The effects of catalase and kisspeptin overexpression on amyloid peptide toxicity

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Abstract

Amyloid β (Aβ) is the major component of the senile plaques in Alzheimer's disease (AD). The mechanism underlying cell death in AD includes oxidative stress, apoptosis, impaired mitochondrial function and receptor mediated effects. Compounds that specifically bind to Aβ are neuroprotective. Catalase is an antioxidant enzyme that specifically binds Aβ. Kisspeptin (KP) is a product of the KiSS-1 gene and contains an Aβ binding domain, which also interacts with Aβ. The localization of catalase, KP and Aβ in the AD brain plus the effects of catalase and KiSS-1 overexpression in neuronal cells were investigated in the present study.

Tissue sections from the AD pons region were immunohistochemically stained to determine if catalase or KP colocalized with Aβ deposits. The immunohistochemistry results show that immunoreactive KP or immunoreactive catalase co-localizes with immunoreactive Aβ in AD pons sections. These results suggest that endogenous catalase and KP could play neuroprotective roles in AD.

Catalase and KiSS-1 overexpressing gene models were created by stably transfecting human catalase and KiSS-1 constructs into SH-SY5Y cells and overexpression confirmed by RT-PCR, immunocytochemistry and Western blotting. The effects of Aβ and H_2O_2 on cell viability were determined by either MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) or trypan blue assay.

Catalase overexpressing SH-SY5Y neurons showed a reduced susceptibility to Aβ and H_2O_2 toxicity compared to vector control cells. The catalase overexpression neuroprotection could by reduced by the catalase activity inhibitor 3-Amino-1,2,4-triazole and an inhibitor of catalase-Aβ interactions benzothiazole aniline-tetra (ethylene glycol).

The KiSS-1 overexpressing SH-SY5Y neurons also showed a reduced susceptibility to Aβ and H_2O_2 toxicity compared to vector control cells. The KiSS-1 overexpression neuroprotection could by reduced by an anti-KP antibody, the oyxtocin antagonist atosiban and the cyclooxygenase inhibitor SC-560.

In conclusion, both catalase and KP colocalize with Aβ in AD pons sections and overexpression of catalase or KiSS-1 is neuroprotective against Aβ toxicity.

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Declaration

This thesis is a presentation of my original research work carried out during my studies for a PhD in accordance with the guidelines and regulations of the University of Westminster. The contributions of others involved, are fully cited and referenced with appropriate acknowledgement.

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List of publications:

1. Chilumuri, A., Markiv, A. & Milton, N.G.N. (2014). Immunocytochemical staining of endogenous nuclear proteins with the HIS-1 anti-poly-histidine monoclonal antibody: A potential source of error in His-tagged protein detection. *Acta Histochem.* **116** (6), 1022-8.

2. Chilumuri, A., Odell, M. & Milton, N.G.N. (2013). Benzothiazole aniline-tetra (ethylene glycol) and 3-amino-1,2,4-triazole inhibit neuroprotection against amyloid peptides by catalase overexpression in vitro. *ACS Chem. Neurosci.*, **4** (11), 1501-1512.

3. Chilumuri, A., Odell, M. & Milton, N. (2013). The neuroprotective role of catalase overexpression in SH-SY5Y cells against beta-amyloid and H2O2 toxicity. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, **9** (4 (Supplement)), P361.

4. Chilumuri, A. & Milton, N.G.N. (2013). The role of neurotransmitters in protection against amyloid-β toxicity by KiSS-1 overexpression in SH-SY5Y neurons. *ISRN Neuroscience,* **2013**, 253210.

5. Chilumuri, A., Ashioti, M., Nercessian, A.N. & Milton, N.G.N. (2013). Immunolocalization of kisspeptin associated with amyloid-ß deposits in the pons of an Alzheimer's disease patient. *J. Neurodegen. Dis.,* **2013**, 879710.

6. Milton, N.G.N., Chilumuri, A., Rocha-Ferreira, E., Nercessian, A.N. & Ashioti, M. (2012). Kisspeptin prevention of amyloid-ß peptide neurotoxicity in vitro. *ACS Chem. Neurosci.,* **3** (9), 706-719.

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Abbreviations

3-AT	3-Amino-1,2,4-triazole
ABAD	amyloid binding alcohol dehydrogenase
Aβ	Amyloid beta
ABri	Amyloid Bri
ACh	Acetylcholine
AD	Alzheimer’s disease
ADan	Amyloid Dan
ADDLs	Aβ derived diffused ligands
ADP	Anterodorsal preoptic nucleus
AGEs	Advanced glycation end-products
AICD	Amyloid precursor protein intracellular domain
AICD	APP intracellular domain
APLP1	Amyloid precursor like protein 1
APLP2	Amyloid precursor protein like protein 2
ApoE	Apo-lipoprotein E
APP	Amyloid precursor protein
APPsα	Soluble Amyloid precursor protein α fragment
APPsβ	Soluble Amyloid precursor protein β fragment
ARC	Arcurate nucleus
ASCAT	Antisense catalase 400-409 peptide
ATO	Atosiban
ATR	Atropine
AVPV	Anterioventral periventricular nucleus

Abbreviations

BACE1	Beta site APP cleaving enzyme
BCA	Bicinchoninic acid assay
BIC	1(S),9(R)-(−)-Bicuculline methiodide
BTA	Benzothiazole aniline
BTA-EG_4	Benzothiazole aniline-tetra (ethylene glycol)
BTA-EG_6	Benzothiazole aniline-hexa (ethylene glycol)
CAβBD	Catalase Aβ binding domain
ChAT	Choline acetyltransferase
CJD	Creutzfeldt-Jakob Disease
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
CYPR	Cyproheptadine hydrochloride
DS	Down syndrome
ERα	Estradiol receptor α
ERβ	Estradiol receptor β
FAD	Familial AD
FSH	Follical stimulating hormone
GnRH	Gonadotropin releasing hormone
GPR-54	G-protein coupled receptor-54 (aka kisspeptin or KiSS-1 receptor)
GSH-Px	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HAL	Haloperidol

HIF-1α	Hypoxia inducible factor – 1 alpha
HNE	4-hydroxy-2-nonenal
HPG axis	Hypothalamic-pituitary-gonadal axis
IAPP	Islet Amyloid Polypeptide (aka Amylin)
KP	Kisspeptin
KP234	Ac-[(D)-A]-NWNGFG-[(D)-W]-RF
KSO	Kissorphin
LH	Luteinizing hormone
LNMA	N^G-Methyl-L-arginine acetate salt
LRP	low-density lipoprotein receptor related proteins
LTP	Long-term potentiation
MCAT	Mitochondrial targeted catalase
MCI	Mild cognitive impairement
MEC	Mecamylamine
MET	Methysergide hydrochloride
MMP	Matrix metalloprotease
MnSOD	Manganese superoxide dismutase
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAL	Naloxone
NALTR	Naltrexone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs	Neurofibrillary tangles
NPFF	Neuropeptide FF

NPFF1	G protein coupled receptor 147 (aka NPFF receptor 1)
NPFF2	G protein coupled receptor 74 (aka NPFF receptor 2)
O_2	Oxygen
OS	Oxidative stress
PCat	SH-SY5Y neurons stably transfected with the pcDNA™4/TO/myc-His expression vector containing the human catalase-1 gene
PCatTR6	SH-SY5Y neurons stably transfected with the pcDNA™4/TO/myc-His expression vector containing the human catalase-1 gene plus pcDNA™6/TR
PD98059	2'-Amino-3'-methoxyflavone
PEN	Periventricular nucleus
PHB	Phenoxybenzamine hydrochloride
PKiSS	SH-SY5Y neurons stably transfected with the pcDNA™4/TO/myc-His expression vector containing the human KiSS-1 gene
PNPP	p-nitro phenyl phosphate
PR	Prazosin hydrochloride
PROP	Propranolol hydrochloride
PrP	Prion protein
PSEN 1	Presenilin 1
PSEN 2	Presenilin 2
PVect	SH-SY5Y neurons stably transfected with the pcDNA™4/TO/myc-His expression vector
RAGE	Receptor for advanced glycation end products
RF9	2-Adamantanecarbonyl-Arg-Phe-NH_2

ROS	Reactive oxygen species
SC-560	5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
SOD	Superoxide dismutase
SP	Senile plaques
TAMOX	Tamoxifen
TGN	Trans-Golgi network
YO	Yohimbine hydrochloride

Chapter 1

Introduction

1.1 Introduction to Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurological disorder that leads to dementia in the elderly. It has been estimated that more than 25 million people worldwide are suffering from AD, and the number is expected to triple by the year 2050 (Mayeux and Stern, 2012). There is no cure for AD and it can eventually lead to death of the patient. In 1906 a German scientist and neuropathologist Alois Alzheimer described two pathological alterations in the brain of a female patient suffering from dementia. He described the presence of a 'peculiar substance' occurring as extracellular deposits in specific regions of the patient's brain, which are now called amyloid plaques (Masters *et al.,* 1985; Mann *et al.,* 1996; Dickson 1997; Gillardon *et al.,* 2007). Not until 1980s was it discovered that the plaques found in the patient's brain consisted of aggregates of a small peptide called amyloid-β (Aβ) (Glenner and Wong 1984; Masters *et al.,* 1985). Neurofibrillary tangles (NFTs) were the second lesion described by Alzheimer. In the late 1980s it was discovered that the NFTs are composed of aggregates of tau protein, which have become abnormally hyperphosphorylated in AD (Kosik *et al.,* 1986; Grundke-Iqbal *et al.,* 1986; Ihara *et al.,* 1986; Goedert *et al.,* 1988).

1.1.1 Risk factors

Age is the single major risk factor for AD, with a 2% chance of developing AD at the age of 65 increasing to a 40% chance at the age of 85 and over (Gao *et al.,* 1998). The second major risk factor for AD is family history, with a threefold to fourfold risk among individuals having a first-degree relative with AD (Van Duijn *et al.,* 1991). Early onset of AD is rare, affecting only 5% of patients between the ages of 30-60 years (Van Duijn *et al.,* 1991). Early onset familial AD is inherited in an autosomal dominant manner, with genetic mutations on chromosomes 21, 1 and 14, resulting in the formation of abnormal amyloid precursor protein (APP), presenilin-2 (PSEN 2) and presenilin-1 (PSEN 1) precursor proteins (Wu *et al.,* 2012). Three different isoforms of apolipoprotein E (ApoE) exist in humans; they are ApoE2, ApoE3, and ApoE4. The presence of ApoE4 on chromosome 19 appears to be another major risk factor for the development of late AD (Tsai *et al.,* 1994). Inheritance of one or more copies of

ApoE4 is associated with an early and dose dependent risk of AD (Manelli *et al.,* 2007).

Loss of neurons and synaptic alterations are consistent features of AD (Cummings *et al.,* 1998). In the AD brain there is a deficit of choline acetyltransferase (ChAT), an enzyme responsible for the synthesis of acetylcholine (ACh) (Bowen *et al.*, 1976). The ACh plays an important role in learning and memory (Drachman *et al.,* 1974). Due to the loss of ACh in AD brain the "cholinergic hypothesis of AD" was proposed which states that - degeneration of cholinergic neurons in the basal forebrain and the loss of cholinergic neurotransmission in the cerebral cortex and other areas significantly contribute to the deterioration of cognitive function in AD patients (Bartus *et al.,* 1982; Francis *et al.,* 1999). Whilst AD is a multi-neurotransmitter disease the most common alteration in the AD brain is loss of cholinergic markers, ChAT, and acetyl cholinesterase (Bartus *et al.,* 1982). In addition there is also a deficit of other neurotransmitters and neurohormones such as serotonin (Palmer *et al.,* 1987), corticotropin-releasing hormone (CRH) (Bissette *et al.,* 1985), somatostatin (Davies *et al.,* 1980) and noradrenaline (Hammerschmidt *et al.,* 2013) in AD plus animal models of AD.

1.1.2 Biomarkers of Alzheimer's disease

The cerebrospinal fluid (CSF) can be a source of AD biomarkers, as the CSF is in direct contact with the brain and the molecular composition of CSF reflects the biochemical changes in the brain (Tang and Kumar, 2008). The total concentrations of tau or Aβ 1-42 in the CSF are possible biomarkers for AD diagnostics. A threefold increase in the concentration of tau and a 50% decrease in Aβ 1-42 compared to controls have been observed in the CSF of AD patients (Blennow and Hampel, 2003). Increased levels of free or conjugated ubiquitin are found in the CSF of patients with mild cognitive impairment (MCI) progressing to AD (Simonsen *et al.,* 2007). A defect in ubiquitin could impair removal of unwanted proteins via the proteasome (Lopez Salon *et al.,* 2000). Collection of CSF is an invasive procedure; therefore finding peripheral biomarkers would be of great interest. Plasma levels of Aβ 1-42 could be used as an AD biomarker, as a reduction in Aβ 1-42 was observed in patients with MCI progressing towards AD (Rembach *et al.,* 2014). Loss of

neurons in AD leads to atrophy of the brain, thus atrophy could be used as a marker for AD progression (Simonsen *et al.,* 2007). Positron emission tomography (PET) could be used to investigate amyloid deposits; multivalent microscopy can detect amyloid deposition labelled with Thioflavin S or T derivatives (Bacskai *et al.,* 2003). Near-infrared imaging is used for quantification of the cerebral amyloidosis *in vivo* (Raymond *et al.,* 2008).

1.1.3 Amyloid precursor protein

One of the major toxic agents involved in the progression of the AD is thought to be Aβ peptide (Mohamed *et al.,* 2011). The Aβ peptide is cleaved by the secretase complex from the (APP) at the neural membrane (Haass and Selkoe, 2007). In 1987 APP was discovered as the only source of Aβ (Kang *et al.,* 1987), it has been extensively studied due to its role in the generation of plaques in AD (Hardy and Selkoe, 2002). The APP gene belongs to the highly conserved family of the type-1 transmembrane proteins (Walsh *et al.,* 2007; Jacobsen *et al.,* 2009; Guo *et al.,* 2012). The APP family also includes APLP1 (amyloid precursor like protein 1) and APLP2 (amyloid precursor protein like protein 2), however neither APLP1 nor APLP2 contain the Aβ peptide sequence (Walsh *et al.,* 2007). The precise physiological function of APP is unknown but it is thought to play a role in cell adhesion (Soba *et al.,* 2005), cell motility (Chen and Yankner, 1991), neuronal survival and neurite outgrowth (Qiu *et al.,* 1995; Perez *et al.,* 1997). Transgenic mice that overexpressed wild type APP were found to have enlarged neurons (Oh *et al.,* 2009). Knockout of endogenous APP expression in mice showed age dependent deficits in passive avoidance learning, suggesting a role of APP in long-term memory (Senechal *et al.,* 2008). The APP, APLP1 and APLP2 show redundancy and knockout studies suggest that APP has some distinct physiological functions (Heber *et al.,* 2000), which could be mediated by the Aβ peptide sequence that is unique to the APP.

1.1.4 Down syndrome and Alzheimer's disease

The APP protein is coded by the APP gene located on chromosome 21 (Goldgaber *et al.,* 1987; Tanzi *et al.,* 1988). Down syndrome (DS) is the result of trisomy of chromosome 21, and is the most frequent cause of mental retardation (Yang *et al.,* 2002). In DS the APP gene is overexpressed (Wilcock and Griffin, 2013). In the brain, neuritic amyloid plaques were found in both children and adults with DS (Leverenz and Raskind, 1998). A mutation in chromosome 21 has been linked to familial AD (St George-Hyslop *et al.,* 1987) and trisomy of chromosome 21 has been linked to precocious development of AD (Wisniewski *et al.,* 1985). The finding that APP overexpression in DS is associated with the development of AD has contributed to the development of the amyloid-cascade hypothesis (Hardy and Higgins, 1992). This observation suggests that patients with DS could be a potential human group to study AD progression.

1.1.5 Amyloid precursor protein processing

The APP protein is produced in neurons and processed rapidly (Lee *et al.,* 2008). Multiple alternative pathways exist for APP processing, some of which lead to the generation of the Aβ peptide (Figure 1-1). The APP protein has three domains, the N-terminal ectodomain, a single hydrophobic transmembrane domain and a short intracellular domain (Muller and Klemens, 2013). From the endoplasmic reticulum and Golgi apparatus, APP is delivered to the axon and from there it is transported to synaptic terminals by fast axonal transport (Koo *et al.,* 1990). Crucial steps in APP processing occur at the surface of the cell and in the trans-Golgi network (TGN). From the TGN the APP is transported to the cell surface or in an endosomal compartment in clathrin-associated vesicles (O'Brien and Wong, 2011). Under normal conditions, at the surface of the cell the APP is processed directly by α-secretase followed by γ-secretase. This "non amyloidogenic" pathway precludes the generation of toxic Aβ peptide and leads to the formation of soluble APPα (APPsα) and αCTF (C-terminal fragment) upon cleavage by α-secretase. αCTF is further cleaved by γ-secretase to produce the P3 peptide and APP intracellular domain (AICD) (Tian *et al.,* 2010). The P3 peptide is an N-terminal truncated Aβ peptide corresponding to Aβ 17-40 and

Aβ 17-42, its molecular weight is approximately 3 kDa and thus the name P3 (Dulin *et al.,* 2008). The importance of P3 peptide in AD is still unclear, it has been suggested that P3 peptide represents a benign form of amyloid (Dickson 1997).

In AD, the APP is sequentially cleaved by β and γ-secretase to generate Aβ peptide, which plays a causative role in the pathogenesis of AD (Hardy and Allsop, 1991). Beta site APP cleaving enzyme (BACE1) or β secretase is an enzyme encoded by the BACE 1 gene (Vassar 2004). The γ-secretase complex contains either PSEN 1 or PSEN 2 in complex with supporting proteins (Smolarkiewicz *et al.,* 2013). This cleavage by β and γ-secretase generates soluble APPβ (APPsβ) and the C99 fragment (membrane bound C-terminal 99 amino acids of the APP or CTF). It has been suggested that α-secretase competes with β-secretase for the APP substrate to reduce Aβ production (Nitsch *et al.,* 1992). Following β-secretase cleavage C99 becomes a substrate for γ-secretase, which cleaves C99 to generate carboxy terminus Aβ and this peptide is released from the cell (Vassar 2004). The cleavage by γ-secretase is not precise and produces a range of Aβ peptides, which vary in length; the most common forms are Aβ 1-40 and Aβ 1-42 (Fukumoto *et al.,* 1996). The α-CTF produced by α-secretase cleavage of APP has been demonstrated to function as a γ-secretase inhibitor for Aβ production (Tian *et al.,* 2010).

