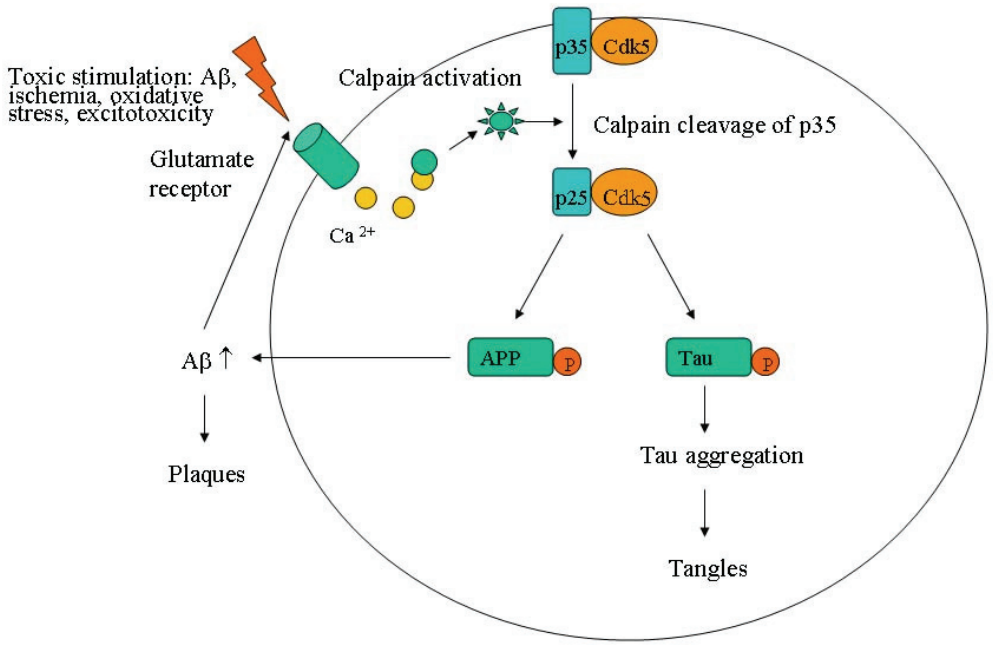

Figure 4. Schematic illustration of a proposed pathway by which Cdk5 deregulation contributes to AD pathology.

There is also some evidence that indicate Cdk5 in amyloid plaque formation. Cdk5 phosphorylates APP at Thr668 (Iijima et al., 2000) and increased phosphorylation of this site is reported to enhance Aβ production (Lee et al., 2003). Increased generation and intraneuronal accumulation of Aβ peptide has been reported in inducible p25 transgenic mice (Cruz et al., 2006). Furthermore, phosphorylated APP is accumulated in AD brain (Lee et al., 2003). Aβ stimulates additional cleavage of p35 to p25 resulting in further aberrant processing of APP and hyperphosphorylation of tau. Figure 4 shows a proposed pathway by which Cdk5 deregulation contributes to AD pathology.

Glycogen synthase kinase 3β

Regulation of Gsk3β

Gsk3β is a Ser/Thr kinase that was originally identified and named for its ability to phosphorylate glycogen synthase (Cohen 1979). Although Gsk3β is widely expressed in all tissues, the highest expression is detected in the brain where it mainly localizes to neurons

(Woodgett, 1990, Hanger et al., 1992). Highest levels of Gsk3β are detected during the development (Leroy and Brion 1999) and the expression declines in the adult brain (Takahashi et al., 1994). Gsk3β can be phosphorylated at Ser9 and Tyr216 in its kinase domain. Serine phosphorylation inhibits Gsk3β activity and can be accomplished by several kinases such as p70 S6 kinase, p90Rsk, Akt, protein kinase C and A (Grimes and Jope, 2001). In neuronal cells, insulin-like growth factor-1, NGF and BDNF cause inhibition of Gsk3β (Quevedo et al., 2000, Pap and Cooper, 1998, Mai et al., 2002). Tyrosine phosphorylation activates the enzyme but the kinases mediating this modification are largely unknown. Tyrosine phosphorylation of Gsk3β is induced by transient increases in intracellular calcium (Hartigan and Johnson, 1999) and proapoptotic stimuli such as NGF deprivation and staurosporine (Bhat et al., 2000). In addition to phosphorylation, Gsk3β is regulated by protein complex formation and by its intracellular localization (Grimes and Jope, 2001). Cleavage of Gsk3β by calpain was recently reported to increase its kinase activity (Goni-Oliver et al., 2007).

Physiolocigal function of Gsk3β

A wide variety of substrate proteins have been identified for Gsk3β reflecting its role in complex functions such as regulation of gene expression, cell survival, cytoskeletal integrity and neuronal plasticity. Gsk3β substrate categories can be divided in metabolic and signalling proteins such as NGF receptor and APP, transcription factors such as activator protein-1 (AP-1) and cyclic AMP response element binding protein (CREB) and structural proteins such as tau, MAP1B, MAP2 and neurofilaments (Grimes and Jope, 2001).

Gsk3β in neurodegeneration

Gsk3β has been linked to many of the major neuropathological mechanisms associated with AD (Grimes and Jope, 2001). These include the participation of Gsk3β in tau phosphorylation, interactions of Gsk3β with presenilin, facilitation of apoptosis and modulation of cholinergic function.

Several studies provide evidence that alteration in the control of Gsk3β occurs in AD. Increased levels of Gsk3β have been found in AD brain and immunohistochemical studies have co-localized Gsk3β with NFT in AD brains (Yamaguchi et al., 1996, Imahori and

Uchida, 1997, Pei et al., 1997). Additionally, active form of Gsk3β, phosphorylated at Tyr216, has been found to accumulate in pretangle neurons (Pei et al., 1999).

In vitro, Gsk3β phosphorylates tau at most sites, which become hyperphosphorylated in AD (Lovestone et al., 1994) and cellular phosphorylation of tau by Gsk3β reduces its affinity to microtubules (Lovestone et al., 1996, Wagner et al., 1996). Overexpression of Gsk3β in mice brain results in hyperphosphorylation of tau and neurodegeneration (Lucas et al., 2001). Gsk3β inhibitor lithium is able to reduce tau phosphorylation both in cultured neurons and in vivo rat brain (Munoz-Montano et al., 1997).

Gsk3β utilizes a phosphate as a part of its recognition requirement and many of the Gsk3β substrates, tau among them, require prior phosphorylation of a priming kinase (Fiol et al., 1988). Part of the Gsk3β-mediated tau phosphorylation may thus be hierarchical. Prior phosphorylation of tau by Cdk5 is reported to enhance the subsequent phosphorylation by GSK3β (Sengupta et al., 1997, Li et al., 2006).

Mitogen-activated protein kinases

MAPK are a family of kinases that are activated in response to extracellular stimuli by dual phosphorylation at conserved Thr and Tyr residues (Robinson and Cobb, 1997). The MAPK module is composed of at least three kinases in a sequential activation pathway (Kyriakis and Avruch, 2001). MAPK are the final kinases in the cascade and phosphorylate substrates on Ser and Thr residues. The majority of MAPK substrates are transcription factors but they can also phosphorylate other substrates including protein kinases, phospholipases and cytoskeletal proteins such as tau (Kyriakis and Avruch, 2001).

