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i) **Title**

Biochemical analysis of genetically-modified mice with learning and memory phenotypes

Florian Plattner

Wolfson Institute for Biomedical Research

University College London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ii) Abstract

Recent advances in molecular genetics have enabled generation of sophisticated genetically-modified mouse models to study specific molecules and their biological function *in vivo*. Here, I investigated biochemical changes in two different genetically-modified mouse lines with previously described learning and memory phenotypes.

Firstly, I analysed biochemical changes in a mouse line carrying a threonine to alanine point mutation at Thr286 of alpha Ca^{2+}/calmoduline-dependent kinase II (αCaMKII), which disenables this phosphorylation site. Autophosphorylation at Thr286 switches αCaMKII into an autonomous activity mode. The T286A mutant mice displayed changes in basal phosphorylation levels. In order to study biochemical changes after activity-dependent synaptic potentiation, an *in vivo* long-term potentiation (LTP) approach was established and validated by assessing activity-dependent changes in phosphorylation levels of well-characterised marker molecules including synapsin I and NR2B. Both αCaMKII and βCaMKII exhibited elevated levels of autophosphorylation after LTP stimulation in hippocampal area CA1 and dentate gyrus (DG). This finding indicates that βCaMKII may compensate for the loss of autonomous αCaMKII activity in T286A mutants. Furthermore, induction of LTP triggered phosphorylation of glycogen synthase kinase 3 (GSK3) at its inhibitory site suggesting a role for GSK3 in synaptic plasticity.

Secondly, I investigated a transgenic (TG) mouse line expressing the cyclin-dependent kinase (Cdk5) activator protein, p25, a protein previously linked to some aspects of Alzheimer's disease (AD). The forebrain restricted expression of p25 started postnatally and stayed constant throughout the life-span of the TG mice. The expression of p25 triggered constitutive over-activation of Cdk5 in the TG mice. The p25 TG mice displayed age-dependent hyperphosphorylation of the microtubule-associated protein

tau and age-dependent alterations in the processing of amyloid precursor protein (APP). Furthermore, p25-induced over-activation of Cdk5 led to inhibition of GSK3. This negative regulation of GSK3 was lost in aged p25 TG mice and correlated with the increased tau hyperphosphorylation. The levels of tau phosphorylation in aged p25 mice were reduced after treatment with lithium, an inhibitor of GSK3. These results indicate that GSK3 directly mediates tau hyperphosphorylation, whereas Cdk5 acts indirectly via inhibitory control of GSK3.

iii) Publications arising from this thesis

- Angelo, M., Plattner, F., Irvine E.E., & Giese K.P. (2003). Improved reversal learning and altered fear conditioning in transgenic mice with regionally restricted p25 expression. *Eur. J. Neurosci.* **18**, 423-431.

- Ris, L., Angelo, M., Plattner, F., Capron, B., Errington, M.L., Bliss, T.V., Godaux, E. & Giese, K.P. (2005). Sexual dimorphisms in the effect of low-level p25 expression on synaptic plasticity and memory. *Eur. J. Neurosci.* **21**, 3023-3033.

- Giese, K.P., Ris, L. & Plattner, F. (2005). Is there a role of the cyclin-dependent kinase 5 activator p25 in Alzheimer's disease? *Neuroreport* **16**, 1725-1730.

- Plattner, F., Angelo, M. & Giese, K.P. (2006). The roles of Cdk5 and GSK3 in tau hyperphosphorylation. *J. Biol. Chem.* **281**, 25457-25466.

- Angelo, M., Plattner, F. & Giese, K.P. (2006). Cdk5 in synaptic plasticity, learning and memory. *J. Neurochem.* **99**, 353-370.

- Cooke, S.F.*, Wu, J.*, Plattner, F.*, Errington, M., Rowan, M., Peters, M., Hirano, A., Bradshaw, K.D., Anwyl, R., Bliss, T.V.P. & Giese K.P. (2006). Autophosphorylation of αCaMKII is not a general requirement for NMDA receptor-dependent LTP in the adult mouse *J. Physiol.* **574**, 805-818. (*contributed equally)

- Irvine, E.E., von Hertzen, L.S.J., Plattner, F. & Giese K.P. (2006). αCaMKII autophosphorylation: a fast track to memory. *Trends Neurosci.* **29**, 459-465.

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vii) Abbreviations

3R	three microtubule binding repeats (in the tau protein)
4R	four microtubule binding repeats (in the tau protein)
2DE	two-dimensional gel electrophoresis
AD	Alzheimer's disease
AICD	APP intracellular domain
ALS	Amyotrophic lateral sclerosis
AMP	adenosin mono-phosphate
AMPA	α-amino-3-hydroxy-5 methyl-isoxazole-4-proprionic acid
ANOVA	analysis of variance
APP	amyloid-beta precursor protein
ATP	adenosine tri-phosphate
Bp	base pair
BCA	acid
BSA	bovine serum albumin
cAMP	cyclic adenosin mono-phosphate
CA1 LTP	NMDA receptor-dependent LTP in CA1 neurons
CA3 LTP	NMDA receptor-independent LTP in CA3 neurons
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CaMKIV	Ca^{2+}/calmodulin-dependent kinase IV
Cdk5	cyclin-dependent kinase 5
CBP	CREB binding protein
CJD	Creutzfeldt-Jacob disease
CNS	central nervous system
CPP	3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid; NMDA antagonist
CRE	cAMP responsive element (binding motif in promoter region of genes)
CREB	cAMP responsive element binding protein
CS	conditioned stimulus
CTF	carboxy-terminal fragment (α-, β- and γ-CTF)
DG	dentate gyrus
DG LTP	NMDA receptor-dependent LTP in DG neurons
DMSO	dimethylsulfoxide
DNA	deoxy ribonucleic acid
dNTP	deoxy nucleotide tri-phosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescent
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E-LTP	early phase long-term potentiation
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
ES	embryonic stem
FAD	familial form of Alzheimer's disease
FTDP-17	frontotemporal dementia with parkinsonism linked to chromosome 17
GABA	γ-aminobutyric acid
GluR	glutamate receptor
GSK3	glycogen synthase kinase 3
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase

Kb	kilo base pair
kDa	kilo Dalton
KO	knock-out
L-LTP	late phase long-term potentiation
LTD	long-term depression
LTM/s-LTM	long-term memory; stable long-term memory
LTP	long-term potentiation
mA	milli ampere
mRNA	messenger ribo-nucleic acid
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MARK	microtubule-affinity regulating kinase
MCI	mild cognitive impairment
MEK	MAPK/ERK kinase
MWM	Morris water maze
NFT	neurofibrillary tangles
MLCK	myosin light chain kinase
Munc	mammalian homologue of C. elegans protein uncoordinated
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NPC	Niemann-Pick disease, type C
NR2A/ NR2B	NMDA receptor subunit 2A/2B
NSE	neuron-specific enolase
PAR-1	protease-activated receptor 1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDPK	proline-directed protein kinase
pI	isoelectric point
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PKI	specific PKA inhibitor peptide
PNS	peripheral nervous system
PP1	protein phosphatase 1
PSD	post-synaptic density
PSP	progressive supranuclear palsy
PVDF	polyvinylidene di-fluoride
RIM	Rab3-interacting molecule
RT	room temperature
sAHP	slow after-hyperpolarisation
SAP	synapse-associated protein
sAPPβ	soluble amino-terminal APP fragment cleaved by the β-secretase
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SNAP	soluble *N*-ethylmaleimide-sensitive factor (NSF)- associated protein
SNARE	SNAP attachment protein receptor
STM/l-STM	short-term memory; labile short-term memory
T286A	point mutation in amino acid 286 from Thr to Ala/ also point mutant mouse line
TBS	tris-buffered saline
TBST	tris-buffered saline, tween-20
TG	transgenic
US	unconditioned stimulus
VDCC	voltage-dependent calcium channel
WT	wild type

CHAPTER 1: Introduction

1.1) General introduction

Learning and memory are key aspects of human cognition and their improper functioning manifests in multiple severe cognitive deficits. Therefore, fully understanding the cellular and molecular mechanisms of how the nervous system acquires, transforms and stores information is a major goal in neurosciences. Anatomical, pharmacological and electrophysiological studies have provided general understanding of the processes important for learning and memory. First insights into the mechanism of learning and memory were gained by psychological examination of patients with lesions in specific brain regions and animal lesion studies. Recent advances in molecular and genetic techniques has helped greatly to broaden the understanding of learning and memory on a molecular and cellular level. Much research focused on neurons, which are the basic functional unit of the nervous system. The study of synapses, specialised sites of contact between neurons important for interneuronal signalling helped greatly in identifying molecules that are involved in learning and memory, and in characterising their functions.

Previously, biochemical processes were investigated in *in vitro* systems, cellular cultures and in single-cellular organisms including bacteria and yeast. This work established many molecular mechanisms of basic biological functions, such as the metabolic mechanisms. However, the elucidation of the complex processes used in learning and memory from the molecular to the systems level required the development of novel methods and research strategies. Over the last decades the advances in biochemistry and molecular biology have made it possible to study biological processes *in vivo* in model organisms using spontaneous mutations, targeted genetic deletion or modification of specific molecules. These conventional transgenic and gene knockout technologies have become invaluable experimental tools for modelling genetic

disorders, testing gene functions, evaluating drugs and toxins, and helping to answer fundamental questions in basic and applied research. These techniques helped greatly the advancement in the field of learning and memory research by enabling behavioural studies combined with targeted molecular manipulations in model organisms. Several model organisms including the worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*) and the mouse (*Mus musculus*) have been exploited to explore the mechanisms underlying learning and memory,

1.2) **Generation of genetically-modified mice and recent advances**

Pharmacological approaches helped greatly to characterise molecular and cellular mechanisms important for learning and memory. However, the drawbacks of pharmacological studies are the lack of specificity of the drugs, the absence of pharmacological reagents for many targets, and the difficulty of confining the pharmacological manipulation. Over the last two decades advances in mouse molecular genetic techniques enabled the generation of knock-out (KO), knock-in, targeted point mutation, region-restricted and conditional transgenic mouse models (see for review Mayford & Kandel, 1999; Tonegawa *et al.*, 2003). The generation of genetically-modified mice made it possible to investigate the effect of a specific molecule on learning and memory at different levels from the molecular and cellular to the systems level. Further, with the advent of region-restricted and conditional transgenic mouse models the function of a specific molecule can be analysed in distinct brain regions and tested at different points in time during life. Together, these approaches opened up new avenues to elucidate the molecular mechanisms underlying learning and memory.

The development of embryonic stem (ES) cell technology and gene targeting methods and their application in mice provided fundamental insights into gene function (see for review Bradley *et al.*, 1998; Capecchi, 2005). This reverse genetic technique allows the

introduction of a mutation into the genome of a mouse ES cell by homologous recombination. The genetically-modified ES cells are injected into blastocysts and then implanted into the uterus of foster mothers. The ES cells from the blastocyst develop into cells of all embryonic tissues resulting in chimeric mice. Chimeras are mice that consist of cells derived from the injected ES cells and from the host blastocyst. Chimeric mice, in which the mutation was transmitted to the germ line, are crossed with wild-type mice. In this way off-spring are generated that carry the mutation in every cell of the body.

As changes in synaptic efficacy are thought to underlie learning and memory, the first studies using conventional KO mice focussed on proteins that have been shown to affect the efficacy of synaptic transmission. Electrophysiological studies had implicated the proteins Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and tyrosine kinases in changes of hippocampal long-term potentiation of synaptic transmission (Malinow *et al.*, 1989; O'Dell *et al.*, 1991). Hence, conventional KO mice, where the expression of αCaMKII or the tyrosine kinase, fyn, was abolished, were studied to evaluate the effects of these proteins on learning and memory. Mutant mice lacking αCaMKII displayed deficits in hippocampal long-term potentiation that correlated with an impairment in spatial learning (Silva *et al.*, 1992a and 1992b). Equally, transgenic mice lacking the expression of the protein kinase fyn were impaired in hippocampal long-term potentiation and spatial learning (Grant *et al.*, 1992). However, these mice also had deficits in hippocampal development, which interfered with their cognitive capacity. Therefore, it was impossible to make conclusions about the involvement of fyn in learning and memory processes.

These studies using conventional KO mice showed that the introduction of genetic changes to the germ line of a mouse can be a powerful tool to track down the effects of a particular gene and a meaningful approach to dissecting the molecular mechanisms of learning and memory. However, they exposed also some of the shortfalls of the conventional genetically-modified mice generated by gene targeting techniques. One of

the major problems is that the genetic changes are present throughout the entire life of the mouse. This makes it difficult to distinguish between acute, direct effects of the genetic changes on the phenotype and consequences elicited as a result of prolonged genetic alteration, developmental effects or compensatory mechanisms. Many proteins are implicated in developmental processes and hence, their genetic alteration may have severe developmental consequences, which complicate or even preclude an experimental analysis as demonstrated by, for example, embryonic lethal KO phenotypes (e.g. glycogen synthase kinase 3β (GSK3β) KO mice (Hoeflich *et al.*, 2000) and presenilin 1/2 KO mice (Donoviel *et al.*, 1999)). Another restriction that arises from the constant presence of the genetic changes is adaptive gene expression. In this case the genetic changes trigger an up or down regulation of some other gene products, which may impinge on various cellular processes and may even lead to cytotoxicity and hence preclude an analysis of the effects mediated by the introduced genetic alteration. This has to be distinguished from a regular compensatory mechanism, where another isoform or a molecule with similar function can compensate for the function affected by the genetic changes. Interestingly, such compensatory mechanisms were observed in numerous KO mouse lines (e.g. microtubule-associated protein tau KO mice (Harada *et al.*, 1994) and presenilin 1 KO mice (Yu *et al.*, 2001)). Though the molecules were thought to be crucial for specific cellular processes, the elimination of their expression resulted in no or only mild phenotypic changes.

Another problem with conventional genetically-modified mice is that the genetic changes are present in every cell of the mouse. Because the genetic alteration of molecules might have a different effect in distinct cell types and tissues, this might impact on vital functions or even the viability of the mouse. For example, a transgenic mouse line expressing the Cdk5 activator p25 in all neuronal cells including the spinal cord displays paralysis and is therefore not suitable for learning and memory studies (Bian *et al.*, 2002). Further, the presence of the genetic changes in every cell makes it difficult to relate an observed alteration in cellular processes to the function in a whole tissue; let alone to a behavioural phenotype.

To overcome these undesired limitations intrinsic to conventional gene targeting approaches more advanced genetic approaches have been developed (see for review Mayford & Kandel, 1999; Bockamp *et al.*, 2002). These conditional transgenic strategies allow more precisely controlled gene expression or ablation of gene function in a spatio-temporal fashion. The Cre/loxP system is commonly used and has been successfully applied to generate mouse lines with tissue and cell type specific inactivation of genes (conditional KO mice), with temporal control of gene activation and with spatially and temporally controlled expression of transgenes (see for review Mayford & Kandel, 1999; Bockamp *et al.*, 2002). Regulating the expression or inactivation of genes in a temporal manner helps to overcome the problems linked with developmental artefacts. The inactivation of genes within specific tissue or cell types is a unique tool for investigating the function of gene products in complex physiological system networks such as the brain. One disadvantage here is that few tissue and cell type specific promoters are known. The temporal control of transgene expression or inactivation can be achieved by the use of inducible Cre-recombinase strategies. Different systems are used including the tamoxifen system (see, for example, Danielian *et al.*, 1998). This reversible inducible regulation by switching transgene expression on and off allows the study of the function of a single gene at various time points. This strategy is particularly helpful in investigating the involvement of gene products in learning and memory processes by performing behavioural tasks with or without induction of the transgene or inactivation of the target gene.

Furthermore, the Cre/loxP system can be used to generate mouse lines carrying targeted point mutations (see, for example, Giese *et al.*, 1998). This so-called "point lox" strategy allows investigating the role of specific residues within a protein. This is particularly useful in the study of signalling pathways, where posttranslational modifications, such as phosphorylation, at single residues play an important role. Hence, with this strategy a specific function of a protein linked to the modification can be blocked while other functions remain intact. The use of mice with single point mutations has the advantage over total KO mice that complete loss of the gene product

can lead to compensatory mechanisms, which may not result from the point mutation. For example, in KO mice lacking αCaMKII expression, the β-isoform has been suggested to compensate for the loss of αCaMKII, whereas in the αCaMKII point mutant mouse line T286A no compensation is observed (Giese *et al.*, 1998; Elgersma *et al.*, 2002; see *section 3.2*).

Along with the gene-targeting techniques, another strategy that is often used for generation of transgenic mouse models is the introduction of genes into the mouse genome through random genomic integration (see for review Bockamp *et al.*, 2002). This technique allows targeted expression of the transgene mRNA in different tissues or even cell types in the transgenic mouse by selection of a specific promoter to drive the expression. Other factors that affect the level of expression are the number of copies of the transgene that are integrated into the mouse genome and the chromosomal integration site of the transgene. Learning and memory studies commonly use transgenic mouse lines where the transgene is driven by promoters that target the expression exclusively to neurons including the neuron-specific enolase (NSE), platelet-derived growth factor (PDGF) and αCaMKII promoter. For example, the αCaMKII promoter targets the expression to excitatory glutamatergic neurons in the forebrain (see, for example, Lui & Jones, 1997). The αCaMKII promoter initiates the expression postnatally and maintains constant expression levels. Hence, the transgene is not expressed during embryonic development and developmental artefacts can be excluded. This makes the αCaMKII promoter an ideal promoter to drive transgene expression in transgenic mouse lines used for learning and memory studies.

The advantages of the transgene over-expression approaches over the conventional KO mice in the study of learning and memory are that the expression of the transgene can be restricted to specific brain regions, whereas in most KO mice the gene is deleted everywhere, and that the transgene expression can be initiated at different time points, for example postnatally. However, transgenic mouse models with random integration

mediated over-expression have also their pitfalls. In transgene over-expression approaches the expression level of the transgene can only be partially regulated by selecting specific promoters and enhancers to drive the expression. But the level of the over-expressed transgene depends as well on other factors that can not be regulated, such as the site of transgene integration and the number of copies inserted. Further, the integration of the transgene into the genome occurs in a random fashion. Thus, the expression may be affected by insertion site artefacts. In addition, the insertion into the genome might confound the function of endogenous genes by disrupting their expression. One has to be cautious when comparing different transgene over-expressing mouse lines, because the number of transgene copies, the integration sites and subsequently the transgene expression can vary between mouse lines and may have different effects on the observed phenotype. Reliable results can be obtained by generating and analysing multiple transgenic mouse lines with the same transgene. Comparison of the different mouse lines with distinct transgene levels allows determining to what extent the insertion site and the transgene level contribute to the phenotype and help to distinguish these from the effects mediated by the expression of the transgene.

1.3) **Memory processes: Short-term and long-term memory**

Cognitive studies distinguish a number of different forms of learning and memory involving distinct neuronal systems. From the neuropsychological view, human memory is characteristically divided into non-declarative (or implicit) and declarative (or explicit) memory (see for review Squire *et al.*, 2004). Non-declarative memory involves unconscious recall and includes motor skills, reflexes and emotional responses. Declarative memory involves recollection of factual information through a conscious effort. This form of memory has been attributed to a neuronal circuit, including all

association areas of the neocortex, the parahippocampal region and the hippocampus (see for review Squire *et al.*, 2004). Psychological examination of patients with hippocampal lesions revealed deficits in declarative memory, such as spatial cognition, whereas their non-declarative memory remained intact (see, for example, Scoville & Milner, 1957; see for review Squire *et al.*, 2004). These studies also demonstrated that memory formation is a step-wise temporal process involving distinct mechanisms and that memories pass through characteristic stages over time. Patients with hippocampal lesions can recall old declarative memories, which they acquired long before their lesion, but are unable to form new declarative memories.

In neuropsychology, two forms of memory storage are characteristically distinguished: short-term memory (STM) describes the first period after memory acquisition, whereas long-term memory (LTM) can last for days or years (see for review Milner *et al.*, 1998; McGaugh, 2000a). The study of retrograde amnesic patients suggests that the hippocampus plays a role in the consolidation of some forms of LTM (see for review Squire *et al.*, 2004). It is hypothesised that the hippocampus acts as a modulator that is implicated in the transformation of STM into LTM. One model of how the hippocampus achieves the transformation, is the transfer of acquired information and the relevant contextual associations to other areas of the brain, possibly the cortex. (see for review Frankland & Bontempi, 2005).

The hippocampus is an area of primitive cortex medially located on the underside of the temporal lobe (see for review Amaral & Witter, 1989). It is connected to several neighbouring cortical areas, including the perirhinal, entorhinal and parahippocampal cortices. The human hippocampus receives direct or indirect inputs from all of the main neocortical and thalamic areas, as well as from some limbic structures, including the amygdala. This high level of connectivity indicates a central role of the hippocampus in memory processes and is suggestive of a structure that might integrate high-order information processed in other brain regions.

From a neurobiological view, it is hypothesised that the molecular and cellular mechanisms underlying the different forms of memory may be temporally organised in a way similar to the neuropsychological concept described above (see for review McGaugh, 2000). Indeed, the molecular processes for memory formation can be grouped similarly in a STM and LTM phase. The main distinction between the two phases is that new memories are initially labile during the STM phase, until they are consolidated to form LTM. Hence, the two phases have been termed labile STM (l-STM), which can last from seconds to hours and is sensitive to disruption, and stable LTM (s-LTM), which lasts from hours to months (see for review McGaugh, 2000; McGaugh & Izquierdo, 2000). On a molecular level the stabilisation process is thought to involve changes in synaptic structure and consequently synaptic connectivity. Blocking experiments using inhibitors of transcription or protein synthesis inhibitors demonstrated that l-STM does not require *de novo* protein synthesis (see for review Davies & Squire, 1984). In contrast, s-LTM can be blocked by application of such inhibitors demonstrating the requirement for transcription and protein synthesis. These results are consistent with the observation of changes in gene expression and *de novo* protein synthesis after learning. Hence, it was proposed that the changes in gene expression and protein synthesis occurring in s-LTM are a critical step to establishing and maintaining synaptic changes. However, once the s-LTM is established, the application of these inhibitors does not erase the memory. Thus, it was hypothesised that in a first phase memories are modulated, which requires transcription and protein synthesis. Once memories are established, they are permanently stored within the brain and depend no longer on transcription and protein synthesis.

1.4) **Behavioural studies of learning and memory**

Psychological examination of patients with different brain lesions has revealed selective memory deficits (see for review Squire *et al.*, 2004). Comparably, in animals including primates and rodents brain lesion studies combined with behavioural studies distinguished different forms of memory and helped to dissociate distinct memory systems (see for review Squire, 1992). This work identified the hippocampus as an important brain structure for the processes of learning and the formation of declarative memory both in humans and in animals. It has been found that the hippocampus, in humans and animals, is involved in the transition from immediate to permanent memory and supports specific domains of memory including spatial memory (see for review Eichenbaum, 2000). In common with the observation in amnesic humans, the sensory, motor, motivational and cognitive processes remained intact in animals after hippocampal lesion, as seen, for example, in the visible platform learning task (see for review Squire, 1992; Eichenbaum, 2000). Further, in common with observations in some human amnesic patients, animal studies showed that memories acquired shortly before the hippocampal damage/lesion were lost, whereas memories acquired a long time before the damage were intact. Taken together, these findings demonstrated the similarity of the main characteristics of hippocampal learning and memory processes between humans and animals such as primates and rodents (see for review Squire, 1992). Hence, these studies validate the use of animal models in specific behavioural paradigms to further characterise the neural circuitry and to assess the mechanisms underlying learning and memory.

Hippocampal lesion studies in animals demonstrated that some behavioural tasks can be performed only when hippocampal function is normal (see for review Eichenbaum, 2000; Squire *et al.*, 2004). These tasks are called hippocampus-dependent tasks. Commonly used behavioural tasks to assess hippocampal-dependent learning and memory include the radial arm maze, novel object recognition, social transmission of

food preferences and contextual fear conditioning (see, for example, Eichenbaum, 2000).

1.4.1) Spatial learning in the Morris water maze

One of the most frequently used tasks to test hippocampus-dependent spatial learning in rodents is the Morris water maze (MWM) (see for review D'Hooge & De Deyn, 2001). In this place navigation task the mouse learns to escape from submersion in a pool by swimming towards an invisible platform located just underneath the surface. The animal uses visual cues positioned around the pool to locate the platform. The training protocol consists of several repeated trails over several days. The learning progress can be tested by comparing the escape latency and the length of the swim path of the individual trials. As the animal learns the location of the platform the latency to find the platform decreases and the swim path gets shorter. Memory retention and search strategy can be assessed in probe trials where the platform is removed. The amount of time the mouse spends looking for the platform in the target quadrant, where the platform is normally located, is indicative of memory. Further, memory can be assayed by reversal training in the MWM, where the platform is placed in a new location. At first, the animal searches specifically where the platform was previously located, but in the course of the trials learns the new location. Rats and mice with hippocampal lesions are impaired in the MWM implying the specific involvement of the hippocampus in the place learning component of the task (see for review Eichenbaum, 2000). However, the sensory-motor and procedural components of the task are independent of the hippocampal function as indicated by the normal performance in the MWM if a local cue is attached to the platform. Because animals with impaired hippocampal function may still be able to learn procedural aspects, they can develop a non-spatial strategy to locate the hidden platform. The search strategy of the animal is most appropriately assessed in a probe trial.

1.4.2) Cued and contextual fear conditioning

Contextual fear conditioning is a single trial hippocampus-dependent learning task (see for review Anagnostaras *et al.*, 2001). The animals are placed in a novel environment (context) and given some time to explore and familiarise with it. Later, they receive a brief electric foot shock in this new context. Rats and mice trained in this manner associate the novel context with the aversive shock and develop fear for the context. This learned fear is expressed as defensive behaviour, where the animal displays freezing, increased heart rate and increased blood pressure. The time the animal spends freezing when re-exposed to the novel context is indicative of some forms of memory. Freezing is a natural response manifested in the presence of predators, in order to avoid being detected and becoming prey.

Lesion studies in rodents showed that the hippocampus and the amygdala are involved in the acquisition and expression of learned fear responses in the contextual fear conditioning paradigm (see for review LeDoux, 2000; Anagnostaras *et al.*, 2001). However, the hippocampus is not essential for cued fear conditioning, where the animal has to associate a single cue, in this case a tone, with a foot shock. The animal is presented with a neutral tone (conditioned stimulus = CS), which is paired with a brief electric foot shock (unconditioned stimulus = US) in a novel context. The animals are expected to freeze in response to the tone presentation independently of the novel context. This form of memory is amygdala-dependent as indicated by lesion studies in rats that showed that the amygdala is required for both the acquisition and expression of learned fear responses to explicit cues or a context (see for review LeDoux, 2000).

1.4.3) Behavioural tests in genetically-modified mice as tools for learning and memory research

The advent of gene targeting and transgenic technologies opened up new avenues for the examination of the molecular and cellular mechanisms underlying learning and memory. The generation of transgenic mice combined with their behavioural characterisation helped greatly to study the specific roles of gene products in learning and memory processes (see for review Martin & Morris, 2002; Silva, 2003). Initially, behavioural experiments in knockout mice lacking αCaMKII demonstrated that deficits in long-term potentiation (LTP) of synaptic transmission observed in the hippocampal area CA1 could be correlated with memory impairments as tested in the MWM (Silva *et al.*, 1992a and 1992b). Since then, many more mutant mice have been generated and tested in behavioural tasks to assess the implication of the mutations on brain function. These studies demonstrate that many molecules which have been involved in synaptic plasticity, have also been implicated in the expression of behaviour (see for review Martin & Morris, 2002). Taken together, these studies established the behavioural analysis of transgenic mice as an important method to assess learning and memory mechanisms. The behavioural analysis of more sophisticated transgenic mouse models including region-restricted and inducible mouse lines will help to characterise the role of specific molecules in distinct brain regions and start elucidating the role of individual molecules in the various stages of learning and memory.

1.5) **LTP: An experimental model for learning and memory**

Neuroscience has long attempted to address questions concerning memory acquisition and storage at the level of individual neurons and synapses. One hypothesis for a simple neural model for learning and memory is Donald Hebb's rule. It states: "When an axon of cell A is near enough to excite cell B and repeatedly or consistently takes part in

firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency, as one of the cells firing B, is increased." (Hebb, 1949). One neurophysiological phenomenon in accordance with Hebb's rule is LTP. LTP is a long-lasting increase in synaptic strength between two connected neurons, first discovered in the hippocampus by Bliss and Lømo (1973), resulting from a brief high-frequency electrical stimulation of the afferent fibres to ensure depolarisation of post-synaptic target neurons. Subsequently, it has been shown that LTP can be induced by pairing pre and post-synaptic depolarisation using intracellular patch-clamp electrophysiology techniques (see, for example, Wigström *et al.*, 1986; Markram *et al.*, 1997), demonstrating, in accordance with Hebb's hypothesis, that it is a matching of depolarisation that governs LTP induction. LTP is characterised by four basic properties: Longevity, cooperativity, associativity and input-specificity (see for review Bliss & Collingridge, 1993). Longevity is an essential feature of any memory storage mechanism and LTP has been shown to last for long periods in animals, even up to one year (see, for example, Abraham *et al.*, 2002). Cooperativity describes the existence of an intensity threshold for induction, so that stimulation at lower intensities does not elicit LTP because too few inputs are co-active (see, for example, McNaughton *et al.*, 1978). Associativity of LTP describes the phenomenon whereby a weakly activated input pathway can be potentiated, if it is simultaneously delivered with a strong stimulus to a separate but convergent input pathway (see, for example, McNaughton *et al.*, 1978). Input-specificity of LTP means that only tetanised pathways induce potentiation but not other inputs to the same cell that are inactive at the time of stimulation (see, for example, Andersen *et al.*, 1977).

LTP has been most extensively studied in the mammalian hippocampus, but has also been observed in neocortical regions, subcortical nuclei, such as the amygdala, and in the peripheral nervous system. The hippocampal trisynaptic circuit is an ideal system to investigate activity-dependent synaptic plasticities because of its laminar organisation (see for review Amaral & Witter, 1989; Fig. 1-1). Pre-synaptic pathways can be stimulated in an isolated fashion whilst simultaneously recording from the cell body of

the post-synaptic target neuron or populations of post-synaptic neurons. LTP was first observed in the perforant pathway that connects the entorhinal cortex to the granule cells in the dentate gyrus (DG) (Bliss & Lømo, 1973). LTP has also been observed in the mossy fibre pathway from the DG to the CA3 pyramidal cells and the Schaffer collateral pathway arising from CA3 and contacting CA1 pyramidal cells (see, for example, Alger & Teyler, 1976). These electrophysiological studies demonstrated that different types of synapses exhibit mechanistically distinct forms of LTP, due to the involvement of different molecular mechanisms. In the hippocampus, LTP induction at Schaffer collateral-CA1 pyramidal cell synapses (CA1 LTP) and at perforant path-granule cell synapses (DG LTP) requires N-methyl-D-aspartate (NMDA) receptors (see also *section 1.6.2*). Hence, CA1 LTP and DG LTP are termed NMDA receptor-dependent. In contrast, LTP induction at mossy fibre-CA3 pyramidal cell synapses (CA3 LTP) is NMDA receptor-independent. The molecular mechanisms underlying induction of NMDA receptor-independent LTP probably involve pre-synaptic changes including activation of kainate receptors and modulation of cAMP-dependent protein kinase (PKA) signalling.

LTP has several different phases, one transient phase maintained by protein phosphorylation, another longer phase by the synthesis of new proteins from existing mRNAs and another even longer-lasting phase dependent upon triggered gene transcription and subsequent *de novo* protein synthesis (see for review Bliss & Collingridge, 1993; Reymann & Frey, 2006). The transient form, or early LTP (E-LTP), can be induced by a single train of high-frequency stimulation. E-LTP can last 3-6 hours and is independent of gene transcription and protein synthesis. However, E-LTP requires protein phosphorylation, as it is blocked by kinase inhibitors. Several trains of tetanic stimulation induce late LTP (L-LTP), which can persist for days and requires protein synthesis and *de novo* gene transcription. It has been demonstrated that L-LTP can be blocked by inhibitors of protein synthesis and gene transcription (see, for example, Krug *et al.*, 1984; Mackler *et al.*, 1992; Impey *et al.*, 1996). Interestingly,

LTM formation can also be blocked by application of inhibitors of protein synthesis and gene transcription (see for review Davies & Squire, 1984).

Activity-dependent synaptic potentiation may persist from milliseconds to hours or days (see for review Bliss & Collingridge, 1993; Malenka & Bear, 2004). Based on their temporal characteristics, different forms of synaptic plasticity can be classified as short or long-term. Short-term varieties include paired pulse facilitation, which is a transient enhancement of synaptic responses after priming the response with an stimulatory pulse, and post-tetanic potentiation (PTP), describing the initial synaptic potentation after tetanic induction, which decays within minutes. Short-term potentiation (STP) can occur normally in the first 15 minutes and decays within one hour after stimultaion. Long-term varieties include LTP and long-term depression (LTD), a plasticity resulting in a weakening of the efficacy of synaptic transmission either by prolonged, low frequency stimulation or mismatching of pre and post-synaptic depolarisation using intracellular recording techniques (see for review Braunewell & Manahan-Vaughan, 2001; Malenka & Bear, 2004).

Since its discovery, LTP of synaptic transmission in the hippocampus has been widely used as primary experimental model to study molecular processes in neurons. Electrophysiological LTP studies have demonstrated that the long-lasting enhancement in synaptic efficacy is a manifestation of multiple, complex molecular mechanisms. The elucidation of molecular and cellular processes involved in the induction, expression and maintenance of LTP potentially provides insight into the mechanisms underlying learning and memory. Recent advances suggest that many of the molecular mechanisms of synaptic plasticity are also engaged during learning and in memory formation (see, for example, Martin & Morris, 2002).

1.6) **Molecular basis of synaptic plasticity**

In recent years electrophysiological, molecular and pharmacological studies have implicated over a hundred proteins in synaptic plasticity, learning and memory (Sanes & Lichtman, 1999). These proteins can be divided broadly into categories including neurotransmitter receptors and ion channels, signalling enzymes, cell adhesion molecules, cytoskeletal proteins and adaptor proteins. The proteomic characterisation of a multi-protein signalling complex has demonstrated the clustering of a network of proteins to the NMDA receptor (Husi *et al.*, 2000). Many proteins in this network are directly related to both synaptic plasticity, learning and memory. This work has revealed the complex structural and functional organisation of synapses in which there is a clustering of large numbers of molecules into macromolecular complexes both pre- and post-synaptically at precise zones. Membrane proteins, such as receptors, channels and adhesion molecules, are attached to a network of intracellular scaffold, signalling and cytoskeletal proteins. These synaptic complexes are specialized input devices for neurons, which allow them to detect and discriminate different patterns of neural activity and, in turn, regulate downstream events via discrete signalling pathways. One of the functions of the signalling cascades is to regulate *de novo* gene transcription and protein synthesis and thereby possibly initiate long-term changes in the organisation, structure and function of the synapse (see, for example, Dosemeci *et al.*, 2001). In dendritic spines activity-dependent morphological changes, such as enlargement of the spine head and shortening of the spine neck become apparent (see for review Yuste & Bonhoeffer, 2001). These recent findings on the molecular structure, organisation and regulation at the synapse begin to provide insight into the complex mechanisms involved in learning processes. However, further elucidation of the signal transduction mechanisms regulated via multiple protein kinases and the molecular mechanisms that drive the activity-dependent reorganisation and maintenance of synapses, both pre- and post-synaptically, are required for a deeper understanding of brain function and, potentially, cognition.

1.6.1) Pre-synaptic mechanisms

It has been postulated that activity-dependent changes in synaptic strength are central to the processes of learning and memory. Much research has focussed on the events occurring post-synaptically that contribute to different forms of synaptic plasticity (see for review Kandel, 2001). The pre-synaptic mechanisms underlying synaptic modulation and synaptic plasticity are less well understood. This is partly due to methodological difficulties in discerning pre-synaptic from post-synaptic effects.

The arrival of an action potential at the pre-synaptic terminal triggers neurotransmitter release into the synaptic cleft, and thereby initiates the synaptic transmission (see for review Südhof, 2004). An action potential induces the opening of voltage-gated calcium channels (VGCC) at the pre-synaptic terminal. The resulting Ca^{2+} influx into the pre-synaptic terminal triggers molecular mechanisms that lead to synaptic vesicle exocytosis and subsequent neurotransmitter release. These processes of vesicular exo- and endocytosis are regulated by an intricate protein machinery. A molecular complex including the SNARE proteins (including synaptobrevin, syntaxin 1, SNAP-25) is required for docking the vesicles close to the active zones in the pre-synaptic terminal (see for review Chen & Scheller, 2001). Furthermore, this complex prepares the vesicles for fusion with the plasma membrane, in a step called "priming". The increase in Ca^{2+} levels promotes a conformational or electrostatic change of Ca^{2+}-sensor proteins, which in turn trigger the fusion reaction with the plasma membrane. Several pre-synaptic Ca^{2+}-binding proteins have been proposed to act as a Ca^{2+}-sensor including synaptotagmins (see for review Chen & Scheller, 2001; Südhof, 2004).

Different pools of synaptic vesicles can be distinguished. Vesicles of the immediate-releasable pool are docked close to the active zones in the pre-synaptic terminal. They can be either immature or already "primed", which means that they are fully mature and ready for release. Vesicles belonging to the releasable pool are not yet docked and need to be recruited to the docking sites. This recruitment step is also Ca^{2+}-dependent and involves multiple proteins including myosin light chain kinase (MLCK) (see, for

example, Polo-Parada *et al.*, 2005). Many pre-synaptic proteins associated with the vesicle release machinery are directly Ca^{2+}-binding (e.g. Munc13, rabphilin and RIM). Also, several pre-synaptic proteins important for neurotransmitter release and pre-synaptic plasticity are regulated via phosphorylation by protein kinases including PKA, protein kinase C (PKC), MLCK and CaMKII (see for review Leenders & Sheng, 2005). CaMKII has been found in pre-synaptic terminals associated with synaptic vesicles (see for review Lisman *et al.*, 2002). Pre-synaptic activation of CaMKII is required for induction of synaptic plasticity in hippocampal cell cultures (Ninan & Arancio, 2004). A number of pre-synaptic proteins have been described as substrates of CaMKII. However, the precise processes mediated by CaMKII that are necessary for the induction of synaptic plasticity are unknown. One candidate substrate is synapsin I, which is persistently phosphorylated at its CaMKII phosphorylation sites after LTP induction (Nayak *et al.*, 1996). Synapsins are involved in regulating the synaptic vesicle mobilisation and hence, controlling the number of vesicles available for exocytosis at pre-synaptic release sites. Synapsins bind simultaneously to synaptic vesicles and parts of the cytoskeleton, including actin and tubulin, and thereby retain the vesicles away from the vesicle release sites. During synaptic activation synapsins are phosphorylated by CaMKII and dissociate from the synaptic vesicles. This liberates the synaptic vesicles, which, in turn, get recruited to the vesicle release sites, where they are docked and primed for neurotransmitter release.

Several post-synaptic mechanisms have been proposed to enhance synaptic strength, including an increase in the number of synaptic neurotransmitter receptors or an enhancement of their single-channel conductance (see for review Malenka & Bear, 2004). Nevertheless, enhanced synaptic plasticity could also be attributed to alteration of pre-synaptic mechanisms, such as increased neurotransmitter release, structural changes of individual pre-synaptic terminals or the formation of new synapses involving *de novo* protein synthesis (see for review Leenders & Sheng, 2005). It is conceivable that changes in neurotransmitter release by modulation of synaptic vesicle dynamics might indeed be a major mechanism for regulating synaptic strength. This hypothesis is

supported by the observation that retrograde messengers, such as nitric oxide (NO), carbon monoxide (CO) and arachidonic acid are involved in the generation of LTP by modifying pre-synaptic transmitter release (see for review Bliss & Collingridge, 1993). However, more research will be required to resolve the respective contribution of pre- and post-synaptic mechanisms to the molecular processes underlying synaptic plasticity.

1.6.2) Post-synaptic mechanisms

Much research has focused on NMDA-receptor dependent LTP in area CA1 and some of the underlying mechanisms are well-characterised. The excitatory Schaffer collateral-CA1 synapses use glutamate (L-glutamic acid) as a neurotransmitter. Electrophysiological stimulation of the Schaffer collateral fibres triggers pre-synaptic glutamate release. The released glutamate binds to post-synaptically located ionotrophic glutamate receptors, the α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionic acid (AMPA) receptor and the NMDA receptor (see for review Hollmann & Heinemann, 1994). Upon glutamate-binding, the AMPA receptor opens its ligand-gated ion channel, allowing post-synaptic Na^+ and K^+ influx. This inward Na^+-current depolarises the post-synaptic membrane and produces an excitatory post-synaptic potential (EPSP). The NMDA receptor is blocked by Mg^{2+} at resting potential and its ligand-gated ion channel is opened only in response to glutamate binding if the post-synaptic membrane is depolarised. This post-synaptic depolarisation, triggered by either AMPA receptor activation or back-propagation of action potentials, removes the Mg^{2+} block of the NMDA receptor and allows Ca^{2+} to enter post-synaptically. The rise in post-synaptic intracellular Ca^{2+} is the critical trigger for LTP induction (see for review Bliss & Collingridge, 1993; Malenka & Bear, 2004). The NMDA receptor thereby works as a coincidence detector, integrating the pre-synaptic glutamate release with the post-synaptic depolarisation. Consistent with the proposed role of the NMDA receptor in synaptic plasticity and memory, LTP is inhibited in area CA1 by pharmacological blockade of the NMDA receptor, which also affects learning and memory in the MWM

(Collingridge *et al.*, 1983; Morris *et al.*, 1986). Furthermore, transgenic mice lacking the NMDA receptor in area CA1 display impaired LTP and hippocampus-dependent spatial learning (McHugh *et al.*, 1996; Tsien *et al.*, 1996).

The importance of post-synaptic Ca^{2+}-influx through the NMDA receptor in synaptic plasticity has been demonstrated by the finding that the addition of Ca^{2+}-chelators to the post-synaptic cell prevents induction of LTP (see, for example, Malenka *et al.*, 1988). Ca^{2+} has been found to regulate many synaptic proteins, including CaMKII, PKC and fyn tyrosine kinase. Hence, it was hypothesised that some of these Ca^{2+}-dependent kinases become activated and phosphorylate specific substrates, which in turn trigger biochemical pathways and initiate long-term changes in synaptic structure and function (see for review Soderling, 2000; Soderling & Derkach, 2000). In particular, CaMKII is a leading candidate in the search for the molecular basis of learning and memory (see for review Lisman *et al.*, 2002; see also *section 1.7.5*). In response to NMDA receptor activation and subsequent intracellular Ca^{2+}-elevation, CaMKII becomes activated by association with Ca^{2+}/calmodulin (CaM) and translocates to the post-synaptic densities, where it binds to a number of structural and modulatory proteins. There are numerous substrates through which CaMKII may modify neuronal function and exert its influence on synaptic strength (Yoshimura *et al.*, 2000 and 2002; see for review Lisman *et al.*, 2002). In addition, several other protein kinases, including extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), PKC and the tyrosine kinases Src and fyn have been proposed to be important for signal transduction that contributes to synaptic plasticity and for initiation of long-term changes in synaptic structure and function (see for review Soderling & Derkach, 2000; Thiels & Klann, 2001).

1.6.3) Maintenance mechanisms involving gene transcription and protein synthesis

LTP maintenance early after synaptic stimulation is mediated by intracellular signalling cascades. Pharmacological studies have demonstrated that LTM and L-LTP require *de*

novo transcription and protein synthesis (see for review Kandel, 2001). Therefore, it is thought that one important function of these signalling cascades is the activation of critical transcription factors and subsequently the transcription and synthesis of specific proteins. So far, several signalling pathways have been implicated in learning-related transcription.

One model proposes the involvement of the cyclic AMP (cAMP) signalling pathway in the activation of transcription (see for review Bailey *et al.*, 2000; Kandel, 2001). Ca^{2+}-influx and subsequent CaM binding activates adenylate cyclase isoforms, which are responsible for the synthesis of cAMP. Increased levels of cAMP can, in turn, activate PKA, which translocates to the nucleus, where it phosphorylates the transcription factor cAMP responsive element-binding protein (CREB). The phosphorylation of CREB at Ser133 is required for its activation as a transcription factor. Upon activation, CREB binds to a specific motif within the promoter region of target genes, called the cAMP responsive element (CRE) and triggers CRE-mediated transcription. Evidence for the involvement of the cAMP signalling pathway in memory formation comes from studies of mutant mice lacking the Ca^{2+}-regulated adenylate cyclase isoforms, AC1 and AC8 (other isoforms are regulated via the activation of G-protein coupled receptors) (Wong *et al.*, 1999). These KO mutants display both impaired LTM and L-LTP in hippocampal area CA1. Furthermore, cAMP agonists trigger L-LTP in hippocampal slices without tetanic stimulation, whereas cAMP antagonists block L-LTP (Frey *et al.*, 1993). In addition, CREB phosphorylation and CRE-mediated transcription can be induced in hippocampal slices by tetanic stimulation that induces L-LTP, as well as by activation of adenylate cyclase and PKA (Impey *et al.*, 1996).

Other studies implicate alternative signalling pathways in the activation of the transcription factor CREB. The Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) has been found to mediate CREB phosphorylation and CRE-mediated transcription after synaptic stimulation. Studies on transgenic mice expressing a dominant negative isoform of CaMKIV have revealed an impairment of CREB phosphorylation and CRE-

mediated transcription coinciding with impaired LTM formation in hippocampus (Kang *et al.*, 2001). Interestingly, consistent with a role for CaMKIV in the initiation of transcription and the finding that the early phase of LTP is independent of transcription, hippocampal L-LTP was impaired in these mice, whereas E-LTP was normal (Kang *et al.*, 2001).

Along with the phosphorylation of CREB at Ser133, the recruitment and subsequent binding of the transcriptional co-activator CREB binding protein (CBP) is required to trigger CRE-mediated transcription (see for review Johannessen *et al.*, 2004). CBP is thought to link CREB to the other components of the transcriptional machinery. CREB regulates the transcription of genes harbouring the CRE motif within their promoter region. A multitude of genes are under the control of the CRE promoter, including ion channels, growth factors and immediate-early genes (see for review Lonze & Ginty, 2002). Importantly, CREB initiates the expression of immediate-early genes, such as the transcription factors c-fos and c-jun, and thereby triggers a transcriptional cascade. The synthesis of these immediate-early transcription factors leads in turn to expression of late-effector genes that encode for proteins that could be important for the regulation of, for example, synaptic structure. Such changes in the organisation, structure and function of synapses could lead to increased synaptic strength and could be the basis of cellular learning and memory mechanisms.

1.7) Ca^{2+}/calmodulin-dependent protein kinase II

1.7.1) General characteristics of Ca^{2+}/calmodulin-dependent protein kinase II

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine kinase, which requires binding of CaM for activation (see for review Hudmon & Schulman, 2002; Lisman *et al.*, 2002; Colbran & Brown, 2004). In mammals, CaMKII is encoded by four alternatively spliced genes (α, β, γ and δ), which give rise to approximately 30 similar isoforms. Specific functions have been characterised for only a few of the alternatively spliced sequences including a domain for SR-membrane targeting, a nuclear localisation signal and an SH_3-binding site. The different CaMKII isoforms have distinct tissue, cellular and subcellular localisations (see for review Hudmon & Schulman, 2002). In the brain, αCaMKII (Swiss Prot|P11798|KCC2A_MOUSE) and βCaMKII are the predominant isoforms; the α-isoform displaying high levels in neurons of the forebrain, and the β-isoform having high levels in the cerebellum. The αCaMKII is almost exclusively restricted to excitatory, glutamatergic neurons, whereas the β-isoform is found in neurons and glial cells (Liu & Jones, 1997). Further, the expression of αCaMKII starts postnatally, whereas βCaMKII is already expressed embryonally. The CaMKII γ- and δ-isoforms are broadly expressed in the brain, but at a much lower level than the α- and β-isoforms. In addition, the subcellular distribution of the isoforms is also different. The αCaMKII isoform is enriched at synapses and constitutes the major postsynaptic density protein. The βCaMKII isoform associates with actin filaments, which confers a localisation at the dendritic shaft.

All CaMKII isoforms comprise an amino-terminal catalytic domain, an autoinhibitory domain and a self-association domain (see for review Lisman *et al.*, 2002; Fig. 1-2). The catalytic domain contains the ATP-pocket and the substrate-binding site. The self-association domain is required for the formation of a holoenzyme composed of 12

subunits grouped in two stacks of hexameric rings. The autoinhibitory domain contains a pseudosubstrate region, a CaM binding site, as well as interaction sites for anchoring proteins and regulatory phosphorylation sites (Fig. 1-2). The function of the autoinhibitory domain is the regulation of the kinase activity. Under basal conditions, CaMKII is inactive because the pseudosubstrate region is binding to the catalytic site and thereby inhibiting its enzymatic activity (Fig. 1-3). A rise in intracellular Ca^{2+}-levels leads to association of the CaM complex, which can subsequently bind to CaMKII on a site overlapping with the pseudosubstrate region. This interaction evokes a conformational change of CaMKII that exposes the catalytic domain and renders the kinase active. In this Ca^{2+}-activated state the Thr286 on the autoinhibitory domain of αCaMKII (respectively Thr287 on βCaMKII) can be autophosphorylated by neighbouring subunits (see, for example, Miller & Kennedy, 1986; see for review Lisman *et al.*, 2002). Once this site is phosphorylated CaMKII can not revert back to the original, inactive conformation, even after Ca^{2+}-levels drop and CaM dissociates from the kinase. This results in a persistent, autonomous activity that is Ca^{2+}-independent. This persistently activated state of CaMKII is maintained until the subunits are dephosphorylated by phosphatases, including PP1 and PP2A. Further, control of CaMKII activity occurs via the (auto)phosphorylation of the regulatory sites Thr305/306 that block activation of CaMKII via CaM by hindering the CaM-binding site and prevents the translocation of CaMKII to the PSD (see, for example, Miller & Kennedy, 1986; Elgersma *et al.*, 2002).

1.7.2) CaMKII as a putative sensor of Ca^{2+} oscillations

The autoregulatory properties of CaMKII subunits combined with the structural properties of the holoenzyme complex make CaMKII a specialised sensor of intracellular Ca^{2+}-concentration and oscillations (see for review Lisman *et al.* 2002; Fig. 1-4). In order for autophosphorylation of one subunit to occur, the coincident binding of two CaM molecules at adjacent subunits is required. With low frequencies of

intracellular Ca^{2+}-oscillations, which evoke only a minor rise in Ca^{2+}-levels, the probability of two CaM molecules binding to neighbouring subunits is low. As the frequency of oscillation increases, the probability of coincident binding becomes higher, until one subunit within the holoenzyme is persistently activated by the autophosphorylation. Once a subunit is autonomously activated the coincident binding is no longer essential and propagation of autophosphorylation can proceed more easily within the holoenzyme. Any subunit, adjacent to an already autonomously activated subunit, can now also be autophosphorylated simply by binding CaM. Therefore, the propagation can occur in response to lower levels of Ca^{2+} than in the initial step, where coincident binding is required. Different frequencies of intracellular Ca^{2+}-oscillations will thereby result in distinct levels of autophosphorylated subunits within the holoenzyme.

1.7.3) Synaptic functions of αCaMKII

Post-synaptic Ca^{2+} influx triggers the activation of αCaMKII by association with CaM and leads to translocation of the activated αCaMKII from the synapse into the post-synaptic densities (Ouyang *et al.*, 1997). There, αCaMKII interacts with numerous proteins including the Cdk5 activator p35, α-actinin, densin-180, SynGAP and NMDA receptor subunits (see for review Colbran, 2004). In the active state αCaMKII can associate with the cytoplasmic carboxy-terminus of the NMDA receptor subunits NR2B and with less affinity, NR1 and NR2A. Next to the autophosphorylation at Thr286, this association is another important mechanism by which αCaMKII can be persistently activated even after Ca^{2+} levels drop and CaM dissociates (Fig. 1-3). The association of αCaMKII to proteins located within the post-synaptic densities brings the kinase into proximity with numerous substrates (Table 1-1.; see, for example, Yoshimura *et al.*, 2000 and 2002). Dephosphorylation of Thr286 by protein phosphatase 1 (PP1), which is

immobilized in the PSD, and PP2A, which is excluded from the PSD, is critical for terminating the autonomous activity of CaMKII (Shen *et al.*, 1998).