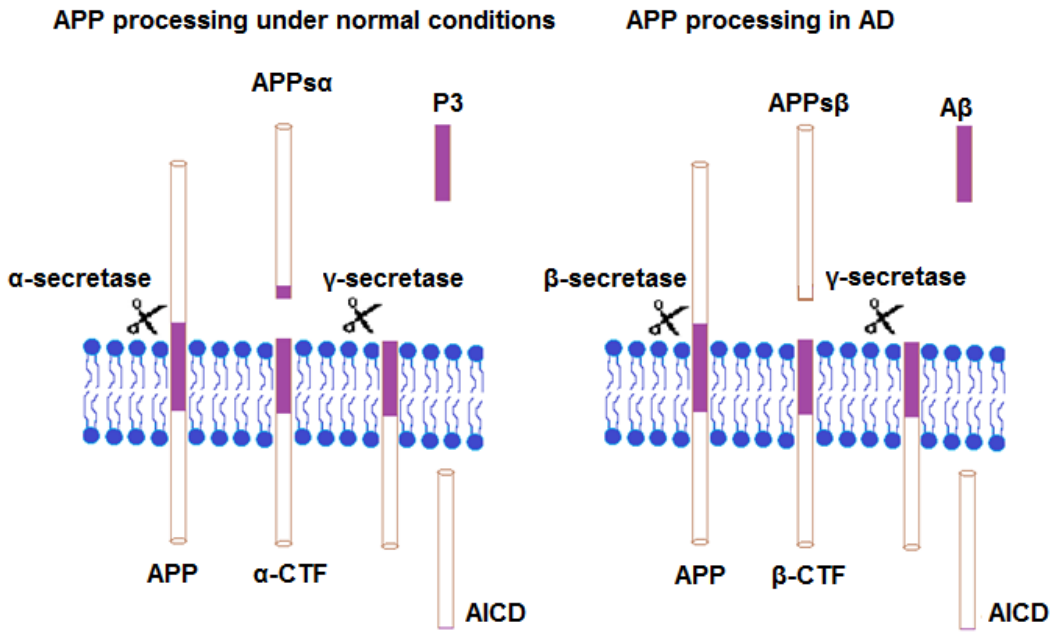

Figure 1-1. Amyloid precursor protein processing. Under normal conditions the APP is cleaved by α- secretase to generate APPsα (soluble APPα ectodomain) and α-CTF (C-terminal fragment). The α-CTF is further cleaved by γ-secretase to generate P3 peptide and AICD (APP intracellular domain). In AD the APP is cleaved by β-secretase to generate APPsβ (soluble APPβ ectodomain) and β-CTF (C-terminal fragment). The β-CTF is further cleaved by γ-secretase to generate Aβ and AICD.

The APP processing could be caspase dependent resulting in the generation of two novel proteolytic fragments which are 25 and 35 kDa in size. These two fragments can be detected by antibodies against Aβ and the c-terminal caspase site (Fiorelli *et al.,* 2013), suggesting a link between APP processing and apoptosis. Both Aβ 1-40 and Aβ 1-42 have been identified in amyloid plaques in AD brain, but Aβ 1-42 is the predominant form and the initially deposited species in plaques, the Aβ 1-40 appears later in the plaque evolution and maturation (Mann *et al.,* 1996). The Aβ 1-42 is particularly important, as it is more prone to oligomerization, forming amyloid fibrils more readily than the shorter forms of Aβ (Burdick *et al.,* 1992). *In vitro* studies have shown that Aβ can easily form fibrils, which could be associated with the mature fibrils found in the neuritic plaques. These plaques can be correlated to the extent of cognitive loss in AD (Cummings *et al.,* 1996). Other forms of Aβ include modified Aβ with a pyroglutamate at amino acid position 3 (Aβ pE3-42) (Wittnam *et al.,* 2012) and Aβ (11-42), which is generated by the β cleavage of APP at position 11 of the amyloid sequence (Willem *et al.,* 2004). The present study will focus only on the major forms of Aβ (Aβ 1-40, Aβ 1-42).

1.1.6 Amyloid cascade hypothesis

The amyloid cascade hypothesis (Figure 1-2) was originally proposed by John Hardy and David Allsop in 1991. According to the amyloid cascade hypothesis, mismetabolism of APP initiates AD pathogenesis, subsequently leading to the aggregation of Aβ (especially Aβ 1-42). Formation of neuritic plaques further leads to the formation of NFTs and synaptic dysfunction. Synaptic dysfunction results in decreased neurotransmitters, ultimately leading to the death of the tangle bearing neurons. Loss of neurons causes dementia, which is the first symptom of AD (Hardy and Allsop 1991). The amyloid cascade theory was supported by the discovery of a mutation in the APP gene that causes

autosomal dominant AD (Goate *et al.,* 1991). This hypothesis was further supported by evidence of autosomal dominant mutations in PSEN 1 and PSEN 2 that can also cause AD (Rogaev *et al.,* 1995), this was observed in families with early onset of AD (Familial AD). Presenilins are multi-pass transmembrane proteins that function as a part of the γ-secretase intra-membrane protease (Zhang *et al.,* 2013). Mutations in ApoE are genetically associated with late onset familial AD (Corder *et al.,* 1993). The presence of Aβ in senile plaques (SP) was interpreted as an effect of these mutations that eventually leads to cell death and dementia. It is still unclear whether the most deleterious form of Aβ peptides in the early stages of AD is represented by deposited fibrillar or soluble oligomeric peptide (Drouet *et al.,* 2000).

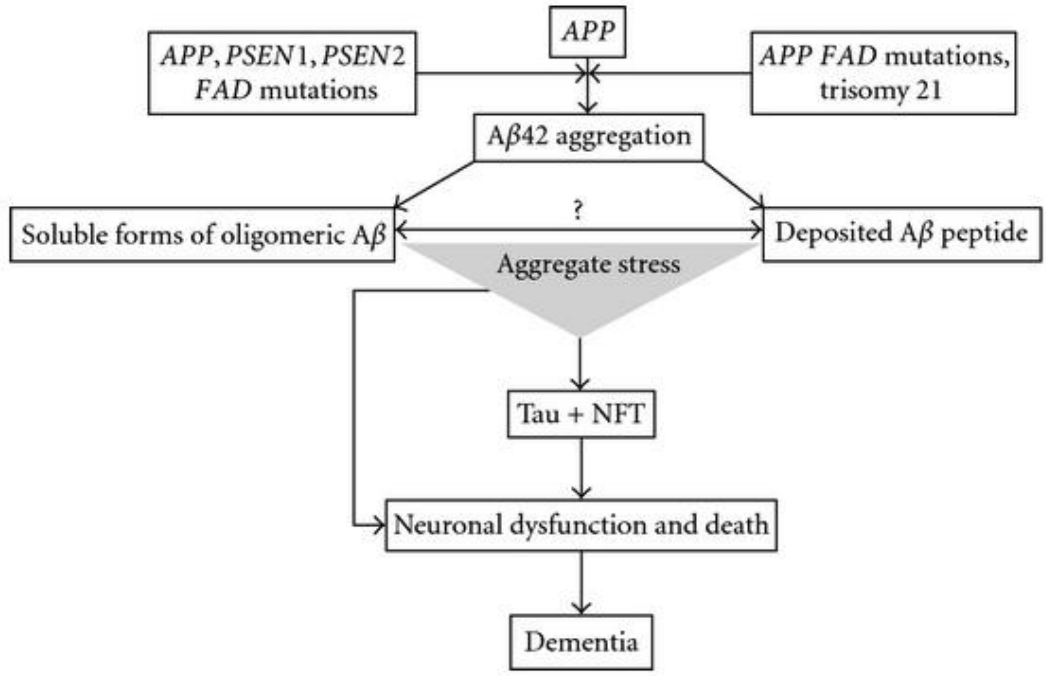

Figure 1-2. Amyloid cascade hypothesis. The flow chart represents the amyloid cascade hypothesis, which suggests that mutations in the APP, PSEN1, PSEN 2 and ApoE (FAD mutations) lead to aggregation of Aβ. Aggregated Aβ (soluble or fibrillar) instigates the formation of NFT and disruption of synaptic connections, leading to neuronal dysfunction and cell death. Loss of neurons ultimately leads to dementia (Reitz 2012).

1.1.7 Limitations of the amyloid cascade hypothesis

The amyloid cascade theory does not explain the interactions between Aβ and tau. The theory suggests that amyloid plaques precede NFTs; the mechanism for this is not explained. Mutation in tau gene can cause autosomal dominant frontotemporal lobe dementia (Hutton *et al.,* 1998). The tau pathology seen in autosomal dominant frontotemporal lobe dementia is similar to the tau pathology seen in AD, suggesting that amyloid plaques are not required for the development of AD and that tau pathology can itself cause neuronal loss. In APP transgenic mice, reduction in endogenous tau levels improved behavioural and other deficits that are mediated by Aβ (Roberson *et al.,* 2007). The theory also suggests that SP and NFTs are the cause of the disease, but SP and NFTs could just be the product of the neurodegeneration rather than the cause (Karran *et al.,* 2011). Apart from Aβ the plaques also contain metal ions (Tiiman *et al.,* 2013), glycosaminoglycans (Ariga *et al.,* 2010), the serum amyloid P component (Pepys *et al.,* 1994), ApoE (Tsai *et al.,* 1994) and collagen (Perlmutter *et al.,* 1991), which could contribute to plaque formation. It has been suggested that the contradictions to the amyloid cascade theory could be removed by including redox-active metals in plaques as ions such as zinc, which can initiate Aβ fibrillization (Tiiman *et al.,* 2013).

1.1.8 Normal function of Amyloid-β.

In primary culture neurons, inhibition of endogenous Aβ or immunodepletion of Aβ caused neuronal cell death (Plant *et al.,* 2003), suggesting that Aβ has a key physiological role in survival of neurons. Endogenous Aβ production participates in a negative feedback that would keep the neuronal hyperactivity in check (Kamenetz *et al.,* 2003). Mice lacking the APP gene showed impairment in long-term potentiation (LTP) and the neurons showed reduced branching of dendrites and fewer synaptic boutons, there was no reduction in neuronal number despite the absence of Aβ (Dawson *et al.,* 1999). These observations suggest that Aβ in moderation may play an important role in maintaining synaptic and cognitive function.

1.1.9 How Amyloid-β is neurotoxic?

The Aβ is stacked as parallel β sheets in mature amyloid plaques (Petkova *et al.,* 2006). Soluble Aβ aggregates ranging from small oligomers (Kayed *et al.,* 2003) to large assemblies called Aβ derived diffused ligands (ADDLs) (Lambert *et al.,* 1998) or protofibrils constitute the plaques. The soluble form of Aβ was found to be more potent in toxicity than the fibrillar form of Aβ (Walsh *et al.,* 2002). The extracellular Aβ from the plaques can be taken up by the cells via low-density lipoprotein receptor related proteins (LRP), which facilitate the endocytosis of Aβ by direct binding or via the LRP ligand ApoE (Bu *et al.,* 2006) or the receptor for advanced glycation end products (RAGE) (Deane *et al.,* 2003). These receptors interact with Aβ and transport it across the blood brain barrier (Deane *et al.,* 2003). The exogenous Aβ also binds to the α7 nicotinic ACh receptor and the cell internalizes the peptide, which results in accumulation of intracellular Aβ (Nagele *et al.,* 2002).

Several lines of evidence show that Aβ is generated intracellularly in AD, with the toxicity of intracellular Aβ preceding the toxicity of senile plaques and neurofibrillary tangles (Gouras *et al.,* 2000). Intracellular Aβ 1-42 can elicit synaptic toxicity similar to amyloid-plaques, even in the absence of amyloid plaques (Mucke *et al.,* 2000). Intracellular accumulation of Aβ is seen in mitochondria, ER, Golgi and cytosol (LaFerla *et al.,* 2007). The impairment of the mitochondrial metabolism in AD has been well documented (Hirai *et al.,* 2001). Progressive accumulation of Aβ in mitochondria leads to impairment of the electron transport chain, ultimately leading to mitochondrial dysfunction and increased oxidative stress (OS) (Bozner *et al.,* 1997; Muirhead *et al.,* 2010). Mitochondrial dysfunction can result in increased production of reactive oxygen species (ROS) and decreased ATP production (Kirkinezos and Moraes, 2001). An increase in OS leads to lipid peroxidation, protein and DNA oxidation (Markesbery 1997). Increased production of ROS can eventually lead to cell death. Neuronal death induced by Aβ displays the characteristics of apoptosis (Loo *et al.,* 1993). Aβ activates caspases, the effector of apoptosis in neuronal cells, and caspase inhibitors can block Aβ induced cell toxicity in synaptosomes (Mattson *et al.,* 1998). The physiological level of Aβ modulates synaptic plasticity and memory (Puzzo *et al.,* 2008) together with activity dependent

regulation of synaptic vesicle release (Abramov *et al.,* 2009). Abnormal accumulation of Aβ can interfere with synaptic transmission or LTP in the hippocampus *in vivo* (Selkoe 2008).

It has been shown that the Aβ peptide is directly incorporated into neuronal membranes and forms cation-sensitive ion channels also known as amyloid channels (Kawahara 2010). Formation of the amyloid channels increases the intracellular concentration of calcium, which could be the primary event for neurodegeneration. Similar channel formation was also seen with the islet amyloid polypeptide (IAPP) and Prion protein (PrP) in a gonadotropin-releasing hormone (GnRH) secreting neuronal cell line (Kawahara *et al.,* 2000). The Aβ interacts with PrP (Laurén *et al.,* 2009; Gunther *et al.,* 2010), and IAPP receptors (Fu *et al.,* 2012) triggering multiple signalling pathways leading to neurodegeneration.

1.1.10 Other neurotoxic amyloid peptides

1.1.10.1 Islet amyloid polypeptide

Type 2 diabetes mellitus (T2DM) is a progressive disease with 90% of the subjects exhibiting islet amyloid deposits (Hull *et al.,* 2004). Islets amyloid deposition involves aggregation of the normal β-cell peptide, IAPP, which is also known as amylin. This contributes to the deterioration of β cell function and reduced β cell mass observed in T2DM (Hull *et al.,* 2004). IAPP is co-secreted with insulin (Kahn *et al.,* 1990). Factors such as insulin resistance that cause insulin secretion can also elevate IAPP secretion (Clark *et al.,* 1990). Both IAPP and Aβ share common features of disease progression, such as increased OS and mitochondrial dysfunction (Gotz *et al.,* 2009). The IAPP peptide, similar to Aβ can induce apoptotic cell death (Hiddinga and Eberhardt, 1999); they both cause a dose, time and cell specific neurotoxicity, supporting the notion that they cause toxicity in similar fashion (Lim *et al.,* 2008).

1.1.10.2 Prion protein

The PrPs are a group of proteins with unique ability to fold into different conformations. The normal prion PrP^{c} (cellular PrP) predominantly consists of two large alpha-helices, whereas the disease causing infectious form PrP^{Sc} (isoform PrP) consists of beta-pleated sheets (Norrby *et al.,* 2011), similar to Aβ. The infectious form is the causative agent of the transmissible spongiform encephalopathies including Creutzfeldt- Jacob disease (CJD) (Aguzzi *et al.,* 2008). A number of neuropathological similarities and genetic links exist between AD and CJD. The coexistence of AD pathology in CJD has been reported (Hainfellner *et al.,* 1998) in CJD patients with AD like pathology. A previous study has shown up regulation of PrP^{c} in neurodegeneration and has suggested that it could be involved in defence against OS (Voigtlander *et al.,* 2001). PrP^{c} has been shown to promote Aβ plaque formation in mice (Schwarze-Eicker *et al.,* 2005) and play a role in the cellular uptake of Aβ (Laurén *et al.,* 2009), suggesting that PrP not only plays a role in CJD but also in AD pathogenesis.

1.1.11 Similarity between amyloid peptide sequences

The Aβ, amyloid-Bri (ABri), amyloid-Dan (ADan), PrP and IAPP, play a key role in the pathology of AD (Buxbaum and Linke, 2012), Familial British dementia (Tsachaki *et al.,* 2010), Familial Danish Dementia (Gibson *et al.,* 2004), CJD (Norrby 2011) and T2DM (Zraika *et al.,* 2010). The Aβ 25-35, IAPP 20-30, PrP 115-125, ABri-11-21 and ADan 11-21 share sequence similarity (Figure 1-3) suggesting that they may have similar mechanisms of fibril formation plus neurotoxicity. Compounds that can inhibit Aβ toxicity could therefore also be useful against neurotoxicity of other amyloid peptides. All the amyloid peptides except IAPP are produced in the brain; IAPP is produced in the pancreatic islet-β cells (Serup *et al.,* 1996). A recent study has shown the deposition of IAPP in the brain of diabetic patients (Jackson *et al.,* 2013), suggesting a need for neuroprotection in the treatment of T2DM.

Aβ (25-35)	**G**	**S**	**N**	**K**	**G**	**A**	**I**	**I**	**G**	**L**	**M**
IAPP (20-30)	S	N	**N**	F	**G**	**A**	**I**	**L**	S	S	T
PrP (115-125)	A	A	A	A	**G**	**A**	**V**	**V**	**G**	G	**L**
ABri (11-21)	F	E	**N**	**K**	F	**A**	**V**	E	T	**L**	**I**
ADan (11-21)	F	E	**N**	**K**	F	**A**	**V**	E	T	**L**	**I**

Figure 1-3. The sequence similarity between Aβ 25-35 and different amyloid peptides. The amino acid sequences of Aβ 25-35, IAPP 20-30, PrP 115-125, ABri-11-21 and ADan 11-21. Amino acids identical to those found in Aβ 25-35 are shown in red, whilst those with similarity are shown in blue. The purple box represents the core region of similarity.

1.2 Oxidative stress in Alzheimer's disease

One of the most well known effects of Aβ is its capacity to induce and be induced by OS (Borghi *et al.,* 2007); Aβ induces OS *in vitro* and *in vivo* (Tamagno *et al.,* 2012). OS is believed to be an early event in AD, as extensive oxidative damage is observed in MCI, which is considered as a transition stage between normal aging and dementia (Lovell and Markesbery, 2001). The membrane damage, cytoskeleton derangement and cell death due to OS suggests that OS plays an important role in AD progression (Perry *et al.,* 2000). The ROS are by-products of cellular metabolism, generated during mitochondrial oxidative phosphorylation and resulting in molecules such as hydrogen peroxide (H_2O_2) or molecules with unpaired electrons such as superoxide. The brain is highly vulnerable to OS because of its high consumption of oxygen, high levels of polyunsaturated fatty acids and low levels of antioxidants (Floyd and Hensley, 2002). The free radical hypothesis of aging postulates that accumulation of ROS in the cell damages mitochondria, the nucleus, cell membranes and cytoplasmic proteins (Harman 1992).