All MAPK pathways, i.e. JNK, ERK and p38, are activated in AD brains suggesting their involvement in the pathogenesis of AD (Pei et al., 2001, Ferrer et al., 2001, Zhu et al., 2001). All these kinases are also able to phosphorylate tau in vitro at many of the sites identified in PHF tau (Reynolds et al., 2000, Yoshida et al., 2004).

Amyloid precursor protein and plaques

The other major histopathological hallmark of AD is the deposition and accumulation of Aβ into plaques in the brain parenchyma and cerebral blood vessels. APP from which Aβ is derived is a transmembrane glycoprotein expressed in most tissues. The precise biological function of APP is not known at present but it has been suggested to regulate trophic

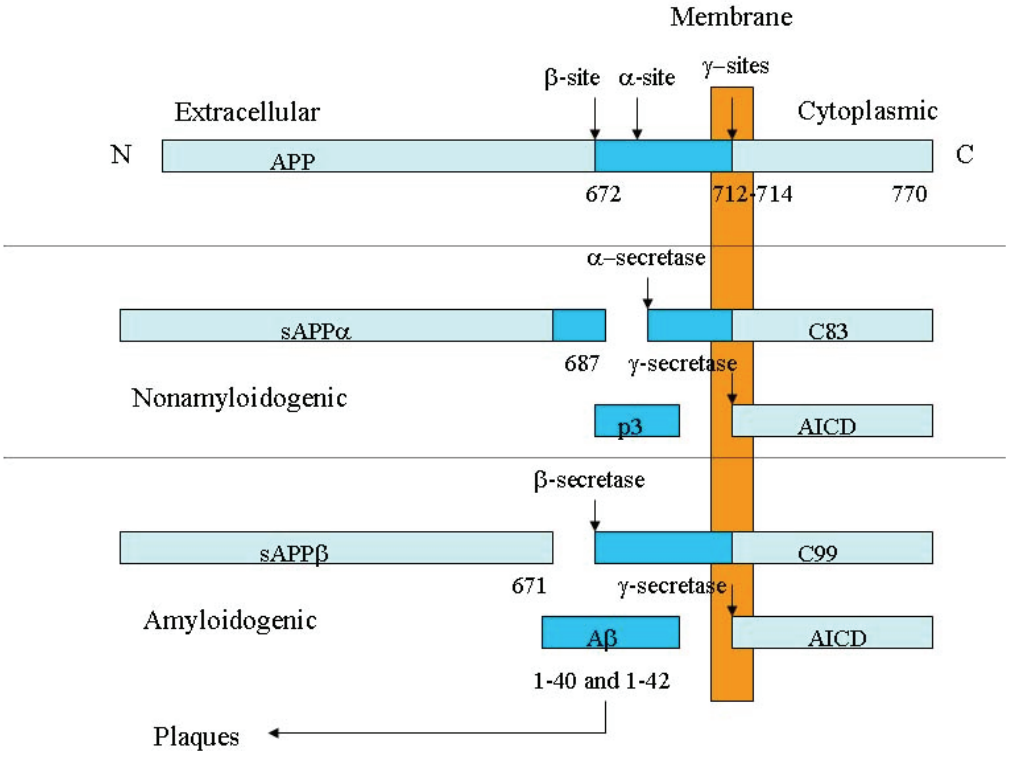

Figure 4. Schematic illustration of the nonamyloidogenic and amyloidogenic APP processing pathways.

functions, cell adhesion, neurite outgrowth, neuronal migration, synaptic function and induction of apoptosis (Russo et al., 2005). All these functions occur via interaction with other components in the nervous system such as kinases and adaptor proteins (Reinhard et al., 2005).

APP can be processed in one of the two pathways: the amyloidogenic that leads to Aβ production and the nonamyloidogenic that precludes Aβ formation (Figure 4). In the nonamyloidogenic pathway, cleavage of APP by α-secretase releases a soluble sAPPα fragment. The remaining membrane bound C83 undergoes subsequent γ-secretase cleavage releasing AICD and the short p3 peptide. In the amyloidogenic pathway, β-secretase cleaves APP releasing the soluble sAPPβ fragment followed by γ-secretase cleavage of the membrane bound C99 fragment liberating AICD and Aβ peptide.

α-secretase activity is mediated by two proteases ADAM 10 and ADAM 17 (Lammich et al., 1999, Buxbaum et al., 1998). Most of the β-secretase activity originates from BACE1 (Vassar et al., 1999). γ-secretase is a multiprotein complex comprising four components: the presenilin proteins, nicastrin, pen-2 and aph-1, with presenilin constituting the active site (Yu et al., 2000, Francis et al., 2002, Takasugi et al., 2003).

Aβ is produced constitutively during cell metabolism. The balance of its synthesis and clearance maintains normally the steady-state level of Aβ but in AD synthesis of Aβ appears to be altered. There are normally two forms of this peptide: Aβ40 accounting for about 90% of all Aβ released from the cells and the longer Aβ42 accounting for only about 10% (Scheuner et al., 1996). The majority of FAD mutations cause an increase in Aβ42 levels, increasing the Aβ42/Aβ40 ratio (Scheuner et al., 1996). Aβ42 is more hydrophobic and more prone to aggregate than Aβ40 (Jarrett et al., 1993). Not only increased Aβ42, but also decreased Aβ40 production has recently been reported to be responsible for the increase of the Aβ42/Aβ40 ratio (Bentahir et al., 2006, Theuns et al., 2006).

Amyloid cascade hypothesis

The existence of two distinct pathological lesions in AD brains has led to debate over which one initiates the disease. The amyloid cascade hypothesis that is the most favoured model states that Aβ deposition is an initiator of AD pathogenesis leading to NFT formation, neuronal dysfunction and finally to dementia (Selkoe 1991, Hardy and Higgins 1992). Supporting this theory, individuals with Down's syndrome, who carry an additional copy of the APP gene, generate more Aβ and develop dementia with AD-like neuropathology early in life (Olson and Shaw 1969). All known APP and presenilin mutations that are considered to be causative of AD increase the production of Aβ42, the longer and more amyloidogenic form of Aβ (St George-Hyslop 2000). These observations were important evidence in formulating the amyloid cascade hypothesis. Additional findings supporting the hypothesis of Aβ pathology occurring upstream of the disease include studies showing that Aβ is toxic in vitro (Yankner et al., 1990) and animal models demonstrating that amyloid deposition precedes tangle formation. For instance, mice expressing mutant tau protein show aggravation of NFT pathology after being injected with Aβ42 (Götz et al., 2001) and in triple transgenic mice that overexpress mutant APP and tau on a PS1 mutant knock-in background, amyloid deposition occurred before accumulation of hyperphosphorylated tau (Oddo et al., 2003). Moreover, administration of Aβ specific antibodies reduced not only the Aβ pathology but also early forms of tau pathology in triple transgenic mice. Mutations in tau gene cause neurodegenerative diseases in form of tauopathies but not AD (Goedert and Jakes 2005) further confirming the importance of Aβ in AD. However, amyloid plaques in the absence of

NFT do not alone produce the disease and NFT pathology seems to be required for clinical expression of AD.

The main objection against the amyloid cascade hypothesis has been the weak correlation between plaque load and severity of dementia. After it was shown that the toxicity of Aβ depends on the state of its aggregation (Pike et al., 1991), it has gradually become clear that soluble oligomers of Aβ and not the insoluble amyloid are the toxic species that are probably responsible for dysfunction in AD brains (Bossy-Wetzel et al., 2004). In fact, when the concentration of Aβ in the brain was measured, it was found to be elevated in early dementia and correlate strongly with cognitive decline (Näslund et al., 2000, Religa et al., 2003) and precede tau pathology (Näslund et al., 2000).