Table 1-1. CaMKII substrates

Receptor and channel proteins	Scaffold proteins	Cytoskeletal and associated proteins	Motor proteins	Enzymes	Proteins involved in neurotrans-mitter release	Others
AMPA receptor	Densin-180	α-actinin	Caldesmon	CaMKII	Synapsin I	Insulin receptor kinase
GABA-modulin	Homer 1b	Clipin C	C protein	Calpain	Synaptotagmin	TOAD64
GABA receptor, type A	PSD95-associated protein	α-internexin	Myosin IIB	Cyclic nucleotide phosphodiesterase	Synaptophysin	TNF receptor-associated factor 3
GluR1 subunit	PSD95		Myosin V	Calcineurin		
IP_3 receptor	SAP90	MAP2		Dynamin 1		
NMDA receptor	SAP97	Neurofilaments H, M and L		Phospholipase A2		
NR2B		tau		Nitric oxide synthase		
N-type Ca^{2+}-channel		α/β-tubulin		SynGAP		
VDAC1						
LRP4						

Abbreviations: AMPA, α-amino-3-hydroxy-5 methyl-isoxazole-4-proprionic acid; GABA, γ-amino butyric acid; GluR, glutamate receptor; IP_3, inositol triphosphate; LDL, low-density lipoprotein; LRP4, LDL receptor-related protein 4; MAP, microtubule-associated protein; NMDA, N-methyl-D-aspartate; NR2B, NMDA receptor subunit 2B; PSD-95, post-synaptic density 95 kDa; SAP, ; TNF, Tumor necrosis factor; TOAD64, turned on after division 64 kDa; VDAC1, voltage-dependent anion channel protein 1 *(After Yoshimura et al., 2002).*

The phosphorylation of specific substrates by αCaMKII is thought to modify neuronal function and exert an influence on synaptic plasticity. CaMKII activity was shown to strengthen synaptic transmission by several mechanisms. One of them involves the direct phosphorylation of the AMPA receptor subunit GluR1, which in turn increases the single-channel conductance of homomeric GluR1 AMPA receptors (Derkach *et al.*, 1999). Another mechanism involves the addition of AMPA receptors to synapses, which is thought to depend on changes in receptor trafficking and on the organised addition of anchoring sites for AMPA receptors (see for review Malinow & Malenka, 2002). Moreover, autophosphorylation increases the affinity of CaMKII for CaM by approximately thousand fold. Hence, it has been proposed that the autophosphorylated

CaMKII pool within the post-synaptic densities might function as a CaM sink (Lisman *et al.*, 2002). However, despite the extensive research there is only a limited knowledge on the specific substrates of αCaMKII and the molecular processes that are regulated specifically by autonomous αCaMKII activity.

1.7.4) CaMKII in synaptic plasticity, learning and memory

The first evidence supporting a role for CaMKII in synaptic plasticity demonstrated that induction of LTP could be blocked by application of CaMKII inhibitors, including peptide inhibitors designed from the autoinhibitory domain of CaMKII (Malinow *et al.*, 1989; Otmakhov *et al.*, 1997). Further it was shown that induction of LTP leads to activation of CaMKII and in turn to autophosphorylation, which results in autonomous, Ca^{2+}/CaM-independent activity (Fukunaga *et al.*, 1993, Ouyang *et al.*, 1997). Studies in different genetically-modified mouse lines demonstrated an important role of CaMKII and its autophosphorylated state in synaptic plasticity, learning and memory processes (see for review Elgersma *et al.*, 2004). KO mice lacking αCaMKII show impairment in CA1 LTP (Silva *et al.*, 1992a; Elgersma *et al.*, 2002). However, some residual CA1 LTP persists in these αCaMKII null mice, which could be attributed to a compensatory translocation of the β-isoform of CaMKII to the post-synaptic density (Hinds *et al.*, 1998; Elgersma *et al.*, 2002). In the αCaMKII T286A point mutant mouse line the autophosphorylation at residue Thr286 is disenabled by replacing endogenous αCaMKII with a mutated form carrying an alanine at residue 286, instead of a threonine (Giese *et al.*, 1998). In these αCaMKII T286A mutants induction of CA1 LTP is completely abolished indicating that the autophosphorylation of αCaMKII is fundamental for the induction of CA1 LTP. Further studies in the αCaMKII T286A mutants implicated αCaMKII in the induction of neocortical LTP (Glazewski *et al.*, 2000; Hardingham *et al.*, 2003). CA1 LTP is also completely absent in the αCaMKII T305D point mutant mouse line, where activation of the kinase by CaM and

translocation to the PSD have been disenabled (Elgersma *et al.*, 2002). No βCaMKII compensation occurs in these T305D mutants (Elgersma *et al.*, 2002). Interestingly, in αCaMKII T305V/T306A point mutant mice, where these inhibitory sites can not be phosphorylated, a 10 Hz tetanus readily induces marked LTP suggesting that the Thr305/Thr306 site is implicated in the control of the threshold of LTP induction (Elgersma *et al.*, 2002). Together, these results indicate that αCaMKII and specifically autophosphorylation of αCaMKII on Thr286 are essential for the induction of NMDA receptor-dependent CA1 LTP and might play an important role in other forms of synaptic plasticity.

The study of αCaMKII KO mice in hippocampus-dependent learning and memory tasks revealed a gross impairment in spatial learning (Silva *et al.*, 1992b; Elgersma *et al.*, 2002). However, as in the LTP studies, it is conceivable that the compensatory mislocalisation of βCaMKII is responsible for the residual learning in the αCaMKII KO mice. The αCaMKII T286A mutants exhibit severely impaired hippocampal learning and memory as tested in the MWM that can not be rescued by over-training (Giese *et al.*, 1998). These results indicate that the autophosphorylation of αCaMKII is essential for hippocampal spatial learning. However, the αCaMKII T286A mutants performed normally in several non-hippocampus-dependent learning tasks including the visible platform water maze suggesting that αCaMKII is not essential in all brain areas (Giese *et al.*, 1998). Investigation of place cell activity in the αCaMKII T286A mutants found that spatial selectivity and stability of place cells require autophosphorylation of αCaMKII on Thr286 (Cho *et al.* 1998). Consistent with an important role for functional αCaMKII in learning processes, the αCaMKII T305D mutants also displayed hippocampal learning and memory impairments (Elgersma *et al.*, 2002). The analysis of the αCaMKII T305V/T306A mutants showed that these inhibitory phosphorylation sites on αCaMKII are not essential for all spatial learning tasks, but in fact are required for flexible fine-tuning in some tasks (e.g. reversal learning in the MWM; Elgersma *et al.*, 2002). Together, these findings demonstrate a central role for αCaMKII and the

autophosphorylation of αCaMKII on Thr286 in learning processes and the formation of memories. However, the molecular mechanisms engaged by αCaMKII activation are poorly understood and no convincing link to other processes important for learning and memory has yet been found.

1.7.5) The CaMKII hypothesis for the storage of synaptic long-term memory

A fundamental question in neuroscience is how memory is stored at the molecular and cellular level. The study of LTP, a promising candidate mechanism for cellular learning and memory, helped greatly to further the understanding of the molecular basis of memory. In line with these advances John Lisman formulated an influential hypothesis suggesting that CaMKII acts as a molecular switch that is capable of storing long-term synaptic memory (Lisman, 1985 and 1994; Lisman *et al.*, 2002). He proposed that, following a synaptic event a group of kinase molecules becomes activated, so that they can autophosphorylate each other. The autophosphorylation switches the kinase into an autonomously active mode, which can persist for prolonged periods even after the synaptic event is over. In this autophosphorylated, autonomous mode the group of kinase molecules could perpetuate its phosphorylation state, even though dephosphorylation by phosphatases occurs or some kinase molecules are replaced by newly synthesised molecules. This group of autonomously active kinase molecules could hence form the basis of stable long term memory of the triggering synaptic event.
The molecular and structural characterisation of αCaMKII showed that its properties are in agreement with the hypothesis and hence made it a prime candidate for the molecular storage of memory. αCaMKII is the major postsynaptic density protein in glutamatergic neurons and it requires association of CaM for activation. In the activated state αCaMKII can undergo autophosphorylation. The autophosphorylation switches CaMKII into a Ca^{2+}-independent, autonomously active mode which can persist for prolonged periods even after the Ca^{2+}-concentration decreases. The structural properties

of the CaMKII holoenzyme complex even suggest that this autophosphorylated state could outlast continuous dephosphorylation and protein turnover (Rosenberg *et al.*, 2005).

Results from electrophysiological and behavioural experiments further support the role of CaMKII as a candidate protein mediating memory storage. Autophosphorylation and autonomous kinase activity of αCaMKII are prolonged following induction of LTP (Ouyang *et al.*, 1997; Fukunaga *et al.*, 1993). Enhanced CaMKII activity partially mimics and/or facilitates LTP (Mayford *et al.*, 1996; Bejar *et al.*, 2002). The T286A point mutant mice, where the autophosphorylation switch is disenabled, display impaired LTP and deficits in spatial learning and memory in the Morris water maze (Giese *et al.*, 1998). These experiments consistently demonstrated that the enzymatic activity of αCaMKII is required for the induction of LTP. However, it is still not known whether persistent, autonomous CaMKII activity is essential for the maintenance of stored information. It is possible that CaMKII acts only as a trigger and that other downstream events are responsible for the maintenance of memory storage. Recent advances have identified novel mechanisms that could serve as memory storage, including atypical PKC isoform, PKMζ, prion-like proteins, gene expression and local protein synthesis (see, for example, Bliss *et al.*, 2006; Si *et al.*, 2003; Dudai, 2004).

1.8) **Neurodegenerative diseases**

1.8.1) Dementia: decline of cognitive functions

Dementia is generally used to describe a chronic and significant decline of cognitive functions (see for review Knopman, 1998; McKeith & Cummings, 2005). Dementia patients show a decline in more than one of the five broad neuropsychological domains including learning and memory, attention and concentration, thinking, language, and visuospatial functioning. In contrast, for example, amnesic patients show a striking deficit only in the area of memory, or aphasic patients in the area of language. Dementia is a collection of diverse conditions evoked by different causes that manifest in similar fashion with the decline of cognitive functions. Many causes have been linked to dementia including brain injury, brain tumors, drug toxicity, syphilis, encephalitis, meningitis, Creutzfeld-Jakob disease (CJD), Huntington disease and Alzheimer's disease (AD). The onset and the progression of the cognitive decline vary between the different types of dementia. Most dementias are progressive, however some are non-progressive (e.g. alcoholic dementia). Further, most dementias, including AD and Huntington disease, have an insidious onset which is followed by a slow and gradual progression of the cognitive decline. In many dementias specific brain regions are affected and characteristic cognitive deficits appear in these dementia patients. Early in the disease memory impairment is often the only clinical sign in dementia patients. With progression of dementia the cognitive decline aggravates and social and occupational functioning is affected. In general, the likelihood of onset of dementia becomes higher with increasing age. Individuals that show first signs of memory impairment are classed as patients with mild-cognitive impairment (MCI), which describes a condition that may or may not eventually lead to dementia (see for review Gauthier *et al.*, 2006).

1.8.2) Neurodegenerative tauopathies

Many sporadic and familial neurodegenerative diseases are characterised neuropathologically by brain lesions that are accompanied by abnormal filamentous depositions of brain proteins (see for review Buee *et al.*, 2000; Lee *et al.*, 2001; Goedert & Jakes, 2005). The so-called neurodegenerative tauopathies consist of a group of heterogeneous dementias and movement disorders including AD (see Table 1-2 for list of diseases). Neurodegenerative tauopathies are characterised by intracellular accumulation of fibrils formed by the microtubule-associated protein tau. The cognitive decline in the tauopathies correlates with the progressive accumulation of the filamentous tau depositions. The study of sporadic and, especially, familial (i.e. genetically-linked) forms of neurodegenerative tauopathies helped greatly to characterise some of the underlying molecular mechanisms. The observation that the frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), a familial form of tauopathy, was caused by a series of mutations within the tau gene, provided evidence that alteration in tau structure and expression are sufficient for the expression of neurodegenerative diseases (see for review Lee *et al.*, 2001; Goedert & Jakes, 2005). These findings inspired further studies to elucidate the physiological functions of tau and its role in the mechanisms of brain dysfunction and neurodegeneration. Much research focussed on the abnormal changes of tau that were characteristically observed in AD and that are not linked to genetic alterations (see also *section 1.8.3*). It was shown that the filamentous tau deposits, termed neurofibrillary tangles (NFT), consist mainly of aberrantly phosphorylated and proteolytically fragmented tau (see, for example, Grundke-Iqbal *et al.*, 1986a and 1986b; see, for review Buee *et al.*, 2000). Hence, it was proposed that increased tau phosphorylation interferes with the physiological role of tau and triggers the formation of fibrillar tau complexes, called paired helical filaments (PHF). The PHF aggregate subsequently into NFT.

Table 1-2. Diseases exhibiting neurofibrillary tangle (NFT) pathology

Disease	NFT as main feature
Alzheimer's disease (AD)	
Amyotrophic lateral sclerosis (ALS)/ parkinsonism-dementia complex	+
Argyrophilic grain dementia	+
Corticobasal degeneration	+
Creutzfeldt-Jacob disease (CJD)	
Dementia pugilistica	+
Diffuse neurofibrillary tangles with calcification	+
Down's syndrome	
Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)	+
Gerstmann-Sträussler-Scheinker disease	
Hallervorden-Spatz disease	
Myotonic dystrophy	
Niemann-Pick disease, type C (NPC)	
Non-Guamanian motor neuron disease with neurofibrillary tangles	
Pick's disease	+
Postencephalitic parkinsonism (PEP)	
Prion protein cerebral amyloid angiopathy	
Progressive subcortical gliosis	+
Progressive supranuclear palsy (PSP)	+
Subacute sclerosing panencephalitis	
Tangle only dementia	+

(After Buee et al., 2000 and Lee et al., 2001)

Several protein kinases and phosphatases have been implicated in the regulation of tau phosphorylation and are thought to be important in the pathogenesis of neurodegenerative tauopathies (see for review Billingsley & Kincaid, 1997; Buee *et al.*, 2000). It was believed that the accumulation of hyperphosphorylated tau and

aggregation into NFT are toxic processes for neurons and therefore might be responsible for the neurodegeneration and subsequently the cognitive deficits observed in patients with tauopathies. A recent study, however, demonstrated that viability of neurons and memory function are not affected by continuous NFT accumulation (SantaCruz *et al.*, 2005). Transgenic mice expressing a repressible human tau variant developed progressively NFT, neuronal cell loss and impaired spatial learning as tested in the MWM. Suppression of the transgenic tau expression resulted in stabilisation of the neuronal cell numbers and recovery of memory deficits, despite the continuous accumulation of NFT. Taken together, these results suggest that NFT are not directly cytotoxic and do not severely impact on memory functions. This is consistent with pathological examinations showing that many older individuals display NFT accumulation in the brain, but did not exhibit any obvious signs of cognitive deficits prior to death (Takayama *et al.*, 2002). Based on these studies, it was hypothesised that it is not the presence of hyperphosphorylated tau and NFT, but more likely the absence of sufficient functional tau supporting its physiological role that impacts on the viability of the neurons (Trojanowski & Lee, 2005).

The study of genetically-linked forms of neurodegenerative tauopathies identified several molecular targets thought to be involved in the ontogeny of the disease. The identification of these molecular targets has been subsequently exploited to generate genetically-modified animal models of tauopathies, characterise the function of these target molecules and explore therapeutic strategies (see for review Lee *et al.*, 2005).

1.8.3) Alzheimer's disease

Alzheimer's disease (AD), a neurodegenerative tauopathy, is the most common cause of dementia (see for review Selkoe, 2001). The prevalence of AD is directly related to age. AD affects about 10% of the population aged over 65 years and rises to at least 25% of the population aged over 85 years. The neuropathological hallmarks of AD are the

senile plaques, NFTs and neuronal cell loss. Senile plaques are extracellular deposits of β-amyloid (Aβ) peptide. The Aβ-peptide is produced by proteolytic cleavage of the amyloid precursor protein (APP) (Fig. 1-7). NFTs are mainly composed of hyperphosphorylated tau at proline-directed serine/threonine sites (see, for example, Grundke-Iqbal *et al.*, 1986b; Paudel *et al.*, 1993; Morishima-Kawashima *et al.*, 1995). NFTs accumulate with the progression of AD (see, for example, Braak & Braak, 1991; Augustinack *et al.*, 2002a). How the accumulation of plaques and NFT interferes with the viability of the neurons is, so far, not known. But there is a striking temporal and spatial relationship between the accumulation of these filamentous deposits and neuronal loss, which is ultimately responsible for the cognitive impairments seen in AD. In the early stages of AD, the areas displaying neuropathological changes spread progressively from the hippocampal formation to the anterior, inferior and mid temporal cortex (Braak & Braak, 1991). In these patients the changes manifest as mild cognitive impairments. With the progression of the disease the association areas of the temporal, parietal and frontal cortex are affected and the patients show increasingly loss of cognitive functions. The last brain regions to be affected are the primary motor area and sensory areas.

AD is broadly classified into an early-onset form (before the age of 50) and a late-onset form (after the age of 65) (see for review Selkoe, 2001; Tanzi & Bertram, 2001). The study of the inherited familial forms of AD has associated several molecules with the early-onset of the disease. Mutations in the genes APP, as well as presenilin 1 (PS1) and presenilin 2 (PS2) have been identified as a cause of familial AD (FAD). Over 100 mutations in these three genes can each trigger the early-onset of AD (see for review Tanzi & Bertram, 2001). It is thought that most of these mutations trigger the aberrant overproduction of Aβ-peptides. Interestingly, the APP gene is located on chromosome 21, and consistently with this the prevalence of AD is increased in patients with Down's syndrome carrying three copies of the chromosome 21. Allelic variation within the apolipoprotein E (APOE) gene on chromosome 19 were linked to the late-onset form of

AD (see for review Herz & Beffert, 2000). Patients that carry the APOE ε4 allele have a predisposition for the late-onset form of AD and hence the ε4 allele acts as a risk factor. Further genetic linkage studies found additional molecules that were weakly linked with the late-onset form of AD, including LDL receptor-related protein and FE65 (see for review Herz & Beffert, 2000). Sporadic AD with a late-onset accounts for the vast majority of AD cases. However, the molecular factors underlying sporadic AD remain unknown and aging is the only known risk factor. Hence, many processes displaying age-related alterations have been proposed to contribute to AD pathogenesis including oxidative stress, changes in gene expression, and altered metabolism (see for review Selkoe, 2001). Because the cognitive deficits are detected before tangle and plaque formation become apparent, it is hypothesised that synaptic dysfunction precedes tangle and plaque formation and could trigger the onset of molecular changes in AD (Selkoe, 2002). A recent study implicated deficient axonal transport in early stages of the pathogenesis of AD long before disease-related pathology was observed (Stokin *et al.*, 2005). The impairment of axonal transport was accompanied by swellings with accumulation of abnormal amounts of microtubule-associated proteins, including tau, molecular motor proteins, organelles and vesicles (Stokin *et al.*, 2005). Interestingly, several molecules associated with the pathogenesis of AD including APP and tau have also been functionally linked to axonal transport. Therefore, it is hypothesised that disruption of proteins with functions important for axonal transport are at the basis of AD (Morfini *et al.*, 2004). Accordingly, tau hyperphosphorylation and increased proteolytical processing of APP could directly affect axonal transport or else may be manifestations of impaired axonal transport. Precise regulation of phosphorylation of tau is important for its proper functioning, for example, for binding to microtubules and hyperphosphorylation of tau could disrupt these functions important for axonal transport (see for review Johnson & Stoothoff, 2004). Interestingly in this context, the proline-directed kinases Cdk5 and GSK3 have been implicated in the regulation of kinesin-driven axonal transport and are prime candidates for mediating tau hyperphosphorylation (Morfini *et al.*, 2004).

APP was found to regulate vesicular transport by linking vesicles to the motor protein kinesin. Proteolytical cleavage of APP is thought to be involved in the release of the vesicles. Further, presenilin 1 and 2, that have also been associated with AD, are implicated in the proteolytical cleavage of APP. Deficits in axonal transport have also been shown to induce oxidative stress and lead to neurodegeneration, characteristic features of AD (Stamer *et al.*, 2002). Future research promises to elucidate the mechanisms involved in axonal transport, which might help greatly to further the understanding of molecular processes underlying AD.

1.9) Microtubule-associated protein tau

The tau protein belongs to the microtubule-associated protein (MAP) family and is evolutionarily conserved amongst invertebrate species including worm and fly as well as vertebrate species such as frog, mouse and human (see for review Buee *et al.*, 2000; Lee *et al.*, 2001). Tau is widely expressed in the mammalian nervous system, but also at low-levels in other tissues including kidney. In the adult human central nervous system (CNS), six different isoforms are generated by alternative splicing from one single gene (Fig. 1-5; Swiss Prot|P10636|TAU_HUMAN). In addition, a peripheral nervous system (PNS) tau isoform and a fetal-tau isoform can be generated from the same gene (Fig. 1-5). The tau protein contains two major functional domains: a projection domain and a microtubule-binding domain (Fig. 1-6). The projection domain is located at the amino-terminal and contains an acidic region and a proline-rich region (Fig. 1-6). The different tau isoforms vary from each other by alternative splicing of two amino-terminal inserts encoded by exons 2 and 3 (Fig. 1-5). The presence or absence of these inserts is thought to affect tau interactions and hence regulate distinct functions of the different isoforms. The carboxy-terminal microtubule-binding domain of tau consists either of three microtubule-binding repeats (3R) or four repeats (4R) (see, for example, Goedert *et al.*,

1989; Goedert & Jakes, 1990). Three of the isoforms lack microtubule-binding repeat number 2 encoded by exon 10 and thus contain 3R, whereas the other three isoforms have 4R (Fig. 1-5). The expression ratios between the tau isoforms vary during development and is different for specific cell types (see, for example, Goedert *et al.*, 1989a and 1989b). The spatio-temporal regulation of tau expression suggests distinct physiological roles of the different isoforms. In contrast to the six tau isoforms generated via alternative splicing in the adult human brain, the homologous mouse *tau* gene is spliced only into 4R isoforms in adult murine brain (Swiss Prot|P10637|TAU_MOUSE). Thus, mice lack the 3R tau isoforms. Furthermore, mouse tau has a different splicing ratio between the isoforms and the amino-terminal region of tau contains an additional 14 amino acid insert (Appendix I).

The tau protein interacts with numerous molecules (see for review Buee *et al.*, 2000). The projection domain is probably important for the interaction with cytoskeletal proteins and the plasma membrane. Interaction of tau with actin filaments and spectrin are thought to interconnect microtubules with other cytoskeletal components and thereby restrict the flexibility of the microtubules (see, for example, Matus, 1994). The amino-terminal proline-rich region of tau has been implicated in the association with the non-receptor tyrosine kinase, fyn (SH3 binding motif (PxxP) residues 231-237, as per 441aa tau isoform), and phospholipase C-γ (PLC-γ) (see, for example, Lee *et al.*, 1998; Hwang *et al.*, 1996). The carboxy-terminal three or four microtubule-binding repeats of tau bind microtubules. Interestingly, the 4R isoforms exhibit a far higher binding affinity to microtubules than 3R isoforms (see, for example, Goedert & Jakes, 1990; Panda *et al.*, 1995). In addition, the microtubule-binding domain of tau interacts with PP2A (residues 224-236, as per 441aa tau isoform) and presenilin 1 (Sontag *et al.*, 1999; Takashima *et al.*, 1998).

Tau undergoes characteristic post-translational modifications, including glycosylation and phosphorylation (see for review Buee *et al.*, 2000; Johnson & Stoothoff, 2004; Fig. 1-7). These post-translational modifications are differentially regulated in specific cell compartments, cell types and during development. Moreover, post-translational modifications at specific sites were found to alter the function of tau and may play a role in subcellular localisation and degradation of tau. For example, phosphorylation of tau at KxGS motifs within the microtubule-binding domain reduces the affinity for microtubule binding (see, for example, Drechsel *et al.*, 1992). There are 79 serine and threonine residues within the 441 tau isoform (Fig. 1-7). Over thirty of these sites have been found phosphorylated on tau (Fig. 1-7). Many of these sites are proline-directed serine/threonine phosphorylation sites and are modified by proline-directed kinases. The proline-directed kinases include cyclin-dependent kinases (Cdks; e.g. Cdc2, Cdk2 and Cdk5), glycogen synthase kinase 3 (GSK3), mitogen activated protein kinases (MAPKs; e.g. ERK and MEK) and stress-activated protein kinases (SAPKs; e.g. c-jun N-terminal kinase (JNK)). Amongst others the following proline-directed phosphorylation sites have been found modified within the 441 residue human tau isoform (Fig. 1-7): Ser46, Thr50, Thr69, Thr153, Thr175, Thr181, Ser199, Ser202, Thr205, Thr212, Thr217, Thr231, Ser235, Ser396, Ser404 and Ser422. However, the function of the phosphorylation at these sites is not yet understood, but it is believed to be important for the association with the microtubules (see for review Johnson & Stoothoff, 2004). Interestingly, none of the proline-directed phosphorylation sites is directly located within a microtubule-binding repeat indicating additional putative functions for these phosphorylation sites. The level of tau phosphorylation is balanced by antagonistic action of phosphatases including PP1, PP2A PP2B and PP5 (see, for example, Liu *et al.*, 2005). Interestingly, both, kinases as well as phosphatases, have been found directly or indirectly associated with microtubules and/or tau, suggesting that the regulation of the phosphorylation levels of tau may be important for its function.

There is still only limited knowledge on the physiological roles of tau and tau phosphorylation. One well-characterised function of tau is the binding and stabilisation of microtubules (for review, see Buee *et al.*, 2000; Johnson & Stoothoff, 2004). Tau interacts directly with microtubules and subsequently promotes their polymerisation (see, for example, Drechsel *et al.*, 1992). Increasing levels of phosphorylation on tau at KxGS motifs within the microtubule-binding domain have been found to induce dissociation of tau from the microtubules and in turn destabilisation of the microtubules (see, for example, Biernat *et al.*, 1993). The interaction of tau and microtubules is also important in the regulation of microtubule-dependent axonal transport (see, for example, Stamer *et al.*, 2002; Mandelkow *et al.*, 2003 and 2004). It has been proposed that local, intermittent dissociation of tau from the microtubules is required to enable the progression of motor-molecules such as kinesin and dynamin (Mandelkow *et al.*, 2004). The dissociation of tau is thought to be regulated via its phosphorylation, specifically mediated by microtubule-affinity regulating kinase (MARK) (at KxGS motifs) and GSK3 (see, for example, Mandelkow *et al.*, 2003 and 2004; Cho & Johnson, 2003; Tatebayashi *et al.*, 2004). These observations prompted the notion that the precise regulation of phosphorylation of tau is important for its normal cellular functions. In contrast, increased tau phosphorylation disrupts cellular processes including microtubule stability and axonal transport. Moreover, tau has been implicated in neurite outgrowth (Caceres & Kosik, 1990). Interestingly, a recent study in a transgenic mouse line expressing mutant tau-P301L reported that LTP is increased in DG, but not in hippocampal area CA1 of young P301L mice before the onset of hyperphosphorylation and tauopathy (Boekhoorn *et al.*, 2006). The increased DG LTP coincided with a higher preference in the novel object recognition task after 3.5h in the P301L mice. In contrast to this finding, old P301L mice exhibit learning and memory impairments, probably due to incipient neuronal cell loss (Ramsden *et al.*, 2005). The changes in LTP and memory in the young P301L mice were not due to alterations in tau phosphorylation, neurogenesis, dendritic morphology or hippocampal morphology (Boekhoorn *et al.*,

2006). These results suggest that expression of mutant tau-P301L directly affects neuronal processes and implicate tau in synaptic plasticity and memory formation.

Much research has focussed on tau and the regulation of its phosphorylation levels, because tau is characteristically hyperphosphorylated in the filamentous aggregates found in many patients with neurodegenerative tauopathies including AD (see, for example, Grundke-Iqbal *et al.*, 1986b; see for review Buee *et al.*, 2000; Lee *et al.*, 2001; Table 1-2). Hence, it was hypothesised that the abnormal phosphorylation of tau may be responsible for their aggregation into intraneuronal filamentous inclusions in neurodegenerative tauopathies. However, many of the sites that are hyperphosphorylated in aggregated filamentous tau, have also been found phosphorylated on fetal and native adult tau (see, for example, Kenessey & Yen 1993; Brion *et al.*, 1993; Matsuo *et al.*, 1994). In addition, despite the fact that fetal tau is highly phosphorylated, no tau aggregation could be observed (see, for example, Matsuo *et al.*, 1994). Furthermore, recombinant tau forms filamentous structures without being phosphorylated in the presence of polyanions (see, for example, Goedert *et al.*, 1996). These findings indicate that phosphorylation of tau may not be involved in the aggregation of tau or additional factors are required. Some results suggest that glycosylation, oxidation, faulty degradation and truncation of tau might trigger the assembly of filaments (see for review Buee *et al.*, 2000). Other studies demonstrate the involvement of co-factors such as glycosaminoglycans, metal ions (i.e. Aluminium) and polyanions (i.e. RNA and lipids) in tau filament formation (see for review Buee *et al.*, 2000).

Native tau and aggregated tau from pathological filaments can be unequivocally distinguished by biochemical means indicating fundamental differences between the two states (see for review Lee *et al.*, 2001). Separation of aggregated filamentous tau by SDS-PAGE produces characteristic electrophoretic profiles. These electrophoretic profiles vary between the different diseases, but are often characteristic within a specific disease. The differences in electrophoretic mobility might result from post-translational

modifications and subsequent conformational changes of tau. Interestingly, the prolyl isomerase Pin1, a molecule that catalyses conformational conversion of proline-directed phosphorylation sites, has been implicated in hyperphosphorylation of tau and neurodegeneration (see for review Lu *et al.*, 2002). Phosphorylation at some proline-directed sites induces a conformational change in the backbone of proteins and thereby restricts the accessibility for phosphatases. Pin1 activity can restore the function of some phosphorylated proteins by reverting the conformation and thereby promoting its dephosphorylation. A KO mouse line lacking Pin1 expression exhibits age-dependent motor and behavioural deficits, tau hyperphosphorylation, tau filament formation and neurdegeneration (Liou *et al.*, 2003). These results indicate that Pin1 plays a role in preventing tau hyperphosphorylation and protecting against neurodegeneration.

1.10) Amyloid-β precursor protein and Aβ-peptides

1.10.1) Amyloid-β precursor protein

The amyloid-β precursor protein (APP) is the product of a single gene located on chromosome 21 and is widely expressed in many tissues (see for review Turner *et al.*, 2003; Swiss Prot|P05067|A4_HUMAN and Swiss Prot|P12023|A4_MOUSE). Three major APP isoforms including APP695, APP751 and APP770 are generated by alternative splicing (Fig. 1-8). The name indicates the number of amino acids within the APP isoforms (i.e. 695, 751 or 770 amino acids). The expression ratio between the three APP isoforms varies within different cell types and tissues. APP is a single membrane-spanning protein with a large extracellular amino-terminal domain and a short intracellular carboxy-terminal portion (Fig. 1-8 and 1-9). The extracellular part contains a kunitz protease inhibitor domain (lacking in APP695), a domain with a neuroprotective function and domains binding copper, zinc, heparin and collagen (Fig.

1-8). The heparin and collagen binding domains of APP are thought to mediate cell adhesion. The short intracellular portion has binding sites for the proteins Fe65, Ib1 (Jip1b), Dab1, PAT1, shc A, shc C and X11 (Fig. 1-9). These protein interactions with the intracellular tail of APP may be involved in various functions (see for review, Turner *et al.*, 2003; Kerr & Small, 2005). Interaction with Fe65 has been implicated in gene transcription, actin binding and protein trafficking. Interestingly, phosphorylation within the binding domain of Fe65 at Thr668 on APP (as per APP695 or Thr743 as per APP770) inhibits the binding of Fe65, but not Dab1 and X11 (see, for example, Ando *et al.*, 2001). The phosphorylation at this proline-directed Thr668 site of APP is mediated by Cdk5 and might be important for the localisation of APP in axons and conferring a specific function on APP (Iijima *et al.*, 2000). Further, association of the APP carboxy-terminus with Ib1, Dab1, shc A and shc C might regulate kinase activation (see for review, Kerr & Small, 2005). Binding of X11 with APP was found to reduce its processing and may be involved in modulation of protein trafficking and synaptic vesicle release (Borg *et al.*, 1998). In addition, APP may function as a cell surface G-protein-coupled receptor, as G_0 was found to associate with APP (Okamoto *et al.*, 1995). Together, APP has been implicated in diverse processes such as axonal transport, cell adhesion, gene transcription and synaptic vesicle release (see for review Turner *et al.*, 2003). Moreover, the study of APP KO mice revealed impaired LTP and memory deficits (Dawson *et al.*, 1999). These findings link APP to synaptic plasticity and memory processes.

The three alternative splice variants of APP undergo extensive post-translational modifications, including glycosylation and phosphorylation, and complex proteolytic processing that gives rise to a large number of APP isoforms and APP fragments (see for review Turner *et al.*, 2003; Fig. 1-9 and 1-10). APP is processed by several proteases, including secretases and caspases. The secretases cleave APP at two extracellular sites in the vicinity of the transmembrane domain. The α-secretase (possibly the metalloproteases: ADAM10, TACE (or ADAM17) and MDC9) cleaves between the residues 687/688 and the β-secretase (BACE1 and BACE 2) between the

residues 671/672 of the APP770 (All further numberings refer to APP770). The proteolytic cleavage site of the γ-secretase complex (including presenilin 1 and 2, Aph-1, Pen-2, and nicastrin) is within the transmembrane domain between residues 711/712, 713/714 or 720/721. Processing at these sites results in the production of a large extracellular domain fragments and smaller fragments composed of the transmembrane domain and the intracellular tail of APP. Depending on the activation pattern of the different secretases, distinct APP fragments are generated. The processing of APP by the α-secretase and β-secretase are thought to be mutually exclusive. The consecutive cleavage of APP by β- and γ-secretases results in the production of the Aβ-peptides, with the predominant fragments $A\beta_{1-40}$ (residues 672 to 711) or $A\beta_{1-42}$ (residues 672 to 713) and the γ-carboxy-terminal fragments (γ-CTFs; depending on cleavage site, the γ-CTF fragment is 59, 57 or 50 aa long). First, the β-secretase cleaves APP within the extracellular domain and releases the soluble APPβ amino-terminal part (sAPP-β) and the β-carboxy terminal fragment (β-CTF or C99; 99aa long). In a second step, the β-CTF can be cleaved by the γ-secretase within the transmembranal domain and produces the Aβ-peptides and the γ-CTFs.

In addition, APP can be cleaved by the α-secretase within the Aβ-peptide domain and thus precluding formation of Aβ-peptides (Esch *et al.*, 1990). Processing by the α-secretase generates the soluble APPα amino-terminal part (sAPP-α) and the α-carboxy terminal fragment (α-CTF or C83; 83aa long). Subsequent processing of α-CTF by the γ-secretase leads to the production of the γ-CTFs and the p3 peptides (depending on the cleavage site the p3 fragment is 24, 26 or 33aa long). This pathway, involving the α-secretase and the γ-secretase, is thought to be the major secretory pathway. It does not produce Aβ-peptides and hence is not amyloidogenic (Esch *et al.*, 1990). Furthermore, caspases have been shown to cleave APP at several sites within the cytosolic domain giving rise to many more different cleavage products (Ayala-Grosso *et al.*, 2002). For example, during neuronal apoptosis caspases cleave APP at the residues 739/740 and

release the neurotoxic C31 peptide. Most of the APP cleavage products listed above seems to have physiological cellular functions, however very little is known about their precise roles. It was demonstrated that sAPPβ is neuroprotective and regulates cell excitability and synaptic plasticity (see for review Turner *et al.*, 2003). Further, the γ-CTFs, also called APP intracellular domain (AICD), were found to relocalise to the nucleus (see, for example, Cao & Südhof, 2004). Because the γ-CTFs bind the protein Fe65, which interacts with transcription factors, it is thought to be a mechanism to regulate gene transcription (Cao & Südhof, 2004). Little is known about the downstream mechanisms that regulate the cleavage of APP into Aβ-peptides. One study reported that the production of Aβ-peptides is regulated by GSK3α activity (Phiel *et al.*, 2003). There, it is shown that therapeutic concentrations of lithium, a GSK3 inhibitor, block the production of Aβ-peptides by interfering with APP cleavage at the γ-secretase step. Further, in transgenic mice with over-expression of APP, the accumulation of Aβ-peptides is also blocked by lithium administration (Phiel *et al.*, 2003; Ryder *et al.*, 2003). Another study confirms the involvement of GSK3 in APP processing (Ryder *et al.*, 2003). However their results implicate the β-isoform of GSK3 in the cleavage of APP. In addition, this study shows that application of roscovitine, a Cdk5 inhibitor, or Cdk5 antisense oligonucleotides stimulates the production of Aβ-peptides. These results indicate that GSK3 activity is increasing the level of Aβ-peptides, possibly by regulating γ-secretase activity, whereas Cdk5 activity is inhibiting Aβ-peptide production. It is thought that Cdk5 regulates the processing of APP via the phosphorylation at Thr668 within the carboxy-terminus of APP. It was observed that in cell cultures over-expression of p35 and subsequent over-activation of Cdk5 led to phosphorylation of both mature and immature APP at Thr668, whereas over-expression of p25 led predominantly to phosphorylation of immature APP (Liu *et al.*, 2003). Curiously, this study implicates increased APP phosphorylation and Cdk5 over-activation with elevated APP processing resulting in enhanced secretion of sAPP-α, sAPP-β and Aβ-peptides contradicting previous findings (see, for example, Ryder *et al.*, 2003).

1.10.2) Aβ-peptides

Much research was focused on the production and function of Aβ-peptides, $A\beta_{1-40}$ and $A\beta_{1-42}$, because of their involvement in Alzheimer's disease (see for review Selkoe, 2001). The Aβ-peptides are the main component in the extracellular senile plaques characteristically found in AD patients. Some early-onset forms of FAD could be directly linked to point mutations near the APP cleavage sites (e.g. Swedish mutation: K670N/M671L, Dutch mutation: E693Q), which alter the processing of APP and lead to increased production of $A\beta_{1-42}$. It was hypothesised that an age-dependent accumulation and aggregation of Aβ-peptides, triggered by increased production and reduced Aβ-peptide clearance, are the primary cause of FAD, as well as sporadic AD. However, the extend to which Aβ-peptides are responsible for the cognitive decline in AD is controversial. Insoluble Aβ-peptide aggregation correlates only poorly with the cognitive state of AD patients. In contrast, NFT formation reflects more accurately the cognitive decline of patients (Braak & Braak, 1991). However, it has been found that the levels of soluble Aβ-peptide oligomers correlate better than senile plaques with the severity of the dementia (Lue *et al.*, 1999). These findings are supported by behavioural studies in transgenic mice expressing human APP 695 carrying the Swedish mutation (Tg2576), where cognitive impairment is observed long before aggregates of Aβ-peptides are found (Chapman *et al.*, 1999). Further, intraventricular injection of soluble mutated Aβ-peptides inhibited the induction of CA1 LTP in anaesthetised rats (Klyubin *et al.*, 2004). Interestingly, application of $A\beta_{1-42}$ peptides impaired DG LTP and mediated inhibition of the phosphorylation of CaMKII at Thr286 and of the AMPA receptor subunit GluR1 at Ser831 (Zhao *et al.*, 2004). These results suggest that Aβ-peptides interfere with molecular mechanisms important for hippocampal synaptic plasticity and thereby induce memory deficits as observed in AD patients.

Furthermore, Aβ-peptides have been implicated in cholinergic neurotransmission. $A\beta_{1-42}$ peptides associate with nicotinic acetylcholine receptors and thereby possibly alter

their conductance (Wang *et al.*, 2000). The dysfunction of acetylcholine receptors and AD is well established and cholinergic neurons are particularly prone to degeneration in AD (see for review Selkoe, 2001). This provides a further possible mechanistic link between the Aβ-peptides and cognitive dysfunction in AD. Moreover, $A\beta_{1-42}$ was linked to increased Ca^{2+} influx through L-type VGCC (see, for example, Ueda *et al.*, 1997). Increased Ca^{2+} influx triggers many molecular processes important for the induction of synaptic plasticity (see *section 1.6*). Hence, the activation of L-type VGCC by Aβ-peptides could lead to the induction of some forms of synaptic plasticity. However, increased Ca^{2+} influx over long periods or at high levels could also induce neurotoxicity.

$A\beta_{1-42}$ has a higher tendency to self-association than $A\beta_{1-40}$ and was shown to be more toxic to neurons (see for review Selkoe, 2001). Interestingly, a study using transgenic mice carrying the Dutch mutation in APP (E693Q) crossed with transgenic mice producing mutated presenilin 1 demonstrated the importance of the ratio of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides for the formation of amyloid pathology at the brain vasculature or in the parenchyma (Herzig *et al.*, 2004). This study demonstrates that neuronally-produced $A\beta_{1-40}$ is key for the vascular amyloid pathology observed in cerebral amyloid angiopathy and emphasises the different roles of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides in amyloid pathology.

In summary, Aβ-peptides play an important role in the pathology of AD and are possibly involved in the cognitive deficits observed in AD. However, recent results indicate that Aβ-peptides serve a physiological role and are not solely a toxic by-product (see, for example, Wang *et al.*, 2004). The basic cellular functions of the Aβ-peptides are not well understood. Therefore, it is important to elucidate the precise biological roles of the Aβ-peptides and its precursor molecule APP, in order to better understand the processes involved in AD.

1.11) **Cyclin-dependent kinase 5**

Cyclin-dependent kinase 5 (Cdk5) is a multifunctional serine/threonine kinase of the cyclin-dependent kinase (Cdk) family (see for review Dhavan & Tsai, 2001; Swiss Prot|P49615|CDK5_MOUSE). Cdk5 is expressed in all tissues with the highest level of expression and kinase activity found in post-mitotic neuronal cells (Hellmich *et al.*, 1992). Cdk5 is a proline-directed kinase, which means that it can phosphorylate serine/threonine residues only immediately upstream of a proline residue resulting in the consensus phosphorylation motif x-(S/T)-P-x. Cdk5 has a broad range of substrates with diverse functions (see Table 1-3).

Table1-3. Cdk5 substrates

Trans-membranal proteins	Scaffolding proteins	Cytoskeletal and associated proteins	Proteins implicated in transcription	Protein kinases and phosphatases	Proteins involved in neurotrans-mitter release	Others
APP	β-catenin	Filamin	β-catenin	Apoptosis-associated tyrosine kinase	Amphyphysin I	Cables
ErbB (neuregulin receptor)	PSD95	MAP1B and MAP2	Histone H1	CPRK	Dynamin I	Canoe
NCAM		Neurofilament H and M	MEF2	DARPP-32	Munc-18 (Sec1 or p67)	cGMP phospho-diesterase
NR2A		NUDEL	mSds3	FAK	Synapsin I	Disabled 1
Presenilin-1		tau	p53	JNK3	synaptojanin	Ezrin
VGCC (P/Q-type and L-type)			Rb	MEK1	Trio	nestin
			STAT3	p35 and p39 (Cdk5 activator)		Pctaire 1
				PAK1 (α-Pak)		RasGRF2
				PP1 inhibitor-1 and 2 (I-1/I-2)		Tyrosine hydroxylase
				Src		

Abbreviations: APP, amyloid precursor protein; CPRK, Cdk5/p35 regulated kinase; DARPP-32, dopamine and cAMP-regulated phosphoprotein molecular mass 32 kDa; FAK, focal adhesion kinase; JNK, c-jun N-terminal kinase 3; MAP, microtubule-associated protein; MEF2, myocyte enhancer factor 2; mSds3, mammalian suppressor of defective silencing 3; NR2A, NMDA receptor subunit 2A; PSD-95, post-synaptic density 95 kDa; RasGRF2, Ras protein-specific guanine nucleotide-releasing factor 2; Rb, ; Src, ; STAT3, signal transducer and activator of transcription 3;VGCC, voltage-gated calcium channel *(After Shelton & Johnson, 2004 and Angelo et al. 2006).*

Cdk5 activity has been implicated amongst other functions in the regulation of the cytoskeleton, axon guidance, axonal transport, apoptosis and synaptic plasticity (see, for example, Morfini *et al.*, 2004; Li *et al.*, 2001; see for review Dhavan & Tsai, 2001). Despite similar substrate specificity to other mitotic Cdks, no function of Cdk5 in cell-cycle regulation was observed (Van den Heuvel & Harlow, 1994). The role of Cdk5 in the development of the CNS is well-established. There it is essential for regulating neuronal migration, axo-dendritic organisation and the development of cortical laminar architecture (Nikolic *et al.*, 1996; Ohshima *et al.*, 1996; Chae *et al.*, 1997). Another intensively studied function of Cdk5 is the phosphorylation of neuronal cytoskeletal proteins, including neurofilaments and the microtubule-associated proteins MAP1B, MAP2 and tau and its potential involvement in neurodegenerative diseases including AD (see for review Shelton & Johnson, 2004). Furthermore, recent studies provide compelling evidence for a role in synaptic plasticity and memory formation (see for review Angelo *et al.*, 2006; see *section 1.11.5*).

1.11.1) Cdk5 activation requires association with activator proteins

As other members of the Cdk family, Cdk5 alone has no enzymatic activity and requires the association with a regulatory subunit for activation. Several activators of Cdk5 have been identified, including p35 and p39 (Ishiguro *et al.*, 1994; Lew *et al.*, 1994; Tsai *et al.*, 1994; Humbert *et al.*, 2000; Swiss Prot|P61809|CD5R1_MOUSE). The interaction of Cdk5 with one of these regulatory subunits is sufficient to activate the kinase (Lew *et al.*, 1994; Tsai *et al.*, 1994). The Cdk5 activators, p35 and p39, are myristoylated at the N-terminus and are in turn membrane-bound (see, for example, Patrick *et al.*, 1999). This restricts the Cdk5 activity to distinct subcellular compartments in the vicinity of membranes. Hence, many Cdk5 substrates are likely to be transmembranal, membrane-bound or membrane-clustered proteins (Table 1-3). So far, it is not clear whether p35 and p39 target Cdk5 activity to a similar or to distinct subsets of substrates. Studies in p39-null mutant mice that lack any specific phenotype indicate that p35 can compensate

for p39 function (Ko *et al.*, 2001; Table 1-4). In contrast, in p35-null mutants p39 can compensate only for some functions of p35 (Chae *et al.*, 1997; Ko *et al.*, 2001).

1.11.2) Formation of the Cdk5 activator p25 by cleavage of p35

The Ca^{2+}-dependent protease calpain can cleave the Cdk5 activator p35 into the carboxy-terminus fragment, p25 and the amino-terminus fragment p10 (Kusakawa *et al.*, 2000). The protein fragment p25 acts also as an activator of Cdk5. Because p25 is more stable than p35, formation of p25 leads to over-activation of Cdk5. The half-life of p35 is short with about 20-30 minutes (Patrick *et al.*, 1998). The degradation of p35 is directly regulated via Cdk5 activity. Phosphorylation of p35 by Cdk5 in the amino-terminal portion leads to degradation of p35 via the ubiquitin-proteosome pathway (Patrick *et al.*, 1998). This negative feedback regulation restricts the formation of the active p35/Cdk5 complex to a limited period of time and constitutes an elegant mechanism to control the kinase activity of Cdk5. The amino-terminus part containing the degradation-inducing phosphorylation site is lacking in p25 and hence it is not subjected to the feedback regulation resulting in longer half-life.

Furthermore, p25 lacks also the amino-terminus myristoylation site of p35 that confers membrane localization. Consequently, p25 displays a broad distribution throughout the cell. Thus, formation of p25 has been suggested to alter the localization of active Cdk5 within the cell (Patrick *et al.*, 1999). In addition, association of Cdk5 with either p35 or p25 may confer discrete substrate specificities to Cdk5. This is indicated by the finding that expression of p25 in p35-null mutants could rescue the phenotypical deficits only partially (Patzke *et al.*, 2003; Table 1-4). In addition, association of Cdk5 with either p35 or p25 resulted in differential phosphorylation of the amyloid precursor protein (APP) and microtubule-associated protein 1B (MAP1B) (Liu *et al.*, 2003; Kawauchi *et al.*, 2005).

Table 1-4. Mouse models with altered expression of Cdk5 or p35.

Mouse line; Reference	Mutation; Genetic background	Phenotype/ Appearance	AD-like molecular phenotype	Remarks
Cdk5 (-/-) (Ohshima *et al.*, 1996)	KO mouse line; C57BL/6	Lesions in brain and spinal cord, not in other tissues, lack of cortical laminar structure and cerebellar foliation; perinatal death	Accumulation of NF immunoreactivity in neuronal cell bodies suggesting defective transport of NF	Heterozygous Cdk5 (+/-) mice appear normal.
p35 (-/-) (Chae *et al.*, 1997)	KO mouse line; 129/Sv and C57BL/6	Cortical lamination defects, altered cell orientation, dendritic and axonal trajectories; sporadic adult lethality and seizures	Not assessed	Study links the Cdk5 activator p35 to proper neuronal migration
p39 (-/-) and **p35/p39 (-/-)** (Ko *et al.*, 2001)	p39 KO mouse line and p35/p39 double KO mouse line; C57BL/6	p39 KO mice have no phenotypic abnormalities, loss of p39 produced no changes in p35 and Cdk5 levels and Cdk5 activity; Double KO mice are perinatal leathal and display similar phenotype as Cdk5 KO mice, double KO display no Cdk5 activity in brain,	No changes in tau phosphorylation during development; Phosphorylation of NF-H is unaltered; Changes in cellular NF-H localisation in spinal cord	Study shows distinct roles for p35 and p39; Regulation of p39 levels by p35 expression; p35 and p39 seem to be only Cdk5 activators important during CNS development.
p35 (-/-) (Hallows *et al.*, 2003)	KO mouse line; C57BL/6	Cortical lamination defects, axonal derangement, redistribution of Cdk5 and cytoskeletal proteins, Cdk5 activity reduced by approx. 60 %.	Increased phosphorylation of NF, MAP2B and tau at Ser202, Ser235, Ser396/404; Increased GSK3β activity	Results indicate a role in neuronal trafficking for CdK5/p35.
p35 (-/-) expressing p25 (Patzke *et al.*, 2003)	Crossing of transgenic p25 mice (Ahlijanian *et al.*, 2000) and p35 (-/-) mice (Chae *et al.*, 1997); C57BL/6	Partial rescue of the p35 (-/-) phenotype, phosphorylation of disabled-1 is partially restored.	No tau hyperphosphorylation, p25 levels increase with age; (spongiform degeneration in the cortex at 2 years)	Study shows that p25 can substitute some but not all functions of p35.

Abbreviations: CNS, central nervous system; GSK3β, glycogen synthase kinase 3β; KO, knock-out; MAP2B, microtubule-associated protein 2B; NF, neurofilament; *(Taken from Giese et al., 2005)*

1.11.3) Formation of p25 under pathological conditions and in disease models

The formation of the Cdk5 activator p25 has been observed so far only under neurotoxic and pathological conditions (see, for example, Lee *et al.*, 2000; see for review Dhavan & Tsai, 2001; Shelton & Johnson, 2004). It is thought that under pathological conditions an alteration in cellular calcium homeostasis occurs, as proposed for example in AD. Changes in intracellular calcium levels would in turn induce calpain activation, and lead to the subsequent cleavage of p35 into the protein fragments p25 and p10. Several post-mortem studies describe the accumulation of the protein fragment p25 in patients with sporadic AD patients, but not in healthy control patients (Patrick *et al.*, 1999; Tseng *et al.*, 2002; Swatton *et al.*, 2004). One problem encountered in post-mortem studies is the spontaneous generation of p25 due to pathological activation of calpain. Other studies did not detect p25 production in AD patients (Yoo & Lubec, 2001; Takashima *et al.*, 2001; Tandon *et al.*, 2003) and hence, there is still controversy as to whether p25 is formed specifically in AD.

However, further support for the specific p25 production in AD comes from the findings that calpain and Cdk5 activity are increased in post-mortem AD brain tissue (Veeranna *et al.*, 2004; Taniguchi *et al.*, 2001; Lee *et al.*, 1999). In addition, the formation of p25 has been described in the transgenic mouse line Tg2576 that models familial AD by expressing the APP carrying the Swedish mutation K670N and M671L (Otth *et al.*, 2002) and in the conditional presenilin double KO mouse line (Saura *et al.*, 2004) (Table 1-5). Interestingly, in patients with Niemann-Pick type C disease, another neurodegenerative tauopathy, accumulation of p25 was observed. Consistent with this, a transgenic mouse line modelling Niemann-Pick type C disease by expressing a mutant form of the protein npc-1 displayed also p25 accumulation (Bu *et al.*, 2002).

Table 1-5. Disease models in which p25 formation occurs.

Mouse line; Reference	Mutation; Mouse genetic background	p25 levels; Cdk5 activity	(AD-like) phenotype; Neurodegeneration	Phenotype	Other remarks
Amyotrophic lateral sclerosis model; (Nguyen *et al.*, 2001)	Mutant SOD (G37R); C57BL/6	Ratio of p25/p35 is about 1:2 to 1:3; 2-fold increase in Cdk5 activity in spinal cord.	Hyperphosphorylation of tau and NF proteins.	Mislocalization of Cdk5 in motor neurons; accumulation of NF in the perikarya	Recent studies indicate p25 expression is not responsible for motorneuronal degeneration in this ALS mouse model (Takahashi *et al.*, 2004)
Niemann-Pick type C model; (Bu *et al.*, 2002)	npc-1 mutation;	p35 signal is several fold stronger than p25; Cdk5 activity is 1.5 to 2-fold increased.	Hyperphosphorylation of NF, MAP2 and tau. Elevated NF levels; neuronal cell loss	Extensive lipid storage accumulation, neuroaxonal dystrophy, neuronal cell loss	p25 formation also observed in human NPC patients; Heterozygous npc-1 (+/-) show no cytoskeletal pathology.
APP transgenic Tg2576 mice; (Otth *et al.*, 2002)	Human APP with Swedish mutation; Mix of C57Bl/6 and SJL	p35 signal is several fold stronger than p25; Cdk5 activity is about 1.5-fold increased.	Hyperphosphorylated tau; small-clustered core plaques in hippo-campus and cortex; dystrophic neurites and astrogliosis		Cdk5 colocalizes with plaques
Presenilin double mutant; (Saura *et al.*, 2004)	Conditional presenilin double KO mouse line; C57BL6/129 hybrid mice	p35 signal is several fold stronger than p25; Cdk5 activity not assessed.	Age-dependent tau hyperphosphorylation and neurodegeneration	Memory impairment and altered synaptic plasticity	Presenilins are components of the γ-secretase protein complex and are thought to affect cellular levels of calcium.
Rat ischemia model; (Wang *et al.*, 2003)	N/A	Ratio of p25/p35 is about 1:2 to 1:3; At different times after ischemia Cdk5 activity was 2 to 4-fold increased.	Apoptosis, neuronal cell loss		Study shows that production of p25 is mediated via Ca^{2+} influx through AMPA receptor channels in ischemic hippocampal CA1 neurons.