1.2.1 Amyloid-β a major link between oxidative stress and Alzheimer's disease

In a murine knock-in model of AD with APP and PS1 mutations, excessive production of Aβ and impairment of antioxidant enzymes with mitochondrial dysfunction was reported (Anantharaman *et al.,* 2006). The brain is rich in polyunsaturated fats; Aβ promotes copper mediated generation of 4-hydroxy-2-nonenal (HNE) from polyunsaturated lipids. HNE is produced from lipid extracts by oxidative lipid damage; HNE covalently modifies the histidine side chain (residues 6, 13 and 14) of Aβ. This modified Aβ has increased affinity for lipid membranes and an increased tendency to aggregate into amyloid fibrils (Murray *et al.,* 2007). This suggests that products of OS such as HNE modify Aβ and increase Aβ misfolding. In addition Aβ can also induce the production of inflammatory mediators such as tumor necrosis factor-α or interleukin-1β, leading to microglia activation, OS induction, and enhanced processing of APP to generate more Aβ (Akiyama *et al.,* 2000).

The expression and activity of the APP cleaving enzyme BACE1 is increased by OS (Tamagno *et al.,* 2002). Increased activity of BACE1 causes overproduction of Aβ and the neurons show morphological signs of apoptotic cell death (Tamagno *et al.,* 2005). OS also increases the activity of γ secretase *in vivo* and the γ secretase regulates expression of BACE1 (Tamagno *et al.,* 2008). This suggests that OS increases the activity and expression of the key secretase enzymes involved in processing APP to Aβ peptides, which results in overproduction of Aβ.

1.2.2 Other risk factors

It has been previously proposed that hypoxia-inducible factor 1α-mediated hypoxia can alter APP processing by increasing the activity of β secretase and γ secretase. This increase in the enzyme activity facilitates increased Aβ production (Zhang *et al.,* 2007). Hyperglycaemia leads to the formation of advanced glycation end-products (AGEs) (Takeuchi and Makita, 2001). The AGEs are involved in the production of ROS, especially superoxide and H_2O_2 (Carubelli *et al.,* 1995). The AGEs also mediate OS production by interacting with RAGE, AGEs can increase BACE1 expression by binding with RAGE,

which is followed by strong production of ROS and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, the NF-κB transcriptional factor can be activated by RAGE (Granic *et al.,* 2009). The NF-κB regulates expression of BACE1, increased BACE1 and ROS leads to Aβ production and accumulation (Bourne *et al.,* 2007). Oxidation products of cholesterol such as oxysterol enhance Aβ production in neuronal cells (Gamba *et al.,* 2011). The sterol regulatory element binding protein 2 transcription factor regulates BACE1 expression, suggesting a role of cholesterol in the pathogenesis of AD (Mastrocola *et al.,* 2011). These results point towards the role of ROS in the regulation of Aβ and aging processes (Figure 1-4).

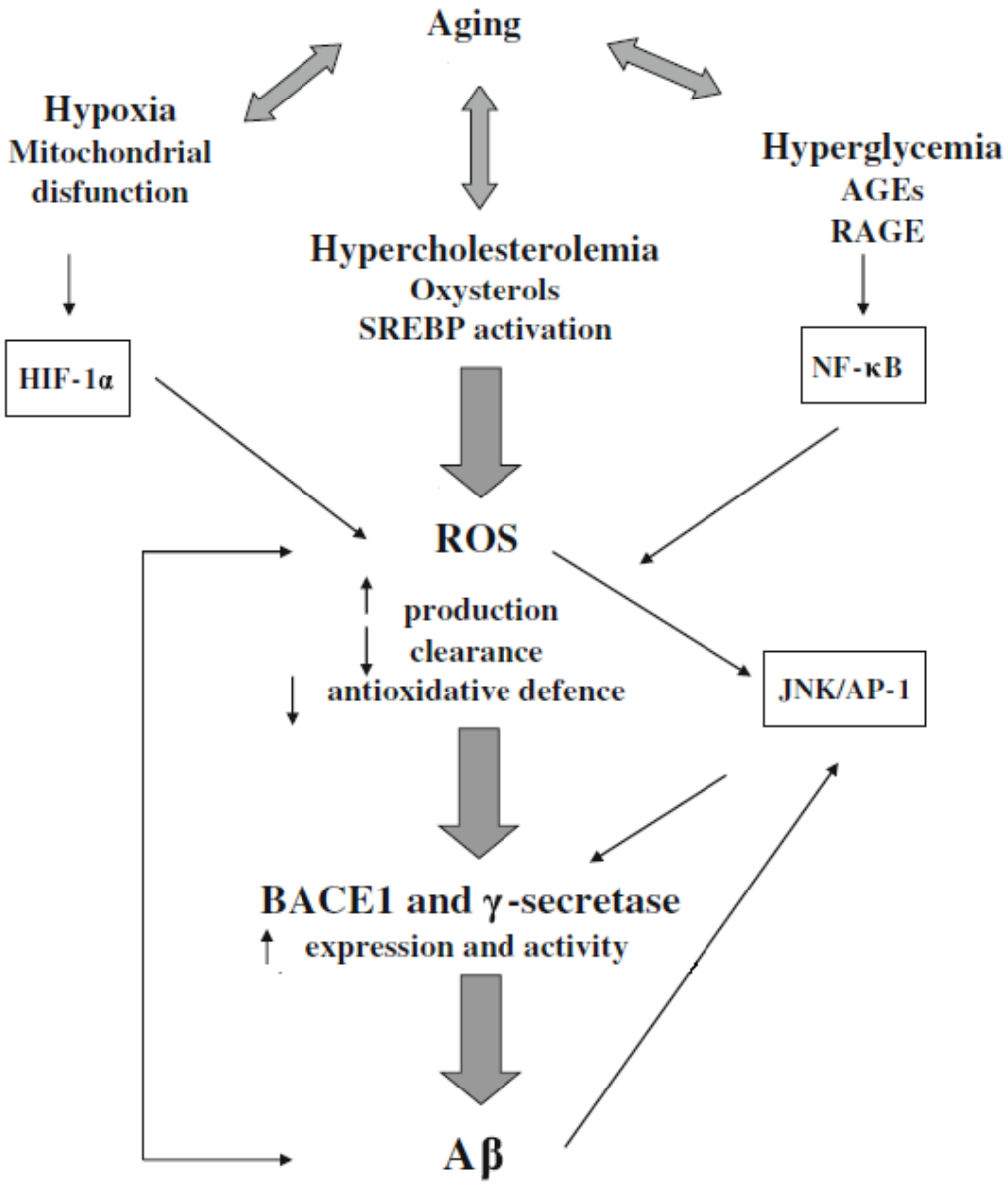

Figure 1-4. Risk factors for the generation of ROS. The flow chart represents the different age related risk factors such as hypoxia, hyperglycaemia and hypercholesterolemia that contribute to the generation of ROS, which leads to increased expression and activity of BACE1 and γ secretase, the end result being increased production and accumulation of Aβ (Tamagno *et al.,* 2012).

1.2.3 Amyloid-β induction of oxidative stress

Aggregation of Aβ leads to the generation of free radicals in AD (Hensley *et al.,* 1994). The Aβ causes increased accumulation of H_2O_2 in cells, which results in free radical induced lipid peroxidation and ultimately cell death (Behl *et al.,* 1994). An inhibitor of neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, diphenylene iodonium (Cross and Jones, 1991) inhibits Aβ in B12 cells and CNS neurons; it also inhibited H_2O_2 production in B12 cells (Kopp and Stanton 1997). Another NADPH oxidase inhibitor neopterin (Kojima *et al.,* 1993) also inhibited Aβ toxicity, suggesting that Aβ generation of H_2O_2 requires the activation of NADPH linked oxidase (Behl *et al.,* 1994).

1.2.4 Oxidative stress and mitochondrial dysfunction

Mitochondria are unique cell organelles and perform key functions in ATP synthesis, calcium homeostasis and also play a major role in cell survival plus cell death. The mitochondrial respiratory chain is the major site for the generation of ROS in cells; mitochondria are also vulnerable to OS (Tan *et al.,* 1998; Grivennikova and Vinogradov, 2006). Mitochondrial dysfunction seems to play an important role in the pathogenesis of AD, as a number of mitochondrial and metabolic abnormalities have been identified in hippocampal neurons of AD compared to controls (Mutisya *et al.,* 1994; Hirai *et al.,* 2001). The mitochondrial abnormalities were also seen in neurons without neurofibrillary tangles, suggesting that mitochondrial abnormalities are an early event in AD (Hirai *et al.,* 2001). A significant reduction in mitochondria, an increase in the mitochondrial DNA and proteins in the cytoplasm and in the vacuoles associated with lipofuscin (a lysosome suggested as the site of mitochondrial degradation by autophagy) were observed in AD brains (Hirai *et al.,* 2001; Zhu *et al.,* 2006). A significant decrease in the mitochondrial cytochrome oxidase

(complex IV) activity in the cortical regions of AD brains was also observed (Mutisya *et al.,* 1994). A decrease in the electron transport enzymes could lead to increase in ROS and reduction in energy stores, contributing to neurodegeneration.

The Aβ peptide was found localized in the mitochondria in the brains of AD patients (Devi *et al.,* 2006; Manczak *et al.,* 2006). The Aβ has been reported to interact with mitochondrial proteins such as amyloid binding alcohol dehydrogenase (ABAD), which have been implicated in AD (Yan *et al.,* 1997; Milton *et al.,* 2001; Muirhead *et al.,* 2010). The ABAD catalyzes the reduction of ketones, aldehydes and oxidation of alcohols. Interaction of Aβ and ABAD inhibits the activity of ABAD. Transgenic mice and neuroblastoma cells stably transfected with human mutant APP, led to an increase in OS and reduced mitochondrial activity (Manckzak *et al.,* 2006). Treatment of isolated mitochondria with Aβ caused oxidative injury to the mitochondrial membrane and inhibited key enzymes of mitochondrial respiratory chain leading to an increase in mitochondrial membrane permeability and cytochrome c release (Casley *et al.,* 2002). Cytochrome c when released in the cytoplasm can activate the apoptotic pathway. Manganese superoxide dismutase (MnSOD) is the primary antioxidant enzyme, which protects mitochondria against superoxide. In a double homozygous knock-in mouse model expressing APP and PS-1 mutants, MnSOD was found to be a target of nitration and inactivation (Anantharaman *et al.,* 2006). Decreased activity of MnSOD further increases ROS levels and leads to loss of mitochondrial membrane potential, caspase activation and apoptosis (Anantharaman *et al.,* 2006). These results all point towards the central role of mitochondrial dysfunction in Aβ toxicity (Figure 1-5).

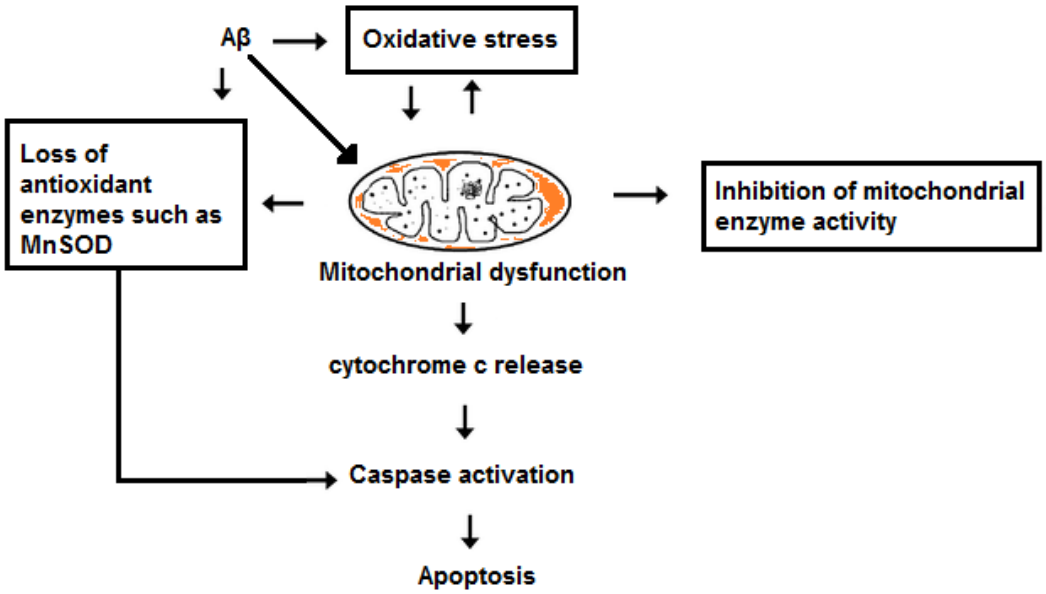

Figure 1-5. The schematic representation of oxidative stress mediated mitochondrial dysfunction and cell death. Oxidative stress (OS) leads to mitochondrial dysfunction, increased Aβ accumulation and reduction in antioxidant enzymes. Increased OS disrupts mitochondrial membrane potential, which leads to release of cytochrome c into the cytoplasm; cytochrome c activates caspase resulting in apoptosis.

1.2.5 Antioxidant enzymes protect against oxidative stress and Amyloid-β toxicity.

Mitochondrial targeted antioxidants were found to be protective against OS and Aβ toxicity (Manczak *et al.,* 2010; Ma *et al.,* 2011). In a recent study, the role of mitochondria-targeted catalase enzyme (MCAT) in life span extension of human APP transgenic mice was investigated. It was found that the APP transgenic mice that carried the human MCAT gene lived 5 months longer than APP mice without the human MCAT gene. Results showed that MCAT prevents abnormal APP processing, reduces Aβ levels and enhances Aβ-degrading enzymes in mice (Mao *et al.,* 2012).

The free radical antioxidant vitamin E protected PC12 cells from Aβ toxicity (Behl *et al.,* 1992). The glutathione peroxidase (GSH-Px) reduces free H_2O_2 to water and superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and H_2O_2. Cell lines genetically engineered to express higher levels

of antioxidant enzymes such as catalase and GSH-Px were found to be resistant to Aβ toxicity, suggesting that Aβ toxicity in part is mediated via H_2O_2 (Sagara *et al.,* 1996). In PC12 cells resistant to Aβ, the mRNA protein levels plus activity of catalase and GSH-Px but not SOD were found to be elevated. The increase in the activity of the catalase and GSH-Px correlate with increased resistance of the cells to peroxide and Aβ toxicity (Sagara *et al.,* 1996).

The Aβ peptide induces H_2O_2 production (Behl *et al.,* 1994), which is one of the major ROS. Both superoxide (Turrens 2003) and H_2O_2 (Cadenas and Davis, 2000) are produced in the mitochondria. Inhibitors of enzymes that are involved in the production of superoxide did not affect Aβ toxicity in primary neuronal cell culture (Behl *et al.,* 1994). The levels of superoxide were not substantially elevated upon exposure to Aβ (Zhang *et al.,* 1996). This suggests that superoxide may not be a major contributor to Aβ toxicity. On the other hand Aβ induced cellular increase of H_2O_2 has been strongly correlated to Aβ toxicity (Behl *et al.,* 1994).

The H_2O_2 is an uncharged molecule, one of the more stable ROS and freely diffusible both between and within the cell. The H_2O_2 can be generated by dismutation of the superoxide anion by SOD (Fridovich 1997) or by direct action of enzymes such as monoamine dismutase and NADPH oxidase (Halliwell 2001). The NADPH oxidase inhibitors can also inhibit Aβ toxicity, suggesting that Aβ increases H_2O_2 production via NADPH oxidase (Behl *et al.,* 1994). *In vivo* studies have shown that Aβ induces an increase in the activity of H_2O_2 generating enzyme MnSOD, and decreases H_2O_2 consuming enzymes catalase and glutathione peroxidase in rat brain neocortex mitochondria (Kaminsky *et al.,* 2008). This suggests that increased levels of H_2O_2 could be due to reduced antioxidant enzyme activities or levels.

Catalase and GSH-Px degrade H_2O_2 in cells both inside and outside mitochondria (Halliwell and Gutteridge, 2007). The activity of both the enzymes was increased in cells resistant to Aβ toxicity, and cells with increased expression of catalase and GSH-Px were resistant to Aβ toxicity (Sagara *et al.,* 1996). Addition of catalase to the extracellular environment of cultured cells was found to be protecting against Aβ toxicity (Behl *et al.,* 1994; Zhang *et al.,* 1996). Catalase was found to be physically associated with senile plaques in human

brain sections from AD patients (Pappolla *et al.,* 1992; Lovell *et al.,* 1995). The H_2O_2 readily diffuses across the cell membrane; the catalase enzyme converts H_2O_2 to H_2O and O_2. The cytoprotective effect of catalase is due to the reduction of H_2O_2 both inside and outside the cells (Halliwell and Gutteridge, 2007). Catalase directly binds Aβ in a cell free assay (Milton 1999), whereas GSH-Px does not bind Aβ (Habib *et al.,* 2010). The binding of catalase to Aβ results in the deactivation of the H_2O_2 degrading activity of catalase (Milton 1999).

1.2.6 Catalase activity and its structure

Catalase is an enzyme found in all living organisms that are exposed to oxygen. Catalase catalyzes the breakdown of two molecules of H_2O_2 to water and oxygen (Chelikani *et al.,* 2004). One molecule of catalase can convert 40 million of H_2O_2 molecules to water and oxygen per second. Human catalase is a tetramer with four polypeptide chains each 500 amino acid long. It contains four porphyrin haem groups that help in converting H_2O_2 to water and oxygen (Chelikani *et al.,* 2004).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase + Aβ}} \text{Oxidative stress}$$

Catalase is found in the peroxisome, cytosol, mitochondria and other cellular organelles (Habib *et al.,* 2010). Catalase binds Aβ with the interaction between catalase and Aβ involving the 25-35 region of Aβ and a specific domain in the wrapping loop of the catalase protein (residues 401-410) (Milton *et al.,* 2001; Rensink *et al.,* 2002). The region of catalase that interacts with Aβ is referred to as catalase Aβ binding domain (CAβBD).