Analogous to the discovery that Aβ oligomers and not the plaques are the toxic species, there is evidence indicating that tau oligomers rather than NFT may be the major toxic species. Results obtained from different tau overexpressing mouse models strongly suggest that accumulation of early-stage aggregated tau species, before the formation of NFT, is associated with neurodegeneration and functional deficits such as memory loss (Santacruz et al. 2005, LeCorre et al., 2006, Berger et al. 2007).

Link between Aβ and tau phosphorylation

In vitro studies have shown that Aβ promotes tau phosphorylation possibly through activation of kinases such as Cdk5 (Alvarez et al., 2001, Town et al., 2002, Li et al., 2003, Zheng et al., 2005) and/or Gsk3β (Takashima et al., 1996 and 1998, Hoshi et al., 2003, Wang et al., 2006). Some in vivo evidence has also been obtained of participation of these kinases in Aβ induced tau phosphorylation. For instance, Cdk5 (Otth et al., 2002) and Gsk3β (Tomidokoro et al., 2001) involvement has been suggested in hyperphosphorylation of tau in Tg2576 mice bearing the Swedish APP mutation. Aβ may promote tau phosphorylation either by direct or indirect activation of tau phosphorylating kinases. Indirect mechanism involves Aβ-induced inflammation leading to increased production of pro-inflammatory cytokines, which in turn activate tau phosphorylating kinases such as Cdk5 and MAPK (Sheng et al., 2001, Li et al., 2003, Quintanilla et al., 2004). Aβ induced transport deficits resulting in mislocalization of tau from axons to somatodendritic compartment is also proposed to contribute to tau pathology (Blurton-Jones and LaFerla 2006). An additional mechanism by which Aβ may facilitate NFT formation is by impairment of proteosomal function leading to accumulation of phosphorylated tau (Tseng et al., 2007).

In addition to hyperphosphorylating tau protein, Cdk5 and Gsk3β have been suggested to participate in amyloid plaque formation. Both Cdk5 and Gsk3β phosphorylate the cytoplasmic domain of APP (Iijima et al., 2000, Aplin et al., 1996) and this phosphorylation affects processing of APP (Liu et al., 2003, Lee et al., 2003, Ryder et al., 2003). Both kinases interact with or phosphorylate presenilin-1, a component of the γ-secretase complex (Tesco and Tanzi 2000, Lau KF et al., 2002). Activation of Cdk5 and/or Gsk3β by Aβ could thus initiate a vicious cycle not only increased tau phosphorylation but also further increase in Aβ production as a result.

Treatment strategies

Acetylcholinesterase inhibitors (AChE-I) and NMDA-receptor antagonists are the most commonly used treatments for AD (Farlow and Cummings 2007). AChE-Is compensate for in AD occurring impairment in cholinergic neurotransmission. They inhibit acetylcholine esterase (AChE), an enzyme responsible for breakdown of acetylcholine (ACh) and thereby increase the synaptic level of this neurotransmitter. NMDA-receptor antagonists protect against excessive activity of the excitatory neurotransmitter glutamate. AchE-I therapy is usually the first hand choice for patients with mild to moderate AD whereas treatment with NMDA-receptor antagonist benefits patients with moderate to severe AD. These drugs only offer symptomatic relief and consequently, there is large need for novel disease-modifying therapies.

Inhibitors of BACE and γ-secretase are potential therapeutic targets in disease-modifying therapies. β-secretase initiates and is the rate-limiting step in the formation of Aβ. BACE1, a major neuronal β-secretase (Cai et al., 2001), provides an attractive drug target since BACE1 -/- knock out mice are viable and fertile suggesting that inhibition of BACE1 is unlikely to be associated with toxicity (Luo et al., 2001). A number of BACE1 inhibitors have been described (John 2006) and some of them have been demonstrated to lower Aβ in APP transgenic mice (Chang et al., 2004, Asai et al., 2006, Hussain et al. 2007). However, these currently available BACE inhibitors are large molecules that poorly penetrate the blood-brain barrier.

The final step in the generation of Aβ from APP involves cleavage by γ-secretase. Treatment with γ-secretase inhibitors (GSIs) has been shown to reduce the amyloid burden in animal model of AD but is accompanied with extensive side effects (Wong et al., 2004). γ -secretase has many biological substrates including Notch and the toxicity of GSIs can be

explained by that they unselectively inhibit cleavage of other substrates as well. A new promising class of compounds γ-secretase modulators, that are actually non-steroidal anti-inflammatory drug analogues, inhibit the amyloidogenic function of γ -secretase without interfering with Notch signalling or processing of other substrates. In murine models of AD, a γ-secretase modulator (R)-flurbiprofen lowered brain levels of $A\beta_{42}$, reduced amyloid pathology and improved learning and memory (Geerts 2007). In clinical trials, AD patients treated with (R)-flurbiprofen demonstrated improvement in cognitive and behavioral performance (Geerts 2007).

In the field of plaque-focused therapies, immunological clearance of Aβ from the brain is an alternative approach. In mice, active immunization with Aβ was shown to reduce number of plaques and improve performance in memory tests (Schenk et al., 1999, Janus et al., 2000, Morgan et al., 2000) but the first trial in humans with this agent had to be terminated due to severe side effects (Orgogozo et al., 2000). However, increased clearance of plaques and improved performance in cognitive tests could be demonstrated even in humans as a result of Aβ immunization (Hock et al., 2003). Owing to these positive effects, the use of shorter Aβ immunogens and passive immunization are currently being investigated (Weiner and Frenkel, 2006).

Abnormal hyperphosphorylation of the tau protein appears to be the most deleterious step in tau aggregation and NFT formation and offers thus a pivotal therapeutic target for treatment of AD. Abnormally hyperphosphorylated tau isolated from AD brain (AD P-tau) self-assembles into bundles of PHF/SF. Dephosphorylation of AD P-tau by PP-2A inhibits its polymerization into PHF and restores its ability to promote microtubule assembly whereas dephosphorylated AD P-tau phosphorylated sequentially with Cdk5 and Gsk3β again self-assemble into PHF similar to those seen in AD brain (Wang et al., 2007). These findings suggest that activation of PP-2A and/or inhibition of both Cdk5 and Gsk3β are promising therapeutic targets for inhibition of NFT formation in AD and related tauopathies. Interestingly, an NMDA receptor antagonist Memantine used to treat AD patients inhibits hyperphopshorylation of tau both in cultured hippocampal slices and in AD brain (Li et al., 2004, Degerman Gunnarsson et al., 2007) probably by increasing PP-2A activity (Chohan et al., 2006).

Assays for tau phosphorylating kinases

Ser-Thr kinases share a high degree of three-dimensional structural fold and sequence homology, which makes development of selective inhibitors a difficult task. Some small molecule inhibitors of Gsk3 that are highly selective over other phylogenetically related kinases have been developed in recent years (Patel et al., 2007). Some of them are shown to reduce tau phosphorylation in cell cultures (Bhat et al., 2003) and in tau transgenic mice (Noble et al., 2005) or in postnatal rat brain (Selenica et al., 2007).