Abbreviations: ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; KO, knock-out; NF, neurofilament; NPC, Niemann-Pick type C disease; SOD, superoxide dismutase. *(Taken from Giese et al., 2005)*

Further, p25 formation was detected in a transgenic mouse line modelling amyotrophic lateral sclerosis (ALS; also known as motor neuron disease) by expressing superoxide dismutase carrying a point mutation (G37R) (Nguyen *et al.*, 2001). However, recently it was demonstrated that p25 and Cdk5 activation are not involved in the ALS-like pathogenesis (Takahashi & Kulkarni, 2004). Finally, ischemic insult in rat after occlusion of cerebral arteries was shown to trigger generation of p25 and a subsequent increase in Cdk5 activity in the hippocampal area CA1 (Wang *et al.*, 2003). This study further demonstrated that the p25 production is mediated via Ca^{2+}-influx through the AMPA receptor channels. For a summary of disease models in which p25 formation occurs, see Table 1-5.

1.11.4) Implication of Cdk5 in tau hyperphosphorylation, tangle formation and neurodegeneration

Enhanced phosphorylation of the microtubule-associated protein tau at proline-directed serine/threonine sites is characteristic for many neurodegenerative diseases including AD (Paudel *et al.*, 1993; Morishima-Kawashima *et al.*, 1995; see for review Lee *et al.*, 2001). Cdk5 has been identified as a prime candidate for mediating tau hyperphosphorylation at proline-directed phosphorylation sites; because Cdk5 co-localizes with the filamentous tau depositions and shows enhanced activity in some neurodegenerative diseases including AD (Patrick *et al.*, 1999; Augustinack *et al.*, 2002b; see for review Shelton & Johnson, 2004). Furthermore, Cdk5 has been involved in phosphorylation of other neuronal cytoskeletal proteins, including neurofilaments MAP1B and MAP2. In addition, Cdk5 activity was found to be detrimental to cell survival. In cultured primary neurons treatment with agents that induce neurotoxicity, such as glutamate, ionomycin and H_2O_2, trigger production of p25, which in turn leads to over-activation of Cdk5 (Lee *et al.*, 2000). Expression of p25 in cultured primary cortical neurons was shown to increase Cdk5 activity and produced drastic effects, including collapse of microtubules and apoptosis (Patrick *et al.*, 1999).

In order to study the physiological effect of p25 formation and Cdk5 over-activation several TG mouse lines expressing p25 have been generated (For a summary of TG mouse lines expressing p25, see Table 1-6). In fact, the reported accumulation of p25 in AD and the possible involvement of Cdk5 in tau hyperphosphorylation made these p25-expressing TG mice an interesting model to test the implication of p25-induced Cdk5 over-activation in neurodegenerative diseases. The study of the different p25-expressing TG mouse lines yielded inconsistent results; for example, on tau hyperphosphorylation. This might be explained by the different strategies that were used to generate the TG mice. In some TG lines, where the transgene was active during embryonic development, paralysis and motor abnormalities were observed (Ahlijanian *et al.*, 2000; Takashima *et al.*, 2001; Bian *et al.*, 2002). This precluded an analysis of memory formation and might have induced artifacts. In all TG mouse lines the expression of p25 led to an over-activation of Cdk5 (Table 1-6). Hence, all studies investigated the impact of the p25-induced over-activation on the phosphorylation levels of tau and neurodegeneration. Most of the studies observed tau hyperphosphorylation and increased phosphorylation of neurofilaments as tested in immunoblot and immunohistological studies (Table 1-6). However, the onset and the level of tau hyperphosphorylation varied markedly between the different TG p25 mouse lines (Table 1-6; for further discussion see *section 4.3.3*). Interestingly, a transgenic mouse line over-expressing the Cdk5 activator p35 did not detect increased tau phosphorylation (Van den Haute *et al.*, 2001). This result suggests that p25 and p35 direct the Cdk5 activity towards distinct substrates and that the active complex p35/Cdk5 is not involved in the phosphorylation of tau (see also Patrick *et al.*, 1999). Two of the TG p25 mouse lines exhibited aggregates of hyperphosphorylated tau, similar to neurofibrillary tangles (NFT) observed in AD patients (Nobel *et al.*, 2003; Cruz *et al.*, 2003). Moreover, in an inducible p25 mouse line immunoblot and immunohistological studies revealed signs of neurodegeneration and gliosis (Cruz *et al.*, 2003).

Table 1-6. Mouse models with p25 expression.

Mouse line; Reference	Transgene; Promoter; Mouse genetic background	Expression pattern, Expression levels; Cdk5 activation	Appearance/ Behaviour/ Synaptic plasticity	AD-like molecular phenotype; Neurodegeneration	Other remarks
p25 TG mice; (Bian *et al.*, 2002)	Human p25; PDGF B chain promoter; FVB/N	Brain and spinal cord; Ratio of p25/p35 is 5:1 ; 1.5 – 2-fold increase in Cdk5 activity	Hindlimb semiparalysis and mild forelimb dyskinesia beginning at 3 months of age	Hyperphosphorylated tau, axonal swelling; no NFTs.	Sciatic nerve and leg muscle do not show axonal abnormalities.
p25 TG mice; (Ahlijanian *et al.*, 2000)	Human p25; Rat NSE promoter; FVB/N	Brain (tested only); Ratio of p25/p35 is 1:1 (lower in cerebellum); Up to 2-fold increase in Cdk5 activity;	At 4-9weeks of age whole body exertion tremors/ increased locomotor activity more time spent on open arms of elevated plus-maze test	Hyperphosphorylation of tau and NF detected by IHC, but not IB, positive Bielschowsky staining; axonal swelling in amygdala.	axonal swelling in spinal cord
p25 TG mice; (Takashima *et al.*, 2001)	Bovine p25; pCMV or PDGF promoter	Cortex, cerebellum and pituitary gland; 3 - 5-fold increase in p25 expression; 2 - 5-fold increase in Cdk5 activity,	Unable to stand upright at 5 month, died at 6 month	No tau hyperphosphorylation (Ser202,Ser396, and Ser404 tested), No neuronal cell loss and apoptosis.	Enlarged pituitary gland with high level of p25 expression, possibly inducing cell proliferation.
p25/mutant tau double TG mice; (Nobel *et al.*, 2003)	p25 mouse line (Ahlijanian *et al.*, 2000) crossed with TG mice expressing mutant (P301L) human tau	Amygdala (tested only); Ratio of p25/p35 is about 1:2 to 1:4; Approx. 2-fold increase in Cdk5 activity	Pre-paralysis and early-stage degeneration mice were analysed	Tau hyperphosphorylation, tau aggregates in sarkosyl-insoluble fractions, NFT as detected by silverstain	P301L TG mice develop NFTs in the brainstem, spinal cord, telencephalon and diencephalons; Neuronal cell loss in spinal cord
Inducible p25 TG mice; (Cruz *et al.*, 2003)	Human p25, GFP and c-myc tagged; TetO-p25 mice crossed with CamKII-tTA TG mice; C57BL/6	Inducible, restricted to forebrain; Ratio of p25/p35 is about 2:1 to 3:1; 2.3-fold increase in Cdk5 activity	Not assessed	Tau hyperphosphorylation, accumulation of sarkosyl-insoluble tau, NFTs; Neurodegeneration and gliosis tested with antibodies in IHC and IB	Raised under presence of doxycycline for 4 - 6 weeks; Leaky transgene; Different substrate specificity of p25/Cdk5 detected.
p25 TG mice; (Angelo *et al.*, 2003; Ris *et al.*, 2005)	Mouse p25; alpha CaMKII; C57BL6 or 129B6F1	Restricted to forebrain; Ratio of p25/p35 is 1:3 in hippocampus;	Normal appearance/ Altered fear conditioning and spatial memory formation/ altered hippocampal LTP	Hyperphosphorylation of NF-M No neurodegeneration or gliosis	Sexual dimorphisms in behavioural phenotype and hippocampal LTP

Abbreviations: CamKIIalpha, calcium/calmodulin kinase II alpha; CamKII-tTA, tetracycline-sensitive transactivator driven by CamKIIalpha promoter; CMV, cytomegalovirus; GFP, green fluorescence protein; IB, immunoblot; IHC, immunohistochemistry; NF, neurofilament; NFTs, neurofibrillary tangles; NSE, neuron-specific enolase; PDGF, platelet derived growth factor; TetO, tetracycline operon; TG, transgenic. *(Taken from Giese et al., 2005)*

Taken together the analysis of the different p25 mouse lines indicates that the protein kinase complex p25/Cdk5 plays an important role in the phosphorylation of tau at proline-directed sites. However, the variation of the onset and level of tau hyperphosphorylation within the different p25 mouse lines suggest that tau hyperphosphorylation is regulated via an intricate molecular mechanism. As there is yet no good evidence that p25/Cdk5 directly mediates tau hyperphosphorylation, it is possible that p25/Cdk5 is indirectly involved in tau phosphorylation by regulating other kinases or phosphatases. Furthermore, it remains to be tested in how far the over-activation of Cdk5 is involved in the aggregation of hyperphosphorylated tau and neurodegeneration. It is likely that additional factors are required to cause tau aggregation, as demonstrated by a study using TG mice expressing p25 and a mutated human tau isoform (Nobel *et al.*, 2003). Expression of p25 alone was not sufficient to induce aggregation of hyperphosphorylated tau and only in combination with expression of a mutated tau isoform NFT-like inclusions were observed.

1.11.5) Cdk5 in synaptic plasticity, learning and memory

Cdk5 and its activators, p35 and p39, are abundant in synaptic terminals in mature neurons. Hence, multiple studies investigated a possible role for Cdk5 in synaptic plasticity, learning and memory (see for review Angelo *et al.*, 2006). Cdk5 has been suggested to influence synaptic plasticity by affecting pre- and post-synaptic processes. Cdk5 is thought to affect pre-synaptic mechanisms including ion channel conductance via phosphorylation of P/Q-type VGCC, exocytosis via phosphorylation of Munc 18 as well as synapsin I and endocytosis via phosphorylation of amphiphysin I, dynamin I and synaptojanin (see for review Angelo *et al.*, 2006). Cdk5 mediates phosphorylation of several post-synaptic proteins including the NMDA receptor subunit NR2A and PSD-95 (Wang *et al.*, 2003; Morabito *et al.*, 2004). Phosphorylation of NR2A at Ser1232 by Cdk5 has been linked to increased NMDA receptor conductance and phosphorylation of PSD-95 has been reported to negatively regulate its clustering, possibly with NMDA

receptors. Furthermore, Cdk5 has been involved in the regulation of several signalling pathways including MAPK and PKA pathways (Sharma *et al.*, 2002; Bibb *et al.*, 1999). These signalling pathways have been shown to be important for synaptic plasticity, especially in a post-synaptic context. The regulation of the PKA pathway by Cdk5 has important implications for dopaminergic signalling in striatum and in turn has been associated with drug addiction and might as well be of importance for learning and memory (see for review Bibb, 2001). Cdk5 phosphorylates dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32), a neostriatum-specific protein known to modulate dopamine signalling (Bibb *et al.*, 1999). Phosphorylation of DARPP-32 by Cdk5 turns it into an inhibitor of PKA and prevents it from acting as a PP1 inhibitor. Phosphorylation of DARPP-32 by PKA makes it an inhibitor of PP1, which in turn triggers dopamine-induced phosphorylation of PKA substrates. Interestingly, Cdk5 has also been reported to regulate the PP1 inhibitors, I-1 and I-2 (Bibb *et al.*, 2001; Agarwal-Mawal & Paudel, 2001).

A direct role for Cdk5 in learning and memory has been investigated for the first time by testing the effect of butyrolactone I, a Cdk5 inhibitor, on contextual fear conditioning in mice (Fischer *et al.*, 2002). Intra-hippocampal and intra-septal injection of butyrolacetone I immediately before or after training has been found to impair contextual, but not cued, fear conditioning. These results suggest that Cdk5 is required for associative learning and memory formation in the hippocampus and septum. As Cdk5 KO mice are perinatal lethal and p35 as well as p39 KO mice have developmental disturbances of the brain, only little insight on the role of Cdk5 learning and memory could be gained through the study of these KO mouse lines (Table 1-4). In fact, the analysis of transgenic mouse lines expressing the Cdk5 activator p25 allowed investigation of the role of Cdk5 in synaptic plasticity, learning and memory (see for review Angelo *et al.*, 2006). First studies analysed a transgenic mouse line expressing postnatally low levels of p25 predominantly in excitatory forebrain neurons (Angelo *et al.*, 2003; Ris *et al.*, 2005). These studies demonstrated that low-level p25 expression does not effect basal glutamatergic synaptic transmission, or short-term synaptic

plasticity. However, p25 expression was associated with enhanced CA1 LTP in female p25 TG mice, whereas CA1 LTP was reduced in male p25 TG mice (Ris *et al.*, 2005). Spatial learning as tested in the MWM was improved in female p25 TG mice, but not affected in male p25 mice (Ris *et al.*, 2005). Moreover, tone fear conditioning was improved in female p25 TG mice in the 129B6F1 background, as well as in male and female p25 TG mice in the C57BL/6 background. A recent study investigating an inducible p25 TG mouse line is in support of these results (Fischer *et al.*, 2005). There, contextual fear conditioning was enhanced by transient high level p25 expression, possibly more in females than in males. The improvement in fear conditioning also coincided with enhanced CA1 LTP in the inducible p25 TG mice. However, after six weeks of high level p25 expression these inducible TG mice develop severe neurodegeneration, which consequently results in impaired fear conditioning and deficient CA1 LTP.

Together these observations indicate that expression of p25 and subsequent over-activation of Cdk5 enhances CA1 LTP, possibly in a sex-dependent manner. The enhanced CA1 LTP could be correlated with improvements in spatial learning and in fear conditioning. These findings indicate that the activated p25/Cdk5 holoenzyme is involved in synaptic plasticity, learning and memory. Further, the effects of p25-induced Cdk5 activation suggest that the formation of p25 is a physiological process with importance for synaptic plasticity, learning and memory.

1.12) Glycogen synthase kinase 3

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase, which exists in two isoforms, termed α and β (see for review Grimes & Jope, 2001; Swiss Prot|Q9WV60|GSK3B_MOUSE). The isoforms are products from two distinct genes, but are highly homologous. GSK3α consists of 482 amino acids and GSK3β of 420 amino acids. GSK3 is widely expressed in all tissues and is found at high levels specifically in brain. GSK3 is subject to several regulatory mechanisms. GSK3 can be inhibited by phosphorylation at Ser9 in the β-isoform and at Ser21 in the α-isoform (Stambolic & Woodgett, 1994). The inhibitory phosphorylation of GSK3α/β at the serine residue (Ser21/9) is regulated by antagonistic kinase and phosphatase activity (see for review Grimes & Jope, 2001). Several kinases have been implicated in this inhibitory regulation of GSK3 including AKT (also known as protein kinase B), p90Rsk, p70 S6 kinase, cyclic AMP-dependent protein kinase A (PKA) as well as some isoforms of protein kinase C (PKC). Further, some phosphatases have been shown to counterbalance the inhibitory phosphorylation, including PP1 and PP2A (Grimes & Jope, 2001; Morfini *et al.*, 2004). The multitude of enzymes that can regulate the inhibitory control of GSK3 indicate the central role of GSK3 by integrating many signalling cascades. Impairment of this inhibitory control of GSK3 results in abnormally high GSK3 activity. In contrast, it was proposed that phosphorylation at Tyr216 in the β-isoform and at Tyr279 in the α-isoform is essential for activation of GSK3 (Hughes *et al.*, 1993). Another mechanism, by which GSK3 activity might be regulated, is the interaction of GSK3 with specific complexes. For example, it was suggested that association of specific GSK3β-binding proteins including FRAT 1 and axin leads to inactivation of GSK3β (see for review Grimes & Jope, 2001).

Table 1-7. Substrates of GSK3

Metabolic and signalling proteins	Structural proteins	Transcription factor
acetylCoA carboxylase	Axin	AP-1 (jun family)
APP	dynamin-like protein	β-catenin
APC	MAP1B	C/EBPα
ATP-citrate lyase	MAP2	CREB
PKA	NCAM	glucocorticoid receptor
cyclin D1	neurofilaments	HSF-1
eIF2B	ninein	c-myc
glycogen synthase	tau	NFAT
IRS-1		NFκB
myelin basic protein		
NGF receptor		
protein phosphatase 1 (PP1)		
PP1 inhibitor-2 (I-2)		
pyruvate dehydrogenase		

Abbreviations: AP-1, activatior protein-1; APC, adenomatous polyposis coli gene product; APP, amyloid precursor protein; C/EBPα, CCAAT/enhancer binding protein alpha; CREB, cyclic AMP response element binding protein; eIF2B, eukaryotic initiation factor 2B; HSF-1, heat shock factor-1; IRS-1, insulin receptor substrate-1; MAP, microtubule-associated protein; NCAM, neuronal cell adhesion molecule; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor κB; NGF, nerve growth factor; PP1, protein phosphatase 1 *(After Grimes & Jope, 2001)*

GSK3 is a proline-directed protein kinase and phosphorylates substrates at serine/threonine residues that are directly followed by a proline residue. In addition, GSK3 phosphorylates substrates at so-called "primed" phosphorylation sites (see, for example, Sengupta *et al.*, 1997; Cho & Johnson, 2003). This means GSK3 recognises on substrates a serine/threonine residue only when the residue located four residues upstream prior has been phosphorylated by a priming kinase. This forms the recognition motif –S/T-x-x-x-S/T(phospho)–. The phosphorylated residue is essential for the

recognition motif and enables the binding of GSK3 to the substrate and its subsequent phosphorylation. GSK3 is a multifunctional protein kinase and has a broad range of substrates including metabolic, signalling and structural proteins, as well as transcriptions factors (Table 1-7). The diversity of the substrates indicates a fundamental role of GSK3 in many cellular processes. Indeed, GSK3 has been implicated in a variety of processes including axonal transport, embryonic development, metabolism, apoptosis and transcriptional regulation (see for review Grimes & Jope, 2001).

1.12.1) Involvement of GSK3 activity in neurodegenerative diseases

Much research focussed on the involvement of GSK3 in the phosphorylation of the microtubule-associated protein tau (see for review Imahori & Uchida, 1997; Grimes & Jope, 2001). GSK3 has been isolated as a protein kinase that associates with microtubules (see, for example, Ishiguro *et al.*, 1993; Agarwal-Mawal *et al.*, 2003). The co-localisation with aggregates of hyperphosphorylated tau (Ishizawa *et al.*, 2003) and increased GSK3 levels in AD patients make it a prime candidate to mediate tau hyperphosphorylation in tauopathies (see for review Imahori & Uchida, 1997; Lee *et al.*, 2001). GSK3 is known to phosphorylate proline-directed serine/threonine sites similarly to Cdk5 (Mandelkow *et al.*, 1992; Reynolds *et al.*, 2000) and generate disease-associated phospho-epitopes on tau (Nishimura *et al.*, 2004). Further, GSK3 has been shown to induce hyperphosphorylation of tau, which subsequently resulted in the loss of microtubule-binding (Hanger *et al.*, 1992; Lovestone *et al.*, 1996). Consistently, pharmacological inhibition of GSK3β with lithium or ATP competitive inhibitors reduced the phosphorylation levels of tau and affected the stability of microtubules (see, for example, Hong *et al.*, 1997; Lovestone *et al.*, 1999).

TG mice conditionally over-expressing GSK3β in adulthood developed tau hyperphosphorylation and neurodegeneration in hippocampal neurons; however, no formation of tau filaments was observed (Lucas *et al.*, 2001; Hernandez *et al.*, 2002). A recent study suggests that GSK3 is involved in kinesin-driven axonal transport and hence, disruption of regulation of GSK3 could represent a molecular mechanism underlying neurodegenerative tauopathies (Morfini *et al.*, 2004). Interestingly, this study proposes that GSK3 activity is regulated by Cdk5 as demonstrated by pharmacological *in vitro* assays.

GSK3 has also been linked to other neuropathological mechanisms that are thought to underlie neurodegenerative diseases including AD. GSK3 has been implicated in the regulation of Aβ-peptide production (Phiel *et al.*, 2003; Ryder *et al.*, 2003). Accumulation of Aβ-peptides could be blocked by inhibition of GSK3 activity. Interestingly, it has been reported that Aβ-peptides activate GSK3 and thereby induce tau hyperphosphorylation (Takashima *et al.*, 1996). Further, increased GSK3 activity was implicated in reduction of acetylcholine synthesis by inactivating pyruvate dehydrogenase, which could explain the loss of cholinergic signalling in AD (Hoshi *et al.*, 1997; see for review Imahori & Uchida, 1997). In addition, GSK3 interacts directly with presenilin 1, a molecule associated with FAD (Takashima *et al.*, 1998).

1.12.2) GSK3 in synaptic plasticity and memory

Because of the presence of GSK3 at the synapse and its involvement in diverse processes including axonal transport and transcription, it is a candidate for regulating processes involved in synaptic plasticity, learning and memory. Studies in TG mice conditionally over-expressing GSK3β in hippocampus and cortical neurons showed that increased GSK3 activity resulted in impaired spatial learning in the MWM (Hernandez *et al.*, 2002). Interestingly, increased GSK3 activity also impairs activation of CREB

(Grimes & Jope, 2001). Therefore, it is likely that GSK3 is involved in synaptic plasticity and learning and memory processes via the regulation of transcription factors, such as CREB, nuclear factor of activated T-cells (NFAT) and activator protein-1 (AP-1; i.e. group of transcription factors including c-jun, c-fos and ATF family members). Consistent with this, over-expression of GSK3 attenuates brain-derived neurotrophic factor (BDNF) induced CREB phosphorylation. Application of lithium and carbamazepine can revert this effect and facilitate activation of CREB (Mai *et al.*, 2002). Further, a pharmacological study demonstrates that serotonergic activity regulates the inhibitory phosphorylation of GSK3β in mouse brain *in vivo* (Li *et al.*, 2004). These results link impaired inhibitory control of GSK3 to conditions in which serotonergic activity is deregulated, such as in mood disorders. This finding is consistent with studies implicating the mood stabilising effect of lithium with its function as a general GSK3 inhibitor (see, for example, Phiel & Klein, 2001).

1.13) **Overview**

This is the end of the introductory *Chapter 1*, which should give the reader a short summary of basic knowledge relevant for the research presented in this doctoral thesis. The following *Chapter 2* contains the materials and methods section. During my doctoral studies I analysed two different genetically-modified mouse lines with previously described learning and memory phenotypes. In *Chapter 3*, the results of the investigation of the αCaMKII T286A point mutation mouse line are shown. Furthermore, *Chapter 3* describes the establishment of an *in vivo* LTP approach in the mouse hippocampus to study activity-dependent molecular changes. *Chapter 4* contains the analysis of a transgenic mouse line expressing the Cdk5 activator p25. Finally, *Chapter 5* is a short general discussion looking at aspects of the analysis of both genetically-modified mouse lines and an outlook on putative follow-up studies in these two genetically-modified mouse lines.

CHAPTER 2: Material and Methods

2.1) Materials

All chemicals were from Sigma, unless stated otherwise. All buffers were prepared using the water purified by the Elix system (Millipore). The Milli-Q system (Millipore) was used to generate ultra-pure water.

2.2) Genetically-modified mouse lines

2.2.1) The αCaMKII T286A point mutant mouse line

The αCaMKII T286A knock-in point mutant mouse line was generated in the 129 genetic background by Dr K. Peter Giese in the laboratory of Dr Alcino Silva in the Cold Spring Harbour Laboratories, USA (Giese *et al.*, 1998). The αCaMKII T286A point mutant mice and WT control littermates were bred with at least 10 backcrosses in the C57BL/6 genetic background. The genotype was determined by polymerase chain reaction (PCR) analysis using 0.2 μM 5′-primer (lox1: 5′-CTGACCAGCAGATCAAAGC-3′) and 0.2 μM 3′-primer (lox2: 5′-ATCACTAGCACCATGTGGTC-3′) The PCR generated a 200 bp WT fragment and a 300 bp fragment from mutants. The mice were housed in groups of 2-5 and treated according to the Animals (Scientific Procedures) Act 1986, UK.

2.2.2) The p25 transgenic mouse line

The p25 transgenic mouse line was generated in the C57BL/6 genetic background by Dr Marco Angelo in the laboratory of Dr K. Peter Giese at the Wolfson Institute for Biomedical Research, University College London (Angelo *et al.*, 2003). Heterozygous

p25 transgenic mice (TG) and wild-type (WT) control littermates were bred in the C57BL/6 genetic background. The genotype was determined by PCR analysis (220 bp WT fragment with primers sp2: 5′-GACAAGAAGCGACTCCTCCT-3′ and WT3: 5′-TTCGCGCAAGGTTCATCAGT-3′ ; 320 bp TG fragment with primers sp2: 5′-GACAAGAAGCGACTCCTCCT-3′ and TG3: 5′-TGCTCCCATTCATCAGTTCC-3′). The mice were housed in groups of 2-5 in the animal facilities of the Wolfson Institute for Biomedical Research, University College London, and treated according to the Animals (Scientific Procedures) Act 1986, UK.

2.2.3) Genotyping of genetically-modified mouse lines

For identification the mice were marked by different combinations of holes in their ears. The tip of the mouse tails was desensitised with ice-spray and a piece of approximately 3 mm was cut off. The protein content of the tail was digested in lysis buffer (0.1 M Tris-HCl pH8, 0.2% sodium dodecyl sulfate (SDS), 0.2 M sodium chloride, 5 mM EDTA and 0.1 µg/µl of Proteinase K) at 55°C overnight. Undigested parts were pelleted by centrifugation and the supernatant removed. One volume of isopropanol was added to precipitate the genomic DNA. The DNA was pelleted by centrifugation, washed with 70% ethanol and air dried. The dried DNA pellet was suspended in 100 µl of 10 mM Tris-HCl, pH 7.6. One microlitre of DNA solution was added to 25 µl PCR mix (PCR-reaction mix: 10% of 10x PCR buffer, 2 mM magnesium chloride, 0.1 mM deoxy nucleotide tri-phosphate (dNTP), Taq-polymerase (Invitrogen) and primer quantities specific to mutant mouse type). The GeneAmp® PCR system 9700 (Applied Biosystems, USA) was used for the PCR run with the following program: 2 minutes at 93°C, followed by 32 cycles of 30 seconds at 93°C, 30 seconds at 58°C and 30 seconds at 72°C. The PCR products were separated on a 1.2% agarose gel containing ethidium bromide (4 µl /100 ml of gel) on a Mini-Sub Cell® system (BioRad). The bands were visualised by UV light and a picture of the gel taken on a imaging system with the software AlphaImager 1220 (Alpha Innotech corporation, USA).

2.3) Lysate preparation

2.3.1) Hippocampal dissection

After removal from the scull the brain was placed in a 40 mm Petrie dish filled with 5ml of ice-cold P2-buffer (10 mM Tris-HCl pH 7.6, 320 mM sucrose and 0.025% sodium azide) containing phosphatase inhibitors (1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 2 mM sodium ortho pervanadate, 0.1 mM ammonium molybdate, 0.2 mM phenylarsine oxide) and protease inhibitor tablets Complete© EDTA-free (1 tablet in 25 ml) (Roche). The cerebellum was removed and the brain halved along the midline. The underlying thalamic structures were removed. The hippocampus rolled out and detached from the cortex.

2.3.2) Lysate preparation

Whole hippocampal protein lysate from mutant mice and WT littermates were prepared using P2-buffer containing phosphatase and protease inhibitors (see above) to preserve the integrity and phosphorylation state of hippocampal proteins. The mice were killed using carbon dioxide in accordance to the Animals (Scientific Procedures) Act 1986, UK. After decapitation, the head was immediately washed in ice-cold TBS buffer (10 mM Tris-HCl pH 7.6 and 150 mM sodium chloride) containing phosphatase and protease inhibitors and the brain quickly removed. The hippocampus was dissected in ice-cold P2-buffer as described above. The hippocampi were dounce homogenised in 700 µl ice-cold P2-buffer, snap-frozen on dry ice and stored at –80°C. After defrosting the samples on ice, detergents (1% TritonX-100 and 1% CHAPS) were added.

2.3.3) Synaptosomes

The preparation of crude synaptosomal fractionation from hippocampus was performed as described in Whittaker (1984). Briefly, the hippocampi were dissected as described

above and dounce homogenised in ice-cold P2-buffer (10 mM Tris-HCl pH 7.6, 320 mM sucrose, 0.025% sodium azide) containing phosphatase and protease inhibitors. The homogenate was centrifuged at 1,000 xg for 15 minutes. The resulting pellet (P1) was snap-frozen and the supernatant re-centrifuged at 30,000 xg for 15 minutes. The resulting supernatant was removed and the pellet fraction (P2) resuspended in P2-buffer containing detergents (1% TritonX-100 and 1% CHAPS) snap-frozen on dry ice and stored at –80°C.

2.3.4) Determination of protein concentration

The protein concentrations of the lysates were determined using the BCA assay (Perbio). In brief, 4 µl of lysate were added to 1 ml of BCA solution in 1.5 ml cuvettes (Fisher). In parallel different amounts (0, 5, 10, 15 20 and 25 µl) of bovine serum albumin (BSA) solution (1mg/ml) were added to 1ml of BCA solution in 1.5 ml cuvettes. Each experiment was carried out in duplicate. The cuvettes were incubated at RT under shaking for 90 minutes. The optical density at 562 nm wavelength of each sample was evaluated with a UV-VIS spectrophotometer (UV mini 1240; Shimadzu). The values of the BSA samples was used to establish a standard curve, from which the protein concentration of the individual lysates was calculated.

2.4) Protein separation

2.4.1) Sodium dodecy sulfate polyacryl gel electrophoresis

Sodium dodecy sulfate polyacryl gel electrophoresis (SDS-PAGE) was performed after Laemmli (1970). Equal amounts of protein were denatured in SDS sample loading buffer (8 x SDS sample loading buffer: 1 M Tris-HCl pH 6.8, 16% SDS, 40% glycerol, 8% β-mercaptoethanol and trace of bromophenol blue) for 15 minutes at 90°C and

separated on 4-16% polyacrylamide gels (BioRad). The gel was placed in the electrophoresis chamber (Ready Gel® Precast Gel System, BioRad) containing running buffer (25 mM Tris, 0.2 M glycine and 0.1% SDS) and the samples loaded. The SDS-PAGE was run with the current set at 10 mA for 30 minutes with constant voltage and thereafter at 20-30 mA for 3-4 hours. The gel was either Coomassie or silver stained or the proteins were transferred from the gel onto polyvinylidene di-fluoride (PVDF) membranes (BioRad) in ice-cold transfer buffer (25 mM Tris, 0.2 M glycine and 20% methanol) at 50 V with constant current for 90 minutes.

2.4.2) Tris tricine gels

Proteins in the low molecular weight range were separated on tris tricine gels according to the protocol from Schagger and von Jagow (1987). Equal amounts of protein were denatured in 2x sample buffer (0.9 M Tris-HCl pH 8.5, 8% SDS, 24% glycerol, 0.1% Coomassie and 0.1% phenol red) at 90°C for 15 minutes. The samples were loaded on 16% tris tricine gels (BioRad) and the chamber filled with tris-tricine running buffer (500 mM Tris base, 100 mM tricine and 0.1% SDS; the buffer is approximately pH 8.3 without adjusting). The proteins were separated at constant voltage with 10 mA current for 30 minutes and thereafter at 20-30 mA for 4-5 hours. The gels were blotted onto PVDF membrane or silver stained.

2.4.3) Two-dimensional electrophoresis (2DE)

Separation of protein according to their isoelectric point (pI) was carried out using thirteen centimeter immobilized pH 3-10 nonlinear gradient strips (Amersham) in the Multiphor IEF cell (Amersham). The strips were rehydrated in 250 µl sample buffer (2 M thiourea, 6 M urea, 4% CHAPS, 100 mM dithiothreitol (DTT), 0.5% Biolytes and trace of bromophenol blue) containing 100 µg of protein for 12 hours at RT. Voltage was gradually increased to 3500 V in the course of 12 hours and then kept constant for

24 hours for isoelectric focussing. The temperature was kept constant at 20°C. After the run the strips were stored at –20°C. For the second dimension run the strips were equilibrated for 10 minutes in equilibration buffer (6 M urea, 4% SDS, 375 mM Tris-HCl pH 8.8, 20% glycerol) containing 130 mM DTT and for 10 minutes in equilibration buffer substituted with 250 mM iodacetamide. The equilibrated strips were quickly dried on a tissue, placed on top of 4-16% polyacrylamide gels and embedded in 1x SDS sample loading buffer containing 1.5% low-melt agarose. Electrophoresis was carried out with constant current at 20 mA for one hour and thereafter at 30-40 mA for 4 hours. The 2DE gel was either silver stained or the proteins transferred to a PVDF membrane. The separation of complex protein lysates from whole tissues on 2DE is difficult to achieve, because the proteins tend to precipitate or do not show good electromotility during the first dimension run. In order to evaluate the applied 2DE methodology, I separated hippocampal synaptosome lysate by 2DE and silver stained the gel (Fig. 2-1). The results show that the proteins displayed a good separation in the range of 20-90 kDa and between the pI of 4 and 8 and indicates that the 2DE methodology is adequate to separate proteins from complex lysates.

2.5) Protein detection

2.5.1) Antibodies and reagents

All primary antibodies used for immunoblotting are listed in Table 2-1. Horseradish peroxidase-conjugated secondary antibodies donkey anti-rabbit IgG (H+L; #31458), mouse anti-goat IgG (H+L; #31400) and goat anti-mouse IgG (H+L; #31430) and goat anti-mouse IgM (#31440) were from Perbio.

Table 2-1. Antibodies used for immunoblotting

Antibody	Epitope	Isotype	Buffer	Dilution	Source
CaMKIIα	total CaMKIIα	mouse IgG	milk	1:100000	Chemicon
22B1	phospho-CaMKIIα (Thr286)	mouse IgG	milk	1:2000	Affinity BioReagents
06-881	phospho-CaMKIIα/β (Thr286/287)	rabbit IgG	BSA	1:1000	Upstate
13-9800	total CaMKIIβ	mouse IgG	milk	1:1000	Zymed
9196	phospho-CREB (Ser133)	mouse IgG	milk	1:2000	Cell Signaling Technology
44-297G	phospho-CREB (Ser129/133)	rabbit IgG	BSA	1:1000	Biosource
38-7000	phospho-NR2B (Tyr1472)	rabbit IgG	BSA	1:2000	Zymed
36-9600	phospho-Synapsin I (Ser603)	rabbit IgG	BSA	1:2000	Zymed
Cdk5 (C-8)	Cdk5 C-terminus	rabbit IgG	milk	1:200	Santa Cruz
p35 (C-19)	p35 and p25 C-terminus	rabbit IgG	milk	1:200	Santa Cruz
2321	phospho-Cdk/MAPK substrate (x-T/S-P-x)	mouse IgM	BSA	1:5000	Cell Signaling Technology
9611	phospho-AKT/CaMKII substrate (R/K-x-R/K-x-x-S/T(phospho))	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9411	Phospho-Tyrosine (PY-100)	mouse IgG	BSA	1:1000	Cell Signaling Technology
4G-1E	total GSK3α/β	mouse IgG	milk	1:1000	Upstate
9331	phospho-GSK3α/β(Ser21/9)	rabbit IgG	milk	1:1000	Cell Signaling Technology
9332	total GSK3β	rabbit IgG	milk	1:1000	Cell Signaling Technology
5G-2F	phospho-GSK3α/β(Tyr279/216)	mouse IgG	milk	1:1000	Upstate
AT-8	tau phospho-S202/205	mouse IgG	milk	1:1000	Pierce
AT-100	tau phospho-Thr212/Ser214	mouse IgG	BSA	1:2000	Innogenetics
PHF1	tau phospho-Ser396/404	mouse IgG	milk	1:100	P.Davies
TG3	tau phospho-Thr231/Ser235; conformation-dependent	mouse IgM	BSA	1:100	P.Davies
BR134	pan-tau	rabbit IgG	milk	1:750	M. Goedert
TAU-5	pan-tau (residues 220-240)	mouse IgG	milk	1:1000	Chemicon
SMI31	phospho-neurofilament antibody crossreacting with PHF-1 site on tau	mouse IgG	milk	1:1000	Sternberger Monoclonals, Inc.
2451	phospho-APP (Thr668)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
2452	APP (around Thr668)	rabbit IgG	BSA	1:1000	Cell Signaling Technology

CT-15	APP carboxy-terminus	rabbit IgG	BSA	1:500	B.M. Austen
ab15272	APP N-terminal (residues 44-62)	rabbit IgG	BSA	1:400	Abcam
ab2084	APP N-terminal (residues 44-63)	goat IgG	BSA	1:1000	Abcam
9562	β-catenin	rabbit IgG	BSA	1:1000	Cell Signaling Technology
06-182	total ERK1/2	mouse IgG	Milk	1:1000	Upstate
9101	phospho-ERK1/2 (Thr202/Tyr204)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
4377	phospho-p44/42 MAPK (Thr202/Tyr204)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9121	phospho-MEK1/2 (Ser217/221)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9251	phospho-SAPK/JNK (Thr183/Tyr185)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9271	phospho-AKT (Ser473)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9275	phospho-AKT (Thr308)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
9344	phospho-p90RSK (Thr359/Ser363)	rabbit IgG	BSA	1:1000	Cell Signaling Technology
PP2Ac	catalytic subunit of PP2A1	mouse IgG	milk	1:1000	S.M. Dilworth
PR65/A	scaffolding/regulatory subunit of PP2A	mouse IgG	milk	1:1000	S.M. Dilworth
sc-7482	catalytic subunit of PP1	mouse IgG	milk	1:4000	Santa Cruz
2581	phospho-PP1 (Thr320)	rabbit IgG	BSA	1:500	Cell Signaling Technology
Clone 42	α-synuclein	mouse IgG	milk	1:1000	BD Biosciences
	flag-tag	Rabbit IgG	milk	1:200	K. Brickley
A5441	β-actin	mouse IgG	milk	1:50000	Sigma
S2177	synaptotagmin	rabbit IgG	milk	1:40000	Sigma

2.5.2) Immunoblot analysis

After the transfer the PVDF membranes were incubated in blocking buffer consisting of TBS with either 3% milk or 3% BSA. Cross-reactivity of phospho-specific antibodies with milk was avoided by using 3% BSA in the blocking buffer. The blots were incubated overnight at 4°C in blocking buffer containing primary antibodies (Table 2-1). After washing in several volumes of TBS with 0.05% Tween-20 (TBST) for 90

minutes, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer at room temperature for 2-4 hours and washed again in several volumes of TBST for 90 minutes. The signals were visualised with the enhanced chemiluminescent (ECL) system (Perbio), captured by apposing the blots to x-ray film (Amersham) and the films were developed with a compact X4 film processor (X-ograph Imaging Systems). The films were scanned with the Densitometer GS800 (BioRad) and band intensities quantified with the software Densitometer Quantity One (BioRad) in the linear range. The immunoblots were stripped with stripping buffer (Perbio) and reprobed with anti-β-actin and anti-synaptotagmin antibody to normalise for amount of loaded protein. After adjusting the immunoblotting protocols, it was assessed whether the ECL system is suitable to detect different protein amounts in the linear range. Further, it was checked whether the stripping affects the quality of the signal on the blots and hence impacts on the reproducibility of the results. The findings show that the ECL system can be used to detect reliably changes in protein concentrations and that the stripping does not affect the quality of the blots (Fig. 2-2).

2.5.3) Colloidal Coomassie staining and silver staining

For the colloidal Coomassie staining the SDS-PAGE gels were rinsed two times with ultra-pure water and fixed in 20% (w/v) trichloroacetic acid fixing solution for 12 hours at RT. The fixing solution was removed and the colloidal Coomassie stain added for 3-4 hours under shaking. The colloidal Coomassie staining solution was prepared by adding 0.1% (w/v) Coomassie blue G250 to 37% water and 37% 1 M sulfuric acid and let dissolve for 3 hours while stirring at RT. The staining solution was filtered to remove any insoluble material and the sulfuric acid neutralised by carefully adding 8% of 10 M sodium hydroxide solution while stirring. After 15 minutes 18% (w/v) of trichloroacetic acid were added to the solution. The Coomassie stain was removed and the gels were washed several times in ultra-pure water for at least 2 hours at RT under shaking. The gels were stored in ultra-pure water.

For silver staining the SDS-PAGE gels the Silver stain plus kit (Biorad) was used according to manufacturer's suggestions (Appendix II). The gels were stored in 5% acetic acid.

2.6) Kinase assays

2.6.1) Cdk5 activity assay

Lysate containing 200 µg protein was diluted in 350 µl IP-buffer (150 mM sodium chloride, 10 mM Tris-HCl pH 7.6, 1% Triton-100, 0.025% sodium azide) containing protease and phosphatase inhibitors, centrifuged and the supernatant transferred to a new tube. Five µl anti-Cdk5 antibody (1 µg) were added per tube and incubated for 2 hours at 4°C under constant shaking. Thirty microlitres Protein-A bead slurry was added and incubated for 90 minutes. After settling of the beads the supernatant was discarded and the beads were washed three times with 400 µl IP-buffer. Forty microlitres of the kinase reaction mix (20 mM Tris-HCl pH 7.6, 25 mM magnesium chloride, 1 mM EDTA, 0.5 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate) containing 20 µg of the Cdk5 substrate Histone 1 (Upstate) and the inhibitor cocktail (Upstate) with 5 µM PKC inhibitor peptide, 0.5 µM PKA inhibitor peptide (PKI) and 0.5 µM Compound R24571 were added to the beads. The reaction was initiated by addition of 10 µl of 100 µM ATP solution containing 5 µCi of radioactive-labelled [γ-32P] ATP (GE Healthcare). The samples were incubated for 30 to 60 minutes at 30°C. The reaction was stopped by spotting 25 µl assay mix on p81 nitrocellulose paper (Upstate). The paper was washed 3-times in 0.75% phosphoric acid and once with acetone. The radioactivity was measured by Cherenkov counting using 25 ml polyethylene scintillation vials filled with 12ml H_2O. Cdk5 activity was calculated as the difference between the activity with and without the presence of 10 µM roscovitine, a Cdk5 inhibitor.

2.6.2) GSK3β activity assay

The GSK3 β activity assay was performed similarly to the Cdk5 activity assay with the following changes: Lysate containing 100 µg protein was immunoprecipitated with 1 µl of anti-GSK3β antibody (1 µg; Cell signalling technology #9332). The kinase reaction mix was completed with 5 µg of the GSK3 substrate phospho-glycogen synthase peptide-2 (Upstate), 0.2 µM okadaic acid, a PP1 inhibitor and the inhibitor cocktail (Upstate). The kinase reaction mix was incubated for 90 minutes at 30°C. The radioactivity count was performed as described above. GSK3β activity was calculated as the difference between the activity with and without the presence of 10 mM lithium chloride, a GSK3 inhibitor.

2.7) Immunoprecipitation

Hippocampal lysate was defrosted on ice and lysate with a protein content of 0.8 to 1 mg was diluted up to 1 ml with ice-cold immunoprecipitation (IP) buffer (150 mM sodium chloride, 10 mM Tris-HCl pH 7.6, 1% Triton X-100, 0.025% NaN3) containing protease and phosphatase inhibitors. The IP mix was vortexed and centrifuged at 7,000 xg for 1 minute at 4°C. The supernatant was transferred to a new eppendorf tube and 5-10 µg of antibody were added. The IP mix was incubated for 2-3 hours on a rotator at 4°C. Fifty to one hundred microlitres of Protein-A bead slurry or IgG bead slurry were added and incubated for 2-3 hours on a rotator at 4°C. After settling of the beads the supernatant was carefully removed and the beads were washed 3-5 times with 1 ml ice-cold IP-buffer containing protease and phosphatase inhibitors. After the last wash 50 µl of 1x SDS sample loading buffer was added to the bead pellet. The sample was vortexed, spun down and denatured at 90°C for 15 minutes. Then the IP sample was separated on a SDS-PAGE as described in *section 2.5.1*. After the washing of the beads, 50 µl of H_2O were added to the bead pellet and denatured at 70°C for 15 minutes in preparation for separating IP samples on 2DE gels. The IP sample was vortexed,

centrifuged and 200 µl rehydration buffer without DTT and Biolytes (Amersham) was added. This solution was directly loaded onto the immobilized pH 3-10 non-linear gradient strips (Amersham) and 2DE was carried out as described in *section 2.5.3.*

2.8) Chronic lithium administration

Aged p25 transgenic mice of twenty months (*n*= 4) were chronically treated with lithium for one month. For the injection a 0.1 M lithium chloride solution was prepared and filter sterilised. The first two days the mice were injected intraperitoneally with 1.5 meq/kg lithium chloride (corresponding to 10.4 mg/kg). From days 3 to 7 a dose of 3 meq/kg lithium chloride was administered. Control aged p25 transgenic mice (*n*= 4) were injected with saline solution. Thereafter, the mice were fed with powdered chow containing 1.7 g lithium chloride per kg of chow for three weeks. Control aged p25 transgenic mice were given regular powdered chow. To prevent hyponatremia, water and 400 mM sodium chloride solution were available *ad libitum* to the mice.

2.9) Contextual and tone fear conditioning

Two to three month-old female αCaMKII T286A mutants (*n*= 2) and WT littermates (*n*= 2) were trained for contextual and tone fear conditioning. Each mouse was placed into the conditioning chamber (Campden Instruments, Loughborough, UK) in a soundproof box. Following a 2 minutes "acclimatisation" period, a tone (80dB, 2.8 kHz) was presented for 30 seconds. The last 2 seconds of the tone presentation coincided with a mild foot-shock (0.7 mA). After the tone/foot-shock pairing the mouse was left for an additional 30 seconds in the conditioning chamber and then was returned to its home cage. The mouse was killed by cervical dislocation one minute after the mouse was taken out of the conditioning chamber. As naïve controls 2-3 month-old female αCaMKII T286A mutants (*n*= 2) and WT littermates (*n*= 2) were exposed to the

conditioning chamber without a foot-shock for 3 minutes. The mice were returned to the home cage and killed by cervical dislocation one minute later. Hippocampal synaptosome lysate was prepared as described in *section 2.3*.

2.10) Electrophysiology in the anaesthetised mouse

The in vivo LTP experiments were carried out by Dr Sam Cooke from the laboratory of Dr Tim Bliss at the National Institute for Medical Research, Mill Hill, London, UK.

All electrophysiological experiments were carried out according to the Animals (Scientific Procedures) Act 1986, UK. Three to six month old mice were anaesthetized with an intraperitoneal injection of urethane (1.8 mg/g). For experiments in area CA1 of the hippocampus, the recording electrode was placed in the stratum radiatum, with the stimulating electrode positioned near the midline in the contralateral ventral hippocampal commissure. In the DG, a concentric bipolar stimulating electrode was positioned in the perforant path, and a glass micropipette was lowered into the hilus of the ipsilateral DG to record evoked field responses. In both cases input-output relationships were assessed using a range of stimulus intensities from 0 μA through to a stimulus strength that evoked a maximal response. Three responses were collected at each intensity and averaged. Pairs of pulses (inter-pulse intervals, 10-100 ms) were used to study paired-pulse interactions at two intensities, one to evoke a pure excitatory postsynaptic potential (EPSP) and the other a population spike of approximately 1 mV. Again, three responses were collected at each intensity and averaged. Test responses were evoked by monophasic stimuli (0.033 Hz; 70-300 μA, 60 μs). Pulse width was doubled during the tetanus. The LTP-inducing tetanus in CA1 consisted of 2 trains of 50 pulses at 100 Hz, with an inter-train interval of 30 seconds. In the DG the tetanus consisted of 6 series of 6 trains of 6 stimuli at 400 Hz, 200 ms between trains, 20 seconds between series. The slope of the field EPSP was expressed as a percentage

change relative to the mean response in the 10 minutes prior to tetanic stimulation in both cases.

The mice were killed by decapitation 15 minutes or one hour after LTP induction in either DG or CA1. The relevant hippocampal subfield was micro-dissected in ice-cold P2-buffer containing protease and phosphatase inhibitors under a magnifying glass using ice-cold steel implements. Lysate was prepared as described in *section 2.4* and samples were stored at -80°C. For the DG LTP experiments the contralateral DG was isolated as unstimulated control tissue, whereas for the CA1 LTP experiments control tissue was isolated from another unstimulated mouse or from a stimulated mouse treated with the NMDA antagonist, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP).

2.11) Data acquisition and analysis

Immunoblot films were scanned with the Densitometer GS800 (BioRad) and band intensities quantified with the software Densitometer Quantity One (BioRad) in the linear range. Band intensities were normalised to amount of loaded protein to band signals from anti-β-actin and/or anti-synaptotagmin antibodies. All changes in band intensities are indicated as x% of 100% control unless stated otherwise.

Images were prepared in Photoshop (Adobe) and assembled into figures in Microsoft PowerPoint. Statistical analysis was performed using one-way analysis of variance (ANOVA) on the Microsoft Excel software. All data are expressed as mean ± S.E.M. unless stated otherwise.

CHAPTER 3: Biochemical analysis of the αCaMKII T286A point mutant mouse line

3.1) Introduction

3.1.1) The αCaMKII T286A point mutant mouse line

Dr K. Peter Giese generated the αCaMKII T286A point mutant mouse line by using knock-in technology (Giese *et al.*, 1998). A mutation was introduced in the endogenous αCaMKII gene resulting in the substitution of threonine at residue 286 by alanine. This mutation disenables the autophosphorylation at Thr286 and in turn prevents αCaMKII from switching into an autonomously active state. For technical reasons, the generation of the T286A knock-in mutants included the introduction of a silent point mutation that gives rise to a diagnostic restriction site and an insertion of a loxP site in an intron of the endogenous αCaMKII gene. As the genetic modifications were directly introduced in the endogenous αCaMKII gene, the expression is still driven by the endogenous promoter, which ideally results in identical expression patterns and levels. Accordingly, the expression pattern of αCaMKII in the hippocampus of the T286A mutants was not altered (Giese *et al.* 1998). Furthermore, identical levels of αCaMKII were detected in the hippocampus of T286A mutants and WT controls (Giese *et al.* 1998; Hardingham *et al.*, 2003). These results show that the point mutation and the intronic insertion of a loxP site into the αCaMKII gene did not impact on the expression pattern of αCaMKII and its protein levels in the hippocampus of αCaMKII T286A mutants. However, in somatosensory cortex αCaMKII protein levels were markedly reduced, which is possibly due to interference of the introduced loxP site with the splicing efficiency of the αCaMKII gene (Hardingham *et al.*, 2003). The genetic modification in the T286A

mutants did not affect the expression levels of the β-isoform of CaMKII (Giese *et al.* 1998). The level of Ca^{2+}/CaM-dependent activity of αCaMKII was also not altered by the mutation (Giese *et al.* 1998). In contrast, the Ca^{2+}-independent αCaMKII activity was significantly reduced in the T286A mutants (Giese *et al.* 1998). Immunohistochemical analysis of the brain did not detect any morphological differences between the brains of T286A mutants and WT mice (Giese *et al.*, 1998). Electrophysiological characterisation of the T286A mutants revealed the importance of the αCaMKII autophosphorylation at Thr286 for the induction of LTP in hippocampal area CA1 and neocortex (Giese *et al.* 1998; Hardingham *et al.*, 2003; Cooke *et al.*, 2006; see also *section 1.7.4*). Induction of LTP was abolished in the CA1 of T286A mutants (Giese *et al.* 1998; Cooke *et al.*, 2006). This striking LTP phenotype was correlated with severe impairments in hippocampal learning and memory, as assayed in the MWM, and altered place cell activity in the T286A mutants (Giese *et al.* 1998; Cho *et al.*, 1998). Importantly, the T286A mutants were able to learn the visible platform version of the MWM indicating that they had the vision, motivation, and motor coordination required to perform this task (Giese *et al.* 1998). Interestingly, a recent study showed that LTP induction was reduced in the DG of the T286A mutants, and not completely abolished as observed with CA1 LTP (Cooke *et al.*, 2006). Thus, autophosphorylation of αCaMKII is essential for NMDA receptor-dependent CA1-LTP, but not required for the maintenance of NMDA receptor-dependent DG-LTP.

3.1.2) Aim of the project

A major goal in neuroscience is the elucidation of the molecular mechanisms underlying learning processes and memory formation. Generally, it has been established that protein phosphorylation, protein synthesis and *de novo* gene transcription are essential for learning and memory (see for review Reymann & Frey, 2006). However, the precise molecular machinery and its integral functioning require further characterisation.

One aim of this study was to identify novel molecular mechanisms involved in learning and memory. I investigated changes in protein phosphorylation levels after behavioural training or electrophysiological stimulation to induce LTP in the hippocampus of WT mice. An important first step for this experiment was the establishment of suitable techniques to induce physiological protein phosphorylation and to detect these changes in phosphorylation levels.