1.2.7 Catalase binding to different amyloid peptides

The Aβ 31-35 fragment is the smallest fragment that inhibits the H_2O_2 degrading activity of catalase (Milton 1999). Catalase can also bind the fibrillar Aβ containing residues 29-32, this binding can be inhibited by the Aβ 31-35 fragment, suggesting an important role for the Ile residues at 31 and 32 of the Aβ peptide (Milton and Harris, 2009). The region of Aβ (25-35) that catalase binds to is a Gly-Ala-Ile-Ile sequence that is similar to the Gly-Ala-Ile-Leu sequence found in human IAPP residues 24-27, PrP fibrils with a Gly-Ala-Val-Val sequence also binds catalase (Milton and Harris 2010). These results suggest that catalase recognizes a Gly-Ala-Ile-Leu–like sequence in amyloid fibril-forming peptides and binds not only to Aβ but also other amyloid peptides such as IAPP and PrP (Figure 1-6). This sequence similarity suggests that catalase can bind to IAPP, Aβ and PrP, which could be a target for anti-amyloid therapeutic development (Milton and Harris, 2010).

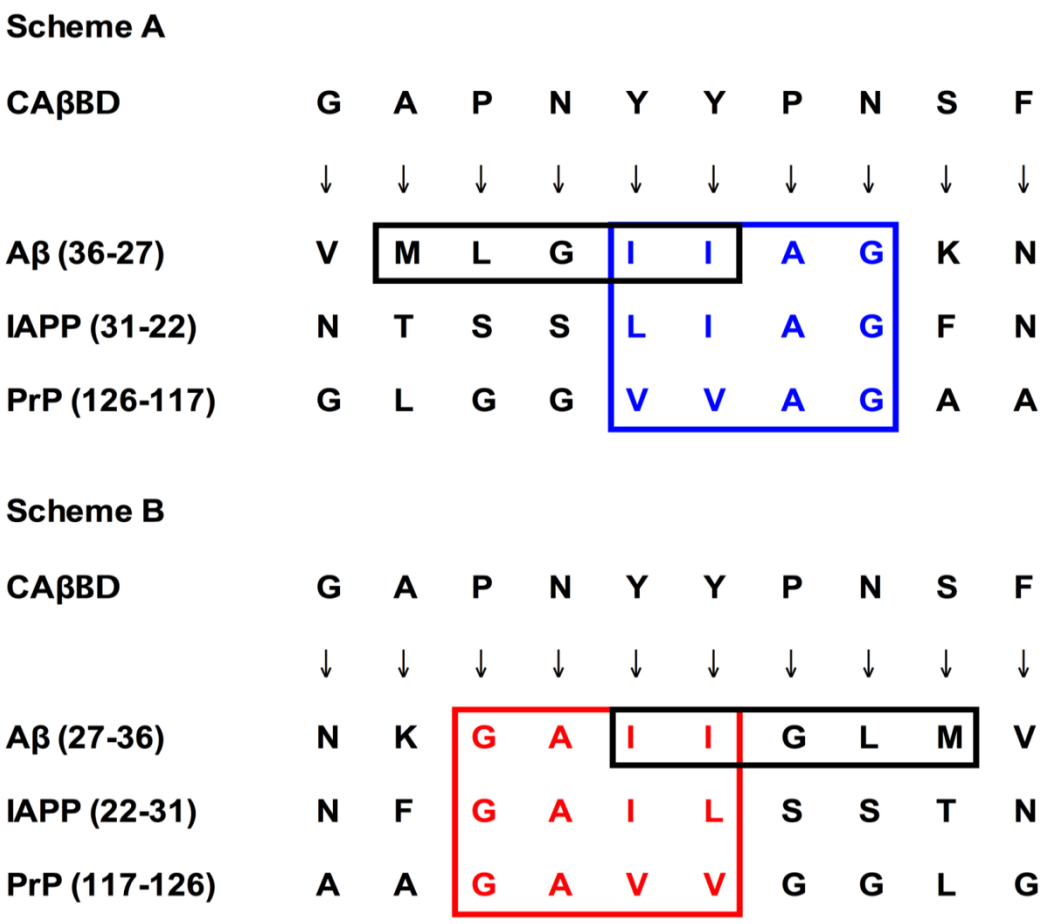

Figure 1-6. The sequence of catalase that binds Aβ, PrP and IAPP. Scheme A, blue box represents the proposed binding of catalase and CAβBD binding to Aβ, PrP and IAPP based on experimental evidence obtained with catalase and CAβBD to Aβ peptides (Milton 1999; Milton *et al.,* 2001; Milton and Harris, 2009; Milton and Harris, 2010). Scheme B green box represents the alternative

binding arrangement which was based on the original experimental results obtained with Aβ and CAβBD (Milton *et al.,* 2001). The black box highlights Aβ 31-35, the smallest sequence of Aβ that can bind catalase. The Aβ 31-35 peptide fragment inhibits Aβ 1-42 peptide binding to catalase (Milton 1999; Milton and Harris, 2009).

1.2.8 Effect of inhibiting the catalase amyloid binding interaction on cells

The Aβ peptide and fibrils both bind catalase and disrupts the ability of catalase to convert H_2O_2 to water and oxygen (Milton 1999). Inhibiting the interaction between catalase and Aβ could reduce H_2O_2 levels and increase cell viability. Benzothiazole aniline (BTA) derivatives with four ethylene glycol units (BTA-EG_4) and six ethylene glycol units (BTA-EG_6) were identified as molecules that could generate a protein resistant surface coating on aggregated Aβ peptides to inhibit catalase amyloid interactions (Inbar *et al.,* 2006, Habib *et al.,* 2010). They are cell permeable, lack toxicity, chemically stable in an oxidizing environment, have intrinsic fluorescent properties and a capability of accessing the same compartments of cells as Aβ. The BTA analogs have high affinity for Aβ oligomers and fibers (Walsh *et al.,* 1999; Maezawa *et al.,* 2008).

Cellular internalization of Aβ by endocytosis or by the non-endocytotic pathway results in accumulation of Aβ within cells, where the Aβ interacts with catalase in the peroxisomes, cytosol and other organelles. The binding inhibits the activity of catalase to breakdown H_2O_2 (Figure 1-7, pathway 1), BTA-EG_x prevents catalase-amyloid interaction by forming protein resistant surface coating on aggregated Aβ. This results in amyloid free catalase which can breakdown H_2O_2 (Figure 1-7, pathway 2) (Habib *et al.,* 2010).

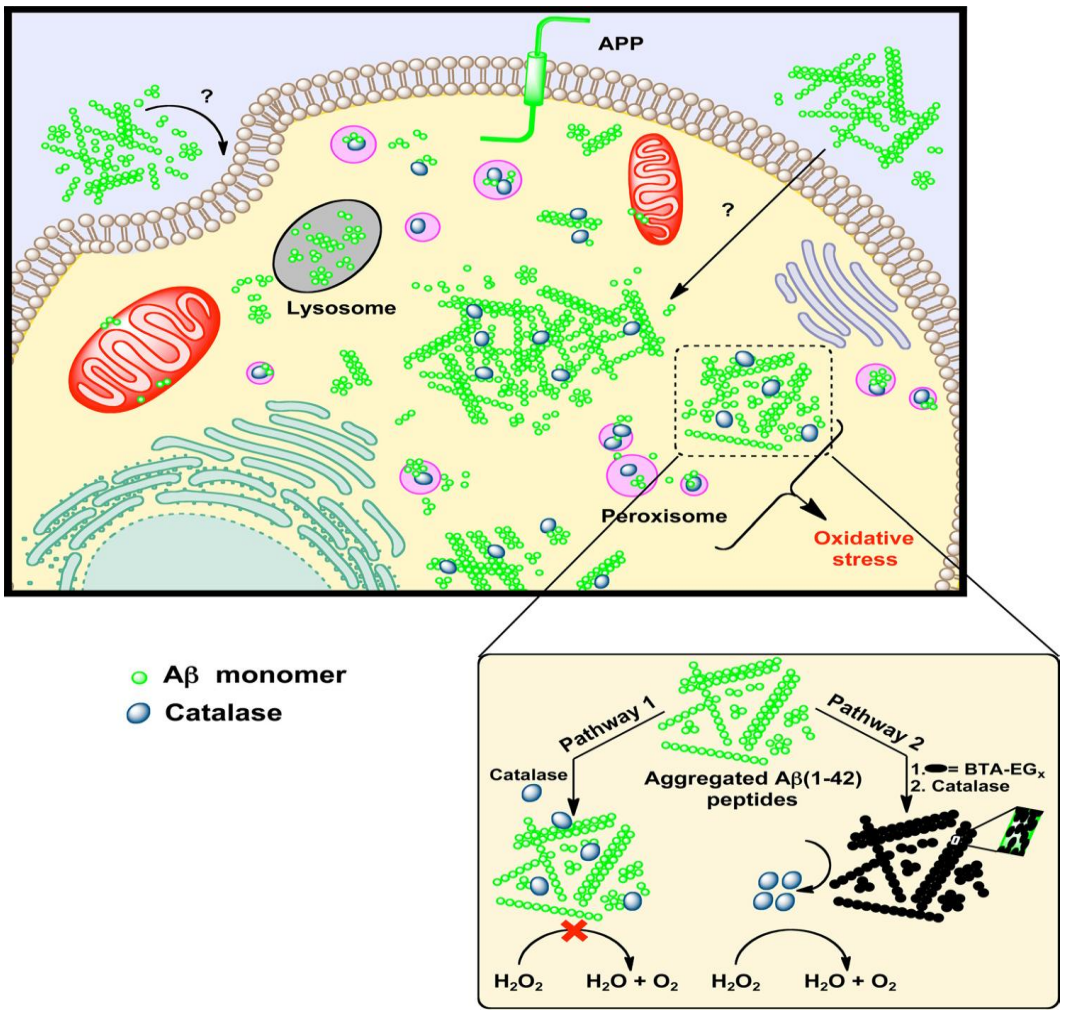

Figure 1-7. Cellular interactions between Aβ and catalase. Cellular internalization of Aβ by endocytosis or by non-endocytotic pathway brings Aβ into contact with intracellular catalase. The Aβ binds catalase, which inhibits catalase activity to breakdown H_2O_2 (pathway 1). The BTA-EG_X forms a protein resistant surface coating on aggregated Aβ, preventing catalase-amyloid interaction (pathway 2). This results in preserving catalase activity to breakdown H_2O_2, reducing OS and allowing cell survival (Habib *et al.,* 2010).

1.2.9 The KiSS-1 gene product kisspeptin shows sequence similarity with the catalase Amyloid-β binding site

The Basic Local Alignment Search Tool (Altschul *et al.,* 1990) was used by Milton (2011) to find peptides that share sequence similarity with catalase Aβ binding site. Results identified a KiSS-1 metastasis suppressor (KiSS-1) gene product as having similarity (Milton 2011; Milton 2013) (Figure 1-8). Kisspeptins (KP) or metastin are peptide products of the KiSS-1 gene, which was first

discovered by Lee *et al.,* (1996). The KP (42-51) sequence was found to show similarity with CAβBD (401-410). The CAβBD binds the Aβ 25-35 region that shows sequence similarity with the PrP and amylin, which plays a major role in the pathology of CJD and T2DM (Milton and Harris 2010). Since, CAβBD binds amyloid peptides; the results suggested that KP could show similar binding interactions with the amyloid peptides, which was confirmed by Milton (2011).

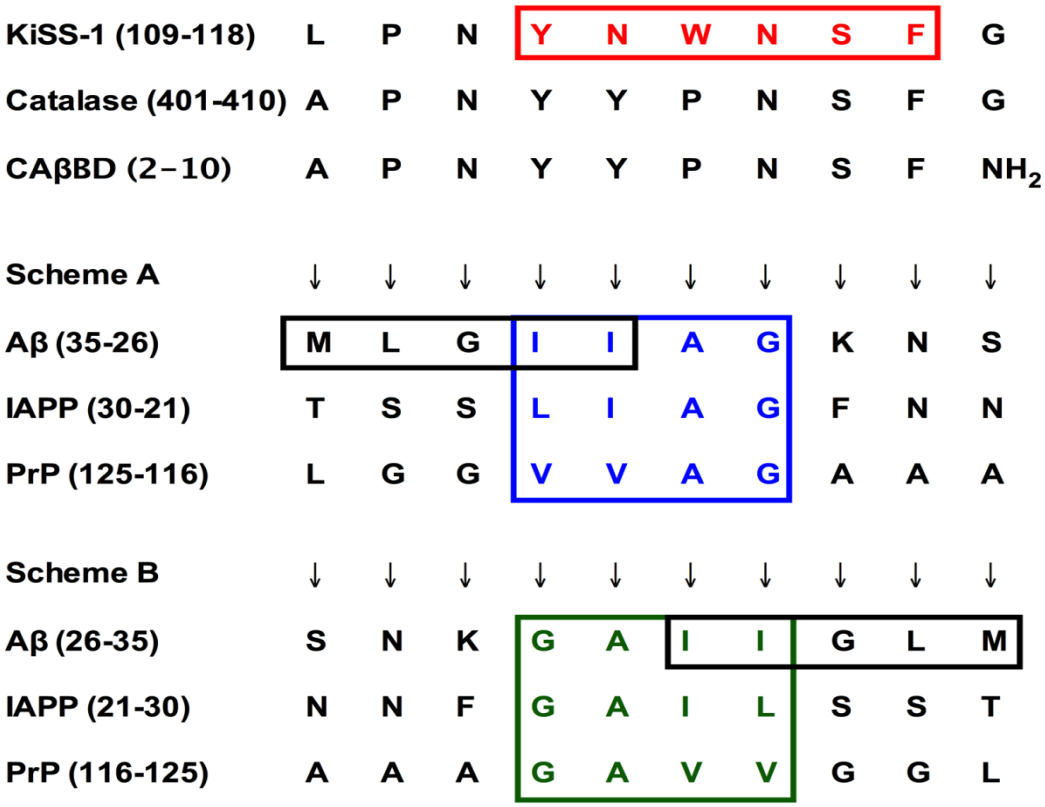

Figure 1-8. Alignment of KiSS-1 gene products with catalase and proposed amyloid peptide interactions. The alignment represents the sequence similarity between human KiSS-1 preproprotein sequence (NP_002247.3) and human catalase sequence (NP_001743.1) - The red box represents the sequence of KP, which binds to Aβ, PrP and IAPP. The blue and green boxes highlights the Gly-Ala-Ile-Ile region that binds catalase in schemes A and B respectively; the black box highlights Aβ 31−35, which inhibits Aβ 1−42 binding to catalase.

In a previous study, administration of KP-10 in young male rats had shown increase in SOD and catalase activities compared to control groups (Aydin *et al.,* 2010). This suggests that KP indirectly has antioxidant properties, which will be investigated in the present study.

1.3 Kisspeptin

The KP peptide was discovered as a product of the metastasis suppressor gene in the human malignant melanoma (Lee *et al.,* 1996). The KiSS-1 gene is located on chromosome 1 (1q32) and the gene codes for a 145 amino acid precursor protein (West *et al.,* 1998). The 145 amino acid precursor is cleaved to 54 amino acids peptide (Figure 1-9), which can be further cleaved to yield 14,13 and 10 amino acid carboxyl-terminal fragments (Kotani *et al.,* 2001) (Figure 1-9). The KP-10 being the smallest biologically active form, which is known to stimulate the GPR-54 receptor (Kotani *et al.,* 2001; Bilban *et al.,* 2004). The biologically active KP peptides belong to the RFamide family of peptides, which share a common Arg–Phe–NH_2 motif at their C-terminus (Kotani *et al.,* 2001).

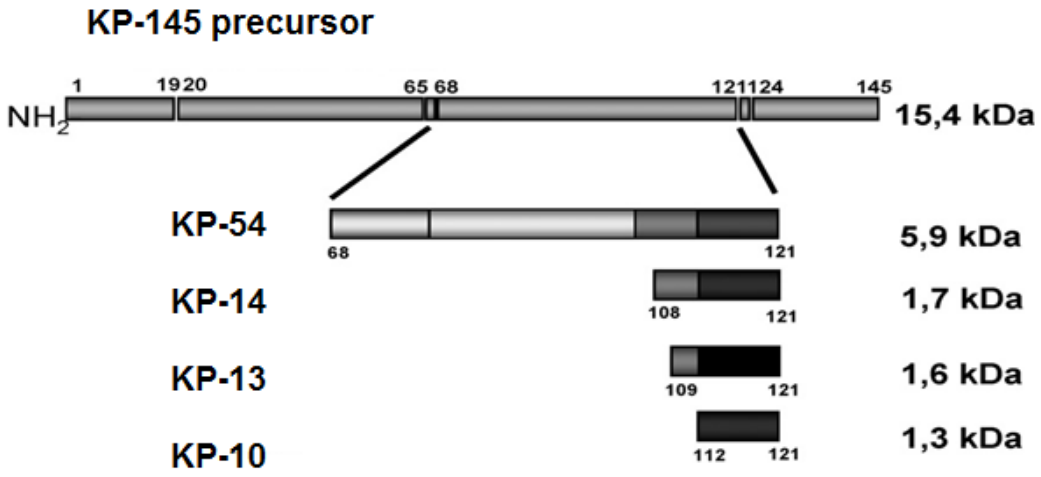

Figure 1-9. The cleavage of the 145 amino acid KP precursor. The KP precursor (145 amino acid form) is cleaved into KP-54 (68-121), which could be further cleaved to KP-14 (108-121), KP-13 (109-121) and KP-10 (112-121) (Roseweir and Millar 2009).

1.3.1 The kisspeptin G-protein coupled receptor 54

The KP peptides are natural ligands of the G-protein coupled receptor 54 (GPR-54) (Kotani *et al.,* 2001). The GPR-54 receptor shares 45 % identity with the galanin receptors (Lee *et al.,* 1999). The GPR-54 receptor sequence was found to be highly conserved in humans and rodents (Clements *et al.,* 2001). The

GPR-54 was found in the brain, stomach, small intestine, thymus, spleen, lungs, testis, ovary, kidney and fetal liver (Kotani *et al.,* 2001; Clements *et al.,* 2001). In the brain GPR-54 is expressed in the cerebral cortex, putamen, medulla, spinal cord, hippocampus and thalamus (Clements *et al.,* 2001). High expression of GPR-54 is found in placenta, pancreas, pituitary and spinal cord, suggesting a role in the regulation of endocrine function (Kotani *et al.,* 2001). This was confirmed by administering KP in rats, which resulted in stimulation of oxytocin release (Kotani *et al.,* 2001). In mice the GPR-54 receptor is essential for the development of the reproductive system (Funes *et al.,* 2003), suggesting that KP peptides regulate reproductive functions.

When KP binds to the GPR-54 receptor, the receptor couples to the Gq/11 class of G-proteins to activate phospholipase C, which hydrolyzes phosphatidyl bisphosphate in the cell membrane to diacyl glycerol (DAG). DAG activates protein kinase C and inositol triphosphate (PI3), which modulates intracellular calcium. This leads to the phosphorylation of ERK1/2 and p38MAPK, cellular reorganization of stress fibers and induction of focal adhesion kinase to inhibit cell movement, which is thought to be important for inhibition of cancer metastasis (Hori *et al.,* 2001; Kotani *et al.,* 2001).