All currently available Cdk5 inhibitors are 'semi-selective' and in addition to Cdk5 they also inhibit both Cdk1 and Cdk2 or only Cdk2 (Sausville 2002, Rzasa et al., 2007). In the process of identifying new compounds within industrial drug research, initial filtering is usually done in biochemical assay and the most promising candidates are then tested further in cellular environment. As Cdk5/p35(p25) complex is almost exclusively localized to the cells of neuronal origin, it has been difficult to find convenient cellular systems for this kinase. Assay development is thus essential to enable validation of compounds designed to inhibit Cdk5.

AIMS OF THE STUDY

The general aim of this study was to develop in vitro models for tau phosphorylating kinases, with an emphasis on Cdk5, and to investigate the possibility to use these in vitro models for screening of kinase inhibitors.

The specific aims for each study were to:

Paper I.

- Investigate the effect of differentiation with retinoic acid alone or sequentially with retinoic acid and brain-derived neurotrophic factor on tau phosphorylation and the levels of tau phosphorylation kinases Cdk5/p35, Gsk3β and JNK in SH-SY5Y cells
- Pharmacologically assess the role of these kinases in tau phosphorylation by using specific kinase inhibitors

Paper II.

- Evaluate the effect of different neurotoxic stimuli on Cdk5/p35 protein levels, Cdk5 kinase activity and tau phosphorylation in differentiated SH-SY5Y cells
- Evaluate the effect of p25 transfection on tau phosphorylation
- Confirm Cdk5 involvement in tau phosphorylation by pharmacological treatment with a Cdk5 inhibitor

Paper III.

- Investigate whether $A\beta_{(25-35)}$ can induce neurodegeneration in organotypic hippocampal slice cultures
- Investigate the effect of $A\beta_{(25-35)}$ on tau phosphorylation and levels of tau phosphorylating kinases Cdk5/p35 and Gsk3β in these cultures

Paper IV.

- Search for Cdk5 substrates in Cdk5/p25 transfected HEK293 cells
- Confirm the role of Cdk5 in substrate phosphorylation by pharmacological treatment with Cdk5 inhibitors
- Evaluate the use of Cdk5/p25 transfected HEK293 cells for assay purposes with phosphorylation of Cdk5 substrate as a read-out

RESULTS AND DISCUSSION

The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau phosphorylation

Increasing knowledge of kinases involved in tau hyperphosphorylation has raised the possibility to treat AD patients with kinase inhibitors. To enable development and screening of selective kinase inhibitors, well-characterized cell models are needed. In paper I, we investigated the potential use of the human neuroblastoma SH-SY5Y cell line differentiated with retinoic acid (RA) alone or sequentially with RA and BDNF.

Morphologically, BDNF was found to enhance the differentiating effect of RA. RA-BDNF differentiated cells displayed a more mature neuronal morphology with longer neurite extensions, that occasionally even connected the cells, than cells differentiated with RA alone.

The tau isoform detected both in undifferentiated and differentiated SH-SY5Y cells was of approximately 48 kDa, which corresponds to the molecular weight of fetal tau. Differentiation with RA increased tau content in the cells although the increase was more prominent in RA-BDNF differentiated cells. Tau phosphorylation occurred at low levels in undifferentiated cells. A modest increase was seen in cells differentiated with RA alone, whereas RA-BDNF differentiation induced a prominent increase in tau phosphorylation in all examined epitopes: Ser199, Ser202, Thr205, Ser396 and Ser404, all of which are hyperphosphorylated in AD brain. Highly phosphorylated tau in RA-BDNF differentiated SH-SY5Y cells coincided with morphologically evident neurite outgrowth, which is in line with previous observations (Brion et al., 1994). The transient hyperphosphorylation of tau that occurs in developing neurons has indeed been found to be very similar to the pathological phosphorylation of tau in NFT (Brion et al., 1993 and 1994).

Examination of tau phosphorylating kinases revealed increased levels of total Gsk3β as well as the activation-associated phosphorylation at Tyr216 both in RA and RA-BDNF differentiated SH-SY5Y cells. Levels of p35, a neuron-specific activator of Cdk5, were increased in RA differentiated cells and an additional increase was seen after subsequent treatment with BDNF. Levels of Cdk5 were only slightly higher in RA and RA-BDNF differentiated cells as compared to undifferentiated controls. Levels of p-JNK and total JNK were unaffected by differentiation.

Although RA alone was able to increase the levels of active Gsk3β and p35, a Cdk5 activator, a protocol with RA and BDNF was chosen for further experiments because this treatment gave highly phosphorylated tau and thus a better signal to noise ratio for screening purposes than RA alone. To pharmacologically validate the assay and to elucidate the contribution of the three examined kinases to tau phosphorylation, three documented reference compounds were used; Gsk3β inhibitor lithium, Cdk5 inhibitor Roscovitine and JNK inhibitor SP600125. Lithium inhibited concentration-dependently tau phosphorylation at Ser199, Ser396, Thr205 and Ser202 in this order of potency but not at Ser404. Similar results have been obtained in rat hippocampal cultures where lithium was able to inhibit tau phosphorylation at the Ser199, Ser396 and AT8 epitopes but not at Ser404 (Takahashi et al., 1999). Roscovitine induced a very modest decrease in tau phosphorylation only in one epitope, Ser404 indicating that Cdk5 plays a minor role in tau phosphorylation in the described system. JNK was not involved in tau phosphorylation in this cell system.

Of the investigated kinases, only Gsk3β was clearly involved in tau phosphorylation. The prominent increase in tau phosphorylation after BDNF treatment cannot solely be attributed to Gsk3β, since BDNF per se did not increase the levels of active Gsk3β. In addition to the three kinases we studied, other Pro directed Ser/Thr kinases could contribute to tau phosphorylation in our cell system. For instance ERK1/2 that is activated by BDNF (Encinas et al., 2000) has been shown to be capable of phosphorylating tau in SH-SY5Y cells (Ekinci et al., 1999).

The Gsk3β-inhibitor lithium reproducibly inhibited tau phosphorylation in a wide concentration range, 0.5-20 mM, indicating that this model can be used to rank compounds. RA-BDNF differentiated SH-SY5Y cells could thus serve as a suitable model for screening of Gsk3β inhibitors.

Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells

One of the tau phosphorylating kinases with relevance in AD has been suggested to be Cdk5. The proposed mechanism leading to pathological Cdk5 activity is through induced cleavage of p35 to a proteolytic product, p25. We investigated whether differentiated SH-SY5Y cells could provide an in vitro model system to study the mechanisms of Cdk5 activation and Cdk5 mediated tau phosphorylation. In paper I we demonstrated increased tau phosphorylation and p35 levels as a result of RA-BDNF differentiation. However, activity of Cdk5 in these cells is low and its involvement in tau phosphorylation minor. Therefore, in paper II, we treated SH-SY5Y cells with neurotoxic stimuli or transfected with a truncated activator, p25 in order to activate Cdk5.

SH-SY5Y cells were treated with calcium ionophore A23187 to increase intracellular levels of calcium. This treatment resulted in calpain-mediated cleavage of p35 to p25, although this was only seen at concentrations that were toxic to the cells. A23187 caused morphologically evident damage to the neurites and dephosphorylation and loss of tau, a finding that is in line with a previous report (Kerokoski et al., 2002). Treatment of the cells with H_2O_2 to cause oxidative stress slightly reduced the p35 levels but no p25 fragment could be detected.