A next aim of this study was to get more information on the function of αCaMKII, which has been characterised as a key molecule for learning and memory. For this I used the T286A mutant mouse line, in which the autonomous αCaMKII activity is blocked and, as a result exhibits impaired synaptic plasticity and hippocampal learning and memory. In the hippocampus of these αCaMKII T286A mutants I analysed changes in protein phosphorylation levels after behavioural training or electrophysiological stimulation.

A final aim was to assess the role of the β-isoform of CaMKII in synaptic plasticity. Compensatory activation of βCaMKII has been suggested to partially rescue activity-dependent potentiation in hippocampal area CA1 in an αCaMKII KO mouse line (Elgersma *et al.*, 2002). To test this possibility I assayed the levels of autophosphorylated βCaMKII after electrophysiological stimulation.

3.2) Results

3.2.1) Evaluation of an approach to detect phosphorylation changes of CaMKII substrates in hippocampus after fear conditioning

Molecular mechanisms including protein phosphorylation, protein synthesis and gene transcription have been identified as underlying processes of learning and memory. It has been observed that behavioural training triggers changes in protein phosphorylation,

protein expression and *de novo* gene transcription. In this experiment, I have investigated changes in protein phosphorylation occurring after contextual fear conditioning, a hippocampus-dependent, single trial learning task (see *section 1.4.2*). As αCaMKII is important for synaptic plasticity, learning and memory, I focussed on phosphorylation of CaMKII substrates by using a phospho-specific antibody raised against the consensus motif of CaMKII and AKT substrates. Analysis from immunoblots of hippocampal synaptosome lysates from naïve and trained WT mice did not reveal any obvious changes in phosphorylation levels between the two conditions (Fig 3-1). Furthermore, an anti-phospho-Cdk5/MAPK substrate antibody did not reveal any changes in phosphorylation between the naïve and trained WT mice. To distinguish specific phosphorylation changes induced by fear conditioning from baseline phosphorylation, I also analysed the phosphorylation levels in naïve and trained αCaMKII T286A mutants. In the T286A mutants the autonomous Ca^{2+}-independent αCaMKII activity is disenabled, which results in the impairment of hippocampal learning and memory (Giese *et al.*, 1998). Hence, it is likely that behavioural training induces phosphorylation changes in αCaMKII substrates in WT mice but not in the T286A mutants. However, as there were no phosphorylation changes in trained WT mice, one would expect to detect no alterations in phosphorylation levels in trained T286A mutants. Consistent with this notion, no changes in phosphorylation levels were observed between naïve and trained αCaMKII T286A mutants (Fig 3-1). Comparison of the immunoblot signals from naïve WT and T286A mutants revealed several changes in phosphorylation levels (Fig 3-1). The intensity of bands with the molecular weight 35, 42 and 50 kDa was reduced in the T286A mutants. Both, naïve and trained T286A mutants displayed reduced signal within in the same subset of bands. Interestingly, an anti-phospho-Cdk5/MAPK substrate antibody did not detect any phosphorylation changes between T286A mutants and WT mice, indicating that the changes observed with the anti-phospho-AKT/CaMKII substrate antibody are due to the lack of autonomous αCaMKII activity in the T286A mice. The band at 50 kDa was absent in the T286A mutants (Fig 3-1). Re-probing the blots with anti-αCaMKII antibody

showed that the 50 kDa band corresponded to phospho-Thr286-αCaMKII (data not shown). Because of the point mutation in the T286A mutants, the anti-phospho-AKT/CaMKII substrate antibody could not bind to the autophosphorylation site of αCaMKII anymore. Consistently, immunoblot analysis of hippocampal lysate from T286A mutants probed with phospho-Thr286-αCaMKII antibody detected no signal, confirming the complete abolition of the Thr286 autophosphorylation in the T286A mutants (Fig. 3-7).

Together, these results indicate that the current conditions used to detect phosphorylation changes in the hippocampus after contextual fear conditioning are not optimal and further studies will be required to improve this approach. The reduction in phosphorylation levels between T286A mutants and WT mice shows that the point mutation affects the basal phosphorylation level of several proteins. As the point mutation disenables autonomous activity of αCaMKII, it is conceivable that these proteins are phosphorylated specifically by αCaMKII in the autonomous activity state.

3.2.2) Evaluation of an in vivo LTP approach to induce and detect phosphorylation changes in hippocampal subfields

The breeding of the T286A mutant mice and in vivo stimulation has been carried out by Dr Sam F. Cooke in the laboratory of Dr Tim V.P. Bliss at the National Institute for Medical Research (NIMR), Mill Hill. At this point, I would like to thank Dr Bliss and Dr Cooke for the fruitful and inspiring collaboration and for their enormous engagement in this project.

Studies in the hippocampus, a brain structure central to the processing and temporary storage of information established LTP as the primary experimental model for investigating the molecular and cellular basis of learning and memory (see, for example,

Bliss & Collingridge, 1993). The use of kinase inhibitors in electrophysiological and behavioural studies demonstrated the requirement of protein phosphorylation in long-term potentiation, as well as learning and memory. Moreover, activity-dependent synaptic potentation has been shown to induce changes in phosphorylation levels of specific molecules such as αCaMKII and CREB (Barria *et al.*, 1997; Ouyang *et al.*, 1997; Impey *et al.*, 1996). In order to investigate changes in protein phosphorylation after synaptic potentiation, an *in vivo* LTP approach was developed in collaboration with Drs Sam F. Cooke and Tim V.P. Bliss. An *in vivo* LTP protocol was employed to induce synaptic potentiation in the hippocampal subfields, CA1 and DG, and subsequently phosphorylation changes were investigated using standard immunoblotting methods. First, it had to be ensured that the sensitivity of the *in vivo* LTP assay was sufficient to observe changes in phosphorylation levels after the induction of LTP. This was tested by monitoring phosphorylation levels of well-characterised marker molecules. I focused on the presynaptic protein synapsin I, the NMDA receptor subunit NR2B and the transcription factor CREB, for which an increase in phosphorylation after LTP induction has been reported (see, for example, Nayak *et al.*, 1996; Rostas *et al.*, 1996; Impey *et al.*, 1996). In anaesthetised WT mice CA1-LTP was induced at Schaffer commissural-pyramidal cell synapses or respectively DG-LTP was induced at medial perforant path-granule cell synapses. The relevant hippocampal subregion, either CA1 or DG, was microdissected at 15 minutes or one hour after LTP induction and used for lysate preparation. As a control, either the unstimulated contralateral DG from the same mouse or CA1 from another unstimulated mouse were used. The lysates were separated by SDS-PAGE and immunoblotted. The blots were probed with phospho-specific antibodies to detect changes in phosphorylation levels of synapsin I at Ser603, NR2B at Tyr1472 and CREB at Ser133. In lysates from the DG of WT mice harvested 15 min after *in vivo* stimulation phosphorylation levels of synapsin I, NR2B and CREB at Ser133 were significantly increased (Fig. 3-2; see also Table 3-1 for overview) (phospho-synapsin I: 158%, n= 8, $F_{1\text{-}14}$= 30.0, p< 0.001, phospho-NR2B: 135%, n= 4, $F_{1\text{-}6}$= 783, p< 0.001; phospho-

CREB: 154%, n= 8; $F_{1\text{-}14}$= 25.8; p< 0.001). In addition, increased phosphorylation levels of synapsin I at Ser603, NR2B at Tyr1472 and CREB at Ser133 were also observed in DG lysates of WT mice harvested one hour after stimulation (Fig. 3-2; see also Table 3-1 for overview) (phospho-synapsin I: 122%, n= 4, $F_{1\text{-}6}$= 9.63, p< 0.02; phospho-NR2B: 146%, n= 5, $F_{1\text{-}8}$= 20.6, p< 0.002; phospho-CREB: 151%, n= 6, $F_{1\text{-}10}$= 26.8, p< 0.001). Moreover, the phosphorylation levels of synapsin I at Ser603 and CREB at Ser129/133 were significantly elevated in hippocampal area CA1 of WT mice harvested one hour after LTP stimulation (Fig. 3-3; see also Table 3-1 for overview) (phospho-synapsin I: 171%, n= 4, $F_{1\text{-}6}$= 8.27, p< 0.05; phospho-CREB: 172%, n= 4, $F_{1\text{-}6}$= 8.58, p< 0.05). Interestingly, the phosphorylation levels of NR2B at Tyr1472 were not increased in area CA1 of WT mice one hour after LTP induction (Fig. 3-3) (phospho-NR2B: 108%, n= 4, $F_{1\text{-}6}$= 0.68, p> 0.05).

Table 3-1. Overview of the results from the *in vivo* LTP approach testing phosphorylation changes in marker molecules.

WT	CA1 1h	DG 1h	DG 15 min
phospho-Ser603-Synapsin I	**171 %** (p< 0.05)	**121 %** (p< 0.05)	**158 %** (p< 0.001)
phospho-Tyr1472-NR2B	**108 %** (p> 0.05)	**146 %** (p< 0.002)	**135 %** (p< 0.001)
phospho-Ser133-CREB	**172 %** (p< 0.05)	**151 %** (p< 0.001)	**154 %** (p< 0.001)

These results show that our *in vivo* LTP approach can be used to induce robust, persistent physiological phosphorylation changes in both CA1 and the DG. Furthermore, the lysate preparation and the immunoblot methods are suitable and the sensitivity of the assay is high enough to detect changes in phosphorylation levels after *in vivo* LTP induction in CA1 and DG. The results confirm that the tested marker molecules can be used as controls for efficient LTP stimulation in CA1 and the DG.

3.2.3) Autophosphorylation of αCaMKII is induced by LTP stimulation in CA1 and DG

The α-isoform of CaMKII is one of the most abundant proteins in the post-synaptic densities of excitatory neurons and is thought to be central to the regulation of Ca^{2+}-mediated signal transduction (see for review Lisman *et al.*, 2002). Autophosphorylation of Thr286 switches αCaMKII into an autonomous Ca^{2+}-independent state of activity, a process shown to be important for synaptic potentiation and learning and memory. Biochemical and immunohistochemical studies on electrophysiologically stimulated brain slices demonstrated that LTP induction increases the levels of autophosphorylated αCaMKII in hippocampal area CA1 (Barria *et al.*, 1997; Ouyang *et al.*, 1997). A disadvantage of studies using stimulated brain slices is that the slice preparation and preservation may induce molecular changes, which lead to an increased background noise and provokes artefacts (see, for example, Taubenfeld *et al.*, 2002). To minimise confounding molecular changes, an *in vivo* LTP approach was used to investigate the autophosphorylation level of αCaMKII. The immunoblots of lysates from *in vivo* stimulated CA1 or DG from WT mice and their relevant controls were probed with a phospho-specific antibody recognising the αCaMKII autophosphorylation site at Thr286. Autophosphorylation of αCaMKII was elevated in CA1 tissue taken from WT mice one hour after the induction of LTP at Schaffer commissural-pyramidal cell synapses as compared to controls (150%, n= 5, $F_{1\text{-}8}$= 13.5, p< 0.02) (Fig. 3-4; see also Table 3-2 for overview). In the DG the αCaMKII autophosphorylation was significantly elevated after *in vivo* LTP induction at medial perforant path-granule cell synapses in anaesthetised WT mice at 15 minutes (145%, n= 8, $F_{1\text{-}14}$= 14.6, p< 0.01). The increased autophosphorylation was sustained for one hour after *in vivo* LTP induction in DG (162%, n= 5, $F_{1\text{-}8}$= 435, p< 0.001) (Fig. 3-4; Table 3-2). These results confirm previous *in vitro* findings in area CA1 showing that the autophosphorylation levels of αCaMKII at Thr286 increase upon stimulation and that this increase is sustained for a prolonged period of time. In addition, I show that LTP induction also triggers autophosphorylation of αCaMKII in the DG.

3.2.4) LTP stimulation in CA1 and the DG induces autophosphorylation of βCaMKII

Along with αCaMKII, the β-isoform of CaMKII is expressed at high levels in neurons (see for review Hudmon & Schulman, 2002; Lisman *et al.*, 2002). As with the α-isoform, βCaMKII can also be switched into an autonomous Ca^{2+}-independent state of activity through autophosphorylation at Thr287.

To test whether the autophosphorylation of βCaMKII is also regulated by LTP induction, I examined the levels of βCaMKII autophosphorylation at Thr287 in CA1 or the DG after LTP stimulation in WT mice *in vivo*. The application of a phospho-specific antibody detecting the autophosphorylation sites (Thr286/287) on both CaMKII isoforms demonstrated that the autophosphorylation of αCaMKII as well as βCaMKII were significantly increased in CA1 and the DG (Fig. 3-5; Table 3-2). I observed a significant increase in βCaMKII autophosphorylation in CA1 lysates from WT mice one hour after the induction of LTP at Schaffer commissural-pyramidal cell synapses (132%, n= 5, $F_{1\text{-}8}$= 7.45, p< 0.05). In the DG the βCaMKII autophosphorylation was elevated 15 minutes after *in vivo* LTP induction in WT mice (153%, n= 8, $F_{1\text{-}14}$= 39.3, p< 0.001). The increased autophosphorylation of βCaMKII was sustained for one hour after LTP induction in the DG (132%, n= 5, $F_{1\text{-}8}$= 14.6, p< 0.01).

These findings demonstrate that the autophosphorylation of βCaMKII is also regulated by LTP induction in both hippocampal subfields, CA1 and DG. Furthermore, the increased βCaMKII autophosphorylation is also sustained for a prolonged period of time, similar to the autophosphorylation of αCaMKII.

3.2.5) LTP induction increases βCaMKII autophosphorylation in the T286A mutants

LTP recordings on transverse hippocampal slices of the αCaMKII T286A mutant mouse line demonstrated that the loss of αCaMKII autophosphorylation completely prevents NMDA receptor-dependent CA1-LTP at Schaffer commissural-pyramidal cell synapses (Giese *et al.*, 1998). Recently, we found that the loss of αCaMKII autophosphorylation did not block LTP at medial perforant path-granule cell synapses in the DG *in vivo* and *in vitro* in the T286A mutants (Cooke *et al.*, 2006). Thus the αCaMKII autophosphorylation is essential for NMDA receptor-dependent CA1-LTP, but is not required for NMDA receptor-dependent DG-LTP at medial perforant path-granule cell synapses. To date it is not known, which signalling pathways are involved in DG-LTP. As described above, I found that LTP induction in area CA1 and DG increases the autophosphorylation of βCaMKII along with αCaMKII. Therefore, it is possible that the spared DG-LTP in the T286A mutants is mediated by autonomous βCaMKII activity in the autophosphorylated state. Furthermore, βCaMKII has been implicated in the partial compensation of LTP deficits in area CA1 in an αCaMKII KO mouse line (Elgersma *et al.*, 2002). To check this hypothesis, I analysed the autophosphorylation of βCaMKII in potentiated DG tissue from T286A mutants. The level of autophosphorylated βCaMKII was significantly increased in the tetanised DG one hour after stimulation as compared to the unpotentiated control tissue (Fig. 3-6) (136%, n= 5, $F_{1\text{-}8}$= 48.7, p< 0.001). The increase of βCaMKII autophosphorylation in the DG of T286A mutants one hour after stimulation (136% of control) was comparable to the levels observed in WT mice (132% of control) (Table 3-2). Based on this result, it could be assumed that that βCaMKII activity compensates for the lack of autonomous αCaMKII activity in the T286A mutants and the T286A mutation does not have a dominant-negative effect on βCaMKII autophosphorylation.

Table 3-2. Overview of the results analysing the level of autophosphorylation of αCaMKII and βCaMKII after LTP induction.

WT	CA1 1h	DG 1h	DG 15 min
phospho-Thr286-αCaMKII	**150 %** ($p< 0.05$)	**162 %** ($p< 0.001$)	**145 %** ($p< 0.01$)
phospho-Thr287-βCaMKII	**132 %** ($p< 0.05$)	**132 %** ($p< 0.01$)	**153 %** ($p< 0.001$)

T286A mutants	DG 1h
phospho-Thr287-βCaMKII	**136 %** ($p< 0.001$)

3.2.6) Normal βCaMKII levels in crude synaptosomal fraction from T286A mutant mice

The βCaMKII compensation observed in αCaMKII null mutants has been attributed to a mislocalisation of βCaMKII into post-synaptic densities (Elgersma *et al.*, 2002). Therefore, I checked whether there is an up-regulation of βCaMKII or autophosphorylated βCaMKII in crude synaptosomal fractions of αCaMKII T286A mutant mice (Fig. 3-7). No differences were observed in βCaMKII levels or autophosphorylated βCaMKII levels in synaptosomal lysates from T286A mutants as compared to WT mice (Fig. 3-7).

This result shows that βCaMKII is not mislocalised and translocated to the post-synaptic densities in the T286A mutants, suggesting no compensatory role for βCaMKII in the T286A mutants. Nevertheless, it is still possible that βCaMKII participates in the compensation of αCaMKII via alternative mechanisms other than translocation to the post-synaptic densities. Further studies will be required to elucidate the role of βCaMKII in synaptic plasticity.

3.2.7) Level of inhibited GSK3 is increased after LTP stimulation in CA1 and the DG

The evaluation of the *in vivo* LTP approach showed that it is suitable to detect changes in phosphorylation levels after induction of LTP. Thus, I wanted to exploit the assay to test the regulation of signalling molecules that might be important for learning and memory. A recent study has demonstrated that mutants with conditional GSK3β over-expression have impaired spatial learning (Hernandez *et al.*, 2002). Hence, I tested whether GSK3β activity is regulated by LTP induction in CA1 and DG. GSK3β is constitutively active, but phosphorylation at Ser9 leads to inhibition of GSK3β (Grimes & Jope, 2001). The phosphorylation levels at the inhibitory Ser9 phosphorylation site were significantly increased one hour after *in vivo* LTP induction in CA1 (134%, n= 4, $F_{1\text{-}6}$= 6.29, p< 0.05) as well as in the DG (156%, n= 6, $F_{1\text{-}10}$= 16.5, p< 0.003) (Fig. 3-8). The same result was observed in DG tissue harvested 15 minutes after LTP induction (134%, n= 8, $F_{1\text{-}14}$= 10.2, p< 0.007) (Fig. 3-8). Interestingly, the phosphorylation at the inhibitory site on GSK3β was also elevated in DG from αCaMKII T286A mutant mice harvested one hour after stimulation (123%, n= 5, $F_{1\text{-}8}$= 5.31, p< 0.05) (Fig. 3-9). Together, these results reveal that inhibition of GSK3β is regulated by LTP induction and therefore may be important for synaptic plasticity, learning and memory processes. Furthermore, it shows that GSK3 inhibition is mediated independently of the autonomous αCaMKII activity in the DG.

3.2.8) Summary

In the present study, I have established an approach in collaboration with Drs Sam F. Cooke and Tim V.P. Bliss to study biochemical changes after electrophysiological *in vivo* stimulation of hippocampal subfields in anaesthetised mice. I demonstrate that well-characterised molecules including synapsin I, NR2B and CREB that exhibit increased levels of phosphorylation after *in vitro* LTP induction, undergo stimulation-

induced phosphorylation changes in this *in vivo* LTP approach. This confirms that the *in vivo* LTP approach is suitable to investigate molecular changes relevant for synaptic plasticity, learning and memory. I then employed this *in vivo* LTP approach to investigate the regulation of the autophosphorylation of αCaMKII and βCaMKII after stimulation in area CA1 and the DG of αCaMKII T286A mutants and WT mice. I find that autophosphorylation of αCaMKII at Thr286 and βCaMKII autophosphorylation at Thr287 are both significantly increased after LTP stimulation *in vivo* in the DG of WT mice at 15minutes and one hour as well as in hippocampal area CA1 at one hour. In T286A αCaMKII mutant mice, in which the autophosphorylation of αCaMKII at Thr286 is disenabled, autophosphorylation levels at Thr287 on βCaMKII were also elevated one hour after *in vivo* LTP induction. These results suggest that autonomous βCaMKII activity could, possibly, compensate for the lack of autonomous αCaMKII activity in the T286A mutants. However, the protein levels of βCaMKII in the crude synaptosomal fraction were unaltered between T286A mutants and WT mice, suggesting that there is no compensation by βCaMKII. Furthermore, I find that the phosphorylation levels at the inhibitory site of GSK3β display a stimulation-dependent increase. This increase in inhibitory phosphorylation is also observed in αCaMKII T286A mutants. These results suggest that regulation of GSK3β is involved in synaptic plasticity and also that this regulation occurs independent of the autonomous αCaMKII activity.

3.3) Discussion

3.3.1) An approach using electrophysiological in vivo stimulation to induce biochemical changes

In order to detect functional molecular changes triggered by learning and memory processes, I investigated changes in protein phosphorylation levels in the hippocampus of WT mice after behavioural training. These pilot experiments using the hippocampus-dependent, single trial learning task, contextual fear conditioning, did not reveal any obvious changes in protein phosphorylation between naïve and trained WT mice. Equally, the analysis of phosphorylation levels between naïve and trained T286A mutants did not detect any changes. Possible explanations as to why no changes have been observed, may include limitations of the stimulation of learning and memory processes by behavioural training. It is likely that only a relative limited number of synapses undergo simultaneously learning-induced changes after the behavioural training. Thus, it may be expected that only small changes in protein phosphorylation levels occur as a result of the behavioural training. Subsequently, the immunoblotting method using phospho-specific antibodies might not provide sufficient sensitivity to detect these subtle changes in protein phosphorylation levels. Furthermore, there is generally a high degree of variation within the results of behavioural studies, which further complicates the identification of changes in phosphorylation levels.

To overcome some of the problems encountered with the behavioural training, I decided to use electrophysiological stimulation to induce biochemical changes. Thankfully, Drs Sam F. Cooke and Tim V.P. Bliss from the National Institute for Medical Research, Mill Hill, were willing to collaborate on establishing an approach to electrophysiologically stimulate CA1 and the DG, and analyse resulting changes in protein phosphorylation. We focussed on phosphorylation changes in the hippocampus, because it is a well-characterised brain region implicated in learning and memory

processes. As LTP has been shown to trigger changes in protein phosphorylation levels comparable to physiological phosphorylation changes observed after behavioural training, we opted to use electrical stimulation to induce long-term synaptic potentiation. A major advantage of using electrophysiological stimulation to trigger activity-dependent phosphorylation changes is that a relatively large number of synapses within a neuronal field can be simultaneously activated. This induction method is likely to result in pronounced changes of protein phosphorylation levels. The results presented here, demonstrate that the *in vivo* LTP induction produced robust and persistent phosphorylation changes in marker molecules including synapsin I, αCaMKII and CREB in both hippocampal subfields, CA1 and the DG (see Tables 3-1 and 3-2). Further advantages are a high level of reproducibility and a low degree of variation between different experiments as many important parameters can be controlled. Consistently, our approach produced reproducible data (see Table 3-1 and 3-2) and the variance within the values is relatively small. This allows us to produce conclusive data with a lower number of samples.

LTP studies are commonly carried out *in vitro* in transverse hippocampal slices. However, the disadvantages of the analysis of activity-dependent biochemical changes *in vitro* in stimulated brain slices include possible introduction of artefacts and background noise resulting from the slice preparation and slice preservation (see, for example, Taubenfeld *et al.*, 2002). A further disadvantage is that the lysate preparation from a single brain slice yields only small amounts of protein lysate and therefore several stimulated brain slices have to be pooled. To overcome some of these limitations we decided to use *in vivo* LTP to induce activity-dependent synaptic potentiation in the hippocampal subfields, CA1 and the DG. The advantages of using the *in vivo* LTP method include the presence of the unstimulated contralateral DG as an intrinsic control for the potentiated DG. Furthermore, there is a reduced likelihood of inducing artefacts and background noise by damaging hippocampal tissue. A previous study on αCaMKII autophosphorylation using rat hippocampal slice homogenates observed an approximate

increase of 120% in phosphorylation levels one hour after *in vitro* CA1-LTP induction (Barria *et al.*, 1997). In comparison, I detect an increase of approximately 150 % in phospho-Thr286-αCaMKII in area CA1 using our *in vivo* LTP approach. This observation indicates that the *in vivo* LTP approach has a higher sensitivity in detecting phosphorylation changes. This is possibly due to less background noise and the fact that the whole hippocampus can be processed and individual hippocampal subfields micro-dissected. Because each hippocampal subfield (either CA1 or the DG) is potentiated individually, the relative amount of protein phosphorylation increases, when the tissue from the stimulated subfield alone is isolated. Furthermore, each of the hippocampal subfields employ distinct activity-dependent molecular mechanisms. Hence, it is crucial to investigate biochemical changes for each subfield separately. A drawback of *in vivo* LTP stimulation is the application of anaesthetic to the mouse during the LTP stimulation protocol. The administration of anaesthesia might interfere with molecular mechanisms underlying synaptic plasticity, learning and memory.

3.3.2) Regulation of the autophosphorylation of αCaMKII and βCaMKII

The present study demonstrates that autophosphorylation of αCaMKII at Thr286 is increased after induction of *in vivo* LTP in both hippocampal subfields, CA1 and the DG. This finding is consistent with *in vitro* hippocampal slice studies that observed a persistent increase in αCaMKII Thr286 autophosphorylation in area CA1 after LTP induction (Barria *et al.*, 1997; Ouyang *et al.*, 1997). Moreover, increased αCaMKII Thr286 autophosphorylation has been observed after contextual fear conditioning (Kouzu *et al.*, 2000). These results, in conjunction with studies in various αCaMKII mutant mouse lines, established a fundamental role for αCaMKII Thr286 autophosphorylation in synaptic plasticity, learning and memory (see for review Lisman *et al.*, 2002). Autophosphorylation of αCaMKII at Thr286 switches the kinase into an

autonomously active state. However, the function of this autophosphorylation switch of αCaMKII is not yet understood.

In vitro and *in vivo* work demonstrated that CA1-LTP was abolished in αCaMKII T286A mutant mice, where the switch into autonomous αCaMKII activity is disenabled (Giese *et al.*, 1998; Cooke *et al.*, 2006). In contrast, DG-LTP at medial perforant path-granule cell synapses can be sustained over days in awake T286A mutant mice (Cooke *et al.*, 2006). Therefore, the αCaMKII Thr286 autophosphorylation is essential for CA1-LTP, but not required for DG-LTP. This raises the question as to which molecular mechanisms sustain DG-LTP. Here I show that phospho-Thr286-αCaMKII is increased in the DG both 15 minutes and one hour after induction of *in vivo* LTP in WT mice. The persistence and the level of the increase in αCaMKII autophosphorylation in the DG resemble the autophosphorylation changes in CA1 (Table 3-2). Thus, it is likely that αCaMKII autophosphorylation can support DG-LTP, but additional molecular mechanisms either are simultaneously activated or can compensate in case αCaMKII is inactivated. Consistent with this idea, application of the Ca^{2+}/CaM-dependent kinase (CaMK) inhibitor, KN62, does not affect the induction of DG-LTP (Cooke *et al.*, 2006). One obvious candidate to support DG-LTP is the β-isoform of CaMKII, as it shares high homology with αCaMKII and is also abundant in neurons (see for review Hudmon & Schulman, 2002). In fact, βCaMKII has been involved in the compensation of αCaMKII by partially rescuing CA1-LTP in an αCaMKII KO mouse line (Elgersma *et al.*, 2002). Consistent with a compensatory role of βCaMKII, I observe an increase in βCaMKII autophosphorylation after *in vivo* LTP induction in both hippocampal subfields; CA1 and the DG. This increase in phospho-Thr287-βCaMKII persists for at least one hour. Further, I find that βCaMKII autophosphorylation is also increased after induction of *in vivo* LTP in the DG of T286A mutants. Together, these results show that βCaMKII autophosphorylation is regulated in an activity-dependent manner, indicating that autonomous βCaMKII activity is implicated in molecular mechanisms underlying

synaptic plasticity. Moreover, these results could support the idea of a compensatory role of βCaMKII. However, there is also evidence against a compensatory role of βCaMKII. The mechanism by which βCaMKII compensation occurs in the αCaMKII KO mouse line is thought to involve translocation of βCaMKII into post-synaptic densities (Elgersma *et al.*, 2002). Here, I show that there is no compensatory translocation of βCaMKII into the post-synaptic densities in T286A mutants. Accordingly, recent pharmacological studies demonstrate conclusively that βCaMKII does not compensate for autonomous αCaMKII activity (Cooke *et al.*, 2006; Wu *et al.*, 2006). Application of the CaMK inhibitor, KN62, had no effect on DG-LTP in both WT mice and T286A mutants, demonstrating that other molecules than CaMKs support DG-LTP. These studies then show that DG-LTP in T286A mutants can be reduced by application of PKA and MAPK inhibitors. However, some residual LTP can still be induced in the DG of T286A mutants. In contrast, CA1-LTP induction is completely blocked in T286A mutants or in WT mice with application of KN62 (Cooke *et al.*, 2006). These findings demonstrate a dissociation between the molecular mechanisms for LTP in CA1 and the DG. These results indicate that CA1-LTP is mediated via αCaMKII. In contrast, DG-LTP at medial perforant path-granule cell synapses can be supported by parallel signalling pathways, one including αCaMKII and the other including PKA and MAPK. In addition, further mechanisms might be involved in the induction of DG-LTP, as suggested by the residual LTP after simultaneous application of KN62 and PKA or MAPK inhibitors (Wu *et al.*, 2006). The finding that there is a dissociation between the molecular mechanisms of LTP in CA1 and DG has important implications for further studies. It shows that future molecular studies need to be performed in a subfield-specific manner. The identification of the distinct molecular mechanisms implicated in LTP of the two subfields will help to understand the role of the hippocampus in learning and memory.

3.3.3) The role of GSK3 in synaptic plasticity, learning and memory.

The present study shows that inhibitory phosphorylation of GSK3β at Ser9 is increased in both hippocampal subfields, CA1 and the DG, after *in vivo* LTP induction. This increase in phosphorylation of GSK3β on Ser9 persists for at least one hour. Increased inhibitory phosphorylation of GSK3 has previously been linked to pharmacological induced activity-dependent conditions. Interference with serotonergic activity by pharmacological means was shown to significantly increase the level of inhibitory phosphorylation of GSK3β in hippocampus, cerebral cortex and striatum of mouse *in vivo* (Li *et al.*, 2004). *In vivo* treatment of mice with pilocarpine, a muscarinic agonist commonly used to induce seizures, resulted also in increased inhibitory phosphorylation in both GSK3β at Ser9 and GSK3α at Ser21 in hippocampus, cerebral cortex and striatum (De Sarno *et al.*, 2006). The induction of the GSK3 inhibitory phosphorylation occurred rapidly between 5 and 15 minutes, persisted for at least 90 minutes and was back to baseline two hours after induction. Interestingly, I observe a comparable time course of inhibitory GSK3β phosphorylation after DG-LTP *in vivo*. The signal is increased after 15 minutes and persists for at least one hour after LTP induction. A similar time course has been observed for molecules involved in processes during the early phase of synaptic plasticity. Thus, it is conceivable that inhibition of GSK3 is involved in molecular mechanisms underlying the early phase of synaptic plasticity. As GSK3 has been implicated in the negative regulation of multiple transcription factors, it is likely that GSK3 may affect synaptic plasticity via the control of transcription. This hypothesis is supported by an elegant study in cell cultures demonstrating that electrical activation of L-type VGCC triggers translocation of the transcription factor NF-ATc4 from the cytoplasm into the nucleus (Graef *et al.*, 1999). The translocation step is regulated via GSK3β activity, which promotes nuclear export of NF-ATc4 and in turn inhibits NF-ATc4-induced transcription (Graef *et al.*, 1999). Furthermore, over-expression of GSK3 in cell culture blocked BDNF-induced CREB phosphorylation and pharmacological inhibition can subsequently revert this effect and facilitate activation of

CREB (Grimes & Jope, 2001; Mai *et al.*, 2002). In addition, these studies highlight a major problem of investigating GSK3 functions. GSK3 is ubiquitously localised within a cell. However, functionally different subcellular pools of GSK3 co-exist, which are probably regulated by alternative complexes, and this complicates the study of one individual GSK3 pool.

To date there are only few data available on the involvement of GSK3 in learning and memory processes. The most conclusive results come from a study analysing TG mice conditionally over-expressing GSK3β in hippocampal and cortical neurons (Hernandez *et al.*, 2002). The GSK3β over-expression resulted in enhanced GSK3β activity, which coincided with impaired spatial learning in the MWM. This result indicates that persistent activation of GSK3 impairs learning and memory processes and suggests that inhibition of GSK3 is important for the expression of learning and memory. Obviously, more detailed research will be required to untangle the diverse functions of GSK3 and to elucidate the involvement of GSK3 in synaptic plasticity and learning and memory.

3.4) Conclusion

In the present study I have established that the *in vivo* LTP approach can be applied to detect protein phosphorylation changes after synaptic potentiation in both CA1 and the DG. The findings show increased levels of protein phosphorylation after *in vivo* LTP induction in much-researched molecules including synapsin I, NR2B, αCaMKII and CREB, which have been consistently reported to exhibit increased phosphorylation after *in vitro* LTP. Thus, this approach may be used to investigate molecular and cellular processes important for synaptic plasticity, learning and memory. Moreover, in this study I show that the *in vivo* LTP approach can be used to assess activity-dependent changes in phosphorylation levels in the T286A mutant mice, where the autonomous

activity of αCaMKII is disenabled. Further characterisation of the T286A mutants with this approach will help to understand the function of αCaMKII in learning and memory processes. In the future application of this *in vivo* LTP approach to analyse other genetically-modified mouse lines with learning and memory impairments promises to yield new insights of the molecular processes underlying learning and memory.

CHAPTER 4: Analysis of a transgenic mouse line expressing the Cdk5 activator p25

4.1) Introduction

4.1.1) The p25 expressing transgenic mouse line

Dr Marco Angelo generated a transgenic mouse line in the C57BL/6 genetic background carrying an autosomal, single-copy integration of the mouse p25 transgene (Angelo *et al.*, 2003). The transgene includes two copies of the coding region head to tail oriented and a FLAG epitope. In this p25 TG mouse line the expression of the transgene is under the control of the αCaMKII promoter, which restricts the expression to excitatory neurons in the forebrain. Consistently, in adult TG mice at 12 month of age the highest level of p25 expression is in the hippocampus, where it averaged at approximately 33% of endogenous p35 expression (Fig. 4-1; Angelo *et al.*, 2003). Further, p25 is detected in neocortex, striatum and low amounts in spinal cord, but not in cerebellum (Fig. 4-1; Angelo *et al.*, 2003). The TG mouse line had low levels of p25 expression as compared to the p25 expression in other TG mouse lines (Table. 1-6). Most of the other TG mouse lines have a p25 transgene expression that is equal or higher than the levels of endogenous p35 expression (Table. 1-6). No p25 expression was observed in brains of TG mice at postnatal day 1. p25 was detected in the hippocampus of 18 days old TG mice, although at slightly lower levels (approximately 25% of endogenous p35 expression) than in adult TG mice (Angelo *et al.*, 2003). These findings suggest that the expression of p25 starts between the first and second postnatal week. The expression of p25 did not interfere with the expression of p35 and Cdk5 (Angelo *et al.*, 2003; see results). Further, p25 and endogenous p35 levels were unchanged between male and female p25 TG mice (Ris *et al.*, 2005). Finally, no

obvious signs of neurodegeneration were observed in the p25 TG mice even at an old age (Angelo *et al.*, 2003). The basic characterisation of the p25 TG mice demonstrates that the mouse line is suitable for further analysis of biochemical changes, synaptic plasticity, learning processes and memory formation. The p25 transgene expression starts postnatally. Hence, embryonic developmental artefacts can be excluded. As the interest focused on the impact of the presence of p25 in the hippocampus, a brain area affected in many tauopathies, including AD, a strategy to restrict the p25 expression to excitatory forebrain neurons was selected. The analysis of the expression pattern in the p25 TG mice showed that the p25 transgene expression is mainly forebrain restricted (Angelo *et al.*, 2003). The electrophysiological and behavioural characterisation of the p25 TG mice revealed that the expression of p25 enhanced CA1 LTP in female, but in contrast CA1 LTP was reduced in male p25 TG mice (Ris *et al.*, 2005). Spatial learning was improved in female p25 TG mice as compared to female WT controls, but it was not affected in male p25 mice (Ris *et al.*, 2005). Furthermore, tone fear conditioning was improved in female p25 TG mice in the 129B6F1 background, as well as in male and female p25 TG mice in the C57BL/6 background. Together, these results implicate the p25/Cdk5 holoenzyme in synaptic plasticity, learning and memory, possibly in a sex-specific manner (see for review Angelo *et al.*, 2006).

4.1.2) Aim of the project

Previous studies have implicated formation of p25 and p25-induced Cdk5 over-activation with molecular changes characteristic for neurodegenerative diseases including AD (see for review Shelton & Johnson, 2004). In fact, formation of p25 has been reported to occur specifically in sporadic AD patients, but not healthy individuals (Patrick *et al.*, 1999). Over-activation of Cdk5 by p25 has been proposed to trigger abnormal tau hyperphosphorylation and alter APP processing (see, for example, Cruz *et al.*, 2003; Lee *et al.*, 2003). Thus, one aim of this study was to investigate whether p25

expression and subsequently p25-induced Cdk5 over-activation leads to tau hyperphosphorylation and altered processing of APP in the p25 TG mouse line. For this I analysed the phosphorylation levels of tau at several disease-associated sites by using phospho-specific tau antibodies in young and old p25 TG mice. Further, I tested whether APP processing was affected in young and old p25 TG mice by using a range of antibodies detecting different parts of APP.

Another aim was the identification of Cdk5 substrates and in turn to acquire more information on what molecular mechanisms p25/Cdk5 is involved in. For this, I assessed changes in phosphorylation levels between p25 TG and WT mice by using a two dimensional gel electrophoresis (2DE) approach.

Finally, as behavioural and electrophysiological analysis of the p25 TG mice indicated an involvement of the p25/Cdk5 holoenzyme in synaptic plasticity, learning and memory, this study aimed to acquire some information on the molecular mechanisms underlying the changes in synaptic plasticity and behaviour in the p25 TG mice.

4.2) Results

4.2.1) Constant p25 expression in p25 TG mice

Previous results have shown that the p25 TG mouse line has postnatal, forebrain-restricted expression of the Cdk5 activator p25 (Angelo *et al.*, 2003). Here, I find that the expression of p25 stayed constant in young (3 months) and old (18 months) p25 TG mice (Fig. 4-2). I observed no expression of p25 in young and old WT controls (Fig. 4-2). This finding indicates that no proteolytic degradation occurred during the lysate

preparation and that the protease inhibitor cocktail used during the lysate preparation is suitable to prevent proteolytic degradation. Furthermore, no change in Cdk5 levels could be observed in young and old TG mice when compared to WT controls (Fig. 4-2). Studies in cell cultures have reported that p25 is not present at the synapse and therefore might delocalise Cdk5 from the synapse (Patrick *et al.*, 1998). However, I find p25 in crude synaptosomal fractions of TG mice and detect no changes in Cdk5 protein levels in synaptosomal lysate of TG mice (Fig. 4-3; Angelo *et al.*, 2003) indicating that Cdk5 is not mislocalised in our TG mice. Taken together, these results demonstrate that the p25 transgene expression and the Cdk5 protein levels are not altered during the lifespan of the TG mouse suggesting a constant over-activation of Cdk5 in TG mice.

4.2.2) Constant increase in Cdk5 activation by p25 expression

I tested whether the low-level expression of p25 in our mouse line resulted in an over-activation of Cdk5. For this I carried out a Cdk5 immunoprecipitation kinase assay. I found that the Cdk5 activity was increased by approximately 2-fold in young and old TG mice as compared to WT littermates (Fig. 4-4). This result shows that Cdk5 is constantly over-activated in the TG mice. This is consistent with the unaltered expression of p25 and Cdk5 protein level in young and old TG mice. Interestingly, in most studies using other TG mouse lines expressing p25 an approximately 2-fold increase in Cdk5 activity was observed, independently of the level of p25 expression (Table 1-6).

4.2.3) 2DE screen for changes in phosphorylation at proline-directed sites in p25 mice

Cdk5 is a proline-directed kinase that phosphorylates a broad range of substrates with diverse functions (Table 1-3). In order to see whether the p25-induced Cdk5 over-

activation is functional *in vivo*, I screened for changes in phosphorylation levels at proline-directed phosphorylation sites. First I focused on changes in phosphorylation levels of synaptic proteins. A 2DE immunoblot of crude synaptosomal protein from hippocampus was probed with a phospho-specific Cdk/MAPK substrate antibody. I observed distinct and reproducible changes in signal intensities of individual spots and spot trains that were controlled for with phospho-tyrosine antibody (see also *section 2.3.4*). Several spots and spot trains displayed higher intensity in TG mice as compared to WT controls (Fig. 4-5). A protein with a molecular mass of 160 kDa and an isoelectric point of 4.5 showed a 2.5-fold increase in TG mice (Fig. 4-5). This protein was identified as neurofilament M, a prime substrate of Cdk5 (Fig. 4-5). Interestingly, our screen could not detect elevated phosphorylation of neurofilament H, even though neurofilament M as well as neurofilament H contain repeats of the same Cdk5 phosphorylation motif. This finding suggests that the activated kinase complex Cdk5/p25 is directed towards a specific subset of substrates. Together, these results show that the over-activation of Cdk5 is functional *in vivo* in the TG mice and leads to increased phosphorylation of the Cdk5-specific substrate, neurofilament M, amongst others.

4.2.4) Age-dependent increase in tau hyperphosphorylation in p25 TG mice

After demonstrating that the expression of p25 induced over-activation of Cdk5 in our TG mice, I studied the phosphorylation levels of the microtubule-associated protein tau at disease-associated sites. Abnormal hyperphosphorylation of tau at proline-directed serine/threonine phosphorylation sites is characteristically found in neurodegenerative tauopathies including AD (see for review Buee *et al.*, 2000, Lee *et al.*, 2001). Previously, we have shown a non-significant trend of increased tau phosphorylation in hippocampal lysates from 13 month-old TG mice (Angelo *et al.*, 2003). Here, I examined the impact of p25-induced Cdk5 over-activation on tau hyperphosphorylation

in young (3 month) and old (18 months) TG mice. For this, I probed immunoblots of hippocampal lysates from TG mice and WT littermates with the phospho-specific tau antibodies AT8 (Ser202/Thr205), AT100 (Ser214 and/or Ser212), PHF-1 (Ser396/404) and TG3 (Thr231/Ser235). These antibodies recognise disease-associated epitopes around proline-directed phosphorylation sites on tau and are commonly used in studies of human pathology to detect NFTs. Protein levels of tau were assessed with the pan-tau antibodies BR134 and TAU5. In the TG mice an age-dependent accumulation of hyperphosphorylated tau was detected with the AT8 and the PHF-1 antibodies (Fig. 4-6). In young TG mice no changes in tau hyperphosphorylation were observed with any of the phospho-tau antibodies, even though Cdk5 activity was constantly elevated (Fig. 4-4). In contrast, old TG mice displayed significantly increased tau hyperphosphorylation by approximately 90% using the AT8 and PHF-1 antibodies (Fig. 4-6). The results of the PHF-1 antibody were confirmed using the phosphorylation-dependent neurofilament antibody, SMI31, which cross-reacts with tau hyperphosphorylated at the PHF-1 site (Lichtenberg-Kraag *et al.*, 1992). The SMI31 antibody revealed increased tau hyperphosphorylation in old TG mice comparable to the PHF-1 antibody (Fig. 4-6). The phospho-specific tau antibodies AT100 and TG3 did not detect differences in tau hyperphosphorylation levels between genotypes at old ages. Interestingly, by 18 months of age the WT mice also had increased tau hyperphosphorylation at the AT8 and PHF-1 site as compared to young age, albeit with larger variation between individuals than in the p25 mutants (Fig. 4-6).

4.2.5) Involvement of other proline-directed kinases in tau hyperphosphorylation

There were no age-dependent changes in expression of p25 and Cdk5, nor in Cdk5 activity in the TG mice that could account for the increased levels of tau hyperphosphorylation at the AT8 and PHF-1 sites. Therefore, I tested the involvement of other proline-directed kinases that have been linked to tau hyperphosphorylation

including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and MAPK/ERK kinase 1/2 (MEK1/2) (see for review Billingsley & Kincaid, 1997; Buee *et al.*, 2000). No changes in the phosphorylation levels at the activatory sites of JNK (Thr183/Tyr185) and MEK1/2 (Ser217/Ser221) were observed between TG and WT mice. The phosphorylation levels at the activatory site of ERK1/2 were reduced in TG mice as compared to WT mice suggesting that ERK activity is decreased in TG mice (Fig. 4-7). This is consistent with the finding that Cdk5 regulates the activity of ERK via the inhibition of MEK1 (Sharma *et al.*, 2002). Together, these results indicate that proline-directed kinases ERK, JNK and MEK are not responsible for the increased tau hyperphosphorylation observed in old TG mice. However, the activity of GSK3, another proline-directed kinase and prime candidate for mediating tau phosphorylation, was differentially regulated in TG mice (Fig. 4-8).

4.2.6) Age-dependent inhibitory regulation of GSK3 activity by p25 expression

GSK3 is known to phosphorylate proline-directed serine/threonine sites similarly to Cdk5 (Mandelkow *et al.*, 1992; Reynolds *et al.*, 2000), and has been linked to tau hyperphosphorylation (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992; for review, see Buee *et al.*, 2000). GSK3 is constitutively active, but can be inhibited by phosphorylation at Ser9 in the β-isoform and at Ser21 in the α-isoform (Stambolic & Woodgett, 1994). I found that the inhibitory phosphorylation at the serine residue of GSK3 was increased by approximately 80% in the young TG mice as compared to WT controls (Fig. 4-8). This result indicates that p25-induced Cdk5 over-activation leads to inhibitory control of GSK3. However, no increased phosphorylation at the inhibitory serine site was observed in old TG mice (Fig. 4-8), suggesting loss of inhibitory regulation in old TG mice. Interestingly, this loss of regulatory control coincides with the increased levels of tau hyperphosphorylation in old TG mice.

4.2.7) Inhibitory regulation of GSK3 is not mediated by AKT and p90Rsk activity

The inhibitory phosphorylation of GSK3α/β at the serine residue (Ser21/9) is regulated by antagonistic kinase and phosphatase activity (Grimes & Jope, 2001). Several kinases, including AKT, p90Rsk, PKA as well as some isoforms of PKC and phosphatases, including PP1 and PP2A have been implicated in this inhibitory regulation of GSK3 (Fig. 4-9; Grimes & Jope, 2001; Morfini *et al.*, 2004). Because Cdk5 does not directly phosphorylate GSK3 at the inhibitory site (Morfini *et al.*, 2004), I checked whether Cdk5 mediates the inhibition of GSK3 via the control of AKT and p90Rsk. I assessed the phosphorylation levels on their activation sites with phospho-specific antibodies and detected no altered regulation in the TG mice (Fig. 4-9). This suggests that the modulation evoked by p25 expression is not mediated by AKT and p90Rsk.

4.2.8) Age-dependent increase in activated GSK3β protein in old TG and WT mice

Phosphorylation of GSK3 at the Tyr279 in the α-isoform and Tyr216 in the β-isoform, respectively, is essential for activation of GSK3 (Hughes *et al.*, 1993). I used a phospho-specific GSK3 antibody recognising the phosphorylated activatory sites, to check whether p25-induced Cdk5 over-activation impacts on the activating phosphorylation. No alterations in phosphorylation at the activating tyrosine site (Tyr279/216) were detected between the genotypes at any age (Fig. 4-10). Interestingly, whereas the protein level of the α-isoform remained constant between young and old mice, the amount of GSK3β protein is increased in both old WT and old TG mice. Further, the phosphorylation at the activating Tyr216 site of the β-isoform was also elevated in old mice as compared to young mice (Fig. 4-10). In contrast, the phosphorylation level of the activating Tyr279 site on the α-isoform remained constant in young and old mice. These findings show that p25-induced Cdk5 over-activation does not impact on the phosphorylation levels at the activating site. Furthermore, it is conceivable that the age-

dependent increase of GSK3β protein phosphorylated at the activatory site confers increased GSK3β activity in old mice.

4.2.9) GSK3β activity is reduced in young, but increased in old TG mice

The phosphorylation level at the inhibitory site of GSK3 was elevated in young TG mice suggesting decreased GSK3 activity. However this inhibitory phosphorylation was lost in old TG mice and coincided with an increase in GSK3β protein. I tested the levels of GSK3β activity by using a GSK3β immunoprecipitation kinase assay. In young TG mice the GSK3β activity was significantly decreased by approximately 25% confirming the immunoblot results of the inhibitory phosphorylation levels (Fig. 4-11). Further, in old TG mice the GSK3β activity was increased by approximately 70% (Fig. 4-11). These results suggest that the inhibitory regulation of GSK3β is altered in old TG mice leading to enhanced GSK3β activity, which correlates with tau hyperphosphorylation at the AT8 and PHF-1 sites in old TG mice. Thus, it is likely that the increase in GSK3 activity is responsible for the tau hyperphosphorylation in the old TG mice.

4.2.10) Chronic lithium treatment reduced the level of tau hyperphosphorylation in old p25 TG mice

To test whether the increased GSK3 activity is responsible for the age-dependent tau hyperphosphorylation at the AT8 and PHF-1 sites, old TG mice were treated chronically with lithium, a commonly used reversible inhibitor of GSK3 (see, for example, Hong *et al.*, 1997; Klein *et al.* 1996) and the level of tau hyperphosphorylation at the AT8 and PHF-1 sites was assessed. At low concentrations lithium is an inhibitor of GSK3, but does not affect significantly the activity of other kinases (Phiel & Klein, 2001; Davies *et al.*, 2000). The phospho-specific tau antibodies, PHF-1 and AT-8, showed a marked

reduction of the tau hyperphosphorylation level in the lithium treated old TG mice as compared to old TG controls (Fig. 4-12). The tau hyperphosphorylation could be reversed close to levels observed in young TG mice. Chronic lithium treatment in 18 to 19 month old WT mice induced a non-significant reduction of phosphorylation levels at the PHF-1 site, but did not alter phosphorylation levels at the AT8 site (Fig. 4-13). The inhibitory effect of lithium on GSK3 was confirmed by assessing the protein levels of β-catenin. Phosphorylation of β-catenin by GSK3 targets the protein for degradation via the ubiquitin-proteosome pathway (Grimes & Jope, 2001). The level of β-catenin was increased in lithium treated WT and TG mice as compared to untreated controls (Fig. 4-12 and 4-13), indicating reduced GSK3 activity. Interestingly, no differences in β-catenin levels were observed between untreated TG and WT mice, both at young and old age, despite the changes in GSK3β kinase activities between genotypes (Fig. 4-14). To assess whether Cdk5 activity was affected by the lithium treatment, the phosphorylation level of amyloid precursor protein (APP) at Thr668 was monitored. APP is phosphorylated at the proline-directed Thr668 in the cytosolic domain specifically by Cdk5 (Iijiama *et al.*, 2000; see also *section 4.2.12*). No differences in APP phosphorylation at Thr668 were observed in lithium treated TG and WT mice as compared to untreated controls (Fig. 4-12 and 4-13) suggesting Cdk5 activity is not affected by lithium treatment.

4.2.11) Interaction of Cdk5, GSK3 and PP2A

The inhibitory cross-talk of Cdk5 and GSK3 suggests that these two molecules are localised in proximity of each other or even aggregate together. To test whether Cdk5, GSK3 and protein phosphatases interact with each other, I carried out co-immunoprecipitation in hippocampal lysates of TG mice using anti-GSK3β antibody. The presence of GSK3β protein in the precipitate fraction showed that the antibody was suitable for pull-down assays (Fig. 4-15). The co-immunoprecipitation with the anti-

GSK3β antibody also pulled down Cdk5 and PP2A as tested by immunoblotting (Fig. 4-15). Interestingly, the immuno-precipitate pulled down with an anti-Cdk5 antibody contained Cdk5, p25 and p35; however, no GSK3, nor PP2A could be detected (data not shown). These results show that GSK3, Cdk5 and PP2A can aggregate within the same complex. However only a small subset of all Cdk5 molecules participates in this complex. Further evidence for association of these molecules comes from GSK3β immunoprecipitation kinase activity assays (Fig. 4-16). The GSK3β activity assay showed similar levels of kinase activity for TG mice and WT littermates when okadaic acid, a specific inhibitor of PP1 and PP2A, was omitted from the kinase reaction buffer. Addition of okadaic acid to the kinase reaction buffer had no significant effect on kinase activity levels in WT mice, but produced a marked reduction in TG mice (Fig. 4-16). These results indicate that protein phosphatases co-immunoprecipitate with GSK3β, as the GSK3β activity is differentially regulated by phosphatase activity in TG mice as compared to WT mice. These observations further demonstrate the necessity of application of suitable kinase and phosphatase inhibitors in immunoprecipitation kinase activity assays. As I found that Cdk5, GSK3 and PP2A co-immunoprecipitate and GSK3 activity is inhibited by Cdk5 over-activation, it is necessary that Cdk5 inhibitors, such as roscovitine, as well as phosphatase inhibitors, such as okadaic acid, are used in GSK3 activity assays.