1.3.2 Kisspeptin also activates neuropeptide FF receptors

The KP peptides also activate two neuropeptide FF (NPFF) receptors, G protein coupled receptor 147 (NPFF1) and G protein coupled receptor 74 (NPFF2) (Lyubimov *et al.,* 2010; Oishi *et al.,* 2010). The NPFF peptide was the first identified mammalian RF amide peptide (Panula *et al.,* 1996) originally isolated from bovine and characterized as a pain modulating peptide with anti-opioid activity (Panula *et al.,* 1999). The NPFF sequence is cleaved from a precursor protein encoded by the NPFF gene and activates two different receptors NPFF1 and NPFF2. Endogenous KP-13 and KP-14 have been shown to activate the NPFF2 receptor (Lyubimov *et al.,* 2010). The KP peptide shares a C-terminal dipeptide RF-NH_2 with NPFF, which activates the NPFF2 receptor and it was suggested that KP has the potential to mediate physiological effects and nociception in man via the NPFF2 pathway (Lyubimov *et al.,* 2010). The actions of KP, mediated via NPFF receptors (Milton 2012), can be inhibited by the NPFF receptor antagonist 2-Adamantanecarbonyl-RF-NH_2 (RF9) (Simonin *et*

al., 2006) but not by the GPR-54 receptor antagonist Ac-[(D)-A]-NWNGFG-[(D)-W]-RF (KP234) (Roseweir *et al.,* 2009).

1.3.3 Kissorphin

Cleavage of KP peptide by matrix metalloproteinase (MMP) to remove the amidated C-terminal tripeptide Leu-Arg-Phe-NH_2 of the KP-10 sequence abolishes the activation of GPR-54 by KP peptides (Takino *et al.,* 2003). The resultant peptide is left with a C-terminal glycine, which could be alpha amidated, a feature of many biologically active neuropeptides. This C-terminally amidated peptide is called kissorphin (KSO) (Figure 1-10).

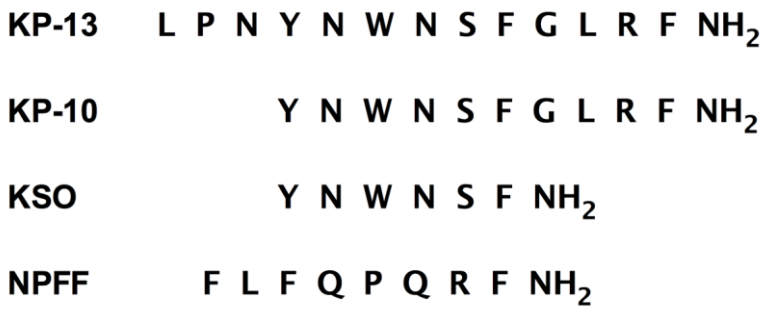

Figure 1-10. Comparison of kisspeptin, kissorphin and neuropeptide FF sequences. Sequence similarity between the biologically active KP-13, KP-10, KSO and NPFF peptides (Milton 2012).

The sequence similarity between KP, KSO and NPFF explains how KP can stimulate NPFF receptors and the NPFF like biological activity of KSO (Milton 2012).

1.3.4 Kisspeptin pathway

The KP peptide not only works as a metastasis suppressor but also plays a pivotal role in regulating the hypothalamic-pituitary-gonadal (HPG) axis. The KP peptide acts on hypothalamic GnRH neurons to stimulate GnRH release (Navarro 2012). The majority of GnRH neurons express the GPR-54 receptor, which KP peptides activate (Kotani *et al.,* 2001). The HPG axis is under the control of steroid hormone feedback from the gonads, the steroid levels fluctuate during the menstrual cycle in females (Moenter *et al.,* 2003). Estradiol feedback controls GnRH release, but the GnRH neurons only express estrogen

receptor β (ERβ) and not estrogen receptor α (ERα) (Shivers *et al.,* 1983), ERβ does not play a role in the feedback. Therefore neurons upstream of the GnRH neurons, which express ERα, mediate the steroid effects on GnRH neurons.

The KP neurons express ERα, progesterone receptor (PR) and androgen receptor (AR) and have the potential to relay the estradiol feedback effect on the GnRH neurons (Rance 2009). The KP neurons reside in the anterioventral periventricular nucleus (AVPV), the periventricular nucleus (PEN), the anterodorsal preoptic nucleus (ADP), the medial amygdala and the arcurate nucleus (ARC) in mouse (Gottsch *et al.,* 2004). In rat and mouse the vast majority of GnRH neurons express GPR-54 (Irwig *et al.,* 2004). The KP immunoreactive fibers make close contact with the GnRH neuronal cell bodies (Clarkson and Herbison, 2006) in the preoptic area and median eminence. The KP neurons form a source of KP ligands to stimulate GPR-54 expressed on the GnRH neurons in the hypothalamus. Binding of KP to the GPR-54 stimulates the GnRH neurons to release GnRH in the hypothalamus. GnRH release stimulates the release of gonadotropins - luteinizing hormone (LH) (Navarro *et al.,* 2004) and follicle stimulating hormone (FSH) (Navarro *et al.,* 2005) in the pituitary. The gonadotropins further stimulate the gonads to secrete estrogen, progesterone and testosterone, which lead to steroid negative or positive feedback (Navarro 2012).

1.3.5 Sex steroids regulate positive and negative feedback of KiSS-1 gene

In rodents, estradiol stimulates KiSS-1 expression in the AVPV, which in turn stimulates GnRH neurons to secrete GnRH resulting in positive feedback regulation of the HPG axis. The estradiol in the ARC inhibits KiSS-1 expression causing reduction in GnRH secretion, and resulting in negative feedback regulation (Smith *et al.,* 2005; Smith 2008) (Figure 1-11). In mouse and rat, KiSS-1 mRNA expression is higher in the AVPV region of females than in males during puberty (Clarkson and Herbison *et al.,* 2006). The increase in the number of KiSS-1 neurons in the AVPV region may contribute to the activation of GnRH neurons during puberty. Kisspeptin signalling appears critical for mice and men to reach puberty, mutations in the GPR-54 receptor results in

hypogonadotropic-hypogonadism (De Roux *et al.,* 2003), whereas treatment with KP advances the onset of puberty (Navarro *et al.,* 2004).

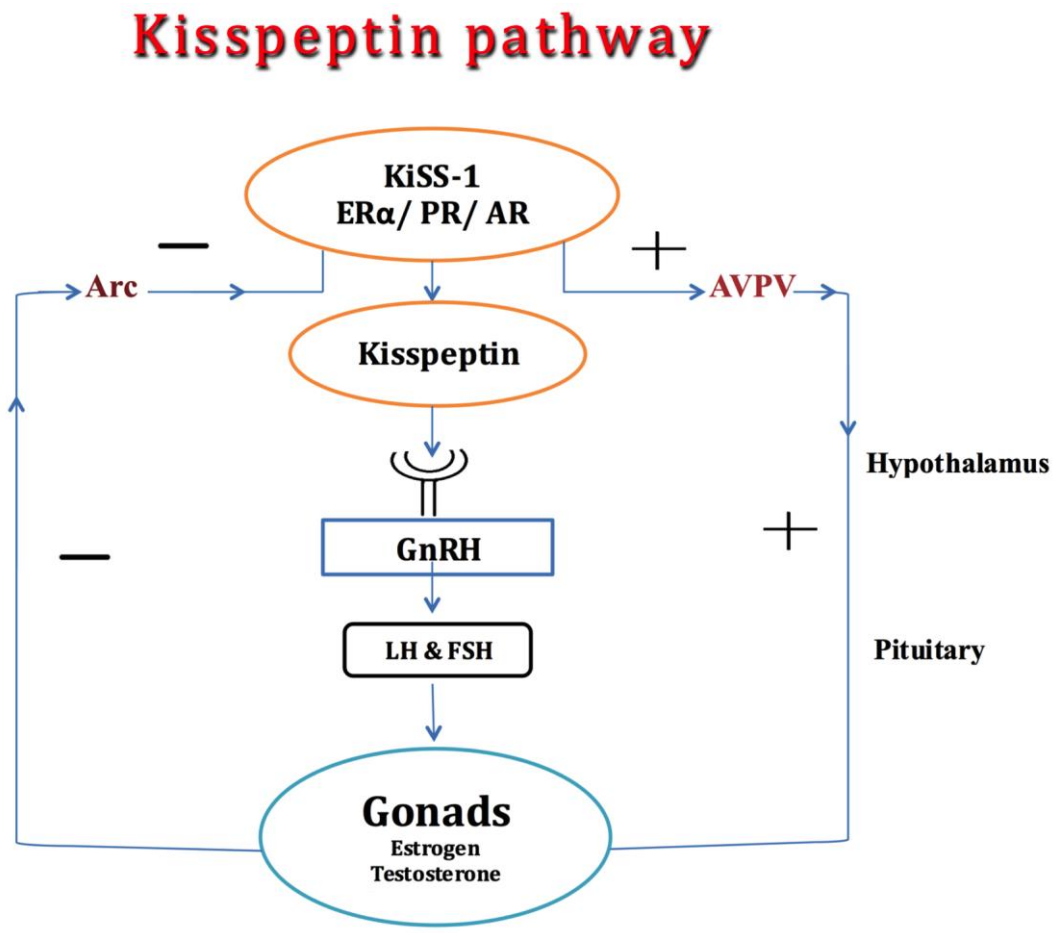

Figure 1-11. The positive and negative feedback inputs in HPG axis. The KP neurons express ERα, AR and PR and generate KP, the ligand for the GPR-54 located on the GnRH neurons. The binding of the ligand to the receptor stimulates GnRH release in the hypothalamus. The GnRH stimulates the release of LH and FSH in the anterior pituitary, LH and FSH in turn stimulates the gonads to release estrogen, testosterone and progesterone. Estrogen positively regulates the neurons in the AVPV region to express KiSS-1 gene, whereas it negatively regulates the neurons in the ARC to inhibit KiSS-1 expression. These actions are mediated by ERα receptors on KP neurons (Roseweir and Millar 2009).

The removal of sex steroid negative feedback results in increased levels of LH and FSH, which occurs post menopause. This increase in LH and FSH levels result in increased gene expression of GnRH and cellular hypertrophy of a

subpopulation of neurons in the human infundibular nucleus, which is the homolog of arcurate nucleus in other species (Rance and Young, 1991; Rance and Uswandi, 1996).

1.3.6 The role of kisspeptin in passive avoidance learning in mice

A recent study has shown that KP-13 could play a role in passive avoidance learning and memory consolidation (Telegdy and Adamik, 2013). In this study the effect of adrenergic, serotonergic, cholinergic, dopaminergic and GABA-A-ergic and opiate receptors plus nitric oxide were investigated in mice. Receptors were blocked using various neurotransmitter antagonists including the α-adrenergic receptor antagonist phenoxybenzamine (PHB); the α_2-adrenergic receptor antagonist yohimbine (YO); the β-adrenergic receptor antagonist propranolol (PROP); the mixed 5-HT_1/5-HT_2 serotonergic receptor antagonist methysergide (MET); the nonselective 5-HT_2 serotonergic receptor antagonist cyproheptadine (CYPR); the nonselective muscarinic ACh receptor antagonist atropine (ATR); the D_2, D_3, D_4 dopamine receptor antagonist haloperidol (HAL); the γ-aminobutyric acid subunit A ($GABA_A$) receptor antagonist bicuculline (BIC); the nonselective opioid receptor antagonist naloxone (NAL); and the nitric oxide synthase inhibitor nitro-L-arginine.

The HAL and NAL did not block the effect of KP-13 on passive avoidance learning and memory consolidation suggesting no involvement of dopaminergic or opioid receptors in the response (Telegdy and Adamik, 2013). The PHB, YO, PROP, MET, CYPR, ATR, BIC and nitro-l-arginine inhibited the action of KP-13 on passive avoidance learning. This suggests that the action of KP-13 at least on passive avoidance learning and memory consolidation is partly mediated through the interaction of α_2-adrenergic, β-adrenergic, 5-HT_2 serotonergic, muscarinic cholinergic and GABA-A-ergic receptor systems plus nitric oxide (Telegdy and Adamik, 2013).

1.3.7 Kisspeptin's antidepressant like effect

A recent study (Tanaka *et al.,* 2013) has shown an anti-depressant like effect of KP-13 in mice. The potential involvement of adrenergic, serotonergic, cholinergic, dopaminergic and gabaergic receptors in KP-13 mediated antidepressant like effect was investigated in a modified forced swimming test (FST) in mice. The forced swimming test (FST) revealed that KP-13 reversed immobility, climbing and swimming times, which represents the antidepressant like effect of KP-13. The PHB, YO and CYPR prevented these effects of KP-13, suggesting that the antidepressant like effect of KP-13 is mediated at least in part by α_2-adrenergic and 5-HT_2 serotonergic receptors (Tanaka *et al.,* 2013). The α1/α2β-adrenergic receptor antagonist, prazosin (PR), PROP, MET, ATR, HAL and BIC did not have an effect on KP-13 mediated antidepressant like effect in mice, suggesting no role for the α_1-adrenergic, β-adrenergic, 5-HT_1 serotonergic, muscarinic acetylcholine, dopaminergic or GABA-A-ergic systems in this response (Tanaka *et al.,* 2013).

1.3.8 Kisspeptin and Alzheimer's disease

In AD, disease specific changes are seen in the neuroendocrine systems (Bao *et al.,* 2008; Tortosa and Clow 2012). The HPG axis changes profoundly with aging and the pathology of AD, CJD and T2DM is also associated with changes in the HPG axis (Verdile *et al.,* 2008; George *et al.,* 2010). The KP peptides are the major regulator of the HPG axis; the level of KP changes during menopause (Rance 2009) and notable sex differences in hypothalamic neurodegeneration is seen in the elderly (Rance 2009; Schultz *et al.,* 1996). This sex dependent neurodegeneration is suggested to be due to impaired neuroendocrine function. A recent study has shown that KP neurons are likely to be among the earliest to undergo aging process while participating in reproductive decline in mice (Zhang *et al.,* 2014). The onset of AD is usually post-menopausal, which could make women more susceptible to AD, raising the possibility that changes in reproductive function could play a role in AD (Bonomo *et al.,* 2009). Changes in the expression of ERα and ERβ in the infundibular nucleus and hypothalamus have been related to the occurrence of AD (Hestiantoro and Swaab, 2004). The estrogen receptors regulate KP levels (Patisaul *et al.,* 2012); this suggests that KP could play a role in AD pathology. Sex steroids such as estrogen and

testosterone were found to be neuroprotective in AD, but the level of sex steroid declines during aging (Pike *et al.,* 2009). Andropause in males is associated with decreased memory function suggesting that changes in the HPG axis may influence memory function (Fuller *et al.,* 2007). This suggests that changes in the reproductive hormones may play a major role in AD pathology.

1.4 Aim

Two major events occur during AD progression (1) accumulation of Aβ and (2) increased OS. During OS the cells fall short of antioxidant defences, partly due to interaction of Aβ with antioxidant enzymes such as catalase. Here the key unanswered question is whether protection against Aβ by catalase is via its antioxidant activity or Aβ binding activity or both. The overexpression of the catalase gene in a human neuronal SH-SY5Y cell line model was used to investigate the mechanism of catalase protection against Aβ toxicity. The KiSS-1 protein is not an antioxidant enzyme and therefore if overexpression of KiSS-1 were neuroprotective this would suggest an Aβ binding action was important. The aim of the present study was to create and characterise a SH-SY5Y human neuroblastoma cell line that is resistant to Aβ and OS. To achieve this, human catalase or KiSS-1 genes were overexpressed in human neuroblastoma SH-SY5Y cells. Both the cell lines were characterized and the mechanism of neuroprotection was investigated.

Chapter 2

Methods

2.1 Binding assay to test peptides binding to Amyloid-β

The NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plates were coated with test peptides (1 µg/ml) in carbonate buffer The carbonate buffer was prepared using 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.01% NaN_3 (w/v) pH 9.6. The test peptides in carbonate buffer were incubated overnight at 4^{o}C on the ELISA plate. The unoccupied sites on the plate were blocked using 0.2% (w/v) casein in carbonate buffer for 30 mins. Biotinylated Aβ (Bachem) was prepared in assay buffer (50 mM Tris-HCl, pH 7.5 plus 0.1% (w/v) Bovine serum albumen Cohn Fraction V (BSA), 0.1% (v/v) Triton X-100, 0.01% (w/v) NaN_3) and was incubated with test peptides on the ELISA plate for 2 hrs. After the incubation, the ELISA plates were washed thrice with assay buffer to remove unbound material. An alkaline phosphatase polymer-streptavidin conjugate (0.5 µg/ml) was added to the plate and incubated for 2 hrs at 37^{o}C. The plates were washed thrice to remove the unbound material after which the p-nitro phenyl phosphate (PNPP) substrate (1.0 mg/ml PNPP and 0.2 ml Tris buffer tablets dissolved in water) (Voller *et al.,* 1976) 100 µl/well was added. The absorbance at 405 nm was determined using an ELISA plate reader.

2.2 Cloning

2.2.1 Preparation of chemically competent cells

The E.*coli* top10 cells (Invitrogen) were grown overnight in 5 ml Lysogeny broth (LB) media. The next day this culture was diluted (1 in 10) in 50 ml of fresh LB media and cells were grown until an OD at 600 nm of 0.4 was reached. The media was centrifuged for 10 mins at 3000 rpm at 4^{o}C. The supernatant was removed and the pellet was resuspended in chilled TSS (transformation and storage solution) buffer (0.0125 M PEG (Polyethylene glycol) 8000, 1 M $MgCl_2$, 2.5 ml DMSO (Dimethyl sulphoxide), 50 ml LB). 100 µl of the resuspended pellet was added to each eppendorf and stored at -80^{o}C.

2.2.2 Polymerase chain reaction amplification of catalase and KiSS-1 cDNA clones.

The cDNA clones for the human catalase gene (NM_001752.3) and the human KiSS-1 gene (NM_002256.2) were obtained from Origene and the genes were amplified from the cDNAs (Origene) using the polymerase chain reaction (PCR) with the following primers (Table 1) (Invitrogen).