Stimulation of SH-SY5Y cells with the excitotoxic agent glutamate increased tau phosphorylation concomitant with an increased Cdk5 activity achieved by upregulation of Cdk5 and p35 protein levels. Previously, increased expression of both Cdk5 and p35 with subsequent Cdk5 activation has been shown to occur after treatment with oxidative stress causing agents (Strocchi et al., 2003). There was no evidence of calcium mediated calpain cleavage of p35 to p25 as a result of glutamate treatment in SH-SY5Y cells, which might be due to low density of NMDA receptors. In primary neuronal cells p35 cleavage to p25 in response to glutamate is shown to be dependent of the time in the culture with more mature cultures being more susceptible to glutamate (Kerokoski et al., 2004). This is probably attributable to increased NMDA receptor subunit expression over time in culture (Cheng et al., 1999).

p25 is reported to activate Cdk5 more efficiently and induce tau phosphorylation to greater extent than p35 (Patrick et al., 1999, Hashiguchi et al., 2002, Zheng et al., 2002). Because p25 could be detected in SH-SY5Y cells only after a treatment that was apparently toxic to the cells, we chose to transfect p25 to the cells. Two epitopes Ser404 and Ser202

were examined in the transfected cells but increased tau phosphorylation was detected only at Ser404. Glutamate induced tau phosphorylation was also detected at Ser404 but not at Ser202 epitope. Ser202, Ser235 and Ser404 are the major reported phosphorylation sites for Cdk5 in vitro and in neurons (Sakaue et al., 2005) but we have not been able to demonstrate Cdk5 mediated tau phosphorylation in SH-SY5Y cells on epitopes other than Ser404 (paper I and II). A different phosphorylation pattern was reported in SH-SY5Y cells transfected with a p25 inducible expression vector (Hamdane et al., 2003). The AD2 epitope (Ser396/Ser404) was unchanged whereas increased phosphorylation was detected at the AT8 epitope (Ser202/Thr205). A possible explanation for this difference might be the fact that Hamdane et al. used SH-SY5Y cells constitutively expressing an adult tau isoform whereas only fetal tau is expressed in our cell model.

Somewhat unexpectedly, the increase in tau phosphorylation induced by p25 transfection could not be inhibited with the Cdk5 inhibitor Roscovitine. This finding prompted us to investigate involvement of additional kinase signalling pathways. We found elevated levels of the active phosphorylated form of ERK kinases, pERK1/2 in Roscovitine treated cells. Cross talk between Cdk5 and the MAPK pathways occurs probably by feedback down regulation of the MAPK pathway by Cdk5 inactivation of MEK1. Vice versa, when Cdk5 is inhibited, ERK1/2 seems to increase its activity both in vitro and in vivo (Sharma et al., 2002, Whittaker et al., 2004, Zheng et al., 2007). Cdk5 and ERK1/2 share some common substrates such as the NF-M subunit of neurofilament (Sharma et al., 2002) but impact of their cross talk on tau phosphorylation has not previously been demonstrated. ERK1/2 has, however, been shown to phosphorylate tau and Ser404 is a reported ERK phosphorylation site in SH-SY5Y cells (Ekinci et al., 1999). Increased ERK1/2 activity and its involvement in tau phosphorylation could thus explain the fact that after Roscovitine treatment, Ser404 phosphorylation does not decrease even though Cdk5 should be inhibited both due to a Roscovitine action and degradation of p25, an additional finding of this study.

p25 was degraded as a result of Roscovitine treatment both in differentiated and undifferentiated SH-SY5Y cells that were transfected with p25. This effect was almost completely blocked by the proteasome inhibitor Lactacystin, indicating that p25 was degraded via a proteasome. Olomoucine, an additional Cdk5 inhibitor, was also able to degrade p25. p35 has previously been shown to be degraded by a proteasome (Patrick et al., 1998) but this has not been reported for p25.

Increased tau phosphorylation at the S^{396} epitope after Aβ-exposure in organotypic cultures

Organotypic cultures retain important anatomical features of the tissue they are derived from. These cultures contain all the cell types of the original tissue, making them more similar to the in vivo brain compared to cell lines or primary cultures. Aβ, a main constituent of amyloid plaques, is reported to induce neurodegeneration and increase tau phosphorylation in organotypic cultures (Johansson et al., 2005). We thus investigated whether Aβ-treated organotypic cultures of hippocampus, the most affected brain area in AD, could constitute a relevant model to study tau phosphorylation. There are two possible culturing methods, as roller-drum or membrane cultures. We chose to culture hippocampal slices on membranes using interface method (Stoppini et al., 1991). The final thickness of membrane cultures provides sufficient material for Western blotting and enables more exact quantification of changes of interest. $A\beta_{(25-35)}$ was used as this peptide has many similarities to full-length Aβ in terms of fibril formation and toxicity (Pike et al., 1995) and is more convenient to handle.

Fluoro-Jade B staining, a histological marker of neurodegeneration, increased in intensity after $A\beta_{(25-35)}$ exposure (50 μM, 96 h) indicating ongoing neurodegeneration in the cultures. Treatment with $A\beta_{(25-35)}$ significantly increased phosphorylation at Ser396 epitope whereas the reverse control peptide $A\beta_{(35-25)}$ did not alter levels of phosphorylated Ser396. The other investigated phosphotau epitopes Ser199, Ser202, Thr205 and Ser404 showed no significant alterations following Aβ exposure. In hippocampal primary cultures, $A\beta_{(25-35)}$ was shown to induce about a 6-fold increase in immunoreactivity at the Ser396 epitope, whereas phosphorylation at other epitopes such as Ser199, Ser202 and Ser404 increased 2-4 fold (Takashima et al., 1998).

According to current opinion, soluble oligomers of Aβ and not the insoluble amyloid are the toxic species (Bossy-Wetzel et al., 2004). As $A\beta_{(25-35)}$ starts to aggregate immediately after addition to the cell culture medium and forms large aggregates within 2 days (Johansson et al., 2005), the time the cultures are exposed to toxic oligomers is probably quite limited. In addition, membrane cultures are not in direct contact with medium. Aβ containing medium was therefore added on top of the slices every day during the exposure period. The effect of $A\beta_{(25-35)}$ in hippocampal slices may thus be local and very circumscribed. After $A\beta_{(25-35)}$ treatment, the slices are homogenized and changes in tau phosphorylation detected with Western blot. Thus, due to properties of $A\beta_{(25-35)}$, culturing method and limitations in

detection method, it may not be possible to detect changes in tau phophorylation in epitopes other than the heavily phosphorylated Ser396.

In organotypic slice cultures, immunohistochemical Ser396-labeling was mainly localized to the dentate gyrus-area of the hippocampus. In 21-day-old rats, the strongest Ser396 immunoreactivity was also seen in dentate gyrus but some fainter staining was detected in the pyramidal cell layer of the hippocampus (Takahashi et al., 2000). Although the age of the rats corresponds well to the age of the cultured slices, our model is still in vitro and cannot fully be compared with in vivo brain. No clear change in Ser396 staining intensity or size of stained areas could be detected with immunohistochemistry.