4.2.12) Constant increase in APP phosphorylation by p25 expression

I assessed whether other well-characterised, specific Cdk5 substrates showed an age-dependent increase in phosphorylation similar to that observed for tau. The membrane-associated protein APP is phosphorylated at the proline-directed site Thr668 (as per APP695) in the cytosolic domain specifically by Cdk5 (Iijiama *et al.*, 2000). The anti-phospho-Thr668-APP antibody predominantly recognised an immature 62 kDa APP isoform (Fig. 4-17 and 4-18). The phospho-Thr668-APP level was significantly

increased by approximately 60% in TG mice as compared to WT mice (Fig. 4-17). This increase was detected in young and old TG mice (Fig. 4-17). No changes in total APP expression were observed with antibodies recognising the unphosphorylated epitope around Thr668 (APP) and the extreme carboxy-terminal region of APP (CT-15) (Fig. 4-17 and 4-18). The constantly elevated phospho-Thr668-APP levels are consistent with the results of the *in vitro* Cdk5 kinase assay that demonstrate increased Cdk5 activity in young and old TG mice. These results confirm that Cdk5 activity is constantly increased in TG mice *in vivo*. Hence, specific substrates of the p25/Cdk5 holoenzyme display elevated phosphorylation levels in the TG mice.

4.2.13) 2DE analysis of APP isoforms and phosphorylated APP at Thr668

APP is the product of a single gene, but a large number of APP isoforms is generated by alternative splicing, post-translational modifications and complex proteolytic processing (Fig. 1-8 to 1-11; see for review, Turner *et al.*, 2003). APP isoforms can be grouped in mature and immature isoforms according to their electromobility in SDS PAGE. Immature APP isoforms are lacking post-translational modifications and run at around 70-90 kDa, whereas mature APP isoforms, which are highly glycosylated have an apparent molecular mass weight of about 110-130 kDa. To investigate the various APP isoforms and explore the impact of the APP phosphorylation at Thr668 on the electromobility of the molecule, 2DE-gels were immunoblotted and probed with different APP antibodies. I used an antibody raised against the Aβ-peptide domain (4G8) and one against the carboxy-terminus of APP (CT-15) on 2DE immunoblots from hippocampal lysates (Fig. 4-18). Both antibodies recognised a similar complex signal pattern of myriads of immature and mature APP isoforms with various molecular mass weights and isoelectric points (Fig. 4-19). The data from the 2DE immunoblot of the two antibodies were combined to generate a composite picture that just included signals recognised by both antibodies. The resulting composite picture is likely to exclusively

represent APP isoforms; however some isoforms (e.g. those lacking the carboxy-terminus) might have been omitted. The composite picture was overlaid with the signal from the immunoblot probed with anti-phospho-Thr668-APP antibody. This overlay reveals that only a well-defined subset of APP isoforms displays phosphorylation at Thr668 (Fig. 4-20). This result implies that only a specific pool of APP isoforms with a characteristic electromobility (possibly due to similar post-translational modifications) undergoes phosphorylation at Thr668, which might correlate with a specific function of this subset of APP isoforms.

4.2.14) Age-dependent alterations in APP processing in p25 TG mice

The alternative splice variants of APP are subjected to numerous post-translational modifications and complex proteolytic processing that give rise to a large number of APP isoforms and fragments, including the Aβ-peptide and carboxy-terminal fragments (CTF) (Fig. 1-9 to 1-11; see for review Turner *et al.*, 2003). Much research has focused on the production of Aβ-peptides, because of their involvement in AD. Aβ-peptides constitute the main component of senile plaques. The APP phosphorylation at Thr668 has been thought to be important for the processing of APP and possibly the generation of Aβ-peptides (Suzuki *et al.*, 1994; Iijama *et al.*, 2000; Lee *et al.*, 2003). As p25 expression caused increased APP phosphorylation at Thr668, I investigated the processing of APP in the TG mice. Levels of different APP isoforms and fragments were assessed in immunoblots of hippocampal lysate probed with an antibody recognising the Aβ-domain of APP (4G8). The anti-Aβ antibody detected a 57 kDa fragment in old (18 and 26 month), but not in young (3 month) TG mice (Fig. 4-21). The quantification of the immunoblot signal revealed a significant age-dependent accumulation of this 57 kDa fragment in old TG mice (Fig. 4-21). Interestingly, the pattern of APP isoforms detected by the anti-Aβ antibody is altered between the tested age-points, implying that the post-translational modifications on APP are affected in an

age-dependent manner (Fig. 4-21). Unprocessed APP runs normally at molecular mass weights higher than 57 kDa. Thus, it is conceivable that parts of the APP have been cleaved off resulting in a 57 kDa fragment. To assess what parts the 57 kDa fragment is lacking, I tested whether the band at 57 kDa can be detected with antibodies against the amino- and carboxy-terminus of APP (Fig. 4-22). Surprisingly, neither the amino-terminus, nor the carboxy-terminus-specific anti-APP antibody recognised a band at 57 kDa (Fig. 4-22).

Taken together, these results indicate that APP processing is altered in old but not in young TG mice. Moreover, the 57 kDa fragment does not contain the amino- and carboxy-terminal portion of APP. Thus, further studies will be required to reveal the identity of the 57 kDa fragment.

4.2.15) No changes in carboxy-terminal fragments of APP in p25 TG mice

To investigate whether the altered APP processing has an impact on the levels of other fragments than the 57 kDa species, I assessed the levels of CTFs and Aβ-peptides in high resolution tris-tricine gels. The analysis of CTF levels is commonly used as an indicator of secretase activity and Aβ-peptide production, because CTFs are released as a by-product of Aβ-peptide processing.

Hippocampal lysates of old TG mice and WT controls were separated on tris-tricine gels, immunoblotted and probed with anti-Aβ antibody (4G8), anti-phospho-Th668-APP antibody and an antibody against the carboxy-terminus of APP (CT-15) (Fig. 4-18). All of the antibodies detected several bands in the molecular mass weight range of 5-25 kDa (Fig. 4-23). Some of the bands were consistently found with all three antibodies (i.e. 16, 19, 23 kDa) indicating that these bands are CTFs of APP. However, the antibodies did not detect any differences in signal intensity of bands between TG mice and WT controls (Fig. 4-23). This suggests that there are no differences in CTF

production/accumulation, and therefore in Aβ-peptide production in old TG mice as compared to old WT littermates. Consistent with this observation, the anti-Aβ antibody (4G8) did not detect any changes in band intensity in the molecular mass weight range, where Aβ-peptides migrate (3-6 kDa; data not shown). However, it is possible that the detection level of tris-tricine immunoblots is not sufficient to assess subtle changes in low Aβ-peptide levels. Hence, a more accurate analysis of Aβ-peptide production in the TG mice using the ELISA technique will be required. The results so far show that there is an age-dependent accumulation of a 57 kDa fragment indicating that APP processing is altered in old TG mice. But no evidence for altered Aβ-peptide generation in old TG mice could be produced. In order to understand the effect of the p25/Cdk5 holoenzyme on APP processing, it will be important to characterise the 57 kDa fragment.

4.2.16) Summary

Here, I analysed biochemical changes in the TG mouse line expressing the Cdk5 activator p25. I examined the impact of p25-induced Cdk5 over-activation on the phosphorylation levels of specific substrates, including the microtubule associated protein tau and APP.

First, I demonstrated that in the TG mouse line p25 is constantly expressed at low-levels. This p25 expression leads to a persistent, two-fold increase in Cdk5 activity and consistently to a constant elevation of the phosphorylation levels of APP at Thr668. However, the p25-induced Cdk5 over-activation did not result in tau hyperphosphorylation in young TG mice. In fact, over-activation of Cdk5 was associated with increased phosphorylation levels at the inhibitory site on GSK3. This result indicates that Cdk5 negatively regulates GSK3 activity. However, in old TG mice the inhibitory regulation of GSK3 is lost. Further, I observed an age-dependent increase in tau phosphorylation at disease-associated sites, including AT8 and PHF-1 sites, that

coincides with enhanced GSK3 activity. These results suggest that the increased tau hyperphosphorylation observed in old TG mice is mediated by GSK3, but not Cdk5 activity. Accordingly, in old TG mice the level of increased tau hyperphosphorylation could be reduced by chronic treatment with lithium, a GSK3 inhibitor.

Moreover, p25 expression was found to influence APP processing such that a 57 kDa fragment of APP accumulates in an age-dependent manner. These results suggest that long-term exposure to low-level p25 expression can induce biochemical changes characteristic of AD. Further, our findings show that key molecular players of AD are linked by regulation through common intersecting signalling pathways involving Cdk5 and GSK3.

4.3) Discussion

4.3.1) The roles of Cdk5 and GSK3 in tau hyperphosphorylation

Hyperphosphorylation of tau is a characteristic feature of neurodegenerative tauopathies including AD. Therefore, many studies tried to identify the protein kinases and protein phosphatases that are involved in the abnormal hyperphosphorylation of tau. Numerous studies, both *in vitro* and *in vivo*, demonstrate that these sites are phosphorylated by proline-directed protein kinases, including Cdk5, ERK, GSK3, JNK and MAPKs (see, for example, Mandelkow *et al.*, 1992; Reynolds *et al.*, 2000; see for review Billingsley & Kincaid, 1997; Buee *et al.*, 2000). In addition, protein phosphatases including PP1, PP2A, PP2B and PP5 have been implicated in the regulation of the phosphorylation level of tau (see, for example, Liu *et al.*, 2005; for review, see Billingsley & Kincaid, 1997; Buee *et al.*, 2000). However, at present, the results implicating the various kinases

and protein phosphatases in the regulation of the many phosphorylation sites on tau are inconsistent.

The proline-directed serine/threonine kinase Cdk5 has been identified as prime candidate for phosphorylating tau at disease-associated sites characteristic for neurodegenerative tauopathies (see for review Shelton & Johnson, 2004). Cdk5 co-localises with filamentous tau deposits and has enhanced activity in several tauopathies, including AD (Augustinack *et al.*, 2002b; Lee *et al.*, 1999). Numerous *in vitro* studies implicate Cdk5 in the phosphorylation of tau at several proline-directed sites (see, for example, Liu *et al.*, 2002; Hamdane *et al.*, 2003). *In vivo* studies using TG mouse lines expressing the Cdk5 activator p25 report tau hyperphosphorylation as tested by antibodies recognising disease-associated phospho-epitopes such as AT8 and PHF-1 (Alijahnian *et al.*, 2001, Cruz *et al.*, 2003; Plattner *et al.*, 2006). Therefore, it is currently believed that Cdk5 directly induces hyperphosphorylation of tau at disease-associated sites and that this may subsequently trigger neurofibrillary tangle formation in tauopathies (see, for example, Cruz *et al.*, 2003).

The results presented in this study do not support a direct role for Cdk5 in tau hyperphosphorylation at disease-associated sites. The p25 TG mice show no tau hyperphosphorylation at young age despite a persistent two-fold increase in Cdk5 activity. Consistently, other TG mouse lines expressing p25 do not exhibit tau hyperphosphorylation, even though Cdk5 activity is enhanced (Takashima *et al.*, 2001; Noble *et al.*, 2003). However, I observe increased tau hyperphosphorylation at the AT8 and PHF-1 epitopes in our p25 TG mice at an old age. This raises the question as to why in the p25 mouse line, tau hyperphosphorylation only becomes apparent with advancing age. One possibility is that long-term exposure to p25 is required to induce tau hyperphosphorylation. Thus, initial Cdk5 over-activation may be compensated by phosphatases and degradation of hyperphosphorylated proteins by proteases. However, this compensation may not be maintained over long periods or in old age.

Another possibility is that Cdk5 is not directly involved in the hyperphosphorylation of tau and additional factors are required, triggered only in old age. In fact, I found that the activity of the proline-directed kinase, GSK3, is reduced in young TG mice, but increased in old TG mice. Hence, the elevated GSK3 activity correlates with the age-dependent increase in tau hyperphosphorylation at the AT8 and PHF-1 site. GSK3 is another major candidate for mediating tau hyperphosphorylation and has been shown to phosphorylate proline-directed serine/threonine sites similarly to Cdk5 (Mandelkow *et al.*, 1992; Hanger *et al.*, 1992; Hong *et al.*, 1997, Lucas *et al.*, 2001;). GSK3 has been observed to generate disease-associated phospho-epitopes on tau (Nishimura *et al.*, 2004) and co-localises with aggregated hyperphosphorylated tau (Ishizawa *et al.*, 2003). In this study I demonstrate that chronic treatment with lithium, a GSK3 inhibitor, reduced the levels of tau hyperphosphorylation at the AT8 and PHF-1 sites in the old TG mice strongly suggesting that GSK3 is a key mediator of tau hyperphosphorylation at these sites. This contention is supported by a study in p35 null mutant mice (Hallows *et al.*, 2003). The p35 null mutants have reduced Cdk5 activity, but enhanced GSK3β activity, which coincides with increased levels of tau hyperphosphorylation. Experiments using the fruit fly, *Drosophila melanogaster*, show that the PHF-1 phospho-epitope on tau contains GSK3-specific phosphorylation sites, but is not modified by Cdk5 (Nishimura *et al.*, 2004). Further evidence for an indirect involvement of Cdk5 in tau hyperphosphorylation via the regulation of GSK3 comes from a recent study using yeast expressing human tau (Vandebroek *et al.*, 2006). In this model it has been observed that Mds1, the GSK3 orthologue in yeast, mediates hyperphosphorylation of tau at disease-associated phospho-epitopes. In contrast, the Cdk5 orthologue Pho85 does not phosphorylate tau, but in fact acts indirectly as a negative regulator of tau hyperphosphorylation. In line with the observation that GSK3 is mediating tau hyperphosphorylation at disease-associated epitopes, the analysis of a TG mouse line over-expressing GSK3β, which displays enhanced GSK3 activity revealed that tau hyperphosphorylation at the AT8 and PHF-1 sites was significantly increased (Lucas *et al.*, 2001). Further, this study localises tau phosphorylated at the

PHF-1 epitope at somatodendritic sites. However, no tau filament formation was observed in the GSK3β over-expressing TG mice (Lucas *et al.*, 2001; Hernandez *et al.*, 2002). Taken together, these results indicate that GSK3 acts as a key mediator of tau phosphorylation at the disease-associated sites, AT8 and PHF-1. Further these results consistently show that Cdk5 negatively regulates GSK3 activity and suggest that Cdk5 indirectly affects tau hyperphosphorylation via the regulation of GSK3.

Some of the GSK3-mediated tau phosphorylation requires priming by other kinases including Cdk5, PKA and PAR-1 kinase (Sengupta *et al.*, 1997; Nishimura *et al.*, 2004; Li & Paudel, 2006). It has been suggested that once a priming site on tau is modified, GSK3 can sequentially phosphorylate neighbouring sites and hence induce hyperphosphorylation (Cho & Johnson, 2003). Therefore, the priming of tau phosphorylation sites may constitute another mechanism, next to the inhibitory regulation of GSK3β, by which over-activation of Cdk5 could affect tau hyperphosphorylation. Recent studies have involved Cdk5 in the priming of tau at the individual sites Ser202 and Ser404 for GSK3β (Li & Paudel, 2006; Li *et al.*, 2006). It is thought that priming of these sites by Cdk5 and subsequent phosphorylation at the neighbouring sites by GSK3β results in the generation of the disease-associated phospho-epitopes, AT8 and PHF-1, respectively. Because Cdk5 and GSK3 are simultaneously over-activated in old p25 TG mice, the two kinases may synergise to trigger tau hyperphosphorylation at these sites. Consistent with this contention, the AT8 and PHF-1 sites are hyperphosphorylated in old TG mice. In this context, I have proposed that the inhibitory regulation of GSK3 by Cdk5 might represent an important mechanism to regulate the level of tau phosphorylation and prevent excessive phosphorylation at primed sites (Plattner *et al.*, 2006).

Numerous protein kinases have been implicated in the phosphorylation of tau at multiple individual serine/threonine residues (see for review Billingsley & Kincaid, 1997; Buee *et al.*, 2000). However, it is not established, which individual residues on

tau are phosphorylated under physiological conditions and which are the kinases that mediate the tau phosphorylation at the different residues. Further, it is unclear what functions, if any, the phosphorylation of the individual sites on tau serves. Here, GSK3 has been specifically implicated in the hyperphosphorylation of tau at the AT8 (Ser202/Thr205) and PHF-1 (Ser396/Ser404) sites *in vivo*, possibly in conjunction with the priming of these sites by Cdk5. Consistent with this finding, TG mice over-expressing GSK3β exhibit tau hyperphosphorylation at the AT8 and PHF-1 site (Lucas *et al.*, 2001). Interestingly, an *in vivo* study where PKA activity was altered by pharmacological means, found that PKA specifically phosphorylates the individual sites Ser202, Ser214 and Ser404 and primes tau for subsequent modification by GSK3 (Liu *et al.*, 2004). Consistent with the notion that PKA mediates phosphorylation at the AT100 (Ser214 and/or Ser212) site, the p25 TG mice do not display increased phosphorylation levels at the AT100 site, suggesting that this site is not phosphorylated by Cdk5 or GSK3 *in vivo*. Together, these data suggest that distinct protein kinases mediate phosphorylation at specific sites on tau. Thus, it is conceivable that distinct kinases may regulate various physiological functions of tau by differential phosphorylation of individual sites.

4.3.2) Regulatory cross-talk between Cdk5 and GSK3

The present study describes a regulatory cross-talk between Cdk5 and GSK3 (for proposed model see Fig. 4-24 and 4-25). In young TG mice this regulation appears to be dependent on phosphorylation of GSK3 at the inhibitory serine site. The phosphorylation of GSK3β at Ser9 is increased resulting in significantly reduced GSK3β activity. Hence, this finding implicates p25-induced Cdk5 over-activation in the inhibitory regulation of GSK3β by affecting the level of Ser9 phosphorylation. Consistently, a study in p35 null mutant mice shows that the reduction in Cdk5 activity coincided with increased GSK3 activity (Hallows *et al.*, 2003). Moreover,

pharmacological inhibition of Cdk5 with olomucine and roscovitine induces activation of GSK3 (Morfini *et al.*, 2004). The molecular mechanisms that lead to inhibition of GSK3 by Cdk5 are not yet characterised. However, it is has been shown that the phosphorylation at the inhibitory serine site on GSK3 is not directly mediated by Cdk5 (Morfini *et al.*, 2004). Hence, it is conceivable that Cdk5 affects the inhibitory GSK3 phosphorylation indirectly via intermediate protein kinases and phosphatases. My results suggest that Cdk5 does not act via AKT and p90Rsk, two kinases directly implicated in the inhibition of GSK3. In fact, my data indicate that Cdk5 mediates its inhibitory control over GSK3 via the regulation of protein phosphatases, including PP1 and PP2A. Here, I observe that application of okadaic acid, a specific PP1 and PP2A inhibitor, reduces GSK3β activity in TG mice, but not WT controls. Consistently, a pharmacological study proposed the involvement of PP1 in the inhibitory regulation of GSK3 (Morfini *et al.*, 2004). This study demonstrated that inhibition of Cdk5 led to increased levels of GSK3 activity, which could be reversed by blocking PP1. Moreover, a functional link between Cdk5, GSK3β and PP2A is further supported by my finding that these molecules associate with each other in a complex. Accordingly, other studies report the association of Cdk5 and GSK3β in a functional complex approximately 450 kDa in mass weight, which contains tau (Li *et al.*, 2006). These data implicate Cdk5 and GSK3 in the formation of protein complexes with PP1, Inhibitor 2, and 14-3-3 (Agarwal-Mawal & Paudel, 2001; Agarwal-Mawal *et al.*, 2003). Within such protein complexes the associated proteins are often regulated via functional cross-talk. In fact, protein phosphatase activity is commonly regulated by association with regulatory partners. For example, for PP1 more than fifty regulatory subunits have been described (see for review Cohen, 2002). Furthermore, it has been demonstrated that the function of several regulatory subunits of PP1 is controlled directly via phosphorylation. Interestingly, Cdk5 and GSK3 have been implicated in the phosphorylation of phosphatase-regulating molecules including Inhibitor 1 and 2 as well as DARPP32 (Bibb *et al.*, 1999; Bibb *et al.*, 2001; Agarwal-Mawal & Paudel, 2001). Similar molecular mechanisms have been implicated in the regulation of PP2A activity (Winder

& Sweatt, 2001). From this, I propose that Cdk5 directly controls the activity of a regulatory subunit, which in turn exerts an effect on PP1 and/or PP2A and ultimately the activity of GSK3.

In contrast to young TG mice, where GSK3 activity is reduced, old TG mice have increased levels of GSK3 activity. Curiously, in old TG mice GSK3 activity is increased, despite equivalent phosphorylation levels at the inhibitory and activatory sites of GSK3 in old TG mice and old WT littermates. This suggests that in old TG mice the inhibitory control of GSK3 by Cdk5 is lost and additional mechanisms, other than phosphorylation of the regulatory sites, regulate the activity of GSK3β. Alternative mechanisms might include differences in protein complex formation involving GSK3 or alterations in subcellular localisation of GSK3. It is conceivable that in young TG mice that the presence of p25 leads to an altered subcellular localisation and complex composition or to differences in the regulation within protein complexes. Consistently, I find that application of okadaic acid, a specific PP1 and PP2A inhibitor, differently affects GSK3 activity in young TG mice and WT controls. This suggests that the protein complex containing GSK3, Cdk5 and PP2A is functionally different in young TG mice as compared to young WT littermates. Based on these results, I propose that the presence of p25 induces functional changes within protein complexes in young TG mice resulting in increased Ser9 phosphorylation and in turn reduced GSK3β activity (Plattner *et al.*, 2006). Long-term exposure to p25 expression disrupts this altered regulatory control of GSK3 resulting in elevated GSK3 activity in old TG mice. Together, these findings suggest that GSK3 activity is regulated by an intricate interplay of protein phosphatases, kinases and possibly other associated proteins. Further studies will be required to characterise the molecular mechanisms by which Cdk5 regulates GSK3. As the age-dependent disruption of the regulatory cross-talk between GSK3 and Cdk5 may underlie tau hyperphosphorylation in neurodegenerative tauopathies, it will be paramount to elucidate this regulatory signalling pathways.

4.3.3) Comparison of the different p25 TG mouse lines

Several TG mouse lines with p25 expression have been generated (for a summary of p25 TG mouse lines, see Table 1-6). The expression of p25 resulted in an over-activation of Cdk5 in all TG mouse lines. Subsequently, these p25 TG lines were used to test the involvement of Cdk5 in tau hyperphosphorylation. The onset and the level of tau hyperphosphorylation varied markedly between the different p25 TG mouse lines. The trend observed in studies examining TG animals at older ages is of increased levels of tau phosphorylation. One study looking at young TG animals did observe no tau hyperphosphorylation (Takashima *et al.*, 2001). These results imply that prolonged p25 expression leads to higher levels of tau hyperphosphorylation. Further, these results show that increased levels of tau phosphorylation occur only after a certain period of p25 expression. Next to the age factor, the difference in p25 expression levels between the TG mouse lines might be responsible for the variation between the results. Transgenic mouse lines expressing higher levels of p25 display higher levels of tau phosphorylation or an earlier onset of tau hyperphosphorylation (Bian *et al.*, 2002; Cruz *et al.*, 2003). The analysis of two TG mouse lines expressing p25 at different levels in young age, revealed tau hyperphosphorylation in the high level p25 expressing line, but not in the low level p25 expressing (Cruz *et al.*, 2003). Interestingly, in most studies the presence of p25 resulted in an approximately two-fold increase in Cdk5 activity, independently of the p25 level (Table 1-6). Hence, higher p25 expression did not correlate with higher Cdk5 over-activation in the TG mouse lines. This raises the concern that high levels of p25 expression might affect other molecular mechanisms next to over-activation of Cdk5. For example, it was suggested that high levels of p25 could also be toxic *per se* (Bian *et al.*, 2002). Accordingly, a recent study has implicated the formation of p25 in excitotoxicity and neuronal cell death via the regulation of nuclear processes (O'Hare *et al.* 2005). Two of the p25 TG mouse lines were reported to form aggregates of hyperphosphorylated tau, similar to NFTs observed in AD patients (Nobel *et al.*, 2003; Cruz *et al.*, 2003). Further, in a inducible p25 TG mouse

line immunoblot and immunohistological analyses revealed neurodegeneration and gliosis (Cruz *et al.*, 2003). However, recent data indicates that the "neurodegeneration" in this inducible p25 TG line is not linked to increased levels of tau hyperphosphorylation (Fischer *et al.*, 2005).

Together, these results indicate that the protein kinase complex p25/Cdk5 plays a role in the phosphorylation of tau at proline-directed sites, where the duration of p25 exposure and the level of p25 are important parameters determining the degree of tau phosphorylation. However, these studies do not provide evidence that p25/Cdk5 directly mediates tau hyperphosphorylation and they cannot exclude that p25/Cdk5 is indirectly involved in tau phosphorylation by regulating other kinases or phosphatases. In fact, my data indicate that GSK3 mediates tau hyperphosphorylation, whereas Cdk5 acts as a modulator of tau hyperphosphorylation via the inhibitory control over GSK3. However, prolonged exposure to p25 deregulates the cross-talk between Cdk5 and GSK3, leading to increased GSK3 activity and in turn to hyperphosphorylation of tau. It is conceivable that higher levels of p25 or prolonged exposure to p25 are more likely to disrupt the regulatory cross-talk between Cdk5 and GSK3. Hence, this could explain why tau hyperphosphorylation was observed predominantly in high level p25 expressing TG mouse lines or in studies analysing p25 TG mouse lines at old ages. Further, this could explain the heterogeneity within the results from different p25 TG mouse lines and other studies on Cdk5 involvement in tau hyperphosphorylation (e.g. cell culture experiments; compare Kerokoski *et al.*, 2002 and Hamdane *et al.*, 2003). As the level of p25 and the duration of p25 exposure vary between the different studies, it is conceivable that different levels of tau phosphorylation are observed. Moreover, as a short period of high level p25 expression and a long period of low level p25 expression lead similarly to tau hyperphosphorylation, it suggest that comparable processes are induced in both cases, independently of ageing factors. Further research will be required to elucidate the role of Cdk5 in intracellular signalling, aggregation of hyperphosphorylated tau and neurodegeneration. This question is complicated by the

fact that the expression of p25 induces numerous other molecular changes, which might impact generally on protein phosphorylation, protein degradation and cell survival. To overcome these confounding factors, new research strategies need to be applied.

4.3.4) Implication of Cdk5 in APP processing

APP is phosphorylated at the residue Thr668 specifically by Cdk5 (Iijima *et al.*, 2000). Consistently, p25-induced Cdk5 over-activation increased the phosphorylation level of APP at Thr668 in the p25 TG mice. Interestingly, the increased phosphorylation occurred predominantly in an immature APP isoform running at approximately 62 kDa and to a lesser extend in mature isoforms running at 110-120 kDa in the p25 TG mice. In line with this, over-expression of p25 in cell cultures induces predominantly phosphorylation of immature APP isoforms, whereas over-expression of p35 triggered phosphorylation of both mature and immature APP at Thr668 (Liu *et al.*, 2003). The phosphorylation at Thr668 has been implicated in protein complex formation and protein localisation (Suzuki *et al.*, 1994; Iijima *et al.*, 2000). Hence, it is possible that p25/Cdk5 and p35/Cdk5 phosphorylate different pools of APP according to their cellular localisation and in turn regulate distinct functions of APP. Further, recent *in vitro* studies link Thr668 phosphorylation with altered processing of APP (Liu *et al.*, 2003; Lee *et al.*, 2003). These studies implicate increased APP phosphorylation and Cdk5 activity with elevated APP processing resulting in enhanced Aβ-peptides production and secretion of sAPPα as well as sAPPβ. However, these studies are contradictory to another finding showing that inhibition of Cdk5 stimulates Aβ production *in vitro* and *in vivo* (Ryder *et al.*, 2003). Consistent with this, I find that despite increased APP phosphorylation levels and Cdk5 over-activation, APP processing is not changed in young p25 TG mice. Nevertheless, APP processing is altered in old p25 TG mice, where both Cdk5 and GSK3 activity are increased; I observed an age-dependent accumulation of a 57 kDa fragment containing the Aβ-

peptide domain. Because p25-induced Cdk5 over-activation did not lead to altered APP processing in young p25 TG mice, it is conceivable that the accumulation of the 57 kDa fragment in old p25 TG is due the enhanced activity of GSK3. Consistent with this assumption are data showing the involvement of GSK3 in APP processing (Phiel *et al.*, 2003; Ryder *et al.*, 2003). These data demonstrate that inhibition of GSK3 (pharmacological agents or siRNA) reduces the production of Aβ-peptides *in vitro* and *in vivo*. Because of the regulatory cross-talk between Cdk5 and GSK3, inhibition of Cdk5 results in enhanced GSK3 activity. Hence, it is possible that in the studies, in which Cdk5 inhibitors are used, the increased APP processing actually is due to enhanced GSK3 activity.

The 57 kDa fragment could not be detected with antibodies against the amino- and carboxy-terminal parts of APP. This observation suggests that the 57 kDa fragment lacks the amino-terminal and carboxy-terminal parts. There are several cleavage sites for proteases and secretases in the carboxy-terminal portion of APP. Thus, it is possible that the carboxy-terminus is cleaved off, but this would not release a 57 kDa APP fragment. Further, in the outer amino-terminal part there are no cleavage sites known, that can lead to the generation of a 57 kDa APP fragment. As this fragment could only be detected with Aβ-specific antibody, it is also possible that the band running at 57 kDa constitutes of an aggregate of multiple Aβ-peptides. Consistent with this idea, a recent study reports the accumulation of an multimeric 56 kDa Aβ-peptide complex in TG mice over-expressing mutant APP (Lesne *et al.*, 2006). This 56 kDa Aβ-complex is stable under normal denaturing conditions used in common immunoblotting protocols. Interestingly, as this study links the presence of the 56kDa Aβ-complex to memory impairments, the authors suggest that this Aβ-complex might be responsible for the cognitive decline in AD.

Together, these results indicate an interplay between Cdk5 signalling and APP processing. Further evidence for such cross-talk comes from the study of the Tg2576

transgenic mice expressing mutated human APP, which display formation of p25 (Otth *et al.*, 2002). Moreover, a recent study describes the formation of p25 in presenilin 1/2 double mutants (Saura *et al.*, 2004). Mutations in APP, as well as in presenilin 1 and 2 have been identified as cause for inherited familial forms of AD. Over 100 mutations in these three genes can each trigger the early onset of pathology apparently via over-production of Aβ-peptides (Tanzi & Bertram, 2001). Thus, it is intriguing to speculate that APP and presenilin share common signalling pathways that involve Cdk5 and GSK3. Further, disruption of these signalling pathways might constitute underlying mechanisms in the onset of AD pathology.

4.3.5) Involvement of p25/Cdk5 in molecular processes underlying synaptic plasticity, learning and memory

Previously, p25/Cdk5 has been implicated in synaptic plasticity, learning and memory (Angelo *et al.*, 2003; Ris *et al.*, 2005). Electrophysiological characterisation of the p25 TG mice revealed that CA1 LTP was elevated in female, but decreased in male TG mice (Ris *et al.*, 2005). Consistent with this observation, spatial learning was enhanced in female p25 TG mice as compared to female WT controls, but it was not affected in male TG mice (Ris *et al.*, 2005). Another study analysing inducible p25 TG mice has reproduced these findings, that p25-expression triggers enhanced CA1-LTP and improved learning and memory (Fischer *et al.*, 2005). However, it is yet not understood how p25/Cdk5 expression might induce enhancement of LTP and memory. One candidate mechanism is the regulation of NMDA receptor function by Cdk5. Phosphorylation of the NMDA receptor subunit NR2A by Cdk5 was shown to enhance the conductance of the channel (Li *et al.*, 2001; Wang *et al.*, 2003). Further, as p25-expression has been implicated in spine density increases, it was hypothesised that Cdk5 affects plasticity and memory-related processes via the regulation of spine numbers (Fischer *et al.*, 2005).

The biochemical characterisation of the p25 TG mice presented here, identifies several alterations in molecular mechanisms, which may underlie the changes observed in synaptic plasticity and memory in the p25 TG mice. I found that ERK activity is significantly reduced in the p25 TG mice. ERK is part of the MAPK signalling pathway that regulates numerous cellular processes such as transcription and ion channel modulation (Sweatt, 2004). In fact, the MAPK pathway including ERK has been involved in synaptic plasticity and memory (Sweatt, 2004). The idea that p25/Cdk5 affects synaptic plasticity and memory via the control of the MAPK pathway are supported by preliminary electrophysiological studies. The deficits in CA1 LTP in male p25 TG mice can be reversed by treatment with anisomycin, which is an activator of ERK (Laurence Ris, unpublished observation). Hence, it is conceivable that inhibition of ERK by Cdk5 is implicated in the impairment of CA1 LTP in male TG mice. Another interesting link is the involvement of ERK in oestrogen signalling pathways. As oestrogens have been implicated in sex-specific plasticity changes, it is conceivable that the male/female divergence in p25 TG mice results from the modulation of oestrogen signalling via the regulation of ERK.

The regulation of GSK3 activity might constitute another mechanism by which p25/Cdk5 affects synaptic plasticity and memory. The inhibitory phosphorylation at Ser9 of GSK3 is significantly increased in young p25 TG mice, which consequently resulted in reduced GSK3β activity. As described in *section 3.3.3*, GSK3 is an emerging molecular player in plasticity and memory-related processes. Consistently, increased activity of GSK3 has been reported to impair spatial learning (Hernandez *et al.*, 2002). Hence, it is conceivable that increased inhibition of GSK3 may lead to an enhancement of some forms of synaptic plasticity and memory. Obviously, further biochemical and electrophysiological characterisation of the p25 TG mice will be required to elucidate the molecular changes underlying the alteration in synaptic plasticity, learning and memory in the TG mice.

4.4) Conclusion

The biochemical analysis of the p25 TG mouse line revealed that p25-induced Cdk5 over-activation, surprisingly, did not generally perturb cellular function, despite the critical involvement of Cdk5 in various processes from synaptic plasticity to cell survival (see for review Dhavan & Tsai, 2003; Angelo *et al.*, 2006). Instead subtle and well-demarcated molecular changes were observed. This underscores the high degree of regulatory and compensatory capacity of the involved molecular mechanisms. The findings presented here, show that long-term exposure to Cdk5 over-activation undermines these well-balanced processes, which subsequently results in increased tau hyperphosphorylation and altered APP processing. The findings that the phosphorylation level of tau as well as the processing of APP are affected by the p25/Cdk5 holoenzyme is indicative that both of these processes are regulated by common underlying molecular mechanisms. This observation may be important to understand neurodegenerative diseases, especially AD. The neuropathological characteristics of AD include the accumulation of hyperphosphorylated tau in tangles and of Aβ-peptides in plaques. Hence, it is conceivable that the derangement of similar common molecular mechanisms are equally responsible for the formation of tangles and plaques in AD. It will be paramount to elucidate the molecular mechanisms that become deranged in the p25 TG mice in order to understand the processes underlying accumulation of hyperphosphorylated tau and the processing of APP and investigate the relevance of these mechanisms in neurodegenerative diseases including AD.

The present study establishes distinct roles of Cdk5 and GSK3 in tau hyperphosphorylation. Increased levels of tau hyperphosphorylation at the disease-associated AT8 and PHF-1 sites was linked to enhanced GSK3 activity. The p25-induced Cdk5 over-activation was not found to directly hyperphosphorylate tau, but was implicated in the inhibitory regulation of GSK3. In fact, these findings indicate that GSK3 acts as a key mediator of tau hyperphosphorylation at the AT8 and PHF-1 sites,

whereas Cdk5 indirectly affects tau hyperphosphorylation via the regulation of GSK3. Moreover, these observations may have important implications for the development of therapeutic treatments of neurodegenerative tauopathies. The application of pharmacological inhibitors of Cdk5 may not be an effective treatment for tau hyperphosphorylation, as the results suggest that inhibition of Cdk5 induces GSK3 activity and this may subsequently lead to enhanced tau phosphorylation. In fact, these findings support the idea that GSK3 inhibitors may be a useful tool for therapeutic intervention to reduce tau hyperphosphorylation and possibly the burden of tau aggregates in neurodegenerative tauopathies. Moreover, inhibition of GSK3 was also reported to lead to reduced Aβ-peptide formation (Phiel *et al.*, 2003). Hence, in conjunction, these data make GSK3 a prime therapeutic target in AD.

CHAPTER 5: General discussion and outlook

5.1) General discussion

The characterisation of genetically-modified mouse strains by behavioural, biochemical, electrophysiological and pharmacological techniques allows investigation of the function of specific molecules at different levels of organisation within the brain. This multilevel analysis of genetically-modified mouse lines has greatly furthered the understanding of the molecular mechanisms underlying synaptic plasticity and helped to explore the molecular processes implicated in neurodegenerative diseases.

In the study presented here, biochemical changes were analysed in two different lines of genetically-modified mice. One mouse line carries a point mutation in the αCaMKII gene, disenabling the autophosphorylation at Thr286. The other mouse line expresses the Cdk5 activator p25 at low levels predominantly in forebrain. Previous evaluation of these genetically-modified mouse lines demonstrated that they are suitable for further analysis, as the genetic modifications did not interfere with their viability or fertility (see, for example, Giese *et al.*, 1998; Angelo *et al.*, 2003). In addition, the two lines displayed no obvious developmental abnormalities and exhibited gross normal anatomy of the brain structures. Subsequently, both mouse strains were assessed in behavioural assays that showed that the genetic alterations had significant effects on behavioural performance of these mouse lines (see, for example, Giese *et al.*, 1998; Angelo *et al.*, 2003). Further, electrophysiological characterisation of the mouse lines revealed that the genetic modifications affected synaptic plasticity (see, for example, Giese *et al.*, 1998; Ris *et al.*, 2005). These findings suggest that αCaMKII as well as p25/Cdk5 are implicated in molecular mechanisms underlying synaptic plasticity as well as learning and memory processes. Accordingly, the analysis of numerous other genetically-

modified mouse lines have correlated synaptic plasticity changes with altered acquisition and storage of memory. Further, these studies greatly helped to advance the knowledge on molecular mechanisms underlying synaptic plasticity and provided ample evidence that the molecular mechanisms implicated in the induction and maintenance of synaptic plasticity changes might also play critical roles in learning and memory processes (see for review Martin & Morris, 2002; Silva, 2003). Many mouse lines have been characterised, where the genetic modification induced LTP deficits that coincided with behavioural impairments (see for review Bockamp *et al.*, 2002; Martin & Morris, 2002; Silva, 2003). As with the p25 TG mice, other genetically-modified mouse lines exhibit increased LTP, correlated with memory enhancements (see, for example, Jeon *et al.* 2003 and Madani *et al.* 1999). Despite the most results from genetically-modified mouse lines support the notion that changes in synaptic efficacy underlie learning and memory processes. However, the relationship of synaptic plasticity and memory is still correlative.

The αCaMKII T286A mutant mouse line as well as the p25 TG mouse line simultaneously display changes in synaptic plasticity and altered learning and memory. Therefore, I assessed these two mouse lines for biochemical changes, in order to get more information on the underlying molecular mechanisms that lead to alterations in synaptic plasticity and in memory. The analysis of basal phosphorylation levels of CaMKII substrates revealed changes between the αCaMKII T286A mutants and WT mice indicating that the autonomous αCaMKII activity mediates phosphorylation of specific substrates. To investigate biochemical changes induced by activity-dependent synaptic plasticity changes, we established an *in vivo* LTP approach. Consistent with previous studies, induction of LTP triggered increased phosphorylation levels of several marker proteins including CREB in the hippocampal subfields, CA1 and DG *in vivo*. This result confirms that the *in vivo* LTP approach is suitable to investigate molecular mechanisms underlying synaptic plasticity changes in different mouse lines.

Furthermore, I assessed phosphorylation changes in p25 TG mice. The p25-induced Cdk5 over-activation induced enhanced phosphorylation on several proteins including ERK, which has been implicated in synaptic plasticity (Sweatt, 2004). Together, the biochemical analysis revealed that both genetically-modified mouse lines exhibit subtle and well-demarcated alterations in phosphorylation levels. The genetic alterations did not affect critical cellular processes in a manner that it would induce severe malfunctions. These observations demonstrate that the two mouse lines are suitable models to investigate the functions of autonomous αCaMKII and p25/Cdk5 activity and their further analysis promises to yield critical new insights. Interestingly, recent advances revealed an intriguing relationship between CaMKII and the p35/Cdk5 holoenzyme, which might have important implications for synaptic plasticity and memory. It was observed that inhibition of Cdk5 resulted in enhanced CaMKII autophosphorylation and subsequently increased CaMKII activation (Hosokawa *et al.*, 2006). Consistent with such molecular interplay is the finding that CaMKII associates with the Cdk5 activators p35 and p39 in an Ca^{2+}-dependent manner (Dhavan *et al.*, 2002). The fact that CaMKII and Cdk5 seem to be involved in common synaptic processes, puts an interesting spin on the analysis of the two genetically-modified mouse lines presented here. Hence, these two mouse lines can be utilised to investigate the role of the molecular relationship between CaMKII and Cdk5 in synaptic plasticity and memory.

In recent years, genetically-modified mouse lines were commonly used to model aspects of neurodegenerative diseases. The identification of molecules carrying mutations responsible for inherited forms of neurodegenerative diseases and the characterisation of pathological proteins from post-mortem tissue allowed the generation of mouse models of neurodegenerative diseases (see for review Ashe, 2001; Lee *et al.*, 2005). Because the Cdk5 activator p25 has been found to accumulate specifically in brains of AD patients but not healthy individuals, it was postulated that p25 is implicated in AD (Patrick *et al.*, 1999). In the present study, I analysed a TG mouse line expressing p25,

in order to asses the involvement of p25/Cdk5 in molecular processes underlying tauopathies including AD. I found that p25-induced Cdk5 over-activation affects both the level of tau hyperphosphorylation and APP processing in an age-dependent manner. These observations show that the p25 TG mouse line models some aspects of tauopathies and hence represent a useful tool to study the molecular mechanisms of tauopathies including AD.

In summary, my studies validate the use of genetically-modified mouse lines for investigating the molecular mechanisms underlying activity-dependent synaptic changes. Further, genetically-modified mice are an adequate tool to model neurodegenerative diseases and hence, study the molecular mechanisms implicated in the ontogeny of the diseases.

5.2) Outlook

The biochemical analysis of the two genetically-modified mouse lines presented here, revealed a number of interesting molecular changes. These data provide a good fundament for future experiments to further characterise the two mouse lines and subsequently get more insights on the molecular mechanisms involving CaMKII and the p25/Cdk5 holoenzyme.

Currently, there is only limited knowledge as to which proteins are phosphorylated by αCaMKII and which of these substrates are critically involved in activity-dependent synaptic plasticity changes. The data presented here, showed that the disenabling point mutation of αCaMKII affects the basal phosphorylation level of several proteins in T286A mutant mice. Hence, the identification of the proteins with altered phosphorylation levels can reveal substrates, which undergo phosphorylation

specifically mediated by autonomous αCaMKII activity and which are possibly implicated in synaptic plasticity and memory processes.

In order to study biochemical changes after synaptic potentiation, I established an *in vivo* LTP approach in collaboration with Dr Sam F. Cooke and Prof. Tim V.P. Bliss. The data presented here, confirmed that this approach is suitable to investigate molecular mechanisms underlying activity-dependent synaptic plasticity changes. The induction of LTP in area CA1 is abolished by the T286A point mutation indicating that autonomous αCaMKII activity is essential for CA1 LTP. Thus, future studies can exploit the *in vivo* LTP approach to detect biochemical changes in T286A mutants and WT mice after CA1 LTP induction. The comparison of protein phosphorylation levels after CA1 LTP induction between T286A mutants and WT mice can be used to characterise proteins, which are regulated by autonomous αCaMKII activity and which may be required for the induction and expression of CA1 LTP. The present study also showed that LTP induction in the two hippocampal subfields DG and CA1 is supported by distinct molecular mechanisms. The *in vivo* LTP approach can therefore be applied to investigate the different molecular mechanisms underlying LTP in DG and CA1.

The analysis of the p25 TG mouse line presented here, revealed several biochemical changes. Low-level expression of p25 interfered with APP processing and tau hyperphosphorylation, two molecular processes affected in neurodegenerative diseases. A 57 kDa APP fragment was observed to accumulate in an age-dependent manner in the p25 TG mice. The role for Cdk5 in APP processing can be studied by characterising the 57 kDa fragment and investigating the molecular mechanisms involved in the formation of this fragment. The data presented here indicate that there is cross-talk between Cdk5 and GSK3, which is involved in the hyperphosphorylation of tau at the AT8 and PHF-1 site. As tau hyperphosphorylation at these sites might serve cellular functions, the analysis of the p25 TG mice may be used to investigate the physiological roles of tau and the hyperphosphorylation of tau. The characterisation of the molecular mechanisms

underlying the inhibitory regulation of GSK3 by Cdk5 will help to elucidate the link between these two kinases and tau hyperphosphorylation. Moreover, the characterisation of these molecular mechanisms might also reveal suitable candidate molecules as targets for drug development.

Electrophysiological and behavioural analysis of the p25 TG mouse line revealed a sex-specific increase in LTP and improved learning and memory (Ris *et al.*, 2005). Thus, further investigation of the p25 TG mice may reveal the molecular mechanisms underlying the alterations in LTP and the behavioural phenotype. A detailed electrophysiological characterisation might be used to evaluate, which signalling processes are altered in the TG mice. Moreover, activity-dependent changes could be assessed applying the *in vivo* LTP approach in the p25 TG mice. The data presented here and elsewhere, support a physiological role for p25, possibly in synaptic plasticity. Hence, the *in vivo* LTP approach can also be employed to investigate whether there is activity-dependent formation of p25, and if so to study its physiological function. Furthermore, analysis of the p25 TG mice might be used to characterise substrates, which are specifically phosphorylated by the p25/Cdk5 holoenzyme.