Table 1. List of sequencing primers

Gene	Primer Direction	Primer Sequence (with restriction site in red)	Restriction Enzyme
Catalase	Forward	5' AAGCTTATGGCTGACAGCC GGGAT 3'	*Hind* III
Catalase	Reverse	5' GCGGCCGCCAGATTTGC CTTCTCCCTTGC 3'	*Not* I
Kisspeptin	Forward	5' TTAGGATCCATGAACTC ACTGGTTTCTTGGCA 3'	*Bam* HI
Kisspeptin	Reverse	5' ATACTCGAGGCCCCGCC CAGCGCTTCT3'	*Xho I*

Table 2. The PCR conditions used were as follows

Step	Description	Temperature	Time
1	Initiation temperature	95^{o}C	1 minute
2	Denaturation temperature	95^{o}C	30 seconds
3	Annealing temperature for catalase gene	60^{o}C	30 seconds
4	Annealing temperature for KiSS-1	58^{o}C	30 seconds

5	Elongation temperature	72^{o}C	45 seconds
6	Repeat 1-5 for 30 cycles		

The PCR products were purified using a Qiagen miniprep kit.

2.2.3 Restriction Digestion

After amplification of the gene the restriction enzyme digestion was set up as follows, 500 µg of plasmid DNA was restriction digested using 5 units of restriction enzyme - *Not* I and *Hind* III for catalase gene, *Bam* HI and *Xho I* for the KiSS-1 gene. The genes were purified using a Qiagen PCR purification kit. The plasmid pcDNA™4/TO/myc-His A (Invitrogen) (Figure 2-1) was also digested with the restriction enzymes to create the sticky ends. The plasmid was gel purified (0.5% agarose gel) using Qiagen gel purification kit.

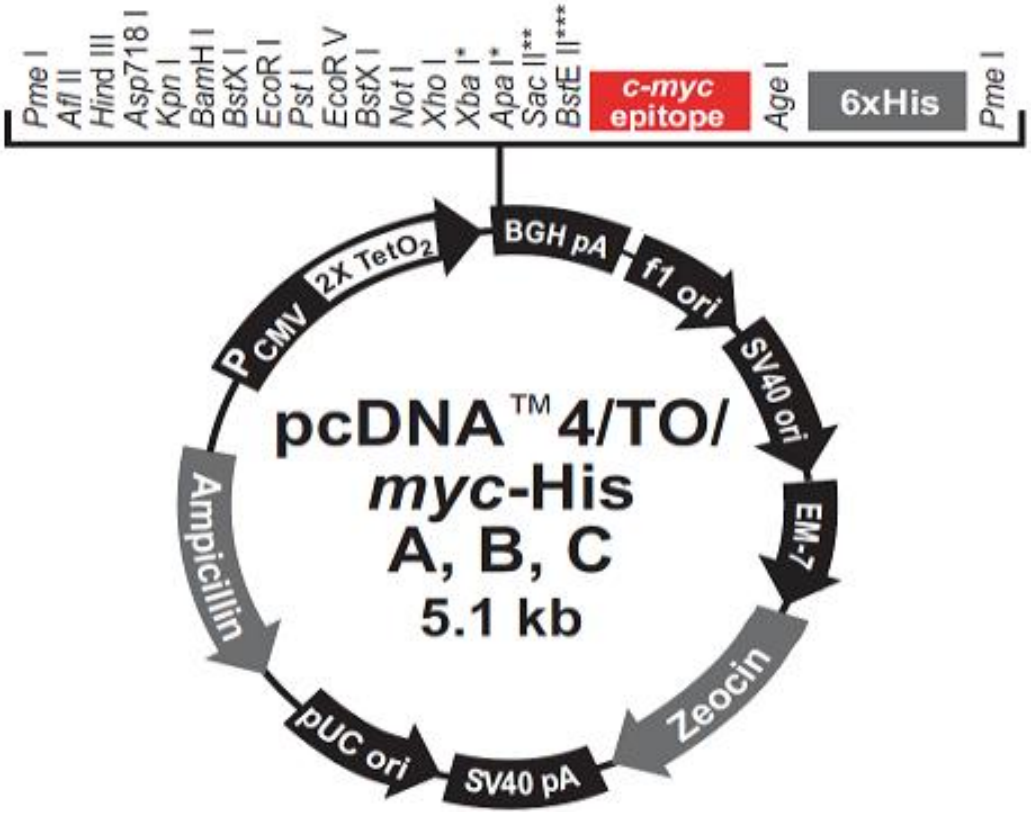

Figure 2-1. Vector map of pcDNA™4/TO/myc-His (Invitrogen).

2.2.4 Ligation

Restriction enzyme digested catalase and KISS-1 PCR products and the vector were ligated together using 5 units of T4 DNA ligase (Invitrogen) at 16^{o}C for 2 hrs. The ligation mixture was prepared in 1:3 ratio of the vector (3 µls) to the gene (9 µls).

2.2.5 DNA sequencing

To confirm the DNA sequence of the gene cloned, the DNA sample was sequenced at UCL (Scientific Support Services at the Wolfson Institute for Biomedical Research and the UCL Cancer Institute). The DNA sequenced data was analysed using bioinformatics programmes Expasy translate and BLAST.

2.2.6 Agarose gel electrophoresis of DNA samples

A 1% agarose gel was prepared by dissolving 0.5 mg of agarose (Promega) in 50 ml of 1X Tris-Borate-EDTA (TBE) buffer 5X TBE (1.1 M Tris; 900 mM boric acid; 25 mM EDTA; pH 8.3). The solution was heated to a boiling point in a microwave. Once cooled to room temperature the solution was poured into an electrophoresis tray (with an appropriate size comb) and allowed to set at room temperature for 30 min. DNA sample was mixed with loading dye (6 x loading dye, Invitrogen) and was loaded into the wells of the gel. The electrophoresis was performed at 140 volts in 1X TBE buffer for 45 mins. The gel was transferred to a tray containing ethidium bromide 0.5 µg/ml for 5 mins. To observe the bands the gel was visualised under ultraviolet light using a transilluminator.

2.3 Cell Culture

A frozen aliquot of the human neuroblastoma SH-SY5Y cell line (Biedler *et al.,* 1978) was thawed and was added to 10 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and HAM's F12 with Glutamax (Invitrogen). The media used for cell culture was prepared by adding 10% v/v foetal calf serum (Sigma), 1% non-essential amino acids, penicillin (100 units/mL), and streptomycin (100 mg/mL). The media with the cells was centrifuged at 1500 rpm for 5 mins at 4^{o}C. The supernatant was discarded and the pellet was

resuspended in fresh media. The cells were grown in 25 cm^2 flasks at 37^oC and 5% v/v CO_2. Media was changed every 3 days, when the cells reached confluency they were split into two flasks and were maintained at the same conditions as mentioned above.

2.3.1 Transfection

SH-SY5Y cells ($1.2x10^6$/ml) were plated into a 6 well plate; the cells were allowed to settle down for 24 hrs, when the cells were 40% confluent, the transfection mix was added to the cells as below.

2.3.2 Transfection mixture preparation

The transfection mixture was prepared by adding 2.5 µg plasmid DNA in sterile dH_2O diluted with 188 µl DMEM (serum and antibiotic free) + 12.5 µl (25 µg) lipofectamine diluted with 313 µl DMEM in a sterile polypropylene tube. The transfection mix was vortexed for 10 seconds and incubated at room temperature for 5 to 10 mins. The media from the 6 well plate was removed and replaced with serum and antibiotic free media. The transfection mixture was dispensed drop wise into each well with gentle shaking. Afterwards the plate was incubated at 37^oC/5%v/v CO_2 for 4 hrs. After this incubation the media was replaced with fresh media and incubated for 24 hrs. Fresh media with the selecting agent zeocin (100 µg/ml) was added after a further 24 hrs, to select the cells with the vector. The media was refreshed twice a week; confluent cells were transferred into a bigger flask.

To create tetracycline inducible cells, the double transfected (pcDNA™6/TR and pcDNA™4/TO/myc-His) cells were cultured in a medium containing zeocin (100 µg/ml) and blasticidin (5 µg/ml) for 4 weeks.

2.3.3 Preparation of mammalian protein lysate

The total protein lysate was made from $1x10^7$ cells. The cells were washed with ice cold phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) (to remove media) and centrifuged at 1500 rpm for 5 mins at 4^oC. The pellet was resuspended (until no clumps were seen) in cell lysis buffer containing 100 µl 1x NP-40 buffer (1% v/v Nonidet P-40 [Perbio], 20

mM Tris-HCl pH 7.8, 150 mM NaCl, 2 mM $MgCl_2$ and 1mM EDTA) containing protease inhibitors (Complete protease inhibitor cocktail tablet). After 30 mins incubation at 4^{o}C the nuclei and cell debris were removed by centrifuging at 1500 rpm for 5 mins at 4^{o}C. The supernatant (protein lysate) was transferred into a fresh tube and stored at -80^{o}C. The protein was quantified using a Bicinchoninic acid assay (BCA) (Sigma).

2.3.4 SDS-PAGE

After the quantification of the proteins using BCA, they were separated by SDS-PAGE using the 10% w/v resolving gel constituted as follows:

- 5 ml acrylamide/bisacrylamide 29:1 (30% w/v) (Protogel® National Diagnostics)
- 3.75 ml resolving buffer (1.5 M Tris-HCl pH 8.8, 0.4% w/v SDS)
- 6.25 ml ddH_2O
- 15 µl N,N,N',N'- Tetramethylethlenediamine (TEMED)
- 150 µl 10% w/v ammonium persulphate (APS) solution.

The 4% w/v stacking gel was constituted as follows:

- 1 ml acrylamide/bisacrylamide (29:1) (30%)
- 1 ml stacking buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS w/v)
- 5 ml ddH_2O
- 15 µl TEMED
- 150 µl of 10% w/v APS solution

The polymerised gel was transferred to the running tank and filled with 1X gel running buffer (23 mM Tris HCl pH 8.3, 192 mM glycine, 0.1% w/v SDS). The loading samples were prepared by mixing 15 µl of the protein lysate (45 µg/ml) and 15 µl of 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris-HCl, pH 6.8) (Sigma) and the samples were heated to 95^{o}C for 5 min. The samples were loaded and the gel was run at 140 V for 45 mins.

2.3.5 Western blotting and antibody detection

After SDS PAGE the proteins were transferred to nitrocellulose membrane in 1X transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% v/v methanol, 0.1% w/v SDS) at 200V for 30 mins. After transfer the membrane (Protran nitrocellulose transfer membrane - Whatman) was blocked in 10% w/v Marvel in 10 ml PBS and 0.1% v/v tween-20 and was left at 4^{o}C overnight (blocking solution). Next day the membrane was incubated with the primary antibody for 1 hr at room temperature. After incubation the membrane was washed thrice (each wash 5 min) with PBS, 0.1% v/v Tween-20. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:1000 dilution in the blocking solution for 1 hr at room temperature. After incubation the membrane was washed thrice with PBS (0.3% v/v Tween -20) and then thrice with PBST (0.1% v/v Tween-20) and the membrane was ready for development.

2.3.6 Enhanced chemiluminescence (ECL) development

For the detection of the HRP secondary antibody, Super Signal® West Femto chemiluminescence system (Pierce Biotechnology) was used. Each reaction reagent was mixed in a 1:1 ratio before incubating with the membrane for 5 mins at room temperature. The membrane was then sandwiched between two sheets of clear plastic and developed using a UVP imaging system.

2.3.7 Western blot stripping buffer

Stripping buffer was used to remove the primary and secondary antibody of KP or catalase and to reprobe the nitrocellulose membrane with rabbit-actin primary antibody. The stripping buffer was prepared using 200 mM glycine, 3.4 mM SDS, 0.001% (v/v) tween-20 was added to 800 ml distilled water, after the pH was adjusted to 2.2 distilled water was added to bring up the volume to 1 L.

2.3.8 Membrane incubation

The stripping buffer was added to the membrane till the membrane was completely covered by the buffer. The membrane was incubated at room temperature for 5-10 mins on a shaker. After the incubation the buffer was replaced with fresh stripping buffer and was allowed to incubate for 5-10 mins. The buffer was discarded and PBS was added to the membrane for 10 mins, this step was repeated twice. After washing the membrane with PBS, TBST (tris buffered saline and tween 20) was added to the membrane and incubated for 5 mins, this step was also repeated twice. After washing with TBST the membrane was blocked with 5% (w/v) marvel and was probed with actin primary antibody.

2.4 Catalase activity

The activity of catalase was measured according to the method described by Góth (1991); the cell lysates for the activity assay were prepared from $1x10^7$ cells. The quantification of the protein in the cell lysates was determined by using nanodrop, 50 µg of the cell lysate was used to measure the activity of catalase using 10 mM H_2O_2, PBS and 32.4 mM ammonium molybdate at 405 nm. When H_2O_2 and ammonium molybdate are added to the cell lysate, the catalase breaks down H_2O_2, which prevents the reaction between H_2O_2 and ammonium molybdate. In the absence of catalase, H_2O_2 reacts with ammonium molybdate and the product of the reaction is visibly seen as a yellow colour and the absorbance can be measured at 405 nm. To ensure the specificity of the assay, the activity of the catalase enzyme was inhibited by an irreversible inhibitor of catalase activity 3-Amino-1,2,4-triazole (3-AT) (Margoliash and Novogrodsky, 1958) (5 mM). The cell lysates were incubated with 3-AT overnight to allow the inhibition of catalase enzyme activity. Inhibition of catalase activity by 3-AT suggests that the breakdown of H_2O_2 was due to catalase. Activity was calculated from a standard curve (0 - 100 kU/l) using purified human catalase (EC 1.11.1.6) from human erythrocytes of known activity. Activity was expressed as U/mg protein (Milton 2001; Milton 2008).

2.5 RNA extraction

To extract the total RNA from the SH-SY5Y cells an RNeasy® Mini Kit (Qiagen) was used. In accordance with the manufacturer's instructions $5x10^6$ cells were harvested, washed once with PBS and the cell pellet was resuspended in 600 µl RLT buffer. The cell pellet was loosened thoroughly by flickering the tube followed by vortexing until no clumps were visible. The lysate was homogenized by vortexing for 1 min. An equal of 70% (v/v) ethanol was added to the homogenized lysate and was mixed by pipetting. The sample was applied to an RNeasy® spin column in a collection tube and centrifuged at 15,000 x g for 15 sec. After discarding the supernatant, the RNeasy® spin column was washed once with 700 µl RW1 buffer (15,000 x g for 15 seconds) and twice with 500 µl RPE buffer (15,000 x g for 15 seconds). Next, the RNeasy® spin column was placed in a 1.5 ml collection tube, 50 µl of RNase free water was added directly to the spin column membrane and centrifuged at 15,000 x g for 1 mins to elute the RNA. The total RNA concentration was determined by measuring the absorption at 260 nm using the nanodrop spectrophotometer. The integrity of the RNA was checked on a 0.5% (w/v) agarose gel.

2.5.1 Reverse transcriptase and cDNA synthesis

The reverse transcription for the cDNA synthesis was carried out using QuantiTect® Reverse transcription Kit (Qiagen). In accordance with the manufactures' instructions, the template RNA was thawed on ice while the gDNA wipeout buffer, Quantiscript® reverse transcriptase, Quantiscript® RT buffer, RT primer mix and RNase free water were thawed at room temperature. The genomic DNA elimination reaction was prepared on ice by mixing 12 µl template RNA with 2 µl gDNA wipeout buffer. The reaction was brought to a total volume of 14 µl using RNase free water. The mix was incubated for 2 mins at 42^oC and immediately placed back on ice. Next, the reverse transcription master mix was prepared on ice, composing 1 µl Quantiscript® reverse transcriptase, 4 µl Quantiscript® RT buffer (5X) and 1 µl RT primer mix. The entire (14 µl) gDNA elimination reaction was added to the reverse transcription master mix. The reverse transcription reaction (RT reaction) was first incubated

at 42^{o}C for 15 mins followed by incubation at 95^{o}C for 3 min, to inactivate Quantiscript® reverse transcriptase. The cDNA sample was stored at -20^{o}C. The RT-PCR was used to qualitatively detect gene expression using the prepared cDNA. The cDNA prepared was used to detect the expression levels of catalase and KiSS-1 gene in SH-SY5Y cells. To determine the expression of the genes, cDNA from the cells was used as a template for the PCR. The PCR conditions used were same as those used for the amplification of the respective genes (Using same primers for the catalase and KiSS-1 as described in section 2.2.2.)

2.6 Immunohistochemistry

Pons sections from a 72-year-old male with AD (Cat No: ab4586; Lot No: B506287) and a 26-year old normal male (Cat No: ab4316; Lot No: A504234) and BAM-10 mouse anti-Aβ antibody were obtained from Abcam PLC, Cambridge, UK. Rabbit anti-KP 45-54 antiserum was purchased from Bachem AG, Switzerland. Goat anti-mouse Alexa-Fluor 568 and goat anti-rabbit Alexa-Fluor 488 were purchased from Chemicon, UK. The VECTASHIELD® mounting media was purchased from Vector laboratories Ltd, UK. The CAT-505 mouse anti-catalase antibody, alkaline phosphatase conjugated goat anti-rabbit IgG, alkaline phosphatase conjugated anti-mouse IgG and all other chemicals were purchased from Sigma-Aldrich, UK.

The Pons tissue sections used were 5 µM in thickness, which were pre-mounted and paraffin embedded. The sections used had been diagnosed and examined by a licensed pathologist and were ethically obtained. To remove the paraffin, the sections were dipped in xylene, 2x5 min. The slides were hydrated with 100% ethanol, 2x3 min. The slides were further hydrated with 95% ethanol, 1 min. The slides were rinsed with distilled water and were blocked using 10% (v/v) goat serum for 1 hour. After blocking the sections, they were incubated with the primary antibody at 1:1000 dilutions at 4^{o}C overnight. Next day, the sections were washed 3x5 mins with PBST before the secondary antibody was added at 1:500 dilution for 1 hour. The sections were washed 3x5 mins with PBST and cover slips were mounted with Vectashield® Mounting Media.

2.7 Immunocytochemistry

The SH-SY5Y cells with vector alone and cells overexpressing the catalase and the KiSS-1 gene were plated in a six well plate, the plate was left in the incubator overnight at 37^{o}C and 5% CO_2. The cells were washed with media (DMEM) without serum followed by 2x PBS washes. The cells were fixed using 4% (w/v) paraformaldehyde followed by 3 washes with PBS. To permeabilize the cells, the cells were treated with ice-cold methanol for 30 min and then washed 3x with PBS. To prevent the non-specific binding of the primary and the secondary antibody to the plate, the plate was blocked with PBS-BSA for 15 mins and was washed 3x with PBS. The cells were incubated with the primary antibody in PBS-BSA for 1 hour. The cells were washed thrice with PBS before adding the secondary antibody in PBS-BSA and incubating for 30 mins in the dark. The cells were washed 3x PBS and were incubated with 100 µg/ml RNase A for 20 mins at 37^{o}C. After washing the cells thrice with PBS, the cells were treated with TO-PRO® - 3 Iodide (642/661; Invitrogen) for 20 min. The cells were washed 3x with PBS and visualized under the confocal microscope.