We also investigated levels of tau phosphorylating kinases in organotypic hippocampal slices. The level of activation-associated Gsk3β Tyr216 phosphorylation was significantly increased in $A\beta_{(25-35)}$ exposed cultures. The level of total Gsk3β was slightly elevated whereas the inactivation-associated Gsk3β Ser9 phosphorylation was unaltered. No change in Cdk5 and p35/p25 levels was detected after $A\beta_{(25-35)}$ exposure.

The inability of $A\beta_{(25-35)}$ to induce the cleavage of p35 to p25 may be due to the phosphorylation of p35 in fetal tissue, that makes it resistant to cleavage (Saito et al., 2003). Aβ treatment has, however, been reported to result in calpain-mediated cleavage of p35 to p25 in primary neuronal cultures (Lee et al., 2000) whereas in animal models mimicking the amyloid pathology, both increased and unaltered levels of p25 have been reported (Otth et al., 2002, Tandon et al., 2003).

Phosphorylation at Tyr216 increases Gsk3β enzyme activity, whereas Ser9 phosphorylation reduces its activity. Since we found no increase in Ser9 phosphorylation that could counterbalance the increased Tyr216 phosphorylation, we conclude that Gsk3β activity was elevated due to $A\beta_{(25-35)}$-exposure. Aβ-treatment has been reported to increase Gsk3β activity in different cell culture systems (Takashima et al., 1998, Cedazo-Minquez et al., 2003). Increased levels of active Gsk3β together with unchanged levels of Cdk5 and p35/p25 and the fact that Ser396 is a Gsk3β phosphorylation site (Ishiguro et al., 1992) suggest that Gsk3β is the major contributor to Aβ-induced tau phosphorylation in organotypic hippocampal cultures.

Identification of non-muscle myosin heavy chain as a substrate for Cdk5 and tool for drug screening

Deregulated activation of Cdk5 is implicated in AD and several other CNS disorders thus making it a potentially important target for drug research. One of the restricting factors for developing specific Cdk5 inhibitors is the lack of cellular in vitro assay systems. Active Cdk5/p35(p25) complex is almost exclusively localized to the cells of neuronal origin making it difficult to find easy-to-handle cell lines for assay purposes. In paper IV, we investigated use of a non-neuronal cell line human embryonic kidney 293 (HEK293) transfected with Cdk5 and p25.

To identify suitable substrates for Cdk5 we utilized an antibody that recognizes phospho serine in a consensus motif for Cdk substrates. A band with molecular weight of approximately 200 kDa appeared in cells that were double transfected with Cdk5 and p25 but did not show up in non-transfected or single transfected cells. This band could be inhibited with Roscovitine as well as other Cdk5 inhibitors. We thus concluded that 200 kDa band is regulated by Cdk5. A 200 kDa band was identified as non-muscle myosin heavy chain II, type B (NMHC-B), by mass spectrometry. Verification that the 200 kDa band indeed was myosin heavy chain was done by immunoprecipitation. When Cdk5/p25 transfected samples were immunoprecipitated with pSerCdk substrate antibody and then run on Western blot, the pSerCdk substrate antibody and NMHC-B antibody detected the same band. Cdk5 was also found to phosphorylate NMHC-B in SH-SY5Y cells.

NMHC are phosphorylated by kinases such as protein kinase C and casein kinase-2 (Redowicz 2001). Phosphorylation of NMHC by Cdk5 has not been reported previously although a link between Cdk5 and non-muscle myosin was recently discovered. A specific interaction was shown to occur between Cdk5 activator p39 and non-muscle myosin essential light chain (Ledee et al. 2005).

Many of the Cdk5 substrates are cytoskeletal proteins and Cdk5 plays thus an important role in regulation of cytoskeletal dynamics (Dhavan and Tsai 2001). Non-muscle myosin identified as a Cdk5 substrate is together with actin a part of an actinomyosin cytoskeleton. Phosphorylation of myosin heavy chains is reported to inhibit filament assembly (Murakami et al., 2000, Rosenberg et al., 2006). Non-muscle myosin functions as a contractile protein when it is in filamentous form and increased phosphorylation of its heavy chains leads to filament disassembly allowing changes in cell shape required for cytokinesis, cell movement and secretion. Both undifferentiated and RA-differentiated SH-SY5Y are dividing cells.

Differentiation of SH-SY5Y cells with RA results in neurite outgrowth, a process that also requires remodelling of the cytoskeleton. Phosphorylation of NMHC in these cells might thus have a function in cell division and neurite outgrowth.

We also suggest that Cdk5/p25 transfected HEK293 cells can be used to screen for Cdk5 inhibitors. Initial filtering of newly synthesized compounds is usually done in biochemical assay such as scintillation proximity assay (SPA) and the most promising candidates need then to be tested in cellular environment. The compounds tested in the present setting represent different chemical classes: the well known Cdk inhibitor Roscovitine, a reference compound from Warner-Lambert Company (Booth et al., 2001), and a compound with an additional distinct structural class identified in AstraZeneca (Malmström and Viklund 2006). A close analogue to AZ compound was co-crystallised with the Cdk5/p25 complex and the X-ray structure showed that the ligand is not directly bound to the backbone in the ATP site of the kinase, as is the usual case. Instead, a water molecule was found to form a bridging interaction between the ligand and the hinge backbone (Malmström et al., unpublished results). The AZ compound is assumed to bind in this unusual fashion, although X-ray crystallography would be required to confirm this.

These three compounds were first run in SPA assay, in which they concentration-dependently inhibited phosphorylation of peptide substrate by a recombinant Cdk5/p25. Thereafter compounds were tested in Cdk5/p25 transfected HEK293 cells where NMHC-B phosphorylation was analysed by Western blot. All the three compounds demonstrated clear concentration dependent response whereas a non-kinase inhibitor had no effect on NMHC-B phosphorylation demonstrating specificity of the measured signal. The described assay with NMHC-B phosphorylation as a read-out in Cdk5/p25 transfected HEK293 cells is sensitive and specific and could be a useful model for pharmacological characterization of Cdk5 inhibitors.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Abnormal hyperphosphorylation of tau is the most deleterious step in NFT formation making the use of kinase inhibitors an attractive treatment possibility in AD. To enable development and screening of selective kinase inhibitors, well-characterized cellular assays are essential. In this thesis, different cell culture systems were investigated as in vitro models for tau phosphorylating kinases with emphasis on Cdk5.

In paper I, investigation of tau phosphorylating kinases in RA-BDNF differentiated SH-SY5Y cells revealed minor involvement of Cdk5 whereas Gsk3β contributed substantially to tau phosphorylation. Lithium, a Gsk3β-inhibitor, also reproducibly inhibited tau phosphorylation in a wide concentration range indicating that this model can be used to screen for Gsk3β inhibitors.

The minor involvement of Cdk5 in tau phosphorylation in RA-BDNF differentiated SH-SY5Y cells led to the experiments in paper II that aimed to increase Cdk5 activity. Even in stimulated cells the Cdk5 mediated increase in tau phosphorylation was found to be very moderate or obscured by involvement of other kinases. Another approach to study the mechanims would be by transfecting the SH-SY5Y cells with both Cdk5 and p25 but this is the possibility that we have not investigated yet. Furthermore, since SH-SY5Y cells differentiated with our protocol express only fetal tau, transfection with a longer tau isoform might change the phosphorylation pattern. Also, as AD-related pathological hyperphosphorylation occurs in mature tau isoforms expressed in adult brain, SH-SY5Y cells expressing adult tau isoforms would probably constitute a more relevant model for tau phosphorylation. Although these approaches might increase the signal strength, involvement of other kinases might not be avoided. This was apparent at least in the present setting, where we detected increased levels of active phosphorylated form of ERK1/2, another tau phosphorylating kinase, in the cells where Cdk5 was inhibited. This probably occurs as a result of reciprocal feedback regulation of these two kinases and might be especially prominent in our cell model since RA per se is able to activate ERK1/2 in SH-SY5Y cells (Pan et al., 2005).