FIGURES: Chapter 1

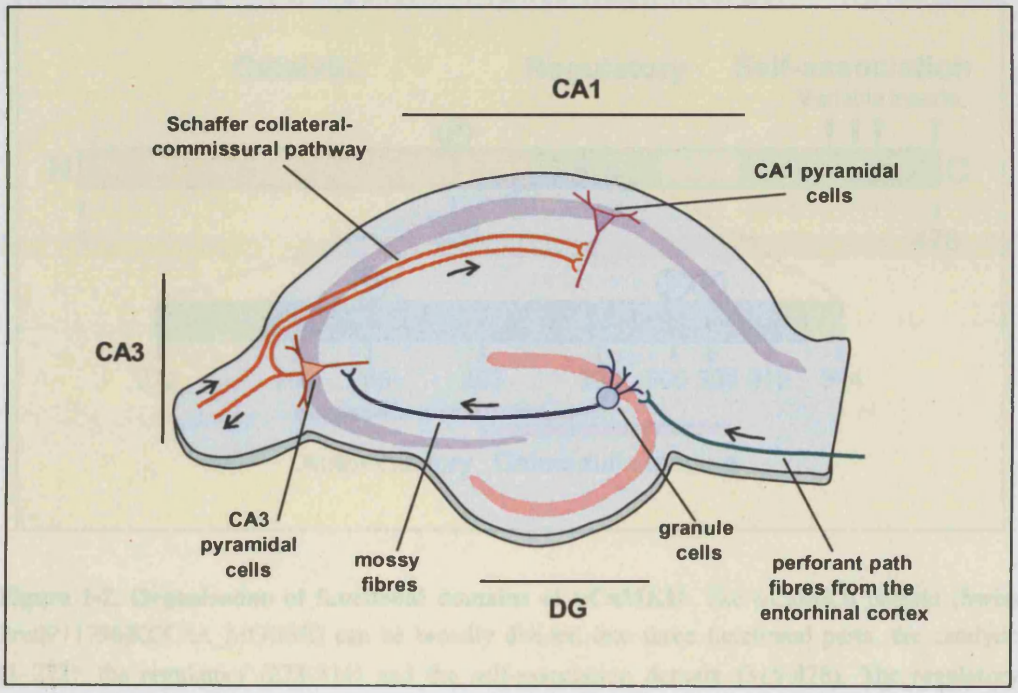

Figure 1-1. The trisynaptic circuit in hippocampus. Schema of a transverse section through the hippocampus of a mouse. The principal hippocampal subfields include the dentate gyrus (DG) with the granule cells, the area CA3 and CA1 with the pyramidal cells. The main excitatory afferent projections include the perforant path from entorhinal cortex to granule cells, the mossy fibre projection from granule cells to CA3 cells and the Schaffer collateral-commissural pathway that connects ipsilateral and contralateral CA3 cells to CA1 cells. *(Adapted from Bliss & Collingridge, 1993)*

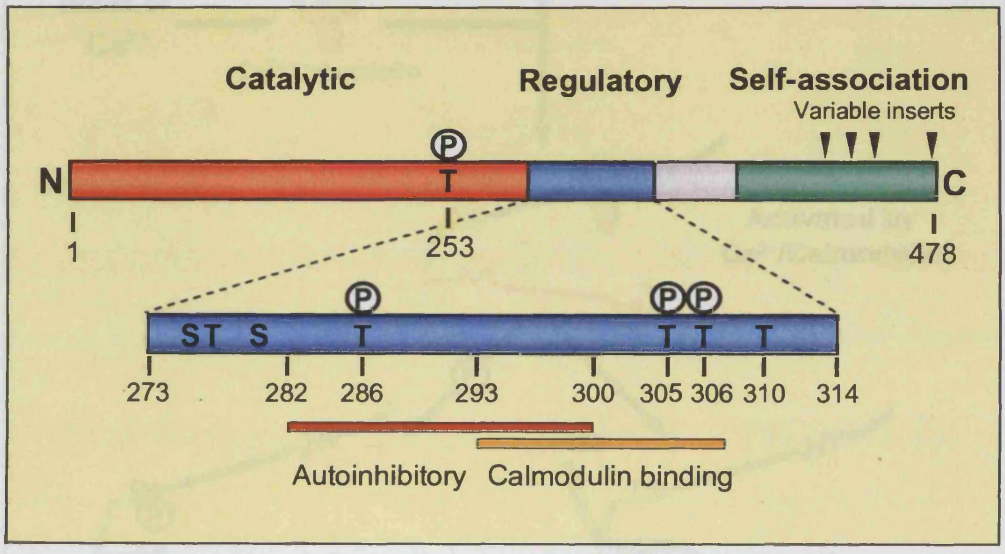

Figure 1-2. Organisation of functional domains of αCaMKII. The αCaMKII protein (Swiss Prot|P11798|KCC2A_MOUSE) can be broadly divided into three functional parts, the catalytic (1–272), the regulatory (273-314) and the self-association domain (315-478). The regulatory domain contains the autoinhibitory site (282-300) and the calmodulin-binding site (293-308). Several variable inserts can be introduced into the self-association domain of αCaMKII by alternative splicing. Phosphorylation at Thr286 switches αCaMKII into an autonomous activity mode that is Ca^{2+}-independent (e.g. Miller & Kennedy, 1986). Phosphorylation at Thr305/306 blocks activation of CaMKII by Ca^{2+}/CaM and prevents the translocation of CaMKII to the post-synaptic density (e.g. Elgersma *et al.*, 2002). Phosphorylation at Thr253 enhances the binding to the post-synaptic density (e.g. Migues *et al.*, 2006) *(Adapted from Lisman et al., 2002)*

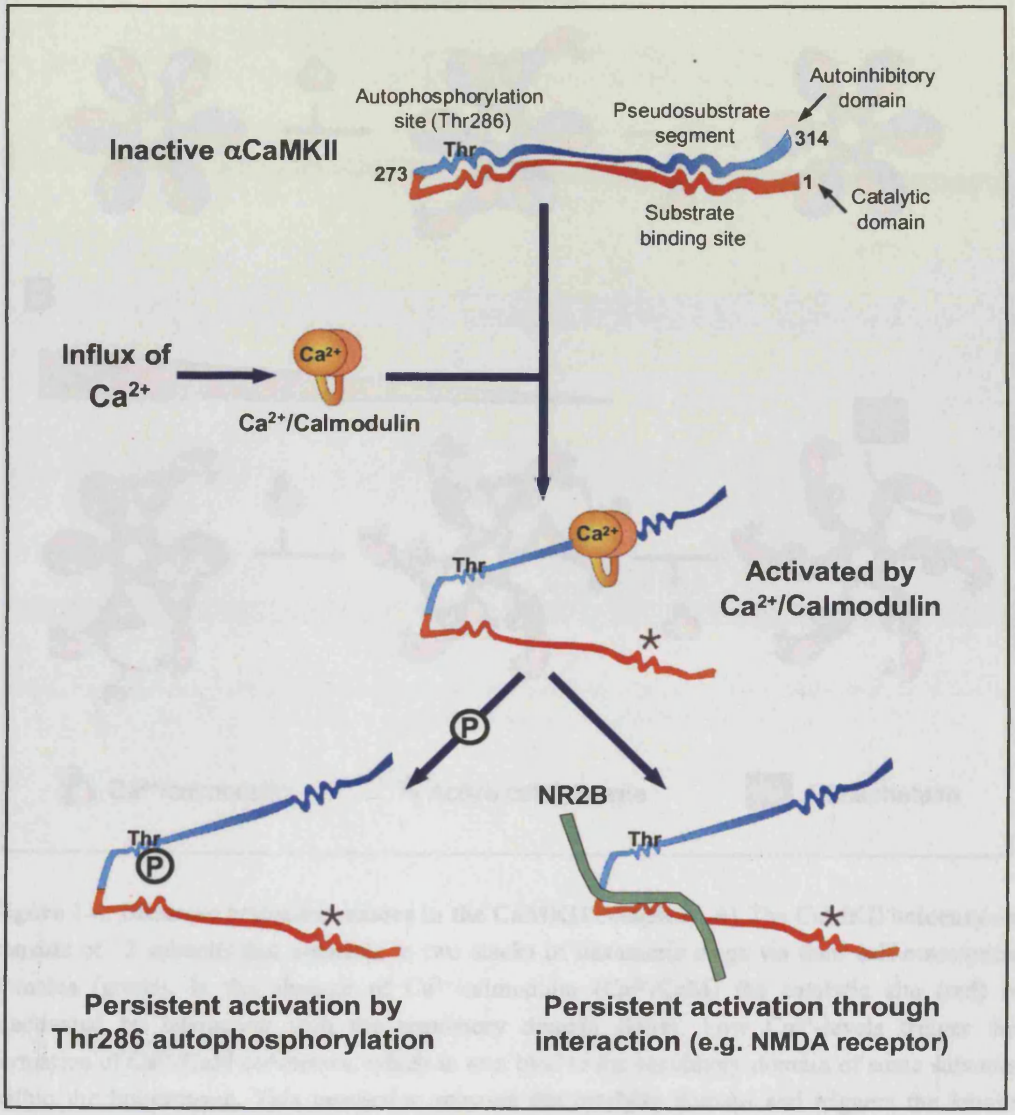

Figure 1-3. The αCaMKII autophosphorylation switch. Under basal conditions the pseudo-substrate region of αCaMKII is binding to its catalytic site and thereby inhibiting its enzymatic activity. Rise in Ca^{2+}-levels triggers association of the Ca^{2+}/calmodulin (Ca^{2+}/CaM) complex, which in turn binds to αCaMKII on a site overlapping with the pseudosubstrate region. This exposes the catalytic domain and renders αCaMKII active (asterix). In this Ca^{2+}-activated state Thr286 can be autophosphorylated by neighbouring αCaMKII subunits. Once Thr286 is phosphorylated αCaMKII can not revert back in the inactive conformation, even after Ca^{2+}-levels drop and Ca^{2+}/CaM dissociates from the kinase. Hence, αCaMKII is in a persistent, autonomous state of activity that is Ca^{2+}-independent and which can only be reversed via dephosphorylation of Thr286 by phosphatases. Alternatively, in the Ca^{2+}-activated state αCaMKII can interact with proteins (e.g. NMDA receptor subunit NR2B), which in turn lock the kinase in the active conformation even after dissociation of Ca^{2+}/CaM. *(Adapted from Lisman et al., 2002)*

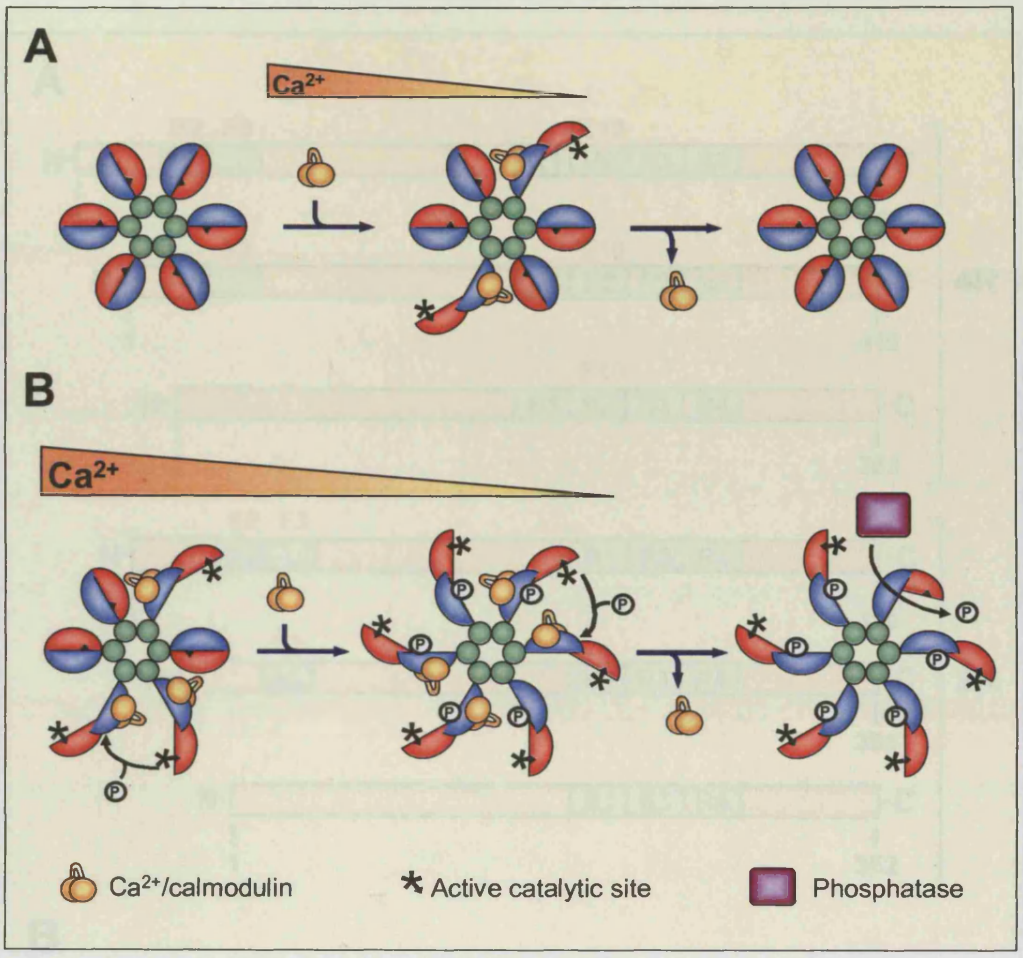

Figure 1-4. Different activation modes in the CaMKII complex. A) The CaMKII holoenzyme consists of 12 subunits that associate in two stacks of hexameric rings via their self-association domains (green). In the absence of Ca^{2+}/calmodulin (Ca^{2+}/CaM) the catalytic site (red) is inactivated by interaction with the regulatory domain (blue). Low Ca^{2+}-levels trigger the formation of Ca^{2+}/CaM complexes, which in turn bind to the regulatory domain of some subunits within the holoenzyme. This interaction releases the catalytic domain and triggers the kinase activity. After the Ca^{2+}-levels drop the CaMKII complex reverts back into the inactive state. **B)** Higher levels of Ca^{2+}-influx and formation of more Ca^{2+}/CaM complexes lead to the activation of several CaMKII subunits within a holoenzyme. This increases the probability that two neighbouring subunits become bound to Ca^{2+}/CaM simultaneously. Coincident binding of Ca^{2+}/CaM to two adjacent subunits allows one subunit to autophosphorylate the Thr286 site of its neighbouring subunit. The phosphorylation at Thr286 switches the subunit into an autonomous activity mode. Even after Ca^{2+}-levels drop, this subunit retains its activity, until it becomes dephosphorylated. One implication of the autophosphorylation switch is that during the next Ca^{2+}-influx the binding of only one Ca^{2+}/CaM, adjacent to the already autophosphorylated subunit, is required to autonomously activate the subunit. Hence, coincident Ca^{2+}/CaM binding is no longer needed. This constitutes a mechanism that allows subunits in the autonomous activity state to switch even at lower Ca^{2+}-levels. Another implication of this Thr286 switch is that an entire holoenzyme can be switched into the autonomous mode, if there is sufficient Ca^{2+}/CaM to bind all subunits within a holoenzyme and trigger their autophosphorylation. This activated state of the CaMKII holoenzyme has been proposed as a mechanism involved in the long-term storage of memory. *(Adopted from Lisman et al., 2002)*

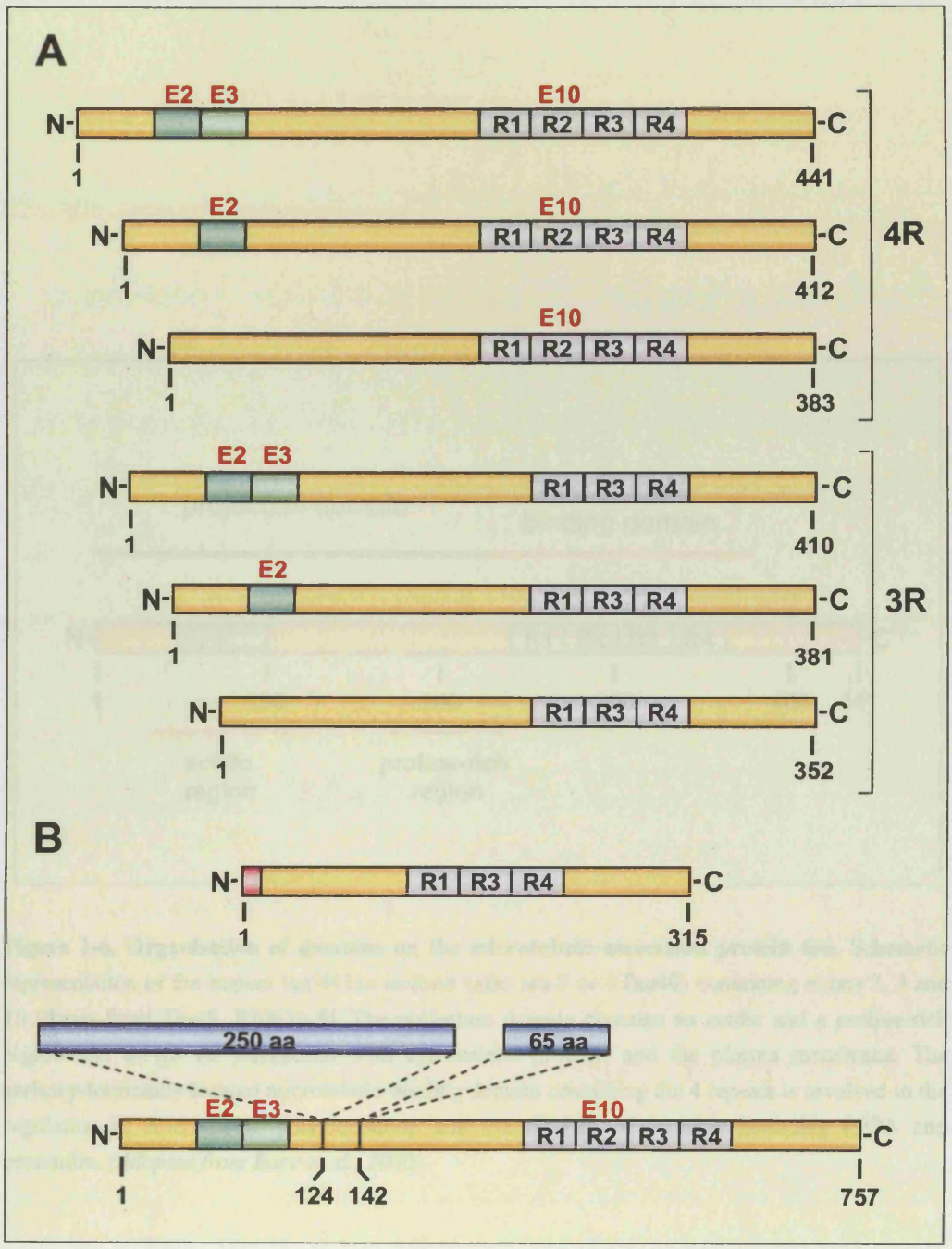

Figure 1-5. The isoforms of the microtubule-associated protein tau. (A) Schematic representation of the six tau isoforms expressed in the central nervous system in the adult human (Swiss Prot|P10636|TAU_HUMAN). In the amino-terminus part exon 2 and 3 (E2 and E3) are alternatively spliced. The carboxy-terminus contains 4 repeats (R1-R4). Alternative splicing of exon 10 (E10) results in the formation of the 3R or 4R isoforms respectively. (B) Schematic representation of fetal-tau and peripheral nervous system (PNS) tau isoforms. Fetal-tau is the shortest isoform with 315aa. It contains an alternative amino-terminus and lacks exons 2, 3 and 10. PNS-tau is the longest isoform with 757aa containing exons 2, 3 and 10. In addition, two large portions can be spliced into PNS-tau, a 250aa part at residue 124 encoded by exon 4A and possibly a 65aa part at residue 142.

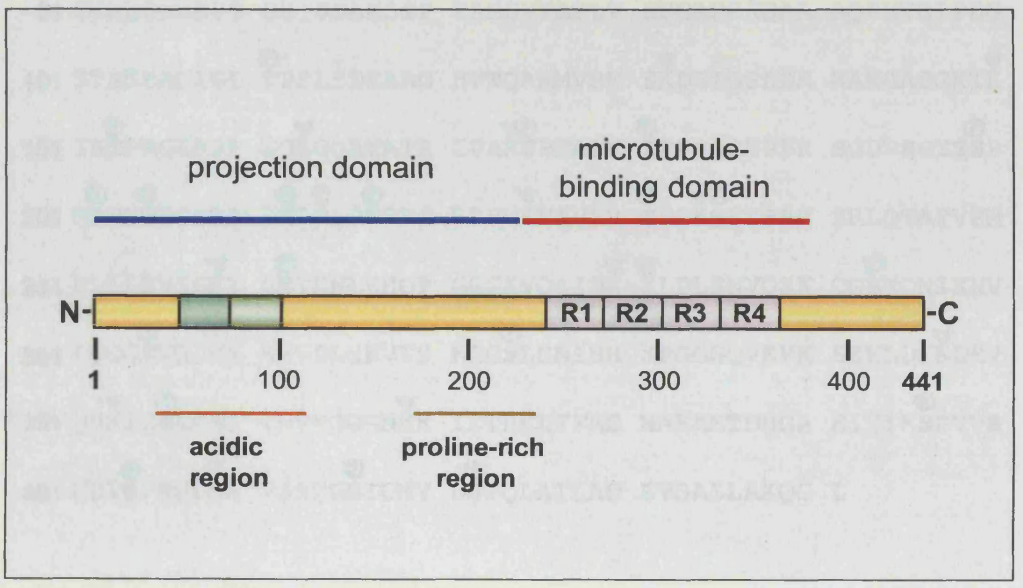

Figure 1-6. Organisation of domains on the microtubule-associated protein tau. Schematic representation of the human tau 441aa isoform (also tau-F or hTau40) containing exons 2, 3 and 10 (Swiss-Prot: Tau-F, P10636-8). The projection domain contains an acidic and a proline-rich region and serves for interaction with cytoskeletal proteins and the plasma membrane. The carboxy-terminally located microtubule-binding domain containing the 4 repeats is involved in the regulation of microtubule polymerisation and the binding of proteins including PP2A and presenilin. *(Adopted from Buee et al., 2000)*

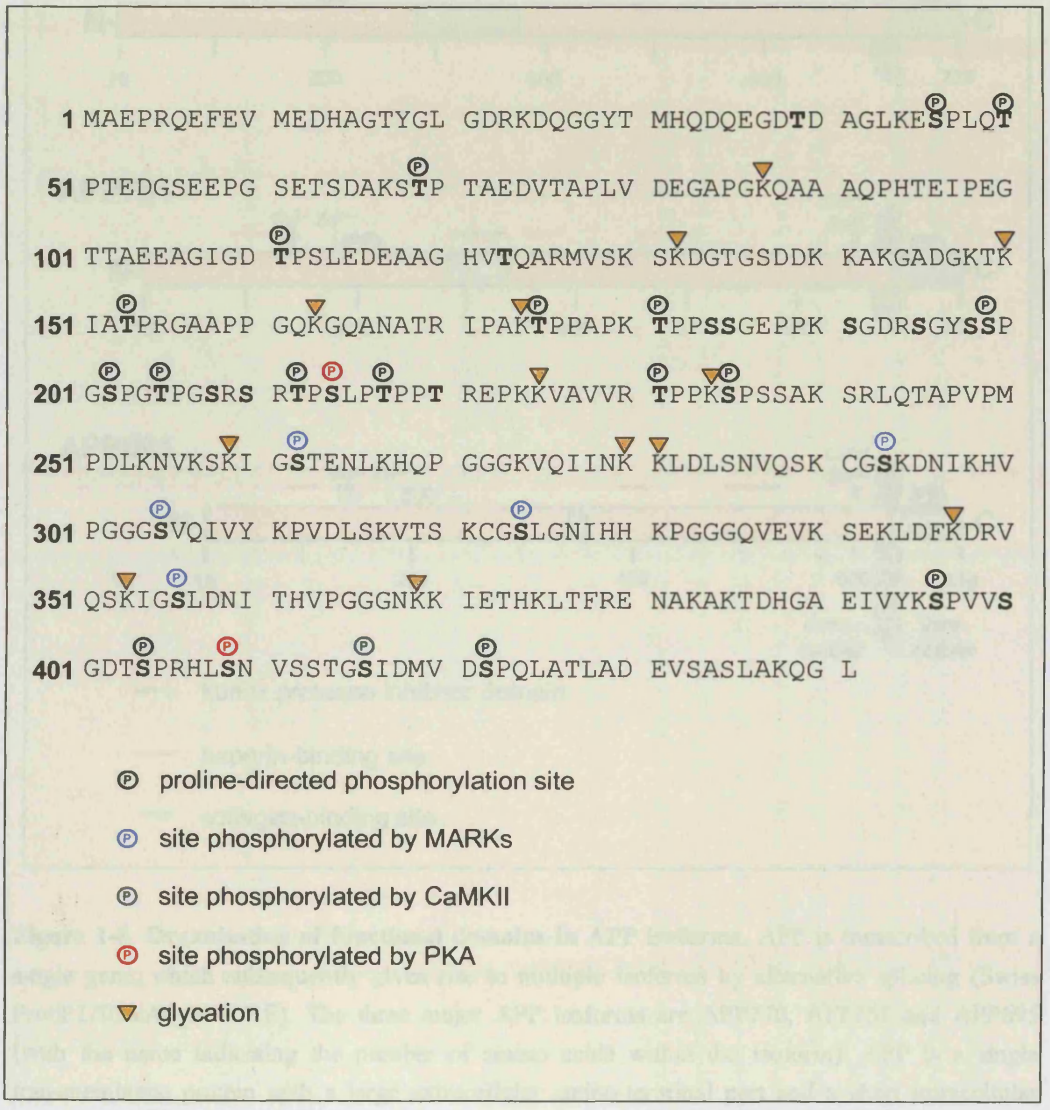

Figure 1-7. Post-translational modifications on tau. Amino acid sequence of the tau-F isoform (Swiss-Prot entry TAU_HUMAN P10636). The tau-F isoform contains 441 amino acids and can be post-translationally modified at numerous sites via phosphorylation and glycation. Letters in bold indicate serine/threonine residues, for which phosphorylation has been reported.

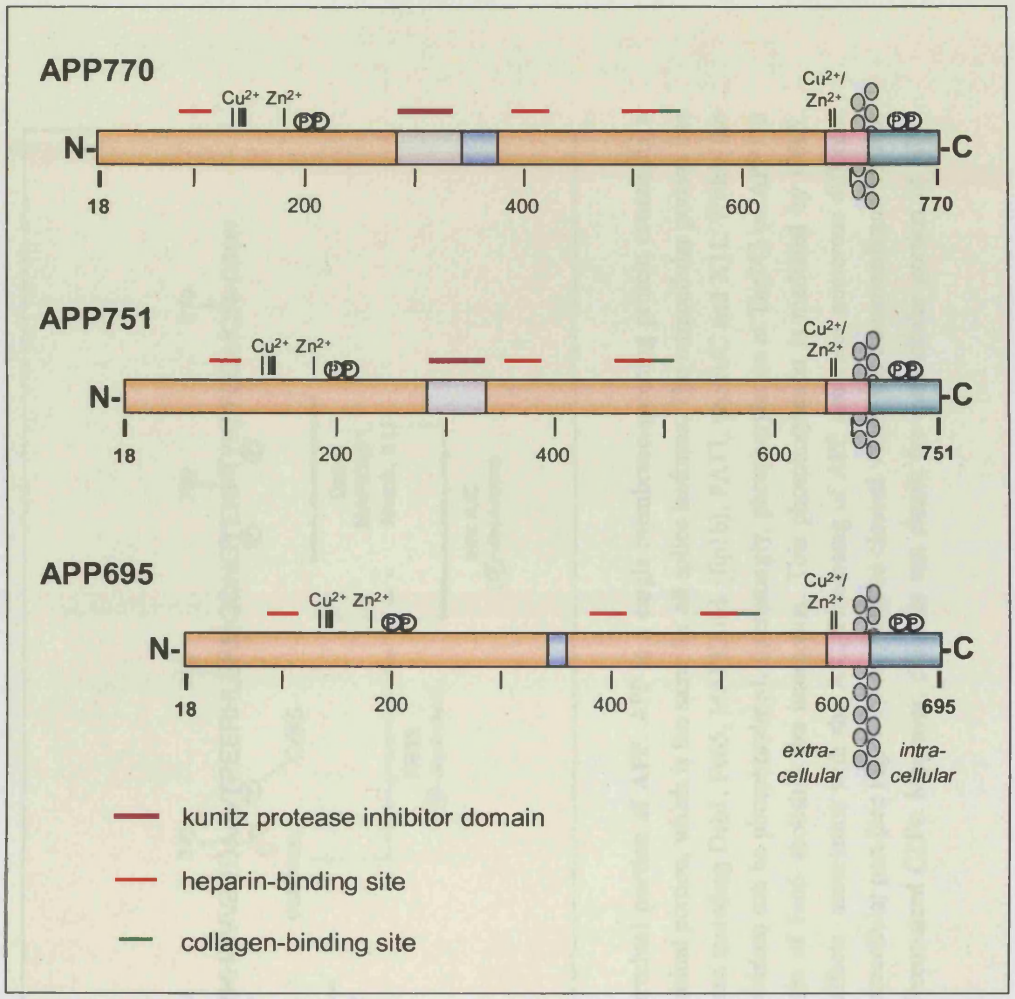

Figure 1-8. Organisation of functional domains in APP isoforms. APP is transcribed from a single gene, which subsequently gives rise to multiple isoforms by alternative splicing (Swiss Prot|P12023|A4_MOUSE). The three major APP isoforms are APP770, APP751 and APP695 (with the name indicating the number of amino acids within the isoform). APP is a single transmembrane protein with a large extracellular amino-terminal part and a short intracellular carboxy-terminal portion. The extracellular part contains domains binding copper, zinc, heparin and collagen. The heparin and collagen binding domains have been implicated in the adhesion function of APP. Furthermore, it contains a kunitz protease inhibitor domain, which is spliced out in APP695 and two phosphorylation sites, possibly modified by casein kinase. There are two extracellular protease cleavage sites immediately adjacent to the transmembrane domain of APP. One site (M672/D673) is cleaved by the β-secretase, the other (K687/L688) by the α-secretase (see also Fig 1-10). The intracellular portion contains also several different functional domains, protease cleavage sites and phosphorylation sites (see Fig 1-9).

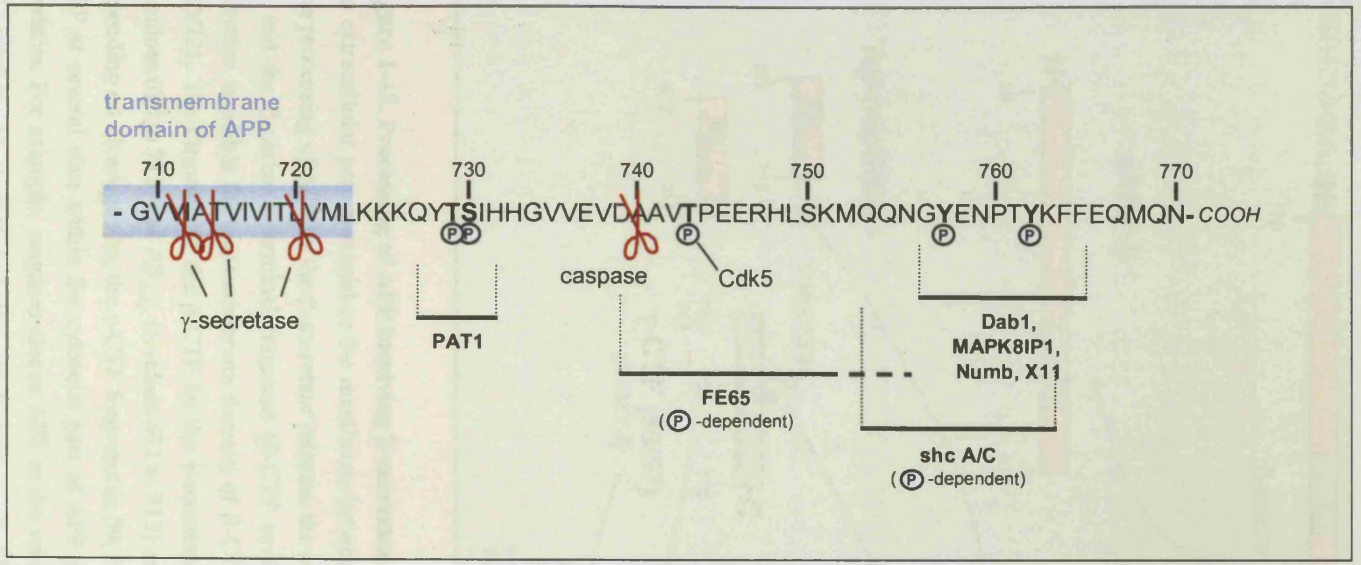

Figure 1-9. Functions within the carboxy-terminal portion of APP. APP is a single membrane-spanning protein containing a short (47 amino acids) intracellular carboxy-terminal portion, which is the same for all splice isoforms. The intracellular portion has been implicated in the binding of several proteins including Dab1, Fe65, MAPK8Ib1 (Jip1b), PAT1, shc A/C and X11. Within the intracellular portion several Ser, Thr and Tyr residues can be phosphorylated. Interestingly, phosphorylation at Thr743 on APP770 (Thr668 in APP695) within the binding domain of Fe65 abolishes the interaction. This phosphorylation is mediated by Cdk5. Likewise, phosphorylation at Tyr757/Tyr762 affects association with shc A/C. Processing of APP generates numerous different carboxy-terminal fragments (CTFs) from the intracellular part (see Fig. 1-7). APP can be cleaved within the transmembrane domain at several sites by γ-secretase resulting in different-sized CTFs. Moreover, caspases can cleave the intracellular portion of APP. *(adapted from Kerr & Small, 2005)*

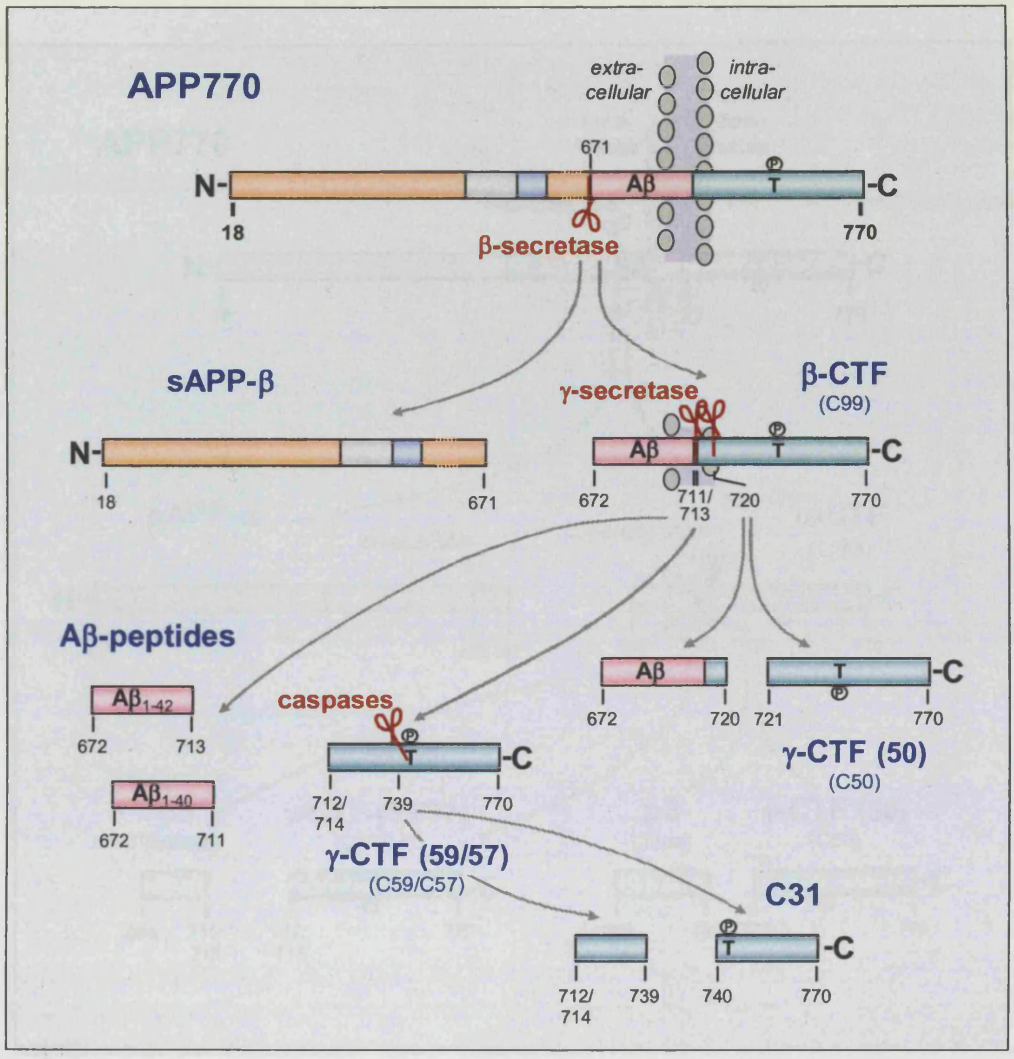

Figure 1-10. Processing of APP involving β-secretase. APP can be cleaved by the β-secretase in the extracellular part proximal to the membrane between the residues 671/672 (as per APP770). The processing of APP by the β-secretase releases the soluble APP-β amino-terminal part (sAPP-β) and the β-carboxy terminal fragment (β-CTF or C99; 99aa). The cleavage site of the γ-secretase is within the transmembrane domain of β-CTF between residues 711/712, 713/714 or 720/721. The cleavage of the β-CTF by the γ-secretase produces Aβ-peptides including $Aβ_{1-40}$ (residues 672 to 711) and $Aβ_{1-42}$ (residues 672 to 713) and γ-carboxy-terminal fragments (γ-CTFs; depending on cleavage site, the γ-CTF fragment is 59, 57 or 50aa). Furthermore, caspases cleave APP at several sites within the cytosolic part of APP giving rise to multiple different cleavage products. For example, caspases cleave APP at the residues 739/740 and release the neurotoxic C31 peptide. As all cleavage sites are present in all APP isoforms, the processing of each APP isoform results in sAPP-α fragments of characteristic size.

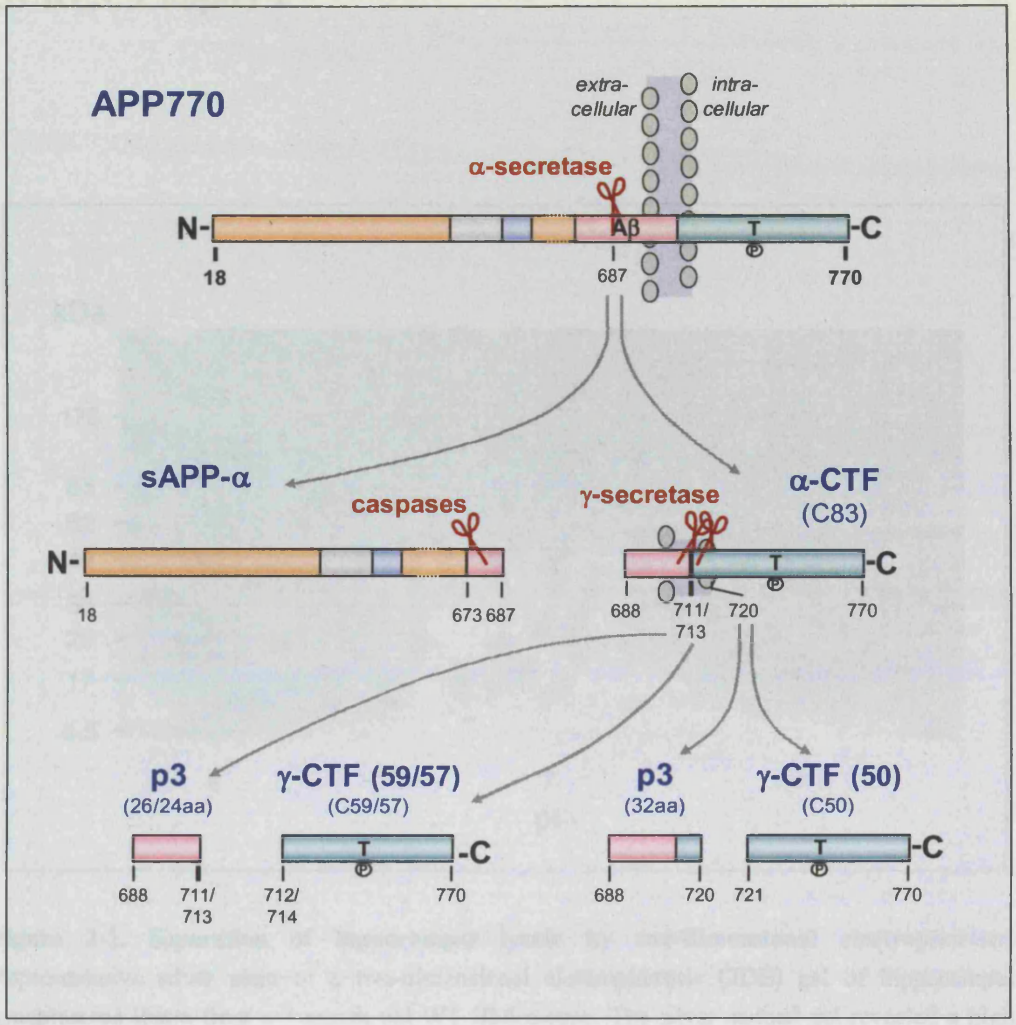

Figure 1-11. Processing of APP involving α-secretase. APP can be cleaved by the α-secretase in the extracellular part proximal to the membrane between the residues 687/688 (as per APP770). The cleavage site lies within the Aβ-domain and consequently prevent the production of Aβ-peptides. Processing by the α-secretase generates the soluble APP-α amino-terminal part (sAPP-α) and the α-carboxy terminal fragment (α-CTF or C83; 83aa). Subsequent cleavage of α-CTF by γ-secretase leads to the production of γ-CTFs (depending on the cleavage site the γ-CTF fragments are 59, 57 or 50aa) and p3 peptides (depending on the cleavage site the p3 fragments are 24, 26 or 33aa). Moreover, sAPP-α can be cleaved by caspases between residues 673 and 674. As all cleavage sites are present in all APP isoforms, the processing of each APP isoform results in sAPP-α fragments of characteristic size.

FIGURES: Chapter 2

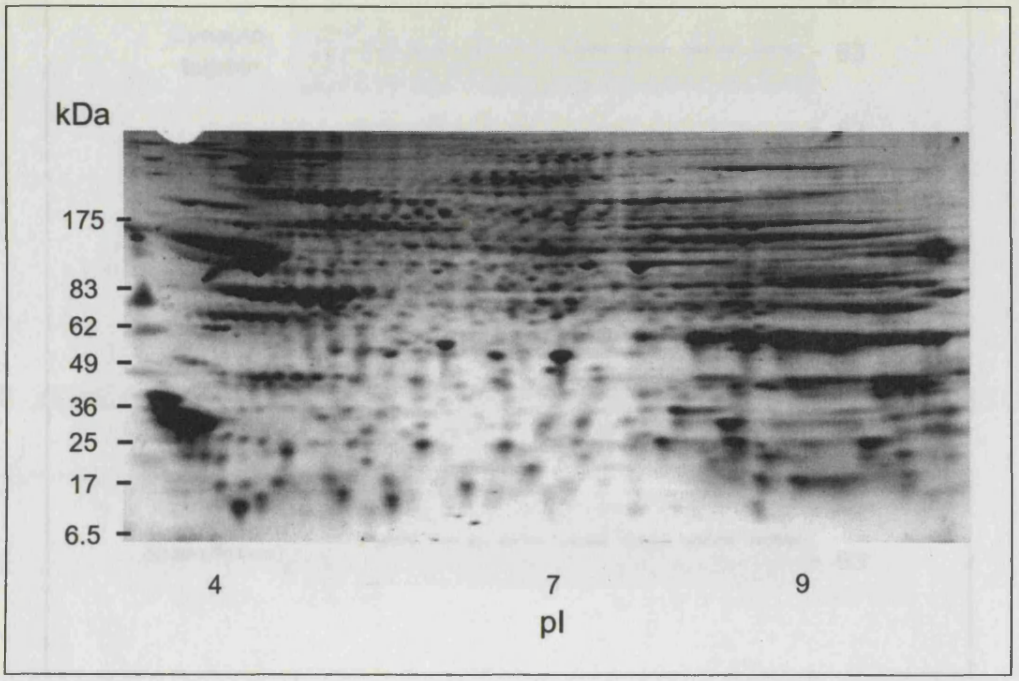

Figure 2-1. Separation of hippocampal lysate by two-dimensional electrophoresis. Representative silver stain of a two-dimensional electrophoresis (2DE) gel of hippocampal synaptosome lysate from a 3 month old WT BL6 mouse. The silver stained gel revealed a high number of protein spots and protein spot trains. The proteins display a good separation in the range of 20-90 kDa and between the isoelectric points (pI) of 4 and 8. In the higher molecular weight range above 90 kDa many spot trains can be detected. These results show that the applied method for the 2DE is adequate to separate proteins from complex lysates. The apparent molecular weight is indicated in kDa on the left side of the gel and the pI range is indicated at the bottom of the gel.

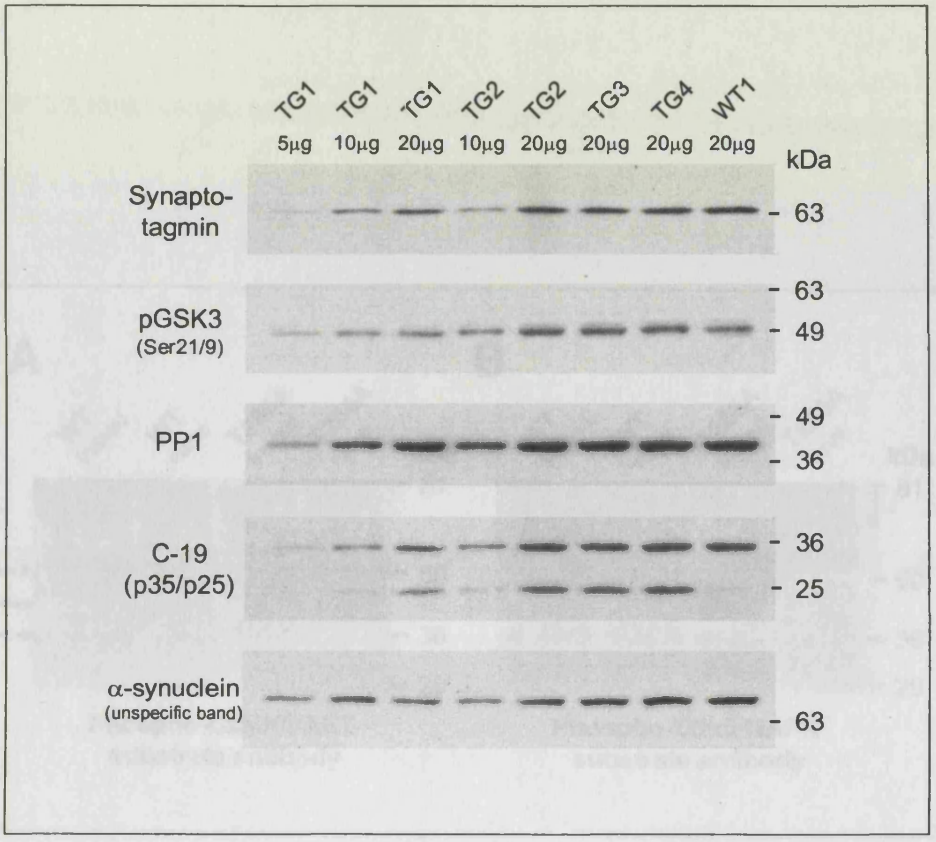

Figure 2-2. Evaluation of the methodology used for immunoblotting. Immunoblots of hippocampal lysates from p25 TG mice and one WT control were probed with antibodies against synaptotagmin, phospho-GSK3α/β (Ser21/9), protein phosphatase 1 (PP1), α-synuclein and C-19 antibody recognising p35 as well as p25. Different amounts of protein were loaded (5, 10 and 20 µg/lane) as indicated on top of the blot. The signal of the bands increased corresponding to the higher protein loading demonstrating that the ECL system is suitable to reproducibly detect changes in protein concentration. These results suggest that the signal intensities are in the linear range. For most blots loading of 10-20 µg of protein per lane resulted in an good signal intensity, where the band is not saturated or too weak. All of the blots shown above have been stripped at least once prior to incubation with the named antibody. This shows that the stripping of the blots does not significantly affect the quality of the immunoblot signal. The blot probed with the anti-phospho-Ser9-GSK3 antibody indicates that changes in phosphorylation levels can be reliably detected with the ECL system also using phospho-specific antibodies. Apparent molecular weight is indicated in kDa on the right side of the blots.

FIGURES: Chapter 3

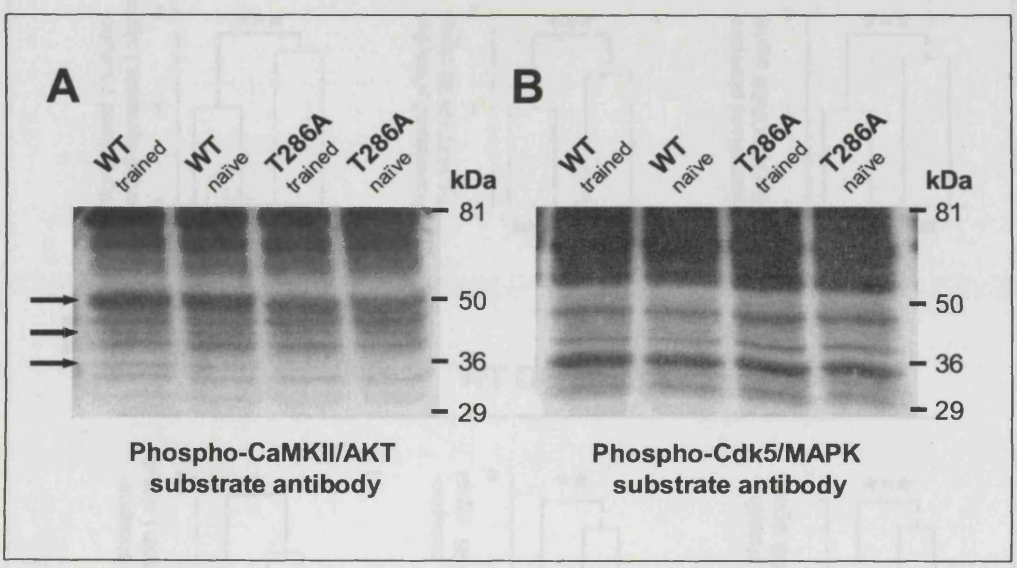

Figure 3-1. Phosphorylation pattern of synaptosomal lysate from naïve and trained αCaMKII T286A mutants and WT mice. Representative immunoblots and quantification from hippocampal synaptosome lysates (20 µg protein/lane) of naive and fear conditioned T286A mutants and WT mice. Immunoblots were probed with phospho-specific antibodies raised against **A)** the CaMKII/AKT substrate motif K-K/L-x-S/T(phospho) and **B)** the Cdk5/MAPK substrate motif x-S/T(phospho)-P-x. No obvious changes could be observed between the naïve and trained mouse groups with either phospho-specific substrate antibody. The phospho-CaMKII/AKT substrate antibody revealed changes in phosphorylation levels between WT and T286A mutants at 35, 42 and 50 kDa (arrow heads). The changes at 50 kDa correspond to the lack of αCaMKII autophosphorylation in the T286A mutants (data not shown).

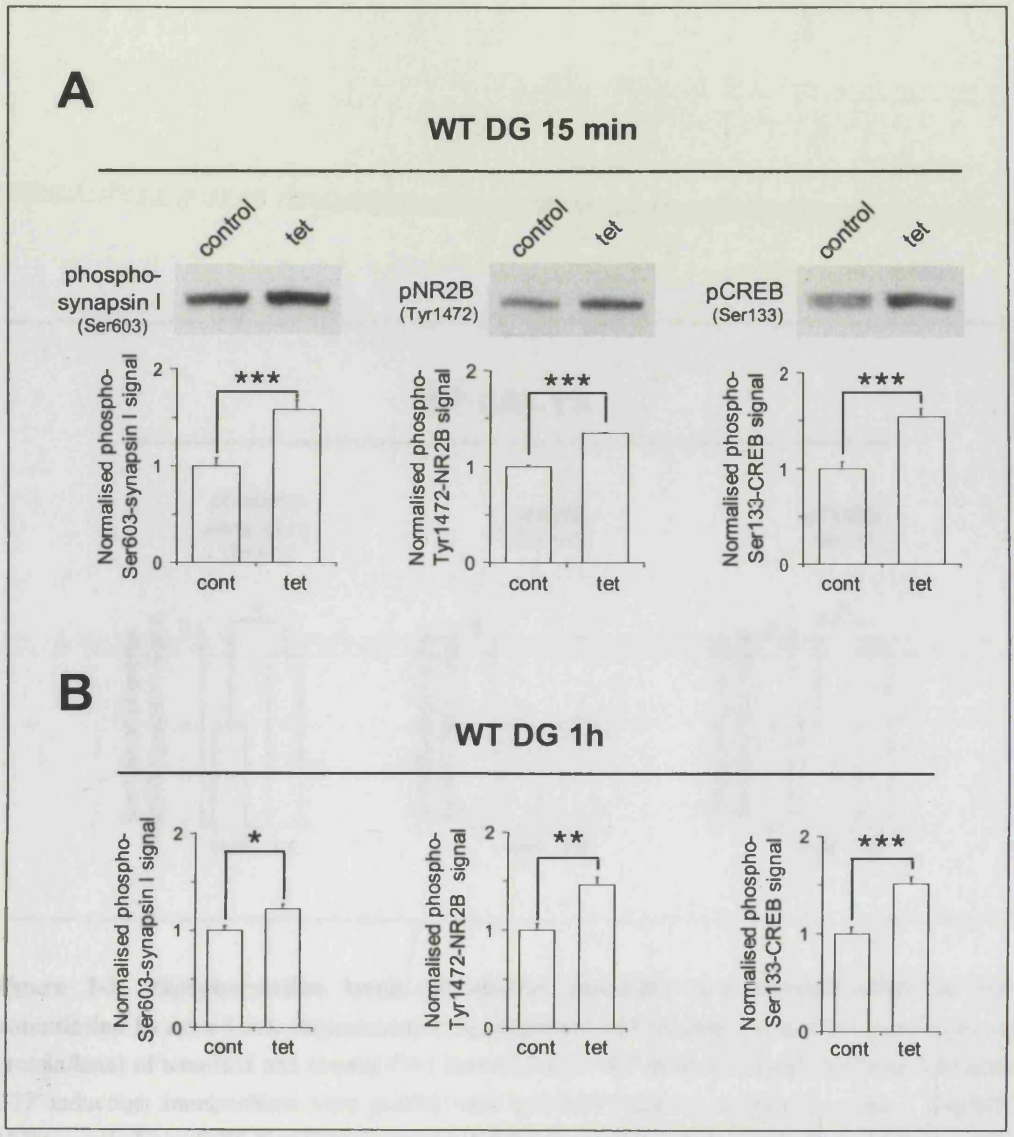

Figure 3-2. Marker molecules exhibit increased phosphorylation levels 15 minutes and one hour after induction of LTP in DG *in vivo*. **A)** Representative immunoblots and quantifications from lysates (20 µg protein/lane) of tetanised and control DG tissue of BL6 WT mice harvested 15 min after LTP induction. Immunoblots were probed with antibodies against phospho-synapsin I (Ser603: 158%, n= 8; $F_{1\text{-}14}$= 30.0, ***p< 0.001), phospho-NR2B (Tyr1472: 135%, n= 4; $F_{1\text{-}6}$= 783; ***p< 0.001) and phospho-CREB (Ser133: 154%, n= 8; $F_{1\text{-}14}$= 25.8; ***p< 0.001). **B)** Quantifications from lysates (20 µg protein/lane) of tetanised and control DG tissue of BL6 WT mice harvested one hour (1h) after LTP induction. Immunoblots were probed with antibodies against phospho-synapsin I (Ser603: 122%, n= 4, $F_{1\text{-}6}$= 9.63, *p< 0.05), phospho-NR2B (Tyr1472: 146%, n= 5, $F_{1\text{-}8}$= 20.6, **p< 0.01) and phospho-CREB (Ser133: 151%, n= 6, $F_{1\text{-}10}$= 26.8, ***p< 0.001). *(taken from Cooke et al., 2006)*

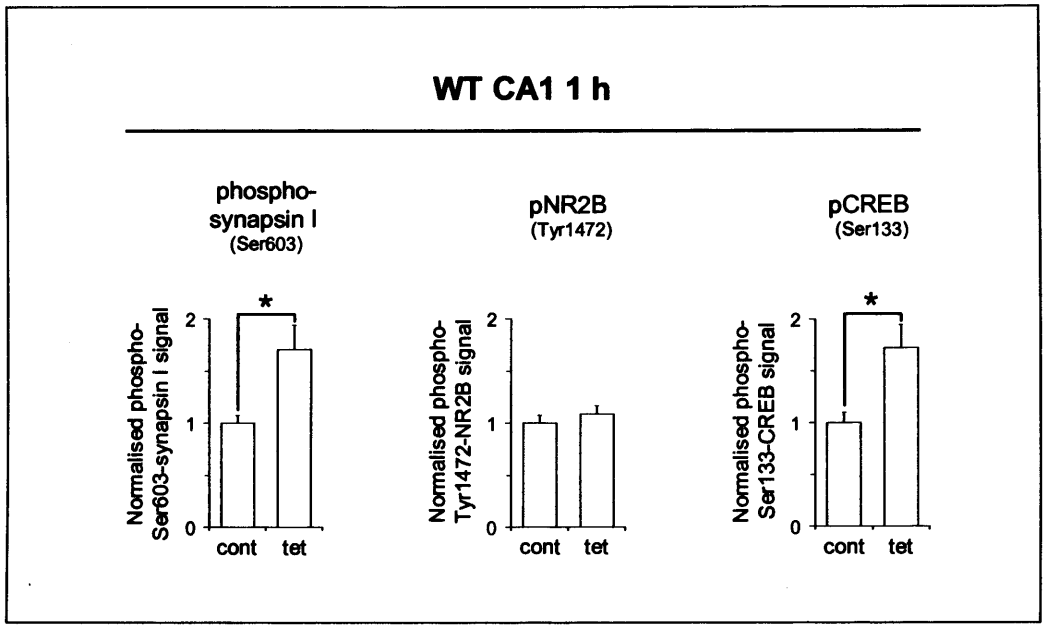

Figure 3-3. Phosphorylation levels of marker molecules are elevated after *in vivo* potentiation in area CA1. Representative immunoblots and quantification from lysates (20 μg protein/lane) of tetanised and control CA1 tissue of BL6 WT mice harvested one hour (1h) after LTP induction. Immunoblots were probed with antibodies against phospho-synapsin I (Ser603: 171%, n= 4, $F_{1\text{-}6}$= 8.27, *p< 0.05), phospho-NR2B (Tyr1472: 108%, n= 4, $F_{1\text{-}6}$= 0.68, p> 0.05) and phospho-CREB (Ser133: 172%, n= 4, $F_{1\text{-}6}$= 8.58, *p< 0.05).