2.8 Confocal Microscopy

The images were acquired by sequential scanning using a Leica TCS SP2 confocal system (Leica Microsystems, Milton Keynes, UK) with a 63× ceramic dipping objective. A 488 nm laser was used for excitation of Alexa-Fluor 488 labelled goat anti-rabbit IgG, while a 543 nm laser was used for Alexa-Fluor 568 labelled goat anti-mouse IgG excitation and a 633 nm laser for the TO-PRO® - 3 Iodide excitation.

2.9 Cell viability assay

To determine the viability of the cells the MTT assay was used. The cells were plated into a 96 well plate (tissue culture treated). The plate was incubated for 24 hrs, allowing the cells to adhere to the plate. The neurotoxins were added to the plate and incubated for 2-4 hrs at 37^{o}C. After incubation MTT (5 µg/ml) 50 µl/well was added. The plate was incubated for 3-4 hrs until purple crystals

appeared. Once the purple colour was observed, the crystals were dissolved using the solvent (50 µl/well) (80% v/v DMSO and 20% v/v ethanol). The plate was read using an MTT plate (absorbance 540-650 nm) reader. Trypan blue (0.4% w/v) was used to confirm the viability of cells exposed to Aβ. The cells were counted and the percentage of cells alive calculated as those cells that excluded the trypan blue dye, while dead cells were stained blue.

2.10 Data analysis

All data are expressed as means ± s.e.m. For cytotoxicity experiments data are expressed as percentage dead (trypan blue stained) cells or percentage control cells MTT reduction. Statistical analysis was performed by one-way analysis of variance (ANOVA) for more than two groups of test samples or students-t test using GraphPad Prism software (version 6). Post-hoc analysis of ANOVA data was carried with Tukey or Dunnett multiple comparison based on the recommendations of GraphPad Prism software for the data sets concerned. For all experiments a P value of < 0.05 was considered statistically significant. The data shown is the best representation of the means of the biological repeats.

Chapter 3

Amyloid-β binding to catalase and kisspeptin

3.1 Introduction

Catalase binding to Aβ has previously been characterized (Milton 1999; Rensink *et al.,* 2002). Catalase was shown to have an Aβ binding domain, which binds Aβ, using an antisense peptide approach (Milton *et al.,* 2001). Peptides that share sequence similarity with the catalase Aβ binding domain could also bind Aβ (Milton *et al.,* 2001). Kisspeptin (KP) shares sequence similarity with catalase Aβ binding domain, and has been shown to bind Aβ (Milton 2011; Milton 2013). Compounds that bind amyloid peptides can potentially modify aggregates in the brain (Novick *et al.,* 2012). One of the characteristics of an amyloid peptide is its ability to interact with Congo red (Buxbaum and Linke, 2012). Thioflavin T is another classic marker for the detection of amyloid fibrils. Thioflavin T and Congo red have similar binding sites on an amyloid fibril (Groenning 2010). Thioflavin derivatives such as BTA-EGx inhibit catalase binding to Aβ (Habib *et al.,* 2010). Therefore the binding of biotinylated-Aβ to KP was determined plus the effect of Congo red on biotinylated-Aβ binding to KP.

An irreversible inhibitor of catalase activity, 3-AT was reported to bind catalase and inhibit the ability of catalase to breakdown H_2O_2 (Margoliash and Novogrodsky, 1958). Another compound BTA-EG_4 can bind Aβ and inhibit the catalase – amyloid interaction (Habib *et al.,* 2010); KP- 45-50 inhibits Aβ toxicity (Milton 2011). Therefore the effect of 3-AT, BTA-EG_4 and KP 45-50 on the binding of biotinylated-Aβ to immobilized catalase was determined.

3.1.1 Aim.

- To confirm the binding interactions of catalase and KP with Aβ.

3.1.2 Objectives

- To characterise the binding interaction of catalase and KP with Aβ using binding assay.
- To investigate the role of 3-AT, BTA-EG_4 and KP 45-50 in catalase-amyloid interaction.

3.1.3 Hypothesis

Proteins that share sequence similarity with Aβ, can bind to Aβ peptide. This binding interaction promotes cell survival and decreased cell death. Compounds such as catalase and KP share sequence similarity with Aβ, based on this sequence similarity the binding interaction between catalase, KP and Aβ will be investigated in the present chapter.

3.2 Results

3.2.1 Binding interaction between KP and Aβ

Binding of Aβ to KP peptides has previously been shown using biotinylated-Aβ binding KP coated ELISA plates (Milton 2011; Milton 2013). ELISA was used to confirm the binding interaction between KP and Aβ. The ELISA plates were coated with KP 1-54, KP-13 and KP-10 peptides (1 µg/ml) overnight, the unoccupied sites on the plates were blocked using 0.2% (w/v) casein in carbonate buffer for 30 mins. Biotinylated Aβ 1-42 (1 µg/ml) was added and incubated overnight at 4^{o}C. An alkaline phosphatase polymer-streptavidin conjugate (0.5 µg/ml) was added to the plate and incubated for 2 hrs at 37^{o}C. Absorbance readings at 405 nm showed significant binding of biotinylated Aβ to KP-54, KP-13 and KP-10 when compared to the binding to control wells (Figure 3-1A).

The binding interaction between KP and biotinylated Aβ could be inhibited by Congo red. The Congo red was used where an ELISA plate was coated with KP-54 to which biotin labelled Aβ alone or labelled Aβ plus freshly prepared Congo red was added and incubated at 4^{o}C overnight. Results showed that biotinylated Aβ bound to KP and that this binding could be inhibited by Congo red in a dose dependent manner (Figure 3-1B).

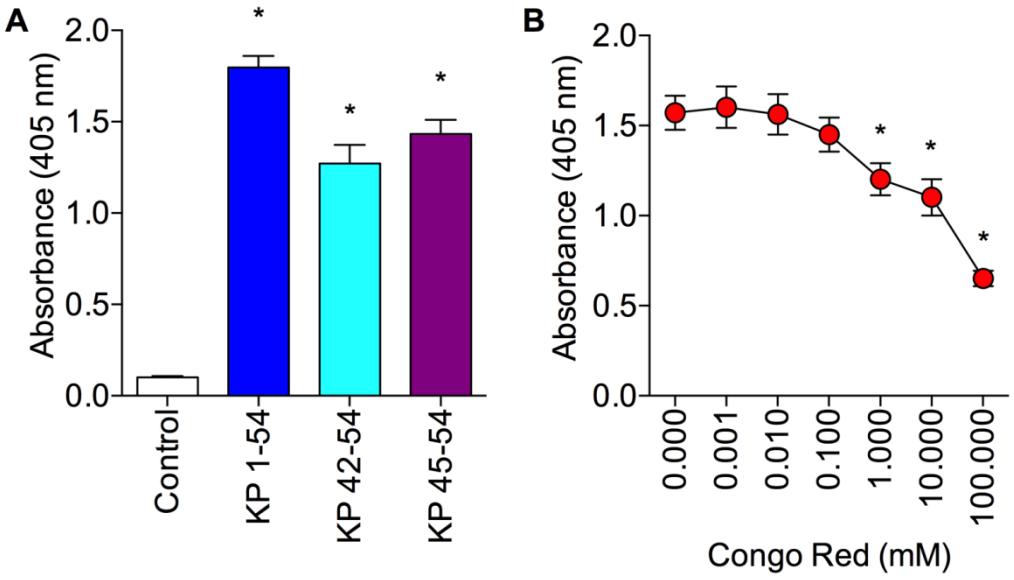

Figure 3-1. Binding interaction between KP and Aβ. ELISA was used to determine the interaction between KP and biotinylated Aβ 1-42 and to investigate if the binding interaction could be modified by Congo red. (A) ELISA plates were coated with KP-54, KP-13 and KP-10 or KP-54 (1 µg/ml) alone or no peptide (control). (B) The plate was coated with KP-54 to which biotinylated Aβ 1-42 alone or biotinylated Aβ 1-42 plus freshly prepared Congo red (0 - 100 mM) was added and incubated at 4^{o}C overnight. Results are mean ± S.E.M (n = 6); * = P < 0.05 vs Aβ (one-way ANOVA).

3.2.2 Catalase and Aβ dose response curve and catalase binding to Aβ 1-40, Aβ 25-35 and Aβ 31-35

Catalase binding to Aβ has previously been shown using biotinylated-Aβ binding catalase coated ELISA plates (Milton 1999). To confirm catalase binding to Aβ, ELISA plates were coated with catalase (1 µg/ml), overnight. Next day, different doses of biotinylated Aβ 1-42 ranging from 0-100 µM were added to the catalase coated ELISA plate and incubated overnight at 4^{o}C. Results showed the catalase-amyloid interaction increased with an increase in the concentration of Aβ (Figure 3-2A). This report supports the previous results that found catalase binds Aβ with high affinity (Milton 1999). To investigate the binding of catalase to Aβ of different peptide lengths, ELISA plates were coated with catalase (1 µg/ml), overnight. Next day, either biotinylated Aβ 1-42 (1 µg/ml) or biotinylated Aβ 1-42 plus unlabelled Aβ 1-40, Aβ 25-35 and Aβ 31-35 (1 µg/ml) was added to the catalase coated ELISA plate, the plate was incubated overnight at 4^{o}C. Results (absorbance at 405 nm) showed that catalase bound to biotinylated Aβ 1-42, this catalase binding to biotinylated Aβ 1-42 could be inhibited by unlabelled Aβ 1-40, Aβ 25-35 and Aβ 31-35 (Figure 3-2B). This shows that the unlabelled and labelled Aβ bound the same site and suggests that the catalase recognizes a region of Aβ containing the 31-35 sequence.

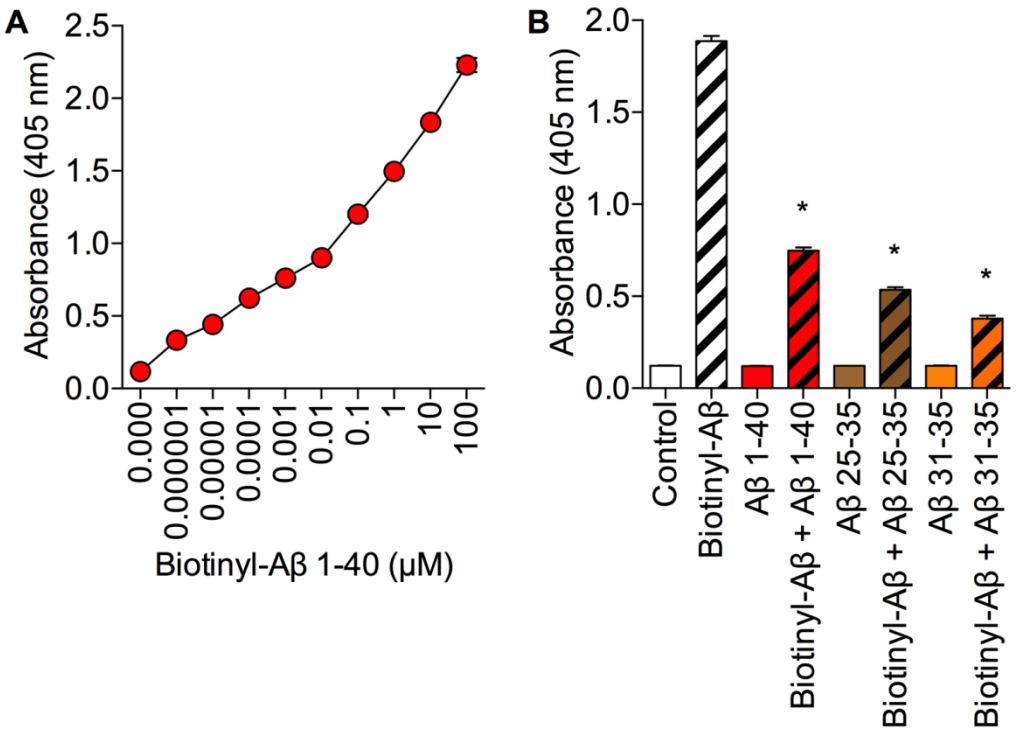

Figure 3-2. Catalase and Aβ dose response curve. (A) ELISA plates were coated with catalase (1 μg/ml) and different concentrations of biotinylated Aβ 1-42 (0 - 100 μM) were added and incubated overnight at 4^{o}C. (B) To investigate the binding of catalase to amyloid β peptides of different lengths, an ELISA plate was coated with catalase (1 μg/ml). Either biotinylated Aβ 1- 40 (1 μg/ml) alone or together with unlabelled Aβ 1-40, Aβ 25-35 and Aβ 31-35 (1 μg/ml) were added to the plate and the plate incubated overnight at 4^{o}C. Results are mean ± S.E.M (n = 6); * = P < 0.05 vs biotinylated Aβ alone (one-way ANOVA).

3.2.3 Effects of BTA-EG$_4$, KP 45-50 and 3-AT on Aβ binding to catalase

To further investigate the binding of catalase to Aβ, the effect of BTA-EG$_4$, KP 45-50 and 3-AT on catalase binding to Aβ 1-42 was tested. ELISA plates were coated with catalase (1 μg/ml). The catalase coated ELISA plates were incubated with (1 μM) biotinylated Aβ in the presence of 0-80 mM 3-AT, 0 – 80 mM BTA-EG$_4$ or 0 – 80 μM KP 45-50 overnight at 4^{o}C. Results (absorbance at 405 nm) showed that BTA-EG$_4$ and KP 45-50 significantly inhibited catalase amyloid binding, whereas 3-AT had no effect on catalase-amyloid interaction (Figure 3-3). KP was more effective in inhibiting catalase-amyloid interaction

(1.25 µM), compared to BTA-EG_4 (2.5 mM) (Figure 3-3). These observations suggests that KP and BTA-EG_4 can bind Aβ, KP can inhibit catalase binding to Aβ and BTA-EG_4 is required in higher concentrations to inhibit catalase-amyloid interaction, these results are consistent with the previous study (Habib *et al.,* 2010). 3-AT does not interfere with catalase-amyloid interaction, suggesting that 3-AT could not enhance Aβ toxicity by inhibiting catalase-amyloid interaction.

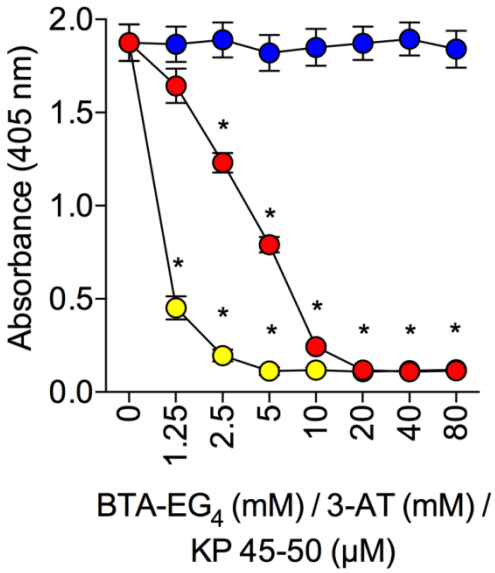

Figure 3-3. Effects of BTA-EG_4, KP 45-50 and 3-AT on Aβ binding to catalase. Catalase coated plates (1 µg/ml) were incubated with 1 µM biotinylated Aβ 1-42 in the presence of 0 - 80 mM 3-AT (blue circles), 0 - 80 mM BTA-EG_4 (red circles), or 0 - 80 µM KP 45-50 (yellow circles). Results are mean ± S.E.M (n = 6); * = $P < 0.05$ vs biotinylated Aβ (one-way ANOVA).

3.3 Discussion

The dose response curve of catalase-amyloid interaction (section 3.2.2) is in agreement with previous experimental studies of the binding interaction between catalase and Aβ (Milton 1999; Habib *et al.,* 2010). In the present study catalase has been shown to bind different peptide lengths of labelled and unlabelled Aβ (section 3.2.2). Due to the sequence similarity between KP and catalase, binding studies with KP and Aβ showed similar results as the binding interaction between catalase and Aβ. The KP 1-54, KP 1-13 and KP-10 were shown to bind biotin labelled Aβ (section 3.2.1), in agreement with Milton (2011). The binding interaction between KP and Aβ was inhibited when Congo red was added. Biotin labelled Aβ 1-42 could not bind to KP in the presence of Congo red resulting in decreased absorbance readings (section 3.2.1). This suggests that Congo red and KP bind to the same region of Aβ and compete for the binding site when added together.

Congo red and Thioflavin T compete for the binding sites on Aβ (Groenning 2010). Thioflavin derivative BTA-EG_4 was shown to inhibit catalase-amyloid interaction (Habib *et al.,* 2010). The effect of BTA-EG_4, catalase activity inhibitor 3-AT and KP 45-50 peptide on the catalase-amyloid interaction was investigated. Results showed that BTA-EG_4 and KP 45-54 significantly inhibited catalase-amyloid interaction, whereas 3-AT did not have any effect on catalase-amyloid binding interaction (section 3.2.3). This confirms the results obtained in the previous study, which showed the inhibition of catalase-amyloid interaction by BTA-EG_4 (Habib *et al.,* 2010). The KP was more effective than BTA-EG_4 as KP could significantly inhibit the interaction at 1.25 µM compared to BTA-EG_4, which was required at higher concentration 2.5 mM to inhibit catalase-amyloid interaction (section 3.2.3). The requirement of milli molar concentration of BTA-EG_4 to inhibit catalase-amyloid interaction is in agreement with previous studies (Inbar *et al.,* 2006; Habib *et al.,* 2010).

3.4 Conclusion

The results presented in this chapter confirm that the Aβ peptide binds to both catalase and KP peptides, in agreement with previous studies (Milton 1999; Inbar *et al.,* 2006; Habib *et al.,* 2010; Milton 2011; Milton 2013). The KP 45-50 fragment can inhibit the binding to catalase, suggesting that both catalase and KP target a similar region of the Aβ peptide. These results provide a basis for looking for co-localization of KP and catalase with the Aβ containing plaques in the AD brain. They also suggest that neuronal overexpression of these Aβ binding compounds has the potential for neuroprotection against Aβ toxicity.