An additional finding from studies in SH-SY5Y cells was degradation of p25 via proteasome following treatment with Cdk5 inhibitors, Roscovitine and Olomoucine. p35 is known to be degraded via a proteasome (Patrick et al., 1998) but this has not previously been reported for p25. In the present study, p25 was transfected to the cells and it remains to be

investigated whether the calpain-produced p25 fragment is also degraded via the proteasome and whether Cdk5 inhibitors enhance its degradation.

In paper III, investigation of $A\beta_{(25-35)}$ treated hippocampal organotypic cultures revealed increased phosphorylation at Ser396 epitope probably through activation of Gsk3β whereas Cdk5 involvement was not detected. When activated by Aβ, Gsk3β is reported to phosphorylate even other tau epitopes (Takashima et al., 1998) not seen in this study probably due to technical limitations. The described organotypic culture model, although very useful for characterization of complex neuropathological processes, is not at least in the present setting sensitive enough for screening purposes.

Although very relevant substrate in AD-related research, tau is phosphorylated by multiple kinases and might not be optimal for screening purposes when it is necessary to get a robust and clear signal. Active Cdk5/p35(p25) complex is almost exclusively localized to the cells of neuronal origin making it difficult to find convenient cell models. In paper IV, we identified a novel Cdk5 substrate NMHC-B in non-neuronal cell line HEK293 transfected with Cdk5 and p25. Only Cdk5 phosphorylates NMHC-B in HEK293 cells and its phosphorylation can be concentration-dependently inhibited with Cdk5 inhibitors. Using NMHC-B phosphorylation as a read-out, we established a specific and sensitive cellular assay that allows validation of compounds designed to inhibit Cdk5. Limitation of this system at present is its relatively low throughput caused by the Western blot based quantification. Faster screening could be achieved for instance by using Image analysis in a high content screening setting such as ImageXpress.

Cdk5 mediated phosphorylation appears to be difficult to detect in the studied cell systems except in HEK293 cells where Cdk5 activity was artificially created by transfection. A possible explanation in SH-SY5Y cells could be the differentiating agents used in this study. Both RA and BDNF activate ERK1/2, which is another PDPK that can contribute to tau phosphorylation (Encinas et al., 2000, Pan et al., 2005). Therefore, the failure of Cdk5 inhibitors to reduce tau phosphorylation can possibly be attributed to cross talk of Cdk5 with MAPK pathways, with Cdk5 inhibition resulting in increased ERK1/2 activity. The fact that Cdk5 is a priming kinase for Gsk3β (Li et al., 2006) may also make it difficult to separately and in isolation study its contribution to tau phosphorylation.

With the current knowledge on Cdk5 and other tau phosphorylating kinases and their roles in regulating many physiological functions it can be questioned whether complete

suppression of their activity would give clinical benefits without causing significant side effects. An approach to fine-tune the suppression instead of complete inhibition is a possibility that has been brought up by some researches (Mazanetz and Fischer 2007). Furthermore, considering the multifactorial nature of AD, clinical efficacy may not be achieved by inhibiting one single target protein. Instead, the ideal therapy might be one that interferes with a more upstream target such as Aβ production. For instance, both APP processing with following Aβ release and tau phosphorylation are affected by multiple kinases (Mazanetz and Fischer 2007). Thus treatment with a suitable profile broad-spectrum kinase inhibitor could affect the upstream event, Aβ release, and at the same time also ameliorate tau pathology. However, this approach with multiple targets would require a different screening strategy. A possible way to do this could be to set up an assay designed to measure Aβ-release from relevant cells and quantifying it with antibody-based techniques.

Nevertheless, although potentially not optimal for therapeutic purposes, a selective Cdk5 inhibitor would still be an important research tool for detailed studies of Cdk5 in physiological and pathological processes. Given the situation with difficulties in cellular screening systems and the extremely close homology to the related Cdk's with selectivity problems, this still remains a challenge for drug discovery researches.

MATERIALS AND METHODS

Cell cultures, transfections and treatments

SH-SY5Y cells

The human neuroblastoma SH-SY5Y cells were cultured in medium with equal amount of Minimum Essential Medium (MEM) and Nutrient Mixture Ham's F-12, supplemented with 1% non-essential amino acids and 0-10% heat-inactivated Fetal Calf Serum (FCS). Cells were plated at a density of $4.0x10^3$ cells/cm^2 using cell medium with 10% FCS. Undifferentiated SH-SY5Y cells were grown in medium with 10% FCS for whole culturing period. For differentiation with all-trans-retinoic acid (RA), the cells were switched day after plating to 1% FCS containing medium supplemented with 10 μM RA in which they were cultured for 6 days. RA-BDNF differentiated cells were first treated like RA differentiated cells for 6 days followed by differentiation with 2 nM brain-derived neurotrophic factor (BDNF) in serum-free medium for 48 hours. Cell cultures were treated with kinase inhibitors lithium (Sigma), Roscovitine (Biomol) or SP600125 (synthesised at AstraZeneca) in paper I and with calcium ionophore A23187 (Biomol), H_2O_2 (Sigma) or glutamate (Sigma) in paper II. In some experiments calpain inhibitor calpeptin (ICN Biomedicals Inc.) was added together with A23187.

SH-SY5Y cells were differentiated with RA for transfection experiments. Cdk5 have high endogenous levels of Cdk5 and therefore only p25 plasmid was transfected to the cells. Lipofectamine^{TM}2000 (InVitrogen) was used as a transfection reagent according to manufacturer's instructions. Transfections were carried out for 24 hours and Cdk5 inhibitors Roscovitine (Sigma) or Olomoucine (Sigma) or the proteasome inhibitor Lactacystin (Calbiochem) were present during last 4 hours of the transfection period.

HEK293 cells

Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's Modified Eagle Medium (D-MEM) with 4.5 g/l glucose, 2 mM glutamine and 110 mg/l sodium pyruvate. The medium was supplemented with 1% non-essential amino acids and 10% heat-inactivated FCS. The cells were plated at a density of $2.0x10^5$ cells/cm^2.

HEK293 cells were transfected day after plating with equal amount of p25 and Cdk5 plasmid. Transfections were carried out in the same manner as for SH-SY5Y cells. Treatments with Cdk5 inhibitors Roscovitine, 7-ethyl-4-[(4-fluorophenyl) amino]-3,5,7-triaza

bicyclo [4.4.0.] deca-1,3,5,9-tetraen-8-one (WL compound) or 4-(6-chlorobenzothiazol-2-yl)thiophene-2-sulfonamide (AZ compound) was carried out for 4 h. WL and AZ compounds were synthesized at AstraZeneca. The p25 and Cdk5 genes were cloned into mammalian expression vectors, pcDNA3 and pcDNA3.1(-), respectively and the expression was under the control of CMV promoter.

Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared from Sprague Dawley rat pups (postnatal day 7-9). The rat pups were decapitated and their brains removed and placed in Gey´s balanced salt solution (GBSS). The hippocampus was dissected out and cut in 400 µm slices with a tissue chopper (Mcllwain). Under light microscope, hippocampal slices containing CA1-CA3 and dentate gyrus (DG) were selected out and placed on a Millicell culture-insert (Millipore). The inserts were placed inside 6-well tissue culture-plates containing 1.1 ml of culture medium, allowing the slices to be exposed to oxygen from above and medium from below. The culture medium consisted of Basal medium eagle, BME, (44%), Earle´s balanced salt solution, EBSS, (23%), horse serum (23%), supplemented with (final concentration) glucose (40 mM); L-glutamine (3 mM); HEPES (20 mM); NaCl (136 mM); $CaCl_2$ (2 mM); $NaHCO_3$ (5 mM); $MgSO_4$ (2.5 mM); insulin (1 mg/l); ascorbic acid (0.5 mM) and antibiotic/antimycotic (0.05%)-solution containing penicillin, streptomycin and amphotericin B. The pH was adjusted to 7.2, followed by filtering of the medium.

$A\beta_{(25-35)}$ or the control $A\beta_{(35-25)}$ peptide (Bachem, no H-1192 and H-2964) was dispersed in dH_2O at a concentration of 1 mg/ml and thereafter further diluted in culture medium. The cultures were exposed to 50 µM $A\beta_{(25-35)}$ for 24 h, 48 h, 96 h or 50 µM $A\beta_{(35-25)}$ for 96 h. $A\beta_{(35-25)}$ containing medium was taken from underneath the membrane and pipetted on top of the slices every day during the exposure period to ensure a direct contact of $A\beta_{(35-25)}$ with cultures.

Western blot

Lysis buffers are described in papers I and II for SH-SY5Y cells, in paper IV for HEK293 cells and in paper III for organotypic hippocampal cultures. In addition to lysis, hippocampal slices were also sonicated (4 pulses x 2). Cell lysates were centrifuged at 14 000 rpm (Eppendorf 5417R) for 15 min, supernatants were collected and the protein content in the supernatants was measured using the BCA Protein Assay kit (Pierce).

Samples containing 30-50 µg protein were resolved in 10% NuPage®Bis-Tris gels (InVitrogen) and the proteins were transferred to Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% nonfat dry milk in PBS with 0.05% Tween 20 (PBS-T) for 1 hour at RT and incubated over night at 4°C with indicated primary antibodies. Membranes were washed in PBS-T and incubated with Horseradish-peroxidase (HRP) conjugated secondary antibodies (Amersham Biosciences) for 1 hour at RT. After washing, blots were developed using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences). When needed, membranes were stripped with Restore Western blot stripping buffer (Pierce) for 30 minutes at 50ºC. Average density of the bands was measured in Fluor-S™MultiImager (Bio-Rad) by using Quantity One software.

Immunoprecipitation

Indicated amounts of protein diluted in lysis buffer was precleared for 1 hour at 4°C with 10 µl protein A/G Plus agarose beads (Santa Cruz Biotechnology Inc.). The samples were centrifuged at 14 000 rpm (Eppendorf 5417R) for 1 min and the supernatants were transferred to new tubes. The indicated antibody was added to the supernatants and incubated rotating over night at 4°C. Thereafter, 20 µl protein A/G Plus agarose beads was added and incubated for 1 hour. The samples were centrifuged at 6000 rpm for 2 min. The supernatants were discarded and the pellets washed three times in lysis buffer. The immunoprecipitates were then diluted in lysis buffer and processed for Western blot.

Scintillation Proximity Assay

The experiments were carried out in duplicate with 10 different concentrations of the Cdk5 inhibitors in clear-bottom 384-well microtiter plates. Recombinant human Cdk5/p25 was added to assay buffer. After incubation for 15 min the reaction was initiated by the addition of a biotinylated peptide substrate, Biotin-Ala-Lys-Lys-Pro-Lys-Thr-Pro-Lys-Lys-Ala-Lys-Lys-Leu-OH (Bachem), [γ^{33}P]ATP, unlabelled ATP and 10 mM $MgCl_2$. After incubation for 40 min at RT, each reaction was terminated by the addition of stop solution containing streptavidine coated SPA beads (Amersham). The microtiter plates were centrifuged for 2 min at 200 g and the radioactivity was determined in a liquid scintillation counter 1450 MicroBeta Trilux (Wallac). The inhibition curves were analysed by non-linear regression using Graph Pad Prism.

Immunohistochemistry

Hippocampal slices were rinsed in PBS and fixed in 4% paraformaldehyde for 4 h at +4°C. The slices were removed from the membranes and immunostaining was performed directly on free-floating slices without sectioning. Cultures were blocked 1 h in 1% BSA, rinsed in PBS and incubated with indicated primary antibody for 72 h at +4°C diluted in PBS containing 0.3% Triton X-100 and 0.5% normal goat serum. After washing, sections were incubated with biotinylated secondary antibody (ABC-Elite Vectastain kit, Vector) for 30 min at RT followed by DAB reaction (FAST™ DAB Tablet Set, Sigma). The sections were dehydrated, cleared in xylene and mounted on glass slides. The sections were analysed by light microscopy (Nicon Eclipse E600).

Fluoro-Jade B staining

The fixed cultures were immersed in a solution containing 1% sodium hydroxide in 80% ethanol for 5 min, followed by 2 min in 70% ethanol and 2 min in dH_2O. The cultures were transferred to a solution of 0.06% potassium permanganate for 10 min on a shaker table. After rinsing in dH_2O the cultures were placed in the staining solution containing 0.0004% Fluoro-Jade B in 0.1% acetic acid for 20 min. After rinsing, the cultures were dried, cleared in xylene and mounted. The sections were analysed by fluorescence microscopy (Nicon Eclipse E600).

Mass spectrometry

Protein spots of interest were excised from Sypro Ruby® (Molecular Probes)-stained gels using a spot cutter robot (Bio-Rad) and transferred to ethanol-washed 96-well plates and the in-gel digestion was performed. Briefly, gel plugs were rinsed and incubated for 30 min with ddH_2O, and then all liquid was removed. Gel pieces were further incubated for 10 min with 25 mM NH_4HCO_3/CH_3CN (1:1v/v). CH_3CN was added to shrink gel pieces, the liquid was discarded and gel pieces dried using a Speed Vac evaporator. Gel plugs were rehydrated by incubating with digestion solution containing 10 ng/µl of trypsin in 25 mM NH_4HCO for 30 at RT. The samples were then placed on a heater at 37°C over night. One-hour incubation with 1% HCOOH stopped the reaction. To concentrate and desalt the samples ZipTip (Millipore) was used according to manufacturers descriptions. The samples were then eluted with matrix solution (Waters Corporation) direct into 96-position MALDI target plate (Waters Corporation) and crystals of matrix and peptide were formed. MALDI-ToF (Waters Corporation) was used to attain the mass of each peptide and the resulting peaklist was

imported into the search engineers Protein Lynx Global Server 2 (Waters Corporation) and MASCOT (Matrix Sciences Ltd). Several databases were selected as information sources.

Statistics

Statistical significance for multiple variables was determined by using one way analysis of variance (ANOVA) followed by Dunnett's or Fisher's PLSD post-hoc analysis. When only two variables were compared Student's unpaired t-test was used. The significance was set at $p<0.05$.

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