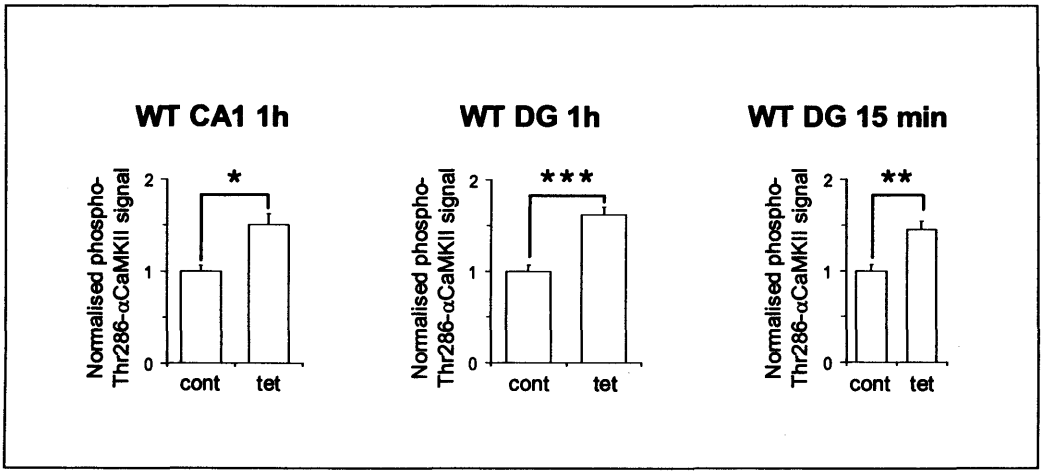

Figure 3-4. Autophosphorylation of αCaMKII is elevated after LTP induction in hippocampal area CA1 and DG *in vivo*. Quantification of immunoblots from lysates (20 μg protein/lane) of tetanised and control CA1 or DG tissue of WT mice. Immunoblots were probed with an antibody recognising phospho-Thr286-αCaMKII. The levels of αCaMKII autophosphorylation at Thr286 were increased one hour after tetanisation in hippocampal area CA1 (150%, n= 5, $F_{1\text{-}8}$= 13.5, *p< 0.05) as well as DG (162%, n= 5, $F_{1\text{-}8}$= 435, ***p< 0.001) and in DG 15 minutes after tetanisation (145%, n= 8, $F_{1\text{-}14}$= 14.6, **p< 0.01). *(taken from Cooke et al., 2006)*

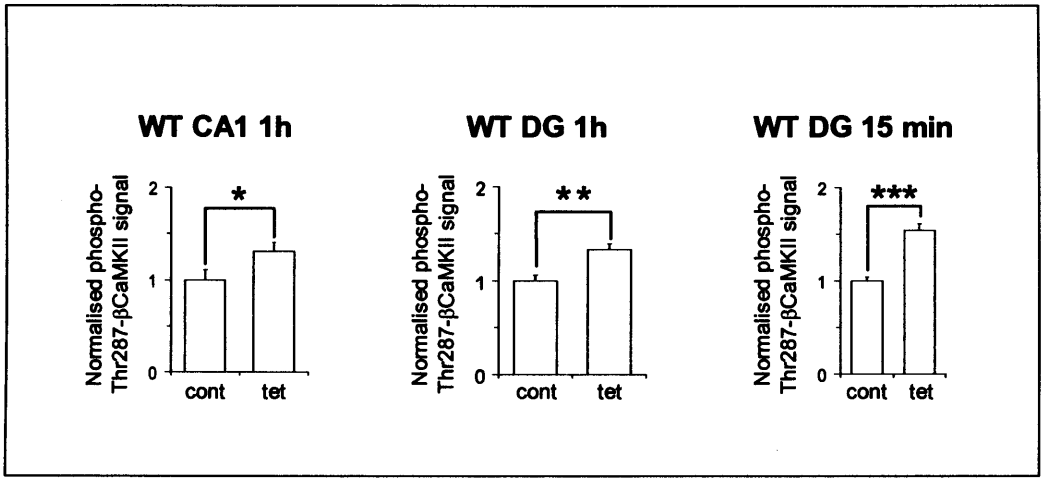

Figure 3-5. Autophosphorylation of β-isoform of CaMKII is increased after LTP induction in hippocampal area CA1 and DG *in vivo*. Quantification of immunoblots from lysates (20 μg protein/lane) of tetanised and control CA1 or DG tissue of WT mice. Immunoblots were probed with an antibody recognising phospho-Thr287-βCaMKII. The levels in βCaMKII autophosphorylation at Thr287 were increased one hour after tetanisation in hippocampal area CA1 (132%, n= 5, $F_{1\text{-}8}$= 7.45, *p< 0.05) as well as DG (132%, n= 5, $F_{1\text{-}8}$= 14.6, **p< 0.01) and in DG 15 minutes after tetanisation (153%, n= 8, $F_{1\text{-}14}$= 39.3, ***p< 0.001). *(taken from Cooke et al., 2006)*

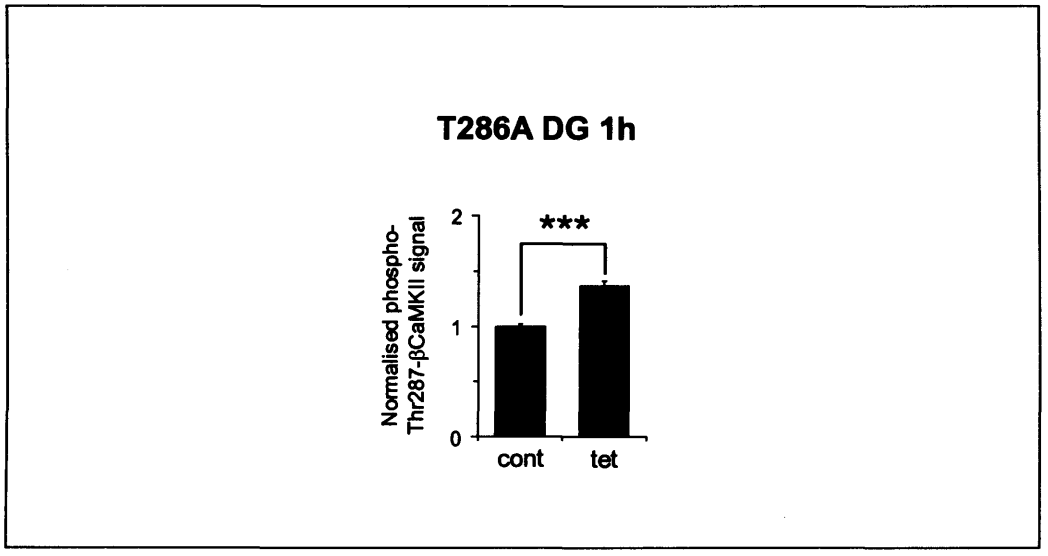

Figure 3-6. LTP induction in DG of T286A mutants leads to increased autophosphorylation of βCaMKII *in vivo*. Quantification of immunoblots from lysates (20 μg protein/lane) of tetanised and control CA1 or DG tissue of WT mice. Immunoblots were probed with an antibody recognising phospho-Thr287-βCaMKII. The levels in βCaMKII autophosphorylation at Thr287 were increased one hour after tetanisation in DG of αCaMKII T286A mutants (136%, n= 5, $F_{1\text{-}8}$= 48.7, ***p< 0.001). *(taken from Cooke et al., 2006)*

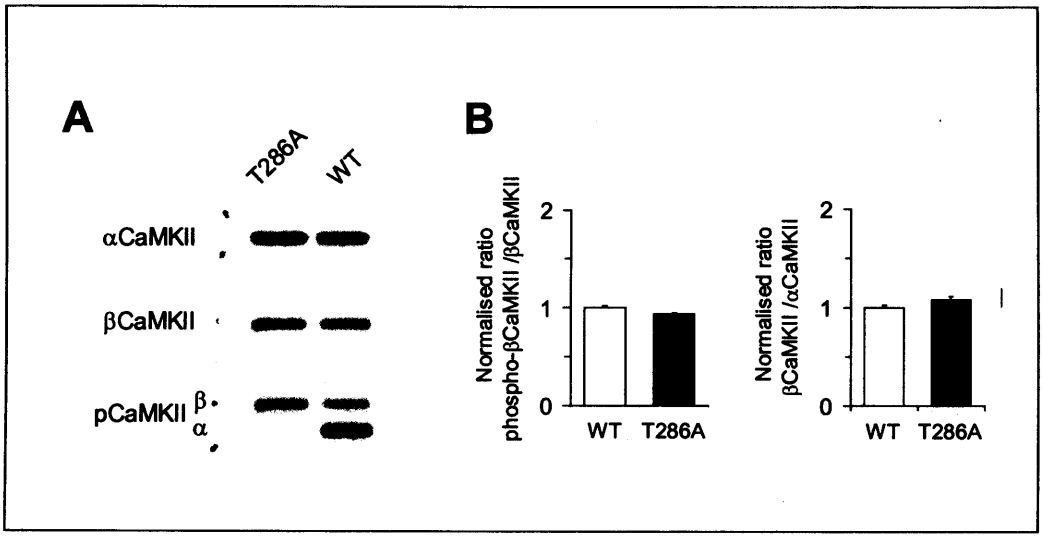

Figure 3-7. Normal βCaMKII levels in crude synaptosomal fraction of αCaMKII T286A mutants. **A)** Representative immunoblots from hippocampal synaptosome lysates (20 μg protein/lane) of T286A mutants and WT mice assayed with antibodies against αCaMKII, βCaMKII and a phospho-specific antibody detecting the autophosphorylation sites on both αCaMKII (Thr286) as well as βCaMKII (Thr287). **B)** Quantification of immunoblots showed no difference between T286A mutants and WT mice in the normalised ratio of phospho-Thr287-βCaMKII /βCaMKII (n= 3, $F_{1\text{-}4}$= 6.90, p> 0.05) and in the normalised ratio βCaMKII /αCaMKII (n= 3, $F_{1\text{-}4}$= 6.27, p> 0.05). *(taken from Cooke et al., 2006)*

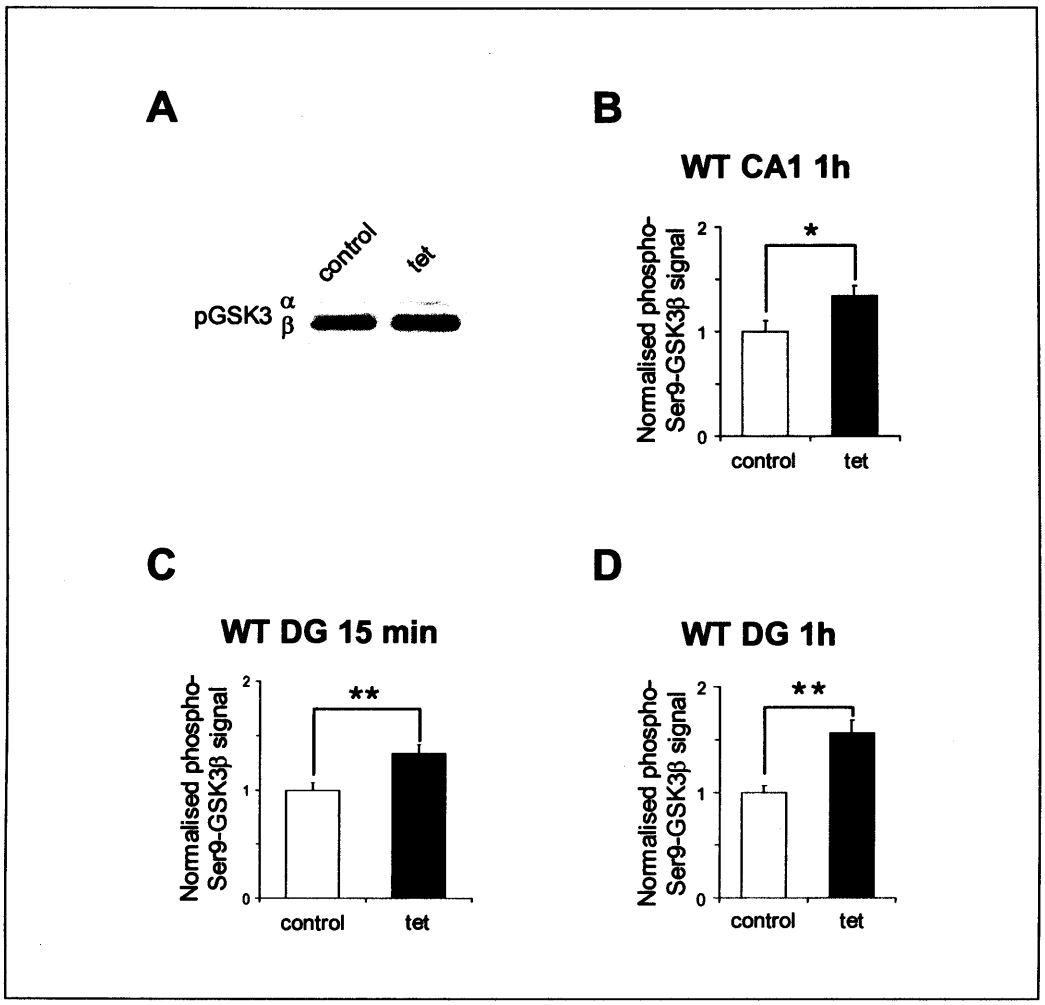

Figure 3-8. Increased phosphorylation at the inhibitory Ser9 site of GSK3 after *in vivo* LTP induction in area CA1 and DG in WT mice. A) Representative immunoblot from hippocampal lysates (20 μg protein/lane) of tetanised and control tissue from DG of WT mice harvested 15 minutes after LTP induction. Immunoblots were probed with an antibody recognising phospho-GSK3α/β (Ser21/9). **B)** The quantification of immunoblots of lysates from hippocampal area CA1 of WT mice harvested one hour after *in vivo* LTP induction revealed a significant increase in phospho-Ser9-GSK3β signal (134%, n= 4, $F_{1\text{-}6}$= 6.29, *p< 0.05). **C** and **D)** The quantification of immunoblots of lysates from DG of WT mice harvested 15 minutes and one hour after *in vivo* LTP induction showed a significant increase in phosphorylation of GSK3β at Ser9 (15 min: 134%, n= 8, $F_{1\text{-}14}$= 10.2, **p< 0.01; 1h: 156%, n= 6, $F_{1\text{-}10}$= 16.5, **p< 0.01)

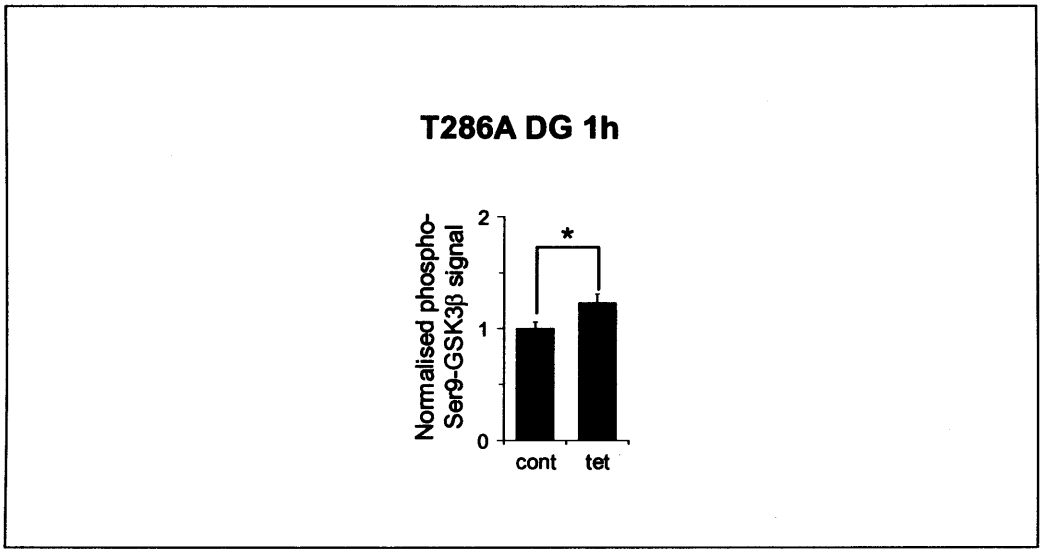

Figure 3-9. The inhibitory site of GSK3 shows enhanced phosphorylation after induction of LTP in DG of αCaMKII T286A mutants *in vivo*. The quantification of immunoblots from hippocampal lysates (20 μg protein/lane) of tetanised and control tissue from DG of T286A mutants assayed with an anti-phospho-GSK3α/β (Ser21/9) antibody revealed a significant increase in phosphorylation of GSK3β at Ser9 one hour after LTP induction (123%, n= 5, $F_{1\text{-}8}$= 5.31, *p< 0.05).

FIGURES: Chapter 4

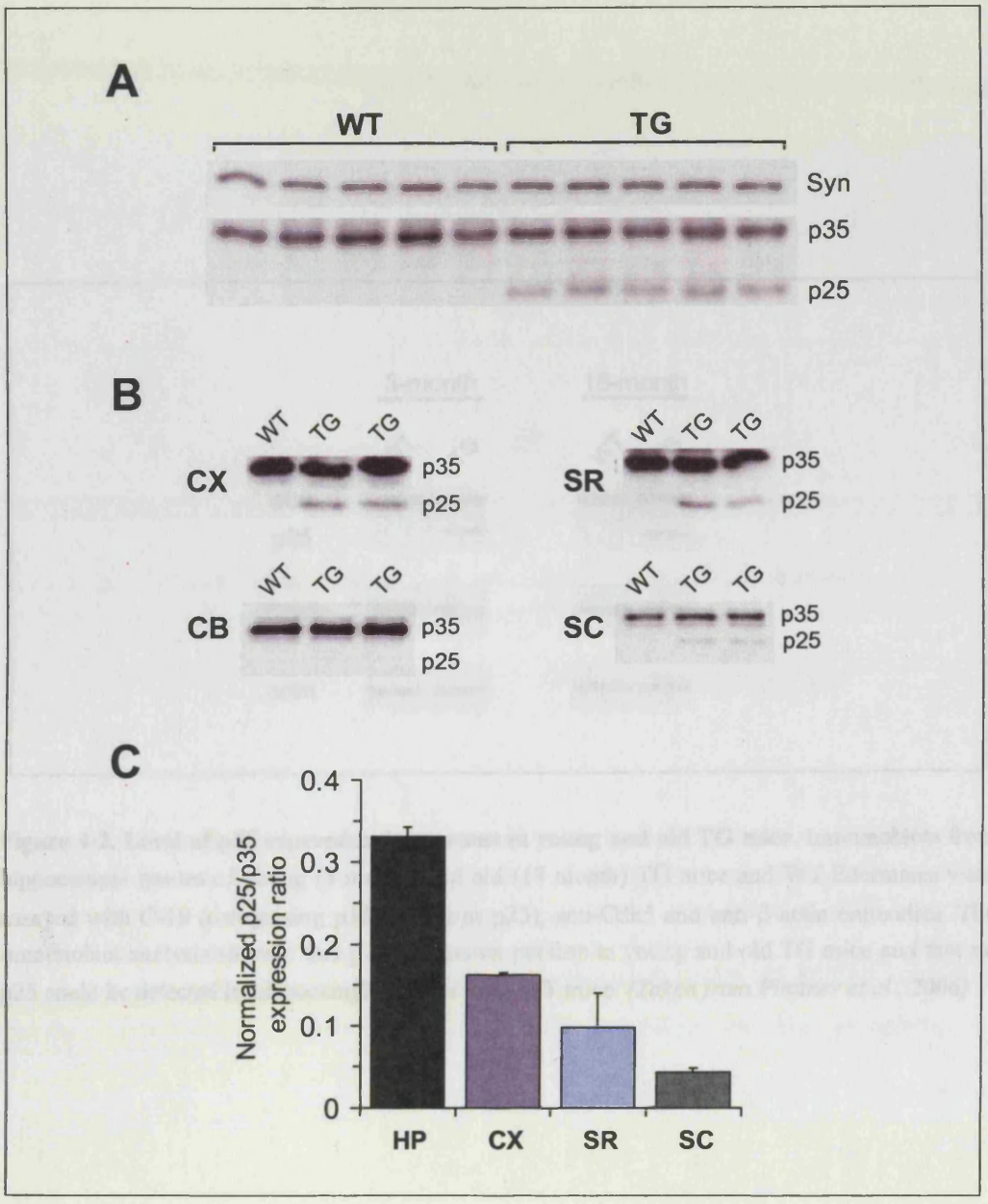

Figure 4-1. Analysis of p25 expression in the p25 TG mice. A) Immunoblots from hippocampal lysates of p25 TG and WT mice probed with antibodies recognising synaptotagmin and the carboxy-terminus of p35 (both p35 and p25). No changes in endogenous p35 expression are detected between genotypes. Expression of p25 is detected in TG mice, but not in WT mice. **B)** In TG mice p25 expression was detected in neocortex (CX), striatum (SR), and spinal cord (SC), but not in cerebellum (CB). **C)** Quantification of p25 immunoblot signal in relation to endogenous p35 signal in hippocampus (HP, n= 5), CX (n= 2), SR, (n= 2) and SC (n= 2). *(Taken from Angelo et al., 2003)*

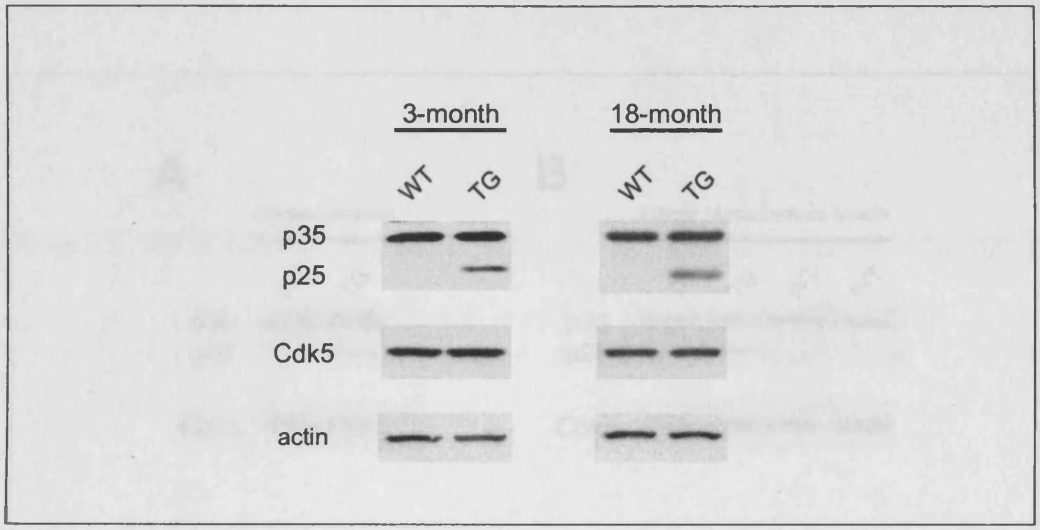

Figure 4-2. Level of p25 expression is constant in young and old TG mice. Immunoblots from hippocampal lysates of young (3 month) and old (18 month) TG mice and WT littermates were assayed with C-19 (recognising p35 as well as p25), anti-Cdk5 and anti-β-actin antibodies. The immunoblot analysis showed that p25 expression persists in young and old TG mice and that no p25 could be detected in hippocampal lysates from WT mice. *(Taken from Plattner et al., 2006)*

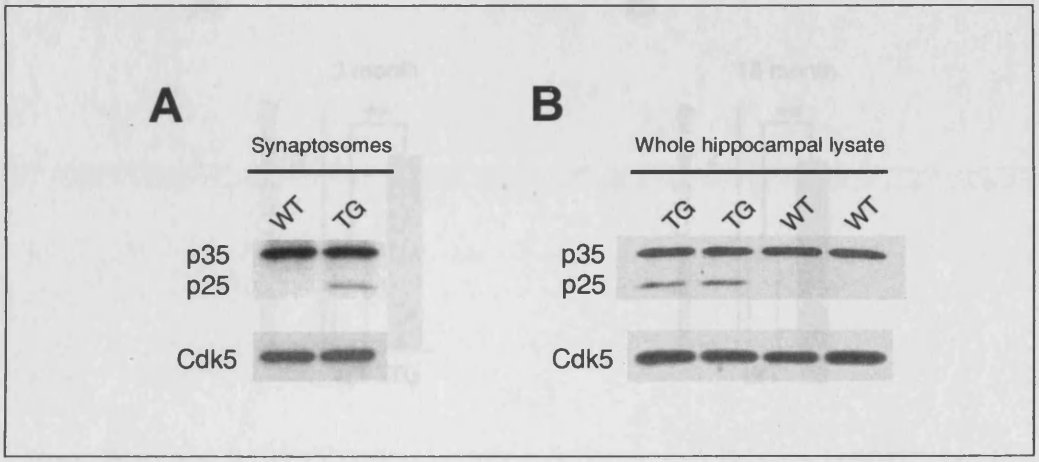

Figure 4-3. Crude synaptosomal fractions from TG mice contain p25 and unaltered levels of Cdk5. Immunoblots of **A)** crude synaptosomal fractions and **B)** whole hippocampal lysates of TG mice and WT littermates probed with an antibody recognising p35 as well as p25 and a Cdk5-specific antibody. The results reveal the presence of p25 in crude synaptosomal lysates from hippocampi of TG mice, but not WT controls. The levels of Cdk5 in the synaptosomal fractions were equal in TG and WT mice, indicating no re-localisation of Cdk5 away from the synapse.

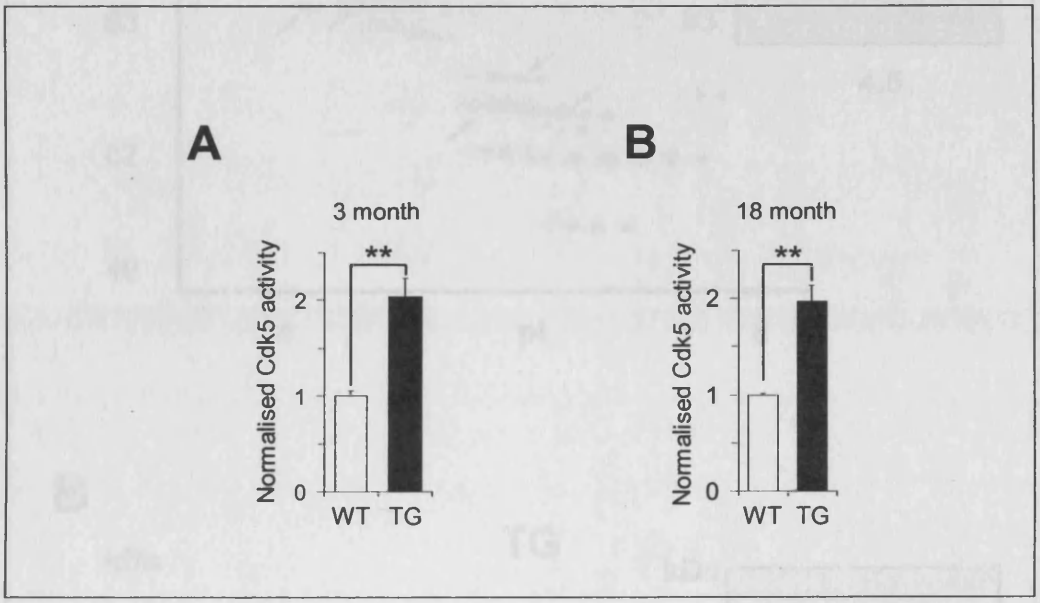

Figure 4-4. Expression of p25 induces overactivation of Cdk5 at 3 and 18 month. Quantification of Cdk5 immunoprecipitation kinase activity assays from hippocampal lysates of TG mice and WT controls at **A)** young (3 month) and **B)** old (18 month) age. Cdk5 activity was increased by approximately 2-fold in both young and old TG mice as compared with WT mice (3 month: $n= 4$, $F_{1\text{-}6}= 29.0$, $^{**}p< 0.01$; 18 month: $n= 4$, $F_{1\text{-}6}= 33.2$, $^{**}p< 0.01$). *(Taken from Plattner et al., 2006)*

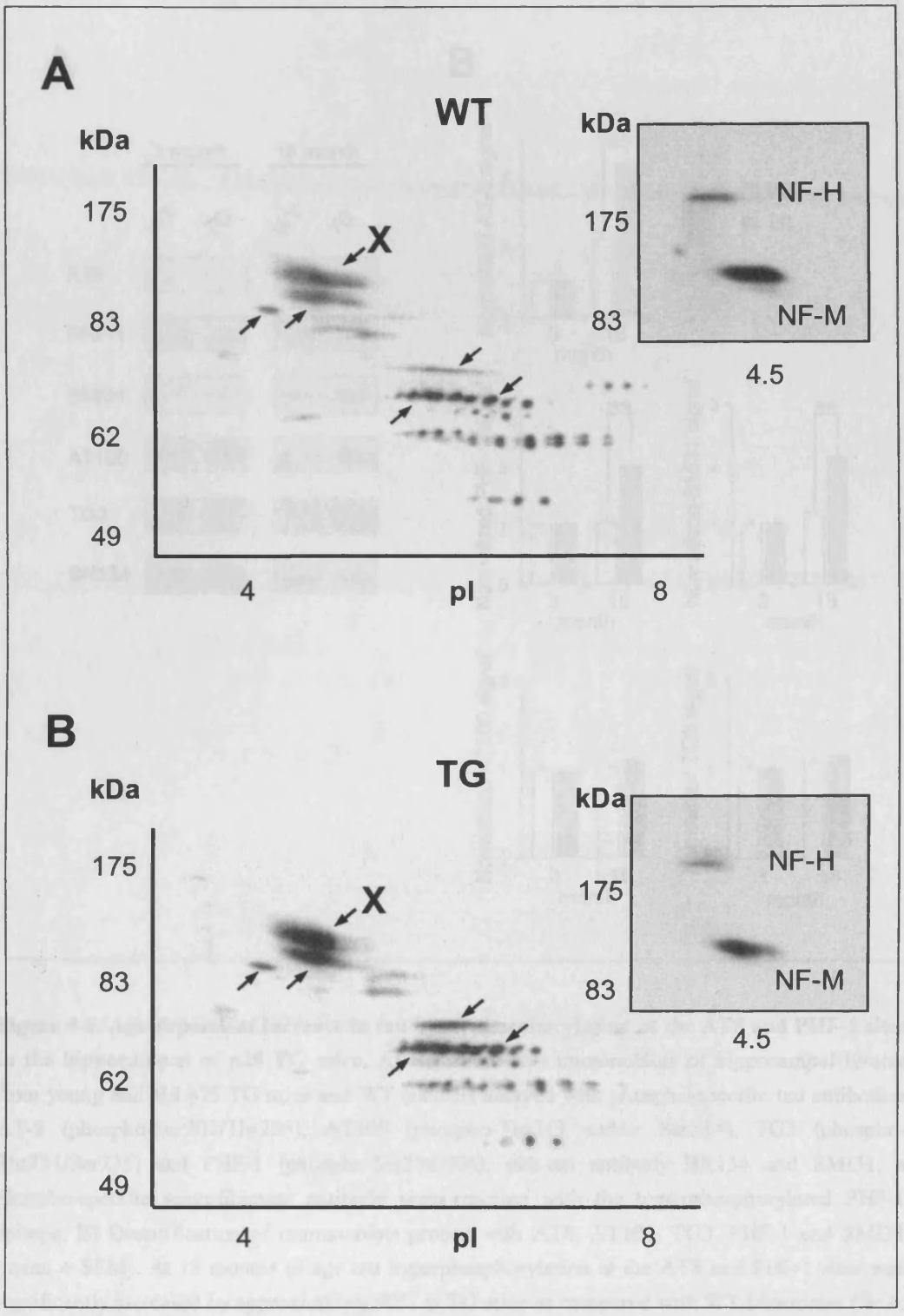

Figure 4-5. Increased phosphorylation of neurofilament M in p25 TG mice. 2-DE immunoblots of crude hippocampal synaptosomes fractions of **A)** WT mice and **B)** p25 TG mice probed with a phospho-specific Cdk/MAPK substrate antibody revealed multiple changes in spot intensities (arrow heads). The spot X (160 kDa, pI 4.5) showed a 2.5-fold change in TG mice as compared to WT controls. Insets: reprobing the 2-DE blots with an antibody (RT97) recognising neurofilament M (NF-M; 160 kDa, pI 4.5) and neurofilament H (NF-H; 200 kDa, pI 4.3) demonstrated that NF-M corresponded to spot X. *(Taken from Angelo et al., 2003)*

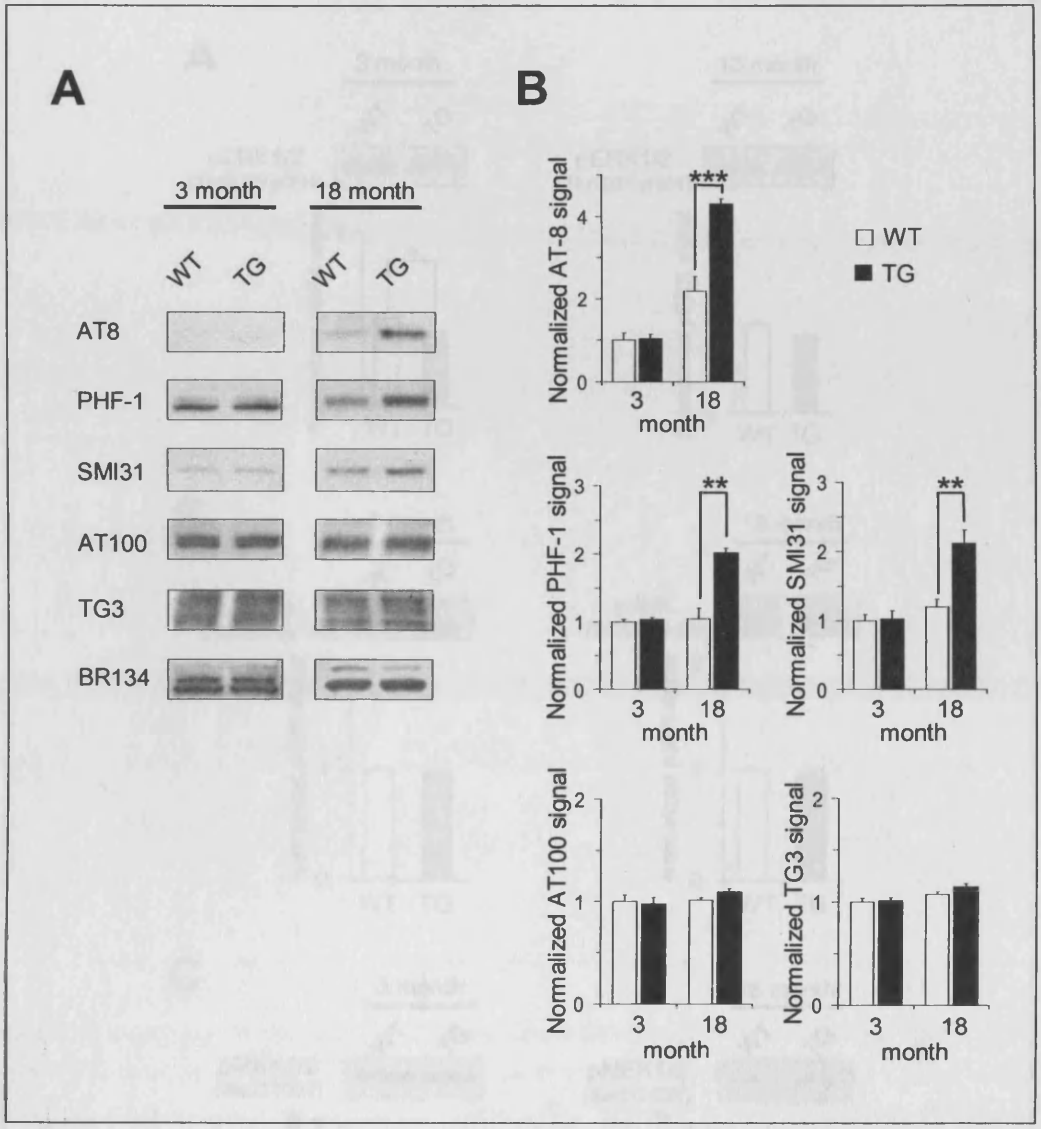

Figure 4-6. Age-dependent increase in tau hyperphosphorylation at the AT8 and PHF-1 sites in the hippocampus of p25 TG mice. A) Representative immunoblots of hippocampal lysates from young and old p25 TG mice and WT controls assayed with phospho-specific tau antibodies AT-8 (phospho-Ser202/Thr205), AT100 (phospho-Thr212 and/or Ser214), TG3 (phospho-Thr231/Ser235) and PHF-1 (phospho-Ser396/404), pan-tau antibody BR134 and SMI31, a phospho-specific neurofilament antibody cross-reacting with the hyperphosphorylated PHF-1 epitope. **B)** Quantification of immunoblots probed with AT8, AT100, TG3, PHF-1 and SMI31 (mean ± SEM). At 18 months of age tau hyperphosphorylation at the AT8 and PHF-1 sites was significantly increased by approximately 90% in TG mice as compared with WT littermates (n= 4; AT-8: $F_{1\text{-}6}$= 55.4, ***p< 0.001; PHF-1: $F_{1\text{-}6}$= 31.2, **p< 0.01, SMI31: $F_{1\text{-}6}$= 27.1, **p< 0.01). Tau hyperphosphorylation at the AT100 and TG3 site were not different between genotypes at young and old age (AT100: 3 month: n= 4; $F_{1\text{-}6}$= 0.02, p> 0.5; 18 month: n= 4; $F_{1\text{-}6}$= 3.31, p> 0.1; TG3: 3 month: n= 4; $F_{1\text{-}6}$= 0.06, p> 0.5; 18 month: n= 4; $F_{1\text{-}6}$= 0.12, p> 0.5). *(Taken from Plattner et al., 2006)*

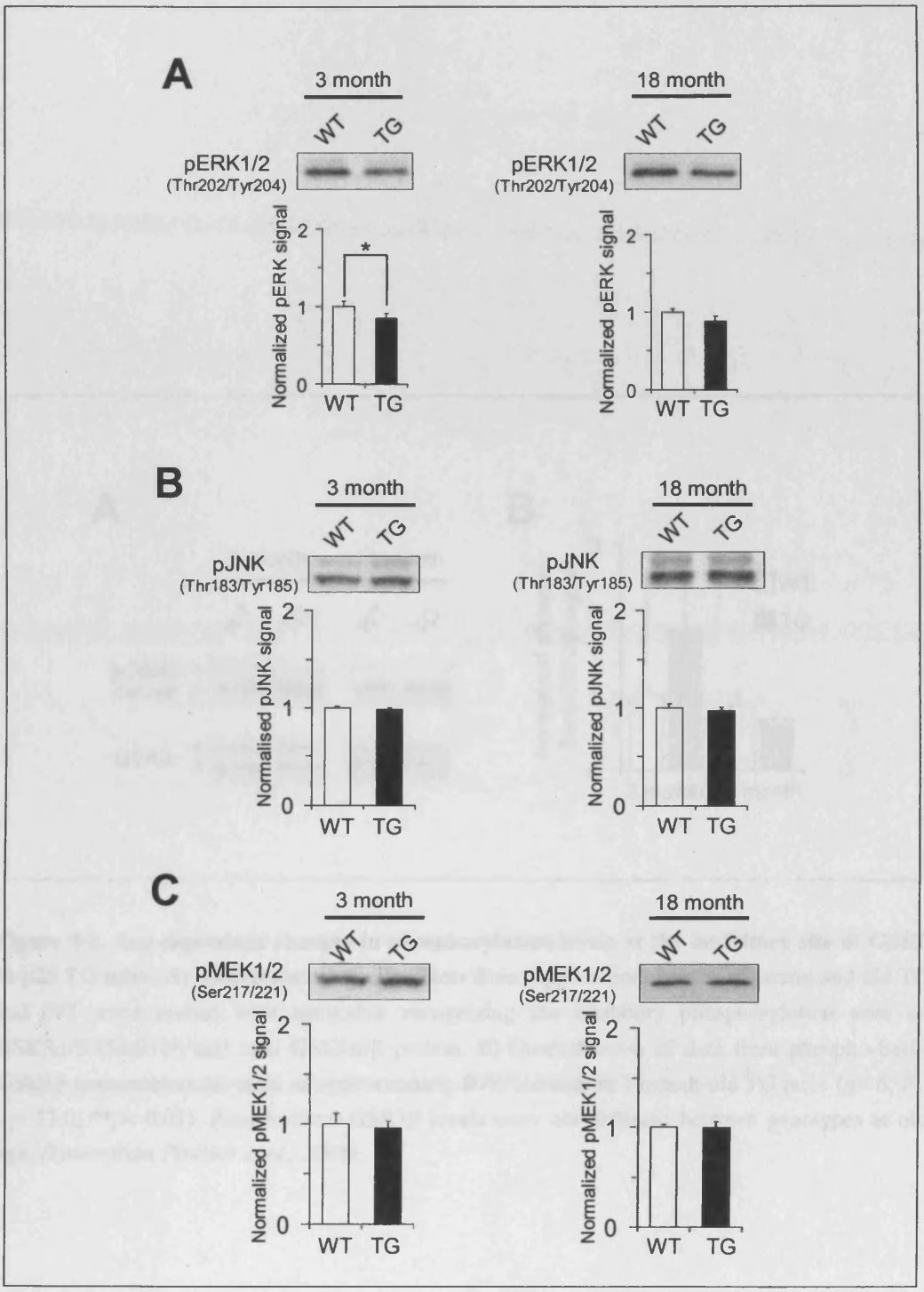

Figure 4-7. Assessment of phosphorylation levels at the activatory sites of the proline-directed kinases ERK, MEK1/2 and JNK in p25 TG mice. Representative immunoblots from hippocampal lysates of young and old TG and WT mice probed with phospho-specific antibodies raised against the activatory phosphorylation sites of ERK1/2 (Thr202/Tyr204), MEK1/2 (Ser217/221) and JNK (Thr183/Tyr185). Quantification of the immunoblot signals revealed a reduction in the phosphorylation levels on **A)** ERK (3 month: n= 8, $F_{1\text{-}14}$= 5.08, *p< 0.05; 18 month: n= 4, $F_{1\text{-}6}$= 3.78, p= 0.07), but no changes in **B)** JNK (3 month: n= 4, $F_{1\text{-}6}$= 0.76, p> 0.1; 18 month: n= 4, $F_{1\text{-}6}$= 0.77, p> 0.1), and **C)** MEK1/2 (3 month: n= 5, $F_{1\text{-}8}$= 0.31, p> 0.1; 18 month: n= 4, $F_{1\text{-}6}$= 0.01, p> 0.1). *(Taken from Plattner et al., 2006)*

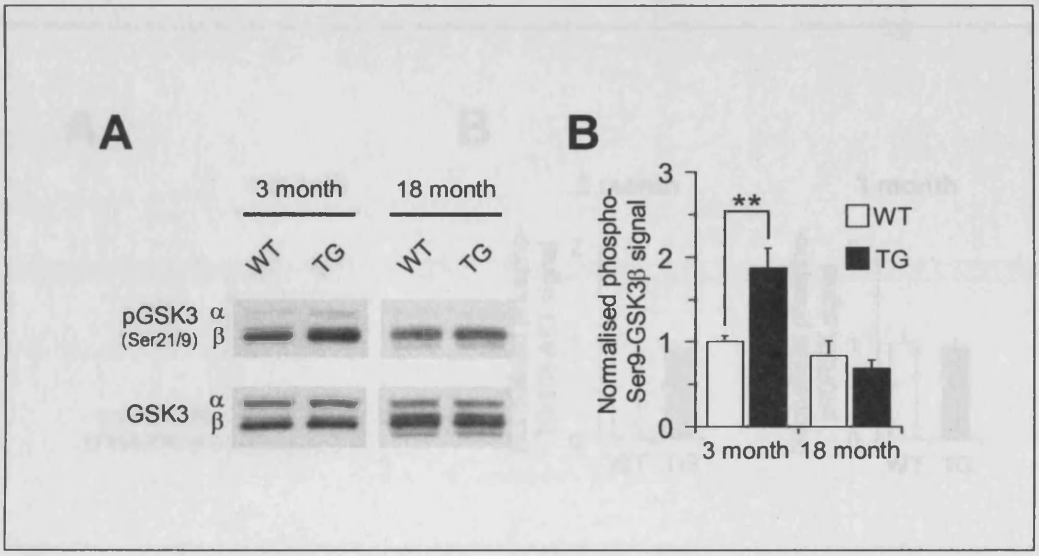

Figure 4-8. Age-dependent changes in phosphorylation levels at the inhibitory site of GSK3 in p25 TG mice. A) Representative immunoblots from hippocampal lysates of young and old TG and WT mice probed with antibodies recognizing the inhibitory phosphorylation sites on GSK3α/β (Ser21/9) and total GSK3α/β protein. **B)** Quantification of data from phospho-Ser9-GSK3β immunoblots revealed an approximately 80% increase in 3 month-old TG mice (n= 6; $F_{1,10}$= 13.0, $^{**}p$< 0.01). Phospho-Ser9-GSK3β levels were not different between genotypes at old age. *(Taken from Plattner et al., 2006)*

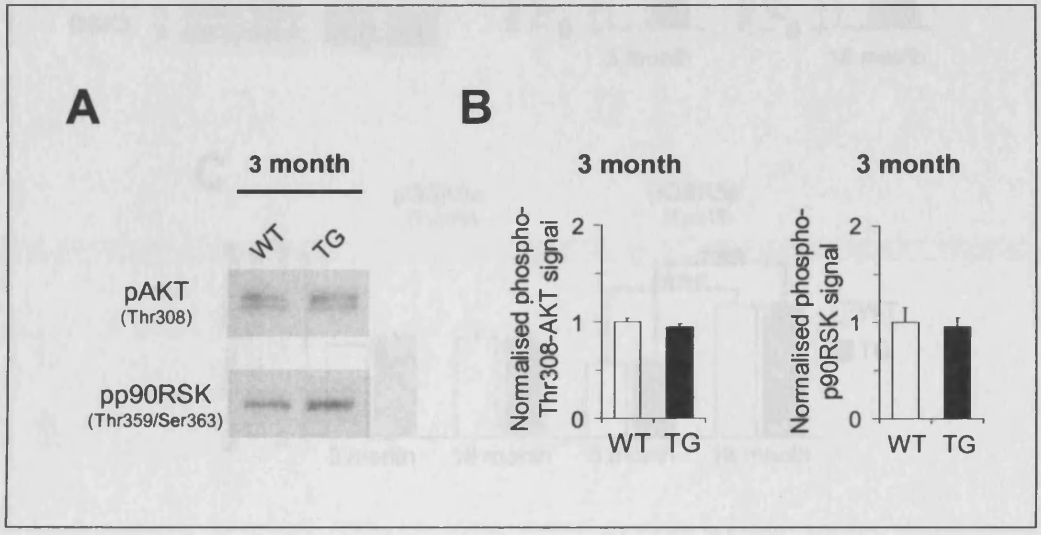

Figure 4-9. Phosphorylation at the inhibitory site on GSK3 is not mediated by AKT and p90RSK in TG mice. A) Representative immunoblots from hippocampal lysates of young TG and WT mice probed with phospho-specific antibodies raised against activatory phosphorylation sites of AKT (Thr308) and p90RSK (Thr359/Ser363). **B)** Quantification of the immunoblot signals showed no changes in phosphorylation levels of AKT (Thr308: n= 4, $F_{1\text{-}6}$= 1.17, p> 0.1) and of p90RSK (Thr359/Ser363: n= 4 , $F_{1\text{-}6}$= 0.06, p> 0.1).

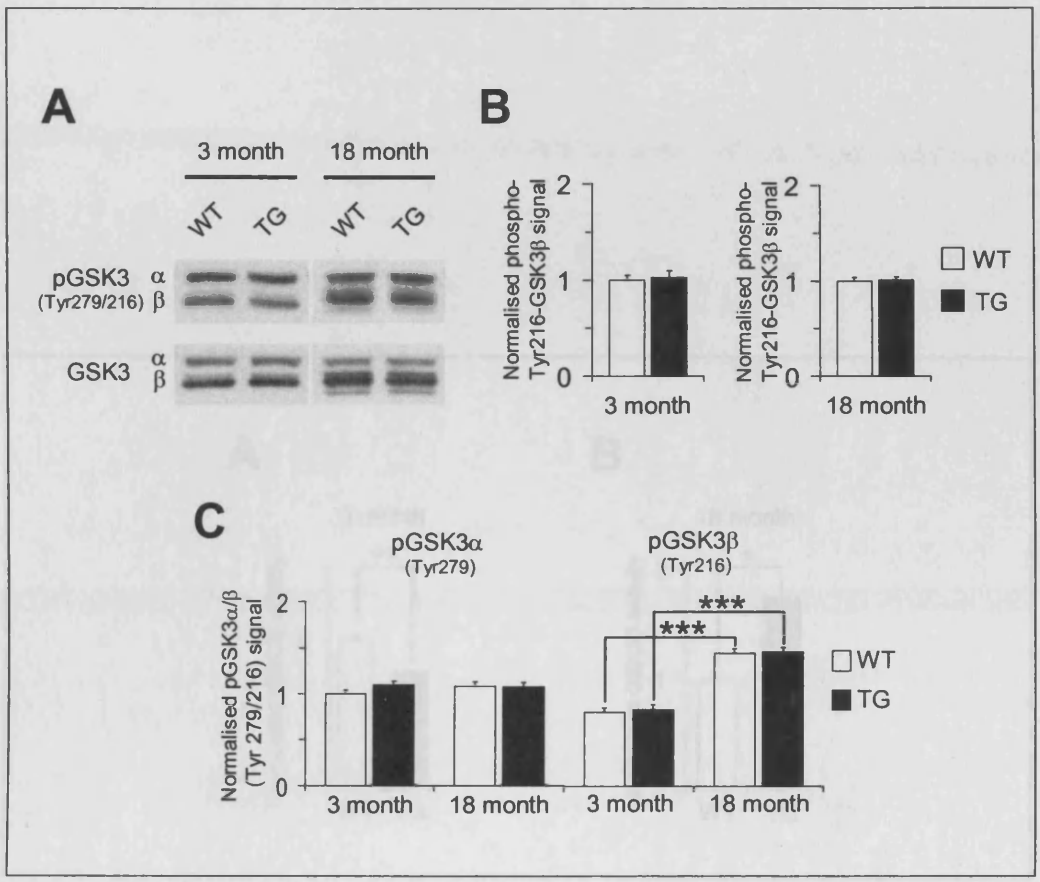

Figure 4-10. Age-dependent increase in GSK3β phosphorylation at the activatory site in TG mice. A) Representative immunoblots from hippocampal lysates of young and old TG and WT mice probed with antibodies recognising the activatory phosphorylation sites of GSK3α/β (Tyr279/216) and total GSK3α/β protein. **B)** Quantification of the immunoblot signals detected no changes in phosphorylation of Tyr216 on GSK3β between genotypes at 3 month (n= 4, $F_{1\text{-}6}$= 0.06, p> 0.5) and at 18 month (n= 4, $F_{1\text{-}6}$= 0.12, p> 0.5). **C)** GSK3β displays higher level of phosphorylation at the activatory sites in old TG as well as old WT mice. Quantification of signals from the activatory sites on GSK3α/β (Tyr279/216) revealed that GSK3α displays a constant level of Tyr279 phosphorylation, whereas the level of phosphorylation of Tyr216 on GSK3β is increased in both TG and WT mice at old age (WT: n= 4, $F_{1\text{-}6}$= 90.7, ***p< 0.001; TG: n= 4, $F_{1\text{-}6}$= 73.9, ***p< 0.001). *(Taken from Plattner et al., 2006)*

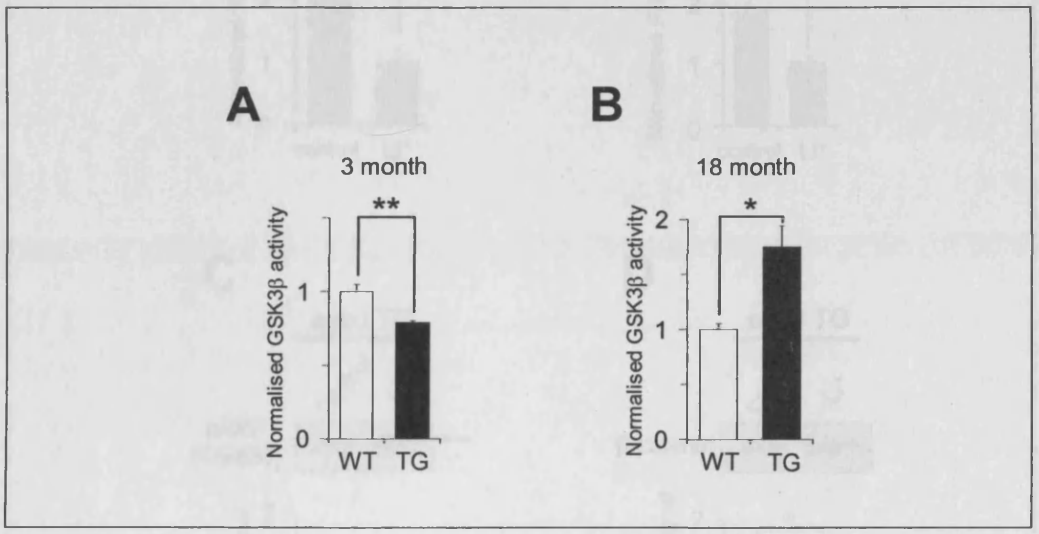

Figure 4-11. GSK3β activity is reduced in young, but increased in old p25 TG mice. Quantification of GSK3β immunoprecipitation kinase assays from hippocampal lysates of TG mice and WT controls at **A)** young (3 month) and **B)** old (18 month) age. GSK3β activity was significantly reduced by approximately 25% in young TG mice (n= 5, $F_{1\text{-}8}$= 16.8, $^{**}p< 0.01$), but it was significantly increased by approximately 70% in old TG mice (n= 3, $F_{1\text{-}4}$= 13.8, $^{*}p< 0.05$). *(Taken from Plattner et al., 2006)*

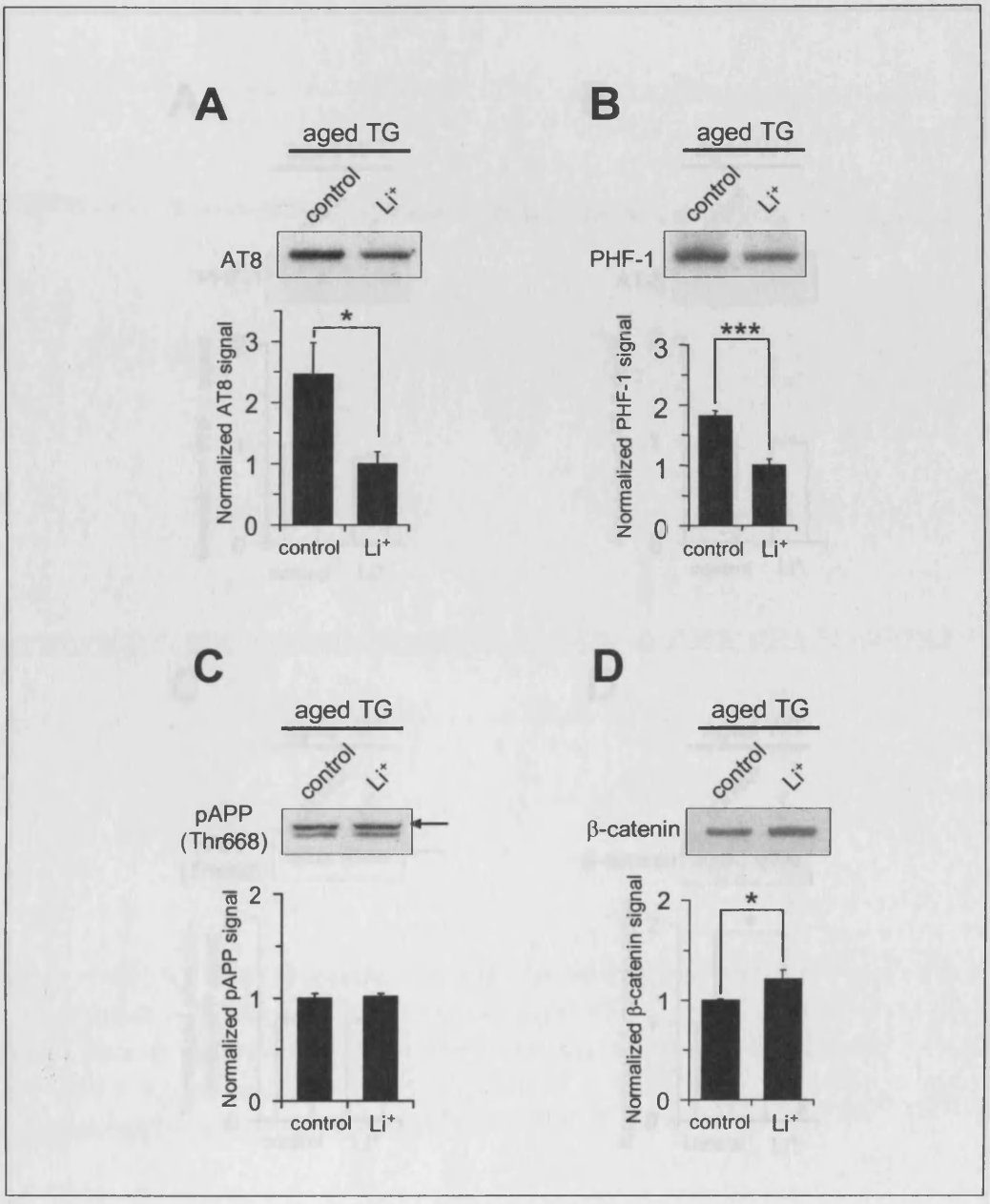

Figure 4-12. Chronic *in vivo* lithium treatment reduced tau hyperphosphorylation in aged p25 TG mice. Immunoblot analysis and signal quantification from hippocampal lysates from aged, 22-24 month old, lithium treated TG mice and untreated aged TG mice (control) probed with PHF-1, AT8, phospho-Thr668-APP and β-catenin antibodies. The quantification showed that tau hyperphosphorylation was significantly reduced in lithium treated TG mice as compared to TG controls **A)** at the AT8 site (n= 4, $F_{1\text{-}6}$= 5.99, *p< 0.05) and **B)** at the PHF-1 site (n= 4, $F_{1\text{-}6}$= 26.4, ***p< 0.001). **C)** Levels of phospho-Thr668-APP were unaltered (n = 4, $F_{1\text{-}6}$= 1.06, p> 0.1) suggesting the lithium treatment did not effect Cdk5 activity. **D)** Levels of β-catenin were increased in lithium treated mice (n= 4, $F_{1\text{-}6}$= 6.23, *p< 0.05) demonstrating reduced GSK3 activity. *(Taken from Plattner et al., 2006)*

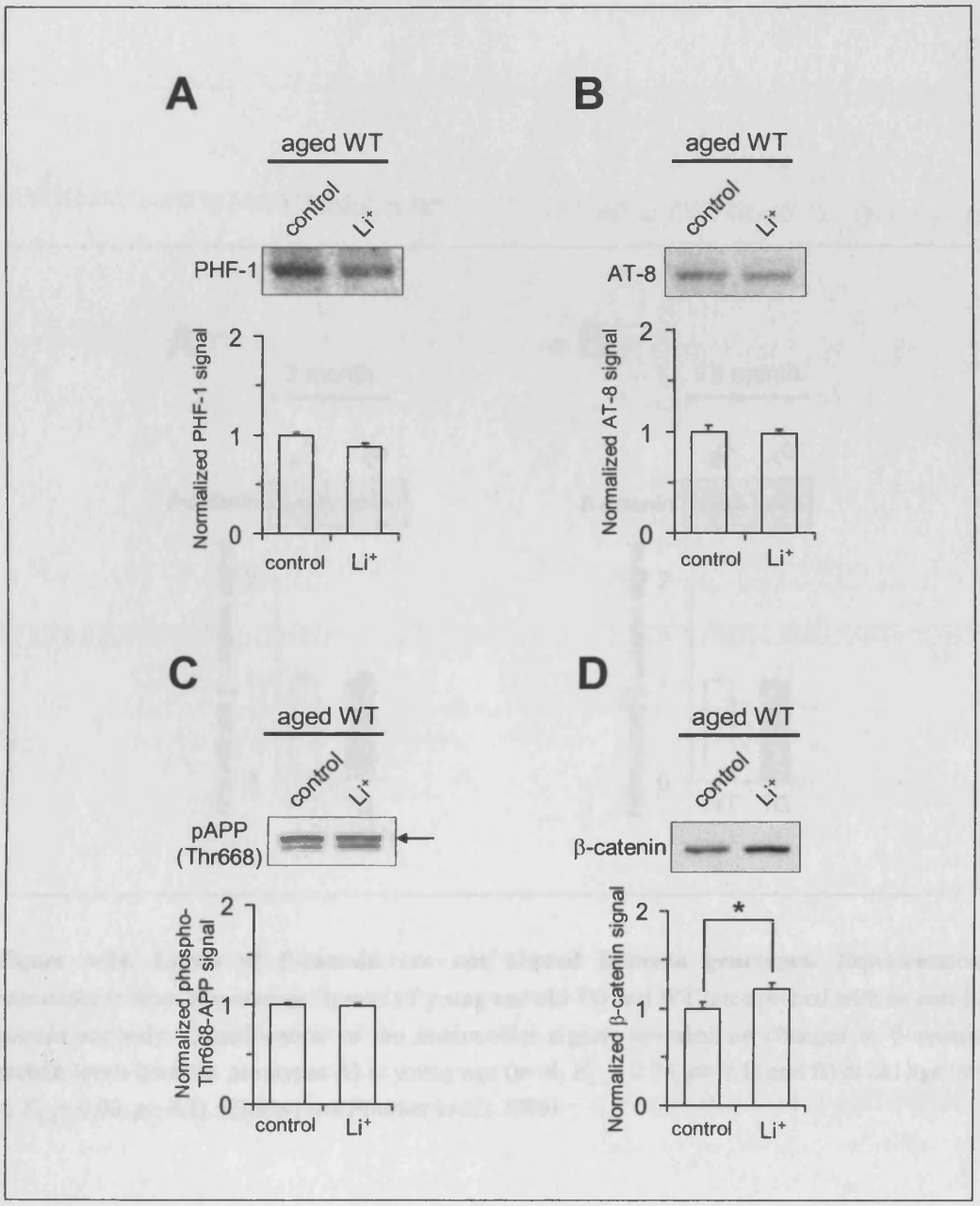

Figure 4-13. Effects of chronic lithium treatment *in vivo* in aged WT mice. Immunoblot analysis and signal quantification from hippocampal lysates from aged, 18-19 month old, lithium treated WT mice and untreated aged WT controls probed with PHF-1, AT8, phospho-Thr668-APP and β-catenin antibodies. The quantification showed that tau hyperphosphorylation was affected non-significantly in lithium treated WT mice as compared to WT controls **A)** at the AT8 site (n= 3, $F_{1\text{-}4}$= 0.64, p> 0.1) and **B)** at the PHF-1 site (n= 3; $F_{1\text{-}4}$= 6.18, p> 0.05). **C)** Levels of phospho-Thr668-APP were unaltered (n= 3; $F_{1\text{-}4}$= 0.02, p> 0.5) suggesting the lithium treatment did not effect Cdk5 activity. **D)** Levels of β-catenin were increased in lithium treated WT mice (n= 3; $F_{1\text{-}4}$= 16.7, *p< 0.05) demonstrating reduced GSK3 activity. *(Taken from Plattner et al., 2006)*

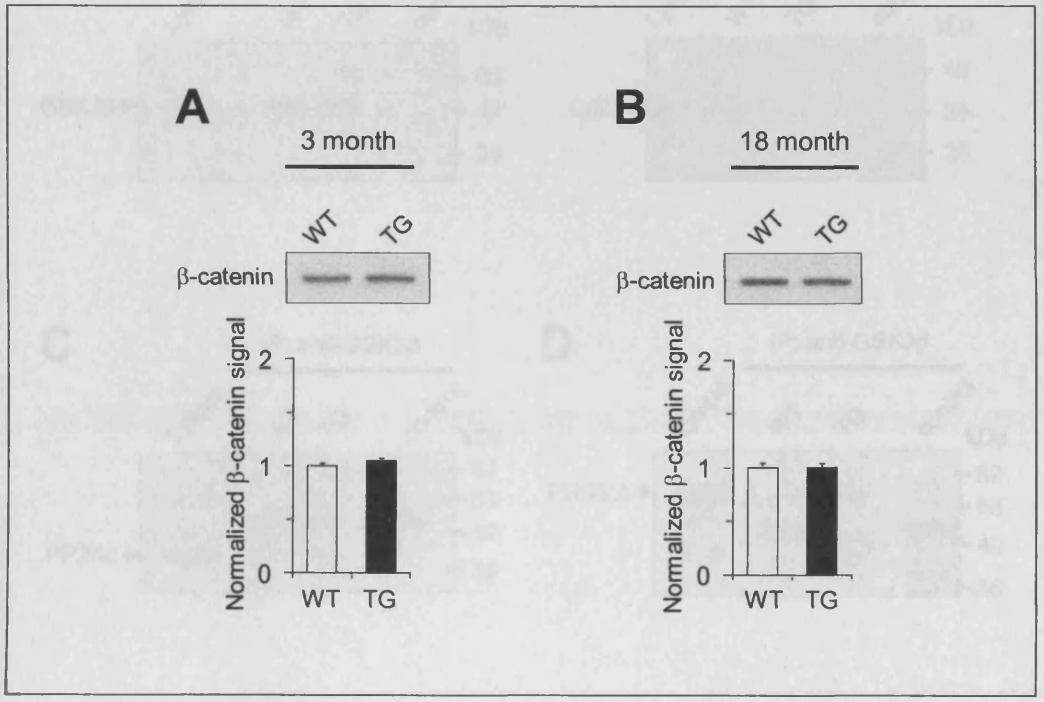

Figure 4-14. Levels of β-catenin are not altered between genotypes. Representative immunoblots from hippocampal lysates of young and old TG and WT mice probed with an anti-β-catenin antibody. Quantification of the immunoblot signals revealed no changes in β-catenin protein levels between genotypes **A)** at young age (n= 4, $F_{1\text{-}6}$= 2.26, p> 0.1) and **B)** at old age (n= 4, $F_{1\text{-}6}$= 0.00, p> 0.1). *(Taken from Plattner et al., 2006)*

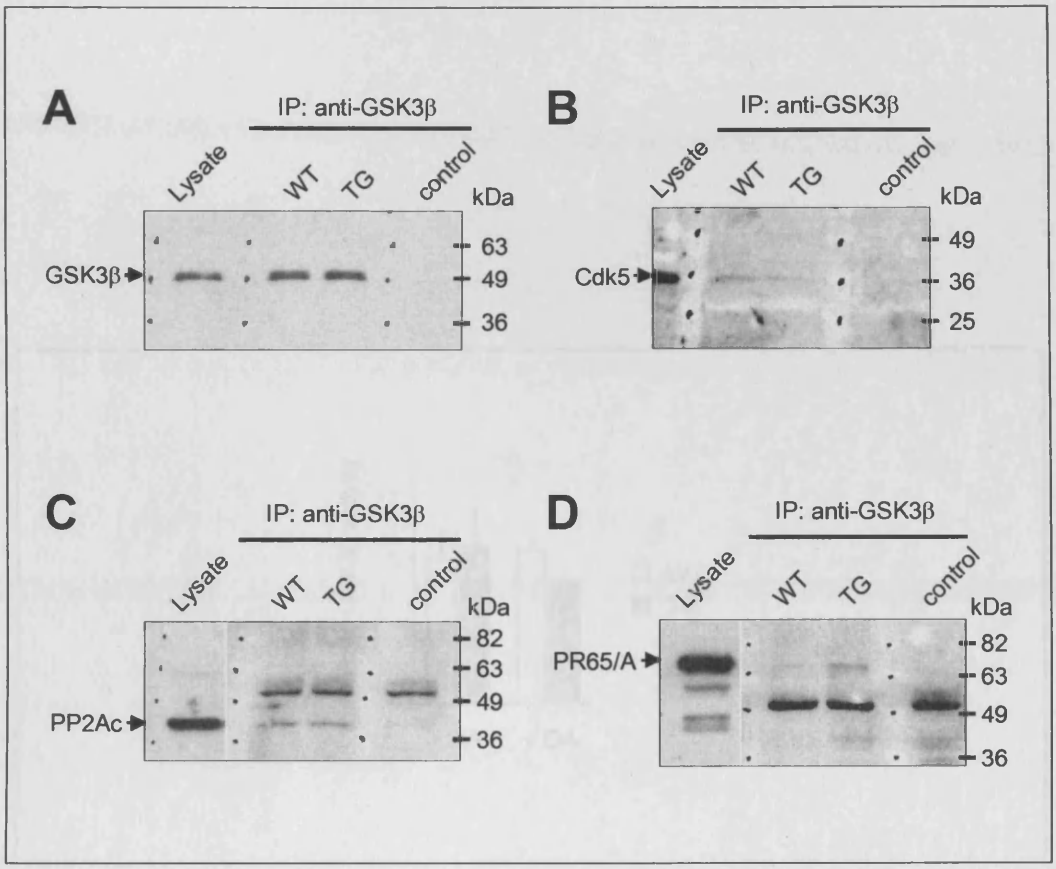

Figure 4-15. Association of Cdk5, GSK3 and PP2A. Co-immunoprecipitation experiments with hippocampal lysates from TG mice and WT littermates were carried out using either anti-GSK3β antibody or non immune antibody as a control. Immune pellets were analysed by immunoblotting using **A)** anti-GSK3β antibody, **B)** anti-Cdk5 antibody as well as antibodies recognizing **C)** the catalytic subunit of PP2A (PP2Ac) and **D)** the scaffolding/ regulatory subunit of PP2A (PR65/A). The molecular weight standards are shown on the right. Immunoreactive bands of interest are indicated by arrow heads. The band running at approximately 50 kDa in *C*) and *D*) and at 28 kDa in *B*) results from cross-reactivity with the immunoprecipitating IgG. These immunoblots are representative of n= 4 independent experiments. *(Taken from Plattner et al., 2006)*

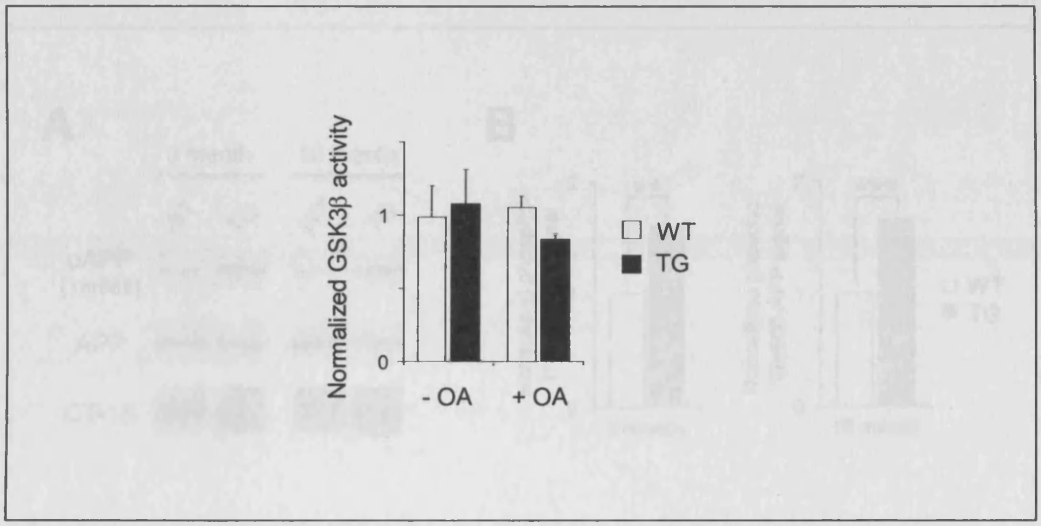

Figure 4-16. Okadaic acid affects GSK3β activity differently in TG mice and WT controls. GSK3β immunoprecipitation kinase assay of hippocampal lysates from young TG (n= 4) and WT mice (n= 4) were carried out in presence or absence of okadaic acid (OA), a PP1 and PP2A inhibitor. Addition of okadaic acid reduced the GSK3β activity in TG mice, but not WT controls. *(Taken from Plattner et al., 2006)*

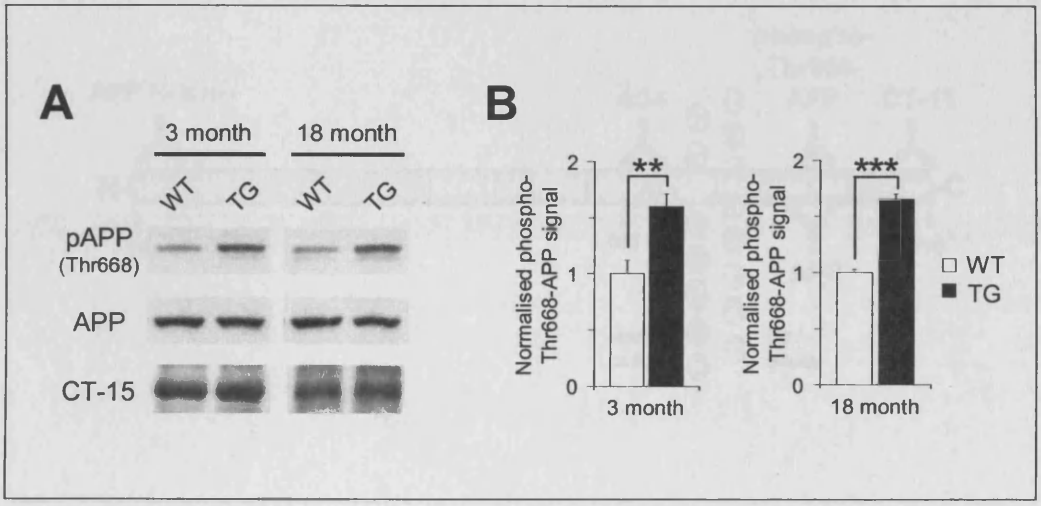

Figure 4-17. Persistent increase in APP phosphorylation at Thr668 in p25 TG mice. A) Representative immunoblots from hippocampal lysates of young and old TG mice and WT controls probed with a phospho-specific antibody recognising APP (pAPP) phosphorylated at Thr668 (as per APP695). Total APP protein levels were detected with antibodies against the unphosphorylated epitope around Thr668 on APP (APP) and the carboxy-terminus of APP (CT-15). **B)** Quantification of phospho-Thr668-APP signal revealed a significant increase of approximately 60% in TG mice at 3 months (n= 6, $F_{1\text{-}10}$= 11.1, $^{**}p< 0.01$) and 18 months (n= 4, $F_{1\text{-}6}$= 175, $^{***}p< 0.001$). *(Taken from Plattner et al., 2006)*

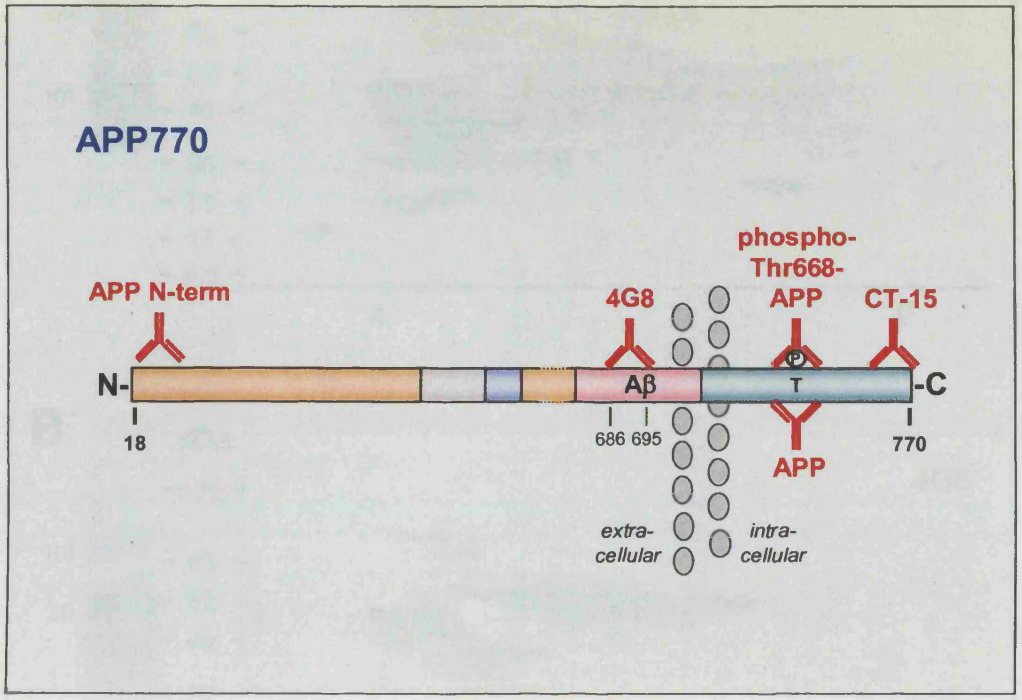

Figure 4-18. Schematic representation of antibody epitopes on APP. APP is a single transmembrane protein. In this study different antibodies were used that recognise distinct epitopes within the extracellular and intracellular part of APP. On the extracellular side are the epitopes of an antibody raised against the amino-terminal sequence of APP (APP N-term) and an antibody recognising a site adjacent to the transmembrane domain within the Aβ-peptide domain at residues 686-695 (4G8). Within the intracellular portion are the epitopes of the anti-phospho-Thr668-APP antibody (as per APP695). Further, an antibody recognising the epitope around Thr668 on APP in the unphosphorylated state (APP) and an antibody raised against the carboxy-terminus of APP (CT-15).

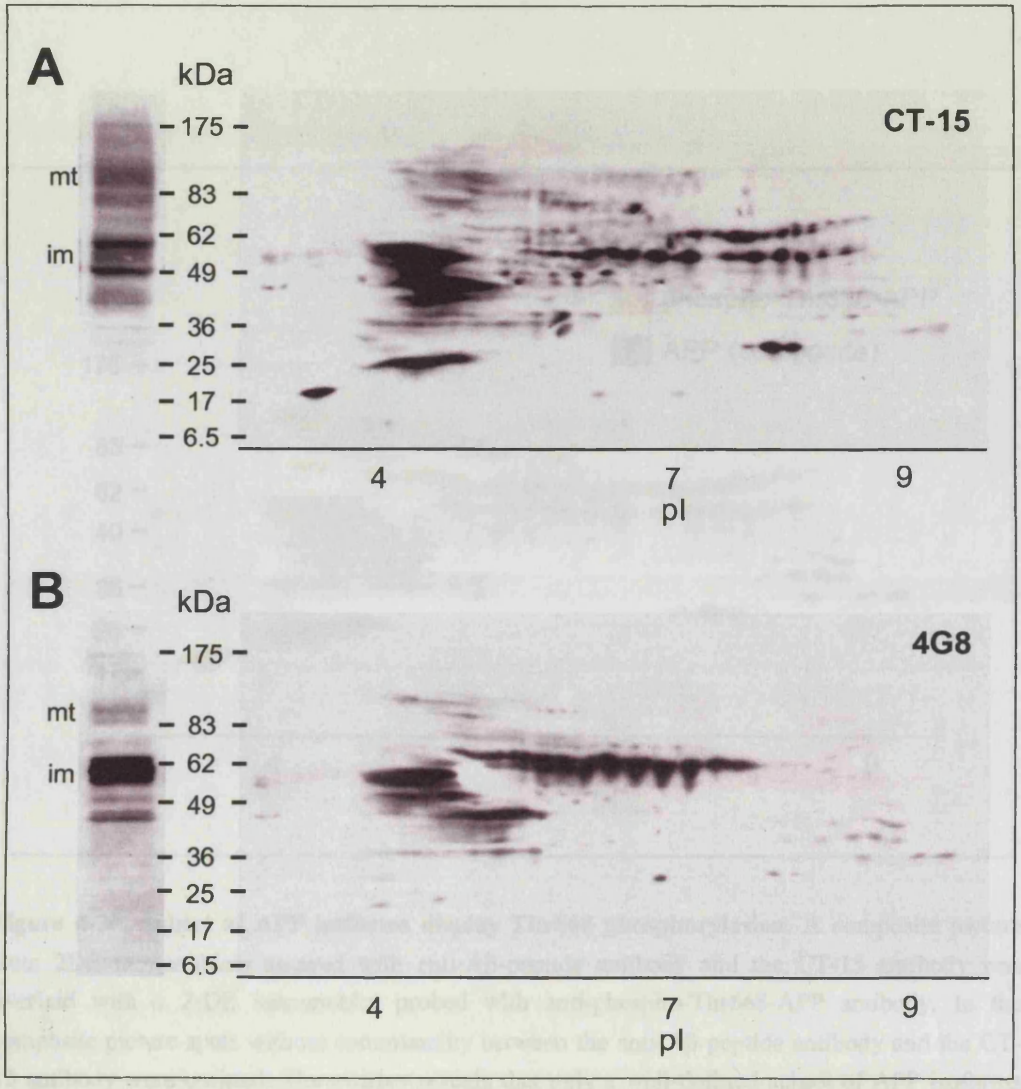

Figure 4-19. 2DE immunoblot analysis of APP isoforms and fragments. Representative one-dimensional SDS PAGE blots (left of marker) and two-dimensional electrophoresis (2DE) immunoblots (right of marker) of hippocampal lysate from a 3 month-old WT mouse probed with **A)** an antibody recognising the carboxy-terminus of APP (CT-15) and **B)** assayed with an anti-Aβ-peptide antibody (4G8). 2DE analysis with both antibodies reveals the existence of a large number of APP isoforms, possibly generated by alternative processing and post-translational modifications. Comparison of the 2-DE blots show that both antibodies detect a similar spot pattern. Protein spots detected by both antibodies are likely to be APP isoforms. Mature (mt) and immature (im) isoforms of APP are indicated left of the one-dimensional blot.

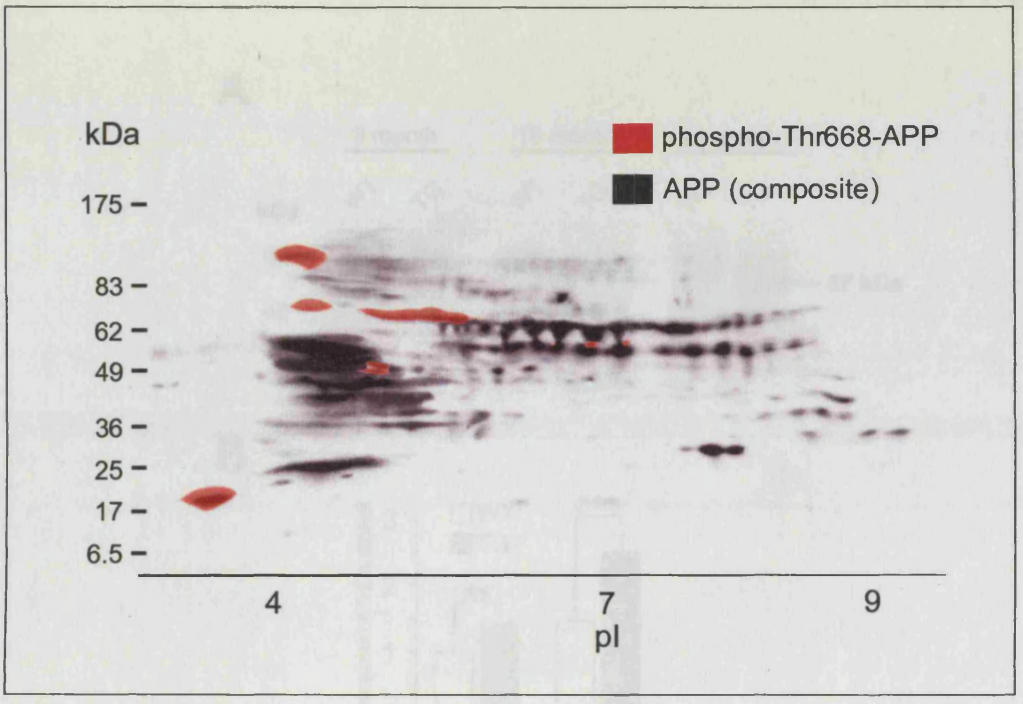

Figure 4-20. Subset of APP isoforms display Thr668 phosphorylation. A composite picture from 2DE immunoblots assayed with anti-Aβ-peptide antibody and the CT-15 antibody was overlaid with a 2-DE immunoblot probed with anti-phospho-Thr668-APP antibody. In the composite picture spots without communality between the anti-Aβ-peptide antibody and the CT-15 antibody were omitted. The overlay reveals that only a well-defined subset of APP isoforms displays phosphorylation at Thr668.

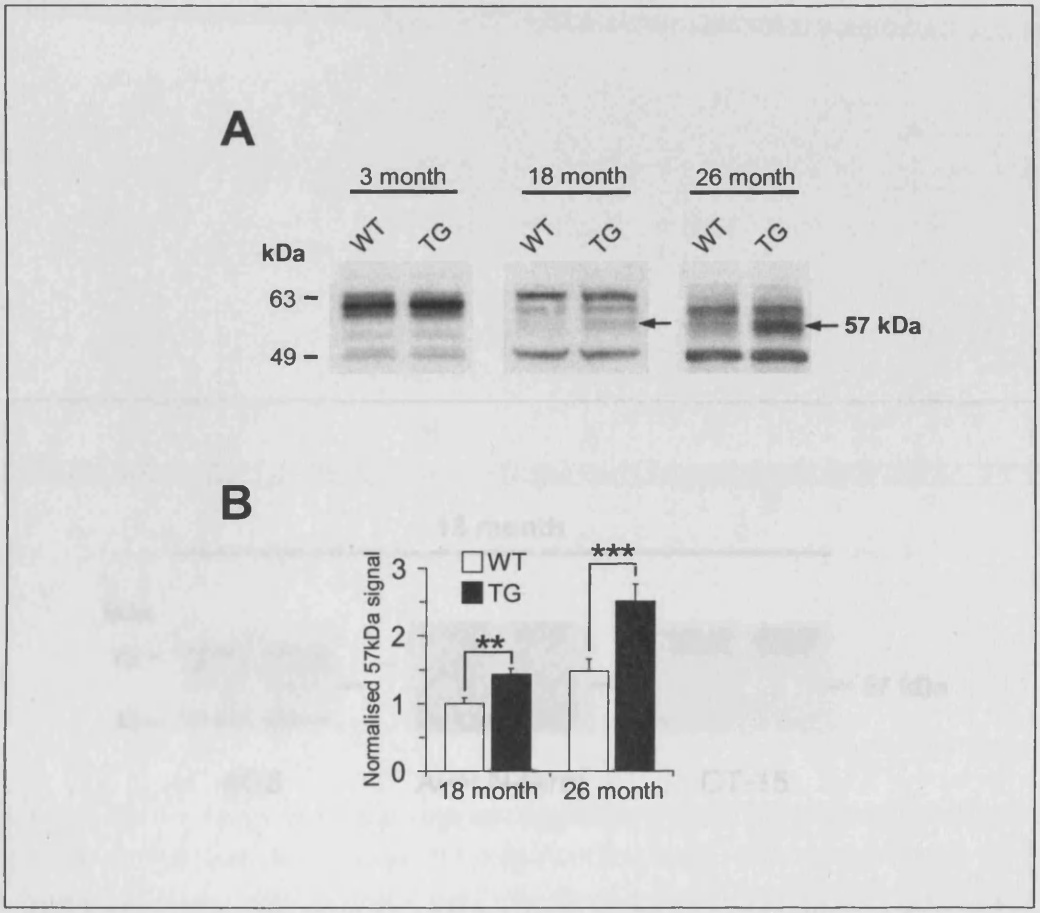

Figure 4-21. Age-dependent changes in APP processing in p25 TG mice. A) Representative immunoblots from hippocampal lysates of 3, 18 and 26 month-old TG mice and WT littermates probed with an anti-Aβ antibody (4G8). The immunoblots reveal an age-dependent accumulation of a 57 kDa fragment (arrow head) in TG mice after 18 months of age. **B)** Quantification of signal from the 57 kDa fragment showed a significant increase in TG mice at 18 month (n= 4, $F_{1\text{-}6}$= 15.9, **p< 0.01) and 26 month (n= 4, $F_{1\text{-}6}$= 44.6, ***p< 0.001). In 3 month-old TG mice and WT controls no signal was observed at 57 kDa.

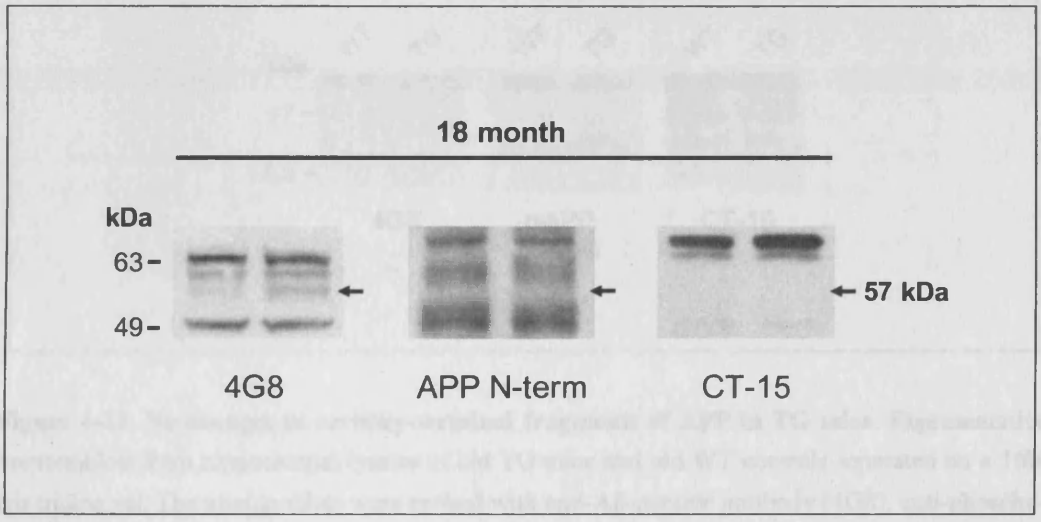

Figure 4-22. The 57 kDa fragment is recognised by anti-Aβ antibody, but not amino- and carboxy-terminal anti-APP antibodies. Representative immunoblots from hippocampal lysates of 18 month-old TG mice and WT littermates probed with antibodies recognising the Aβ-peptide domain (4G8), the amino-terminal part of APP and the carboxy-terminus of APP (CT-15). The 57 kDa band (arrow head) is only detected with the anti-Aβ antibody, but not with antibodies against the amino- and carboxy-terminus of APP.

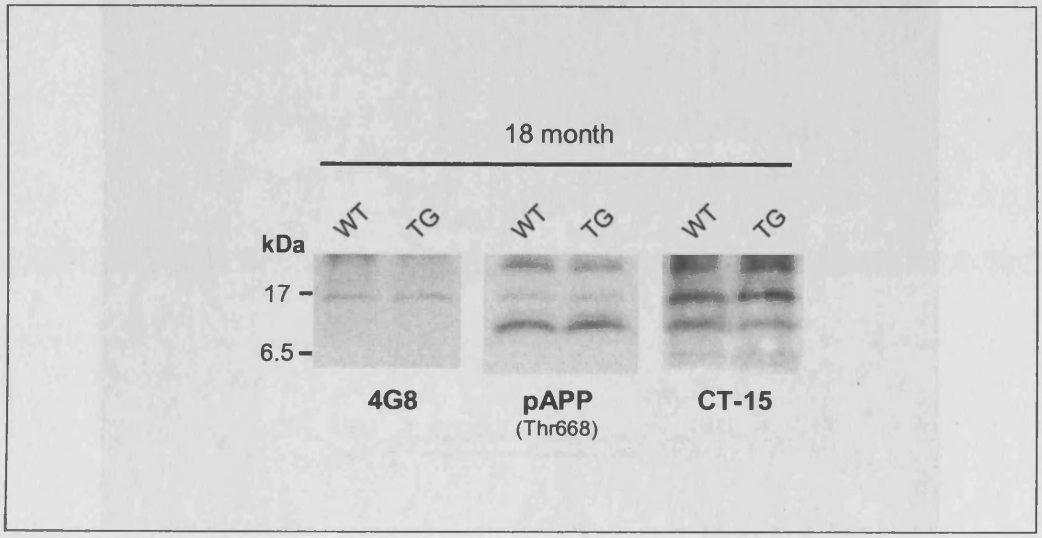

Figure 4-23. No changes in carboxy-terminal fragments of APP in TG mice. Representative immunoblots from hippocampal lysates of old TG mice and old WT controls separated on a 16% tris tricine gel. The immunoblots were probed with anti-Aβ-peptide antibody (4G8), anti-phospho-Thr668-APP antibody (pAPP) and antibody against the carboxy-terminus of APP (CT-15). None of the antibodies detected any changes in signal intensity between genotypes.

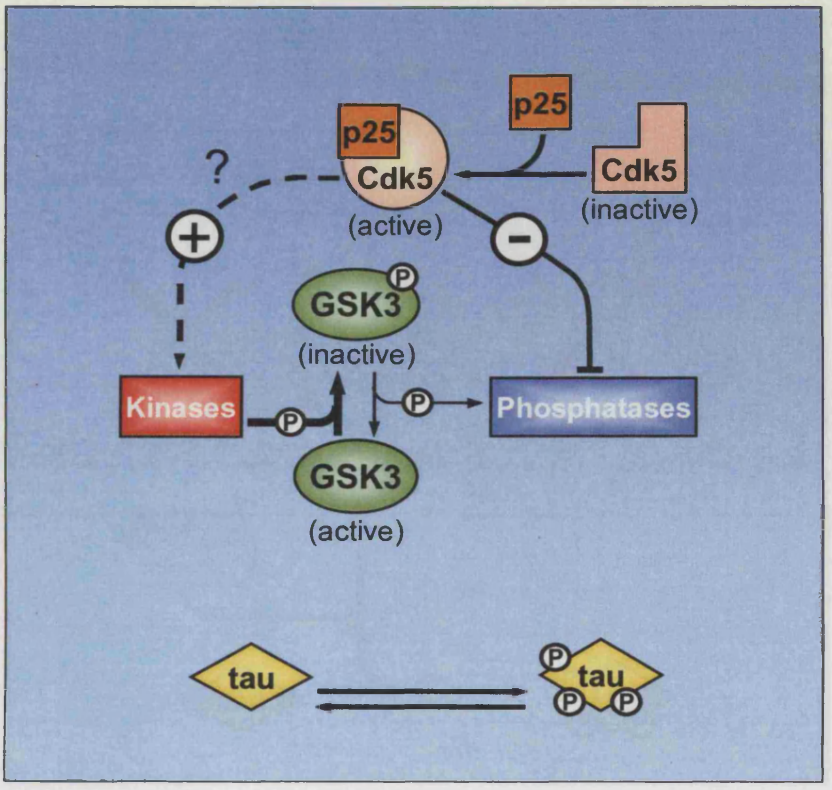

Figure 4-24. Proposed model of the inhibitory cross-talk of p25/Cdk5 and GSK3 as observed in young p25 TG mice. In 3 month-old p25 TG mice Cdk5 is over-activated by association with p25. The p25-induced Cdk5 over-activation leads to the inhibition of GSK3, possibly by indirectly regulating the phosphorylation level at the inhibitory site of GSK3. Numerous protein kinases and protein phosphatases have been directly involved in the regulation of the inhibitory phosphorylation of GSK3 (see for review Grimes & Jope, 2001). Hence, it is conceivable that Cdk5 controls GSK3 activity via some of these protein kinases and phosphatases. In line with this hypothesis, my results indicate that Cdk5 acts via protein phosphatases such as PP1 and PP2A, possibly by affecting their regulatory subunits. However, Cdk5 seems not to act via AKT and p90RSK, two kinases linked to the regulation of GSK3 activity (see for review Grimes & Jope, 2001). Interestingly, in the p25 TG mice at 3 month of age the two-fold increase in Cdk5 activity does not lead to hyperphosphorylation of tau at disease-associated epitopes. This finding suggest that Cdk5 is not directly mediating hyperphosphorylation of tau, but in fact might affect tau hyperphosphorylation by regulation of other kinases, such as GSK3. *(Taken from Plattner et al., 2006)*

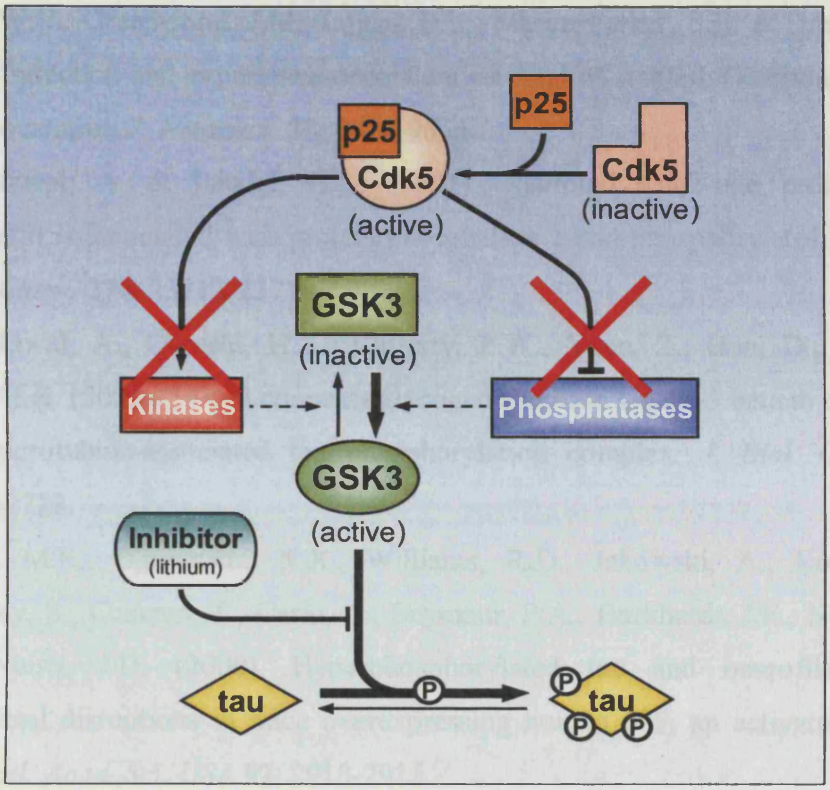

Figure 4-25. Model of the disruption of the cross-talk between p25/Cdk5 and GSK3 in old p25 TG mice. In 18 month-old p25 TG mice Cdk5 is over-activated by the expression of p25. However, the p25-induced Cdk5 over-activation does not inhibit GSK3 any longer, possibly because of the disruption of the regulatory mechanism by which Cdk5 controls GSK3. Consequently, in old TG mice GSK3 activity is increased, which correlates with elevated levels of tau hyperphosphorylation at the disease-associated epitopes AT8 and PHF-1. Pharmacological inhibition of GSK3 by chronic lithium-treatment reduces the levels of tau hyperphosphorylation at the AT8 and PHF-1 sites in old TG mice.

Together, these results suggest that GSK3 is directly hyperphosphorylating tau at the AT8 and PHF-1 sites. Furthermore, the data indicate that pharmacological GSK3 inhibitors, such as lithium, can reduce the levels of tau hyperphosphorylation at the AT8 and PHF1 sites and thus may be useful as a treatment in tauopathies including AD. *(Taken from Plattner et al., 2006)*

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APPENDIX I: Sequence alignment of tau from mouse, human and rat origin

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Aligned sequences:                    length:

   1) sp|P10637|TAU_MOUSE              732aa
   2) sp|P10636|TAU_HUMAN              757aa
   3) sp|P19332|TAU-RAT                751aa

   Please note that only upper-case letters are considered to be aligned.


   Alignment (DIALIGN format):
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sp|P10637|TAU        1   ADPRQEFDTM EDHAGD---- -------YTL LQDQEGDMDH GLKESPPQPP
sp|P10636|TAU        1   AEPRQEFEVM EDHAGtyglg drkdqggYTM HQDQEGDTDA GLKESPLQTP
sp|P19332|TAU        1   AEPRQEFDTM EDQAGD---- -------YTM LQDQEGDMDH GLKESPPQPP

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sp|P10637|TAU       40   ADDGAEEPGS ETSDAKSTPT AEDVTAPLVD ERAPDKQAAA QPHTEIPEGI
sp|P10636|TAU       51   TEDGSEEPGS ETSDAKSTPT AEDVTAPLVD EGAPGKQAAA QPHTEIPEGT
sp|P19332|TAU       40   ADDGSEEPGS ETSDAKSTPT AEDVTAPLVE ERAPDKQATA QSHTEIPEGT

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sp|P10637|TAU       90   TAEEAGIGDT PNQEDQAAGH VTQ------- ---------G RREGQAPDLG
sp|P10636|TAU      101   TAEEAGIGDT PSLEDEAAGH VTQEPESGKV VQEGFLREPG ppgls-----
sp|P19332|TAU       90   TAEEAGIGDT PNMEDQAAGH VTQEPQKVEI FSQSLLVEPG RREGQAPDSG

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sp|P10637|TAU      124   TSDWTRQQVS SMSGAPLLPQ GLREATCQPS GTRPEDIEKS HPASELLRR-
sp|P10636|TAU      146   -----HQLMS GMPGAPLLPE GPREATRQPS GTGPEDTEGG RHAPELLKhq
sp|P19332|TAU      140   ISDWTHQQVP SMSGAPLPPQ GLREATHQPL GTRPEDVERS HPASELLWQ-

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sp|P10637|TAU      173   --------GP PQKEGWGQDR LGSEEEVDED LTVDESS-QD SPPSQASLTP
sp|P10636|TAU      191   llgdlhqeGP PLKGAGGKER PGSKEEVDED RDVDESSpQD SPPSKASPAQ
sp|P19332|TAU      189   --------ES PQKEAWGKDR LGSEEEVDED ITMDESS-QE SPPSQASLAP

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sp|P10637|TAU      214   GRAAPQAGSG SVCG---ETA SVPGLPTEGS VPLPADFFSK VSAETQASQP
sp|P10636|TAU      241   DGRPPQTAAr -------EAT SIPGFPAEGA IPLPVDFLSK VSTEIPASEP
sp|P19332|TAU      230   GTATPQARSV SASGvsgETT SIPGFPAEGS IPLPADFFSK VSAETQASPP

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sp|P10637|TAU     261   EGPGTGPmEE GHEAAPEFTF HVEIKASTPK EQD-----LE GATVVGVPGE
sp|P10636|TAU     284   DGPSVGra-K GQDAPLEFTF HVEITPNVQK EQahseehLG RAAFPGAPGE
sp|P19332|TAU     280   EGPGTGPsEE GHEAAPEFTF HVEIKASAPK EQD-----LE GATVVGAPAE

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sp|P10637|TAU     306   EQKAQtqGPS VGKGTKEASL QEPPGKQPAA GLPGRPVSRV PQLKARVa--
sp|P10636|TAU     333   GPEAR--GPS LGEDTKEADL PEPSEKQPAA APRGKPVSRV PQLKARMVSK
sp|P19332|TAU     325   EQKAR--GPS VGKGTKEASL LEPTDKQPAA GLPGRPVSRV PQLKARVAGV

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sp|P10637|TAU     354   SKDRTGNDEK KAKTSTPSCA KAPSHRPCLS PTRPTLGSSD PLIKPSSPAV
sp|P10636|TAU     381   SKDGTGSDDK KAKTSTRSSA KTLKNRPCLS PKLPTPGSSD PLIQPSSPAV
sp|P19332|TAU     373   SKDRTGNDEK KAKTSTPSCA KTPSNRPCLS PTRPTPGSSD PLIKPSSPAV

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sp|P10637|TAU     404   SPEPATSPKH VSSVTPRNGS PGTKQMKLKG ADGKTGAKIA TPRGAASPAQ
sp|P10636|TAU     431   CPEPPSSPKH VSSVTSRTGS SGAKEMKLKG ADGKT--KIA TPRGAAPPGQ
sp|P19332|TAU     423   CPEPATSPKY VSSVTPRNGS PGTKQMKLKG ADGKTGAKIA TPRGAATPGQ

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sp|P10637|TAU     454   KGTSNATRIP AKTTPSPKTP PGSGEPPKSG ERSGYSSPGS PGTPGSRSRT
sp|P10636|TAU     479   KGQANATRIP AKTpPAPKTP PSSGEPPKSG DRSGYSSPGS PGTPGSRSRT
sp|P19332|TAU     473   KGTSNATRIP AKTTPSPKTP PGSGEPPKSG ERSGYSSPGS PGTPGSRSRT

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sp|P10637|TAU     504   PSLPTPPTRE PKKVAVVRTP PKSPSASKSR LQTAPVPMPD LKNVRSKIGS
sp|P10636|TAU     529   PSLPTPPTRE PKKVAVVRTP PKSPSSAKSR LQTAPVPMPD LKNVKSKIGS
sp|P19332|TAU     523   PSLPTPPTRE PKKVAVVRTP PKSPSASKSR LQTAPVPMPD LKNVRSKIGS

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sp|P10637|TAU     554   TENLKHQPGG GKVQIINKKL DLSNVQSKCG SKDNIKHVPG GGSVQIVYKP
sp|P10636|TAU     579   TENLKHQPGG GKVQIINKKL DLSNVQSKCG SKDNIKHVPG GGSVQIVYKP
sp|P19332|TAU     573   TENLKHQPGG GKVQIINKKL DLSNVQSKCG SKDNIKHVPG GGSVHIVYKP

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sp|P10637|TAU      604   VDLSKVTSKC GSLGNIHHKP GGGQVEVKSE KLDFKDRVQS KIGSLDNITH
sp|P10636|TAU      629   VDLSKVTSKC GSLGNIHHKP GGGQVEVKSE KLDFKDRVQS KIGSLDNITH
sp|P19332|TAU      623   VDLSKVTSKC GSLGNIHHKP GGGQVEVKSE KLDFKDRVQS KIGSLDNITH

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sp|P10637|TAU      654   VPGGGNKKIE THKLTFRENA KAKTDHGAEI VYKSPVVSGD TSPRHLSNVS
sp|P10636|TAU      679   VPGGGNKKIE THKLTFRENA KAKTDHGAEI VYKSPVVSGD TSPRHLSNVS
sp|P19332|TAU      673   VPGGGNKKIE THKLTFRENA KAKTDHGAEI VYKSPVVSGD TSPRHLSNVS

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sp|P10637|TAU      704   STGSIDMVDS PQLATLADEV SASLAKQGL
sp|P10636|TAU      729   STGSIDMVDS PQLATLADEV SASLAKQGL
sp|P19332|TAU      723   STGSIDMVDS PQLATLADEV SASLAKQGL

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APPENDIX II: Protocol for the use of Silver Stain Plus reagents (BioRad)

For all handling use clean containers. After use wash the containers with 50% nitric acid.

1. Rinse Gel with Milipore water and incubate for at least 20 min in fixative solution (200ml for one 20x20cm gel or two mini-gels).

Fixative Enhancer Solution

Methanol	100 ml	50% v/v
Acetic acid	20 ml	10% v/v
Fixative Enhancer		
Concentrate	20 ml	10% v/v
Millipore H2O	60 ml	30% v/v
Total	200 ml	100% v/v

In this solution the gel can be stored for longer period of time.
Therefore, the fixative step is a good stopping point.

2. Rinse Step: Decant Fixative solution and rinse gel with Millipore water for 10 min. Change water and rinse at least for another 10 min. Adjust rinse time for larger and thicker gels (see BioRad protocol).

3. Staining step: Prepare staining solution within 5 min of use (Use special 250 ml bottle). Take Development Accelerator Solution out of fridge and bring to RT.
50 ml of staining solution are enough for one 20x20cm gel or two mini-gels.

Staining Solution

Millipore H2O	18 ml
Add in following order:	
Silver Complex Solution	2.5 ml
Reduction Moderator Solution	2.5 ml
Image Develoment Reagent	2.5 ml
Immediately before use quickly add:	
Development Accelerator Solution	25 ml
	50 ml

Stain until desired staining intensity is reached. It may take at least 10 min before bands become visible.

4. After desired staining is reached, place the gel in 5 % acetic acid for a minimum of 15 min to stop the reaction. Rinse the Gel with Millipore water and photograph or dry it.

Destaining

Silver stain plus gels can be completely destained in 1% Hydrogen Peroxide.

Question?

For all further question please consult BioRad protocol.