Chapter 4

Immunolocalization of catalase and kisspeptin associated with amyloid-β deposits in the pons of an Alzheimer’s disease patient

4.1 Introduction

The KP peptide was found to have sequence similarity with the catalase region that binds Aβ (Milton 2011). Catalase binding to Aβ has previously been shown (Milton 1999) and confirmed in Chapter 3 (Figure 3-2). The binding of catalase has previously been found in amyloid plaques in the AD brain (Pappolla *et al.,* 1992). The deposition of amyloid in the plaques is the central feature of AD pathology (Karran *et al.,* 2011; Reitz 2012). Aβ deposition occurs in different parts of the brain as the disease progresses (Thal *et al.,* 2002; Alafuzoff *et al.,* 2009). A previous study has shown that Aβ deposits localize last in the cerebellum and pons region of the brain (Thal *et al.,* 2002). Overexpression of the APP gene in transgenic mice showed a similar sequential pattern, where the deposition of Aβ was last localized in cerebellum and pons region of the brain (Rijal Upadhaya *et al.,* 2012). This resistance to Aβ deposition in pons and cerebellum suggests the presence of endogenous neuroprotective components that delay the deposition of Aβ in these areas of the brain.

Neuroprotection against Aβ by the CRH peptide has been well established (Facci *et al.,* 2003; Bayatti and Behl, 2005). The CRH is a peptide hormone and neurotransmitter involved in the stress response, and evokes the release of adrenocorticotrophic hormone from pituitary (Arborelius *et al.,* 1999). CRH neuroprotection was found to be receptor mediated (Facci *et al.,* 2003), CRH does not bind Aβ unlike catalase (Milton 1999). The CRH peptide was found associated with Thioflavin-S positive amyloid deposits in the AD brain (Powers *et al.,* 1987) and reduced levels of CRH was found in some parts of the brain in AD (De Souza *et al.,* 1987).

The KP peptide (Brailoiu *et al.,* 2005), CRH peptide (Austin *et al.,* 2003) and catalase (Moreno *et al.,* 1995) are all found in the pons region of the brain. In this study the localization of immunoreactive (ir) KP, CRH and catalase in relation to Aβ deposits have been determined in pons sections from a male AD patient.

4.1.1 Aim

- The pons sections of an AD patient was used to investigate the presence of immunoreactive catalase and KP in the amyloid plaque like deposits.

4.1.2 Objectives

- To characterise the binding of anti-Aβ, anti-KP, anti-catalase and anti-CRH antibodies.
- Staining the pons sections of a normal male patient with characterized antibodies against Aβ and KP.
- Staining the pons sections of the AD patient with characterized antibodies against Aβ, catalase, KP and CRH.

4.1.3 Hypothesis

As KP and catalase bind Aβ in a cell free assay, the localization of catalase, KP and Aβ in an AD patient's pons sections may shed some light on the binding interaction of these peptides in AD. Immunohistochemistry will be used to detect the localization of catalase, KP and Aβ in the AD patient's pons sections.

4.2 Results

4.2.1 Characterization of antibodies

The binding of BAM-10 mouse anti-Aβ antibody (Kotilinek 2002), rabbit anti-Aβ 21–32 antiserum (Milton 2005), rabbit anti-KP 45–54 antiserum, CAT-505 mouse anti-catalase antibody (Hwang *et al.,* 2007), and KCHMB001 mouse anti-CRH antibody (Milton 1990) were tested for binding to Aβ, KP, NPFF, catalase, and CRH. The ELISA plates were coated with Aβ 1–42, Aβ 17–40, KP 1–54, KP 45–54, NPFF, catalase, and CRH. The binding interaction was detected using alkaline phosphatase conjugated secondary antibodies and p-nitrophenylphosphate substrate. Results showed that both BAM-10 antibody (Figure 4-1A) and anti-Aβ 21–32 antiserum (Figure 4-1B) both bound to the full length Aβ 1- 42, but did not bind to KP, NPFF, catalase or CRH. The BAM-10 antibody is specific for Aβ 1–12 (Kotilinek *et al.,* 2002) and did not show binding to Aβ 17- 40, whereas anti Aβ 21–32 antiserum binds to the Aβ 17- 40. The anti-KP 45-54 antiserum (Figure 4-1C) showed significant binding to KP 1- 54, KP 45-50 and NPFF; however it did not cross react with Aβ, catalase or CRH. The CAT-505 anti-catalase antibody (Figure 4-1D) showed significant binding to catalase, the antibody did not cross react with Aβ, KP, NPFF, or CRH peptides. The KCHMB001 anti-CRH antibody (Figure 4-1E) showed significant binding to CRH and showed no cross-reactivity with Aβ, KP, NPFF or catalase.

A second set of plates were coated with Aβ 1- 42 and were pre-incubated with the following peptides or proteins - KP 45–54, NPFF, catalase, or CRH overnight before addition of BAM-10 mouse anti-Aβ antibody (Figure 4-2A), anti-Aβ 21–32 antiserum (Figure 4-2B), anti-KP 45-54 antiserum (Figure 4-2C), CAT-505 anti-catalase antibody (Figure 4-2D) or KCHMB001 anti-CRH antibody (Figure 4-2E). Both anti-Aβ antibodies showed significant binding to Aβ 1- 42 even after pre-incubation with the other peptides (Figure 4-2A & B). The anti-KP 45-54 showed significant binding to the plate coated with Aβ 1- 42 and pretreated with KP 45-54. No cross reactivity of the antibody to NPFF was observed (Figure 4-2C). The CAT-505 mouse anti-catalase antibody showed significant binding to the plate coated with Aβ 1- 42 and pretreated with catalase (Figure 4-2D). The KCHMB001 mouse anti-CRH antibody did not bind

significantly to the plate coated with Aβ 1- 42 and pretreated with CRH (Figure 4-2E).

These results suggest that the BAM-10 mouse anti-Aβ antibody (Kotilinek 2002), rabbit, anti-Aβ 21–32 antiserum (Milton 2005), rabbit anti-KP 45–54 antiserum and CAT-505 mouse anti-catalase antibody (Hwang *et al.,* 2007) are suitable for co-localization studies of these proteins that bind the Aβ peptide to examine whether the proposed binding interactions have the potential to occur in the AD brain.

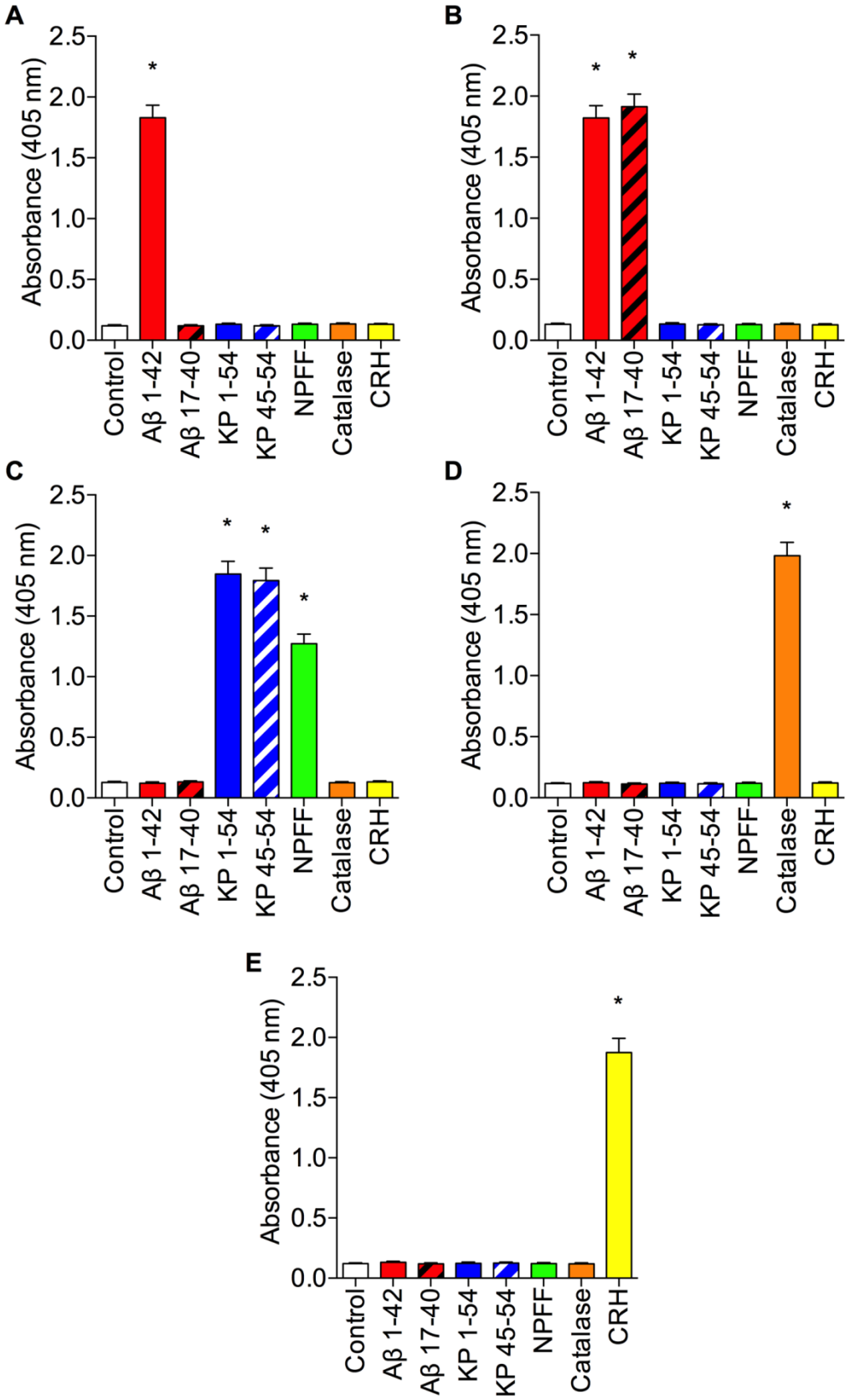
A
B
C
D
E
Absorbance (405 nm)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Aβ 1-42
Aβ 17-40
KP 1-54
KP 45-54
NPFF
Catalase
CRH

Figure 4-1. Characterization of antibodies- To characterise the binding of the antibodies to the respective antigens, plates were coated with Aβ 1- 42, Aβ 17–40, KP 1–54, KP 45–54, NPFF, catalase, and CRH (1 µg/ml overnight). The BAM-10 mouse anti-Aβ antibody (A), rabbit anti-Aβ 21–32 antiserum (B), rabbit anti-KP 45–54 antiserum (C), CAT-505 mouse anti-catalase antibody (D) and the KCHMB001 mouse anti-CRH antibody (E) were added to the plate and binding determined by ELISA. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (one-way ANOVA).

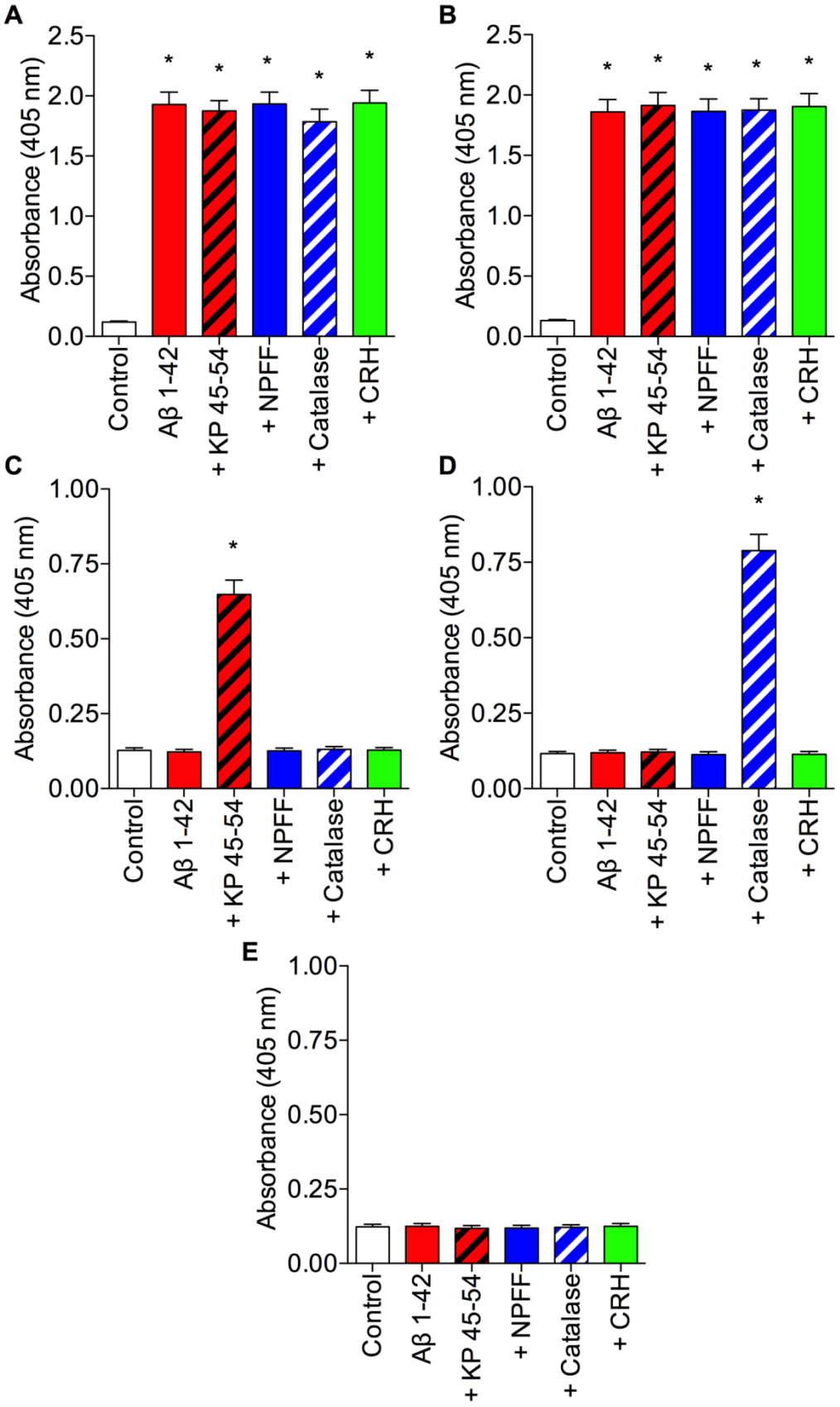
A
B
C
D
E
Absorbance (405 nm)
Control
Aβ 1-42
+ KP 45-54
+ NPFF
+ Catalase
+ CRH
*

Figure 4-2. Characterization of antibody binding to Aβ-protein complexes. To characterise the binding of the antibodies to complexes formed between Aβ and the respective proteins or peptides, plates coated with Aβ 1- 42 (1 µg/ml overnight) and pretreated either one the following peptides or proteins - KP, NPFF, catalase and CRH. The BAM-10 mouse anti-Aβ antibody (A), rabbit anti-Aβ 21–32 antiserum (B), rabbit anti-KP 45–54 antiserum (C), CAT-505 mouse anti-catalase antibody (D) and the KCHMB001 mouse anti-CRH antibody (E) were added to the plate and binding determined by ELISA. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (one-way ANOVA).

4.2.2 Detection of KP and Aβ in a normal pons section

Pons sections from a 26 year old normal Male (Cat No: ab4316; Lot No: A504234) was used to investigate the co-localization of KP and Aβ. The normal pons sections were double labelled with anti-KP 45-54 and BAM-10 anti-Aβ antibodies (Kotilinek *et al.,* 2002). Goat anti-mouse IgG Alexa- Fluor 568 (red) and goat anti-rabbit IgG Alexa-fluor 488 (green) were used as secondary antibodies.

Immunohistochemical analysis with anti-KP 45-54 antibody alone (Figure 4-3A) showed staining that was detectable by green fluorescence and not red fluoresence. Immunohistochemical analysis with BAM-10 anti-Aβ alone showed staining that was detectable by red fluorescence and not green fluorescence (Figure 4-3B). Staining the normal pons sections with the secondary antibodies (goat anti-mouse IgG-Alexa-Fluor 568 and goat anti-rabbit IgG-Alexa-Fluor 488, 1:500) for 1 hour (Figure 4-3D,E,F) alone did not show any background staining. The results from double label immunohistochemistry results showed the staining of tissue with anti-KP45-54 and BAM-10 anti-Aβ antobodies. There was no co-localization of anti-KP45-54 and BAM-10 anti-Aβ label observed in the normal pons sections (Figure 4-3C).

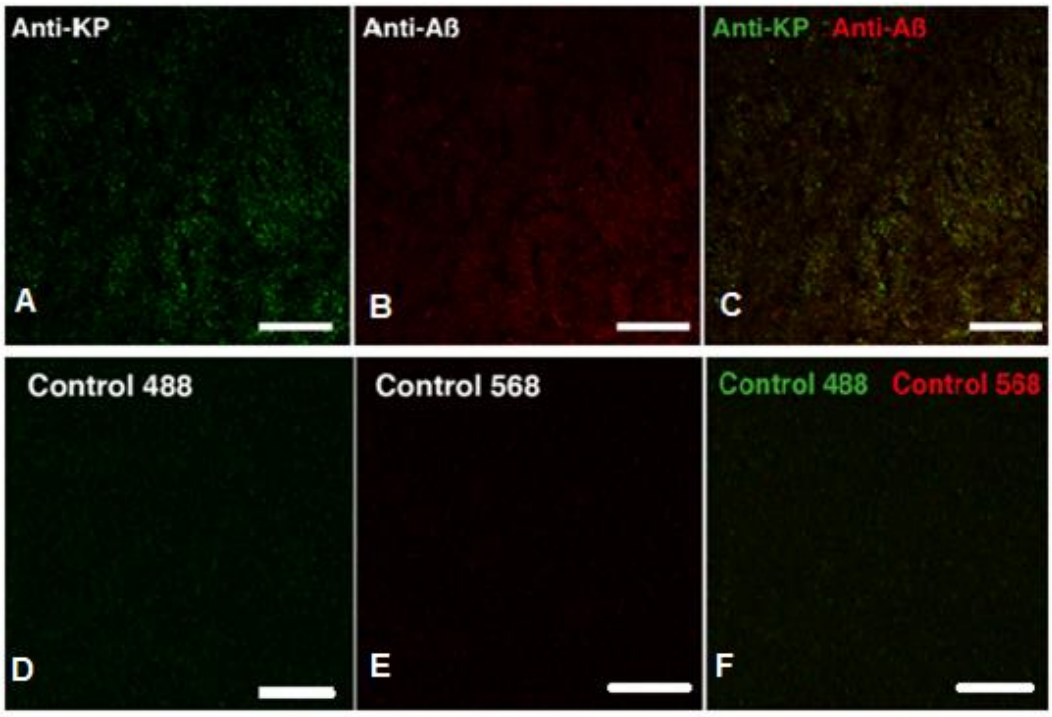

Figure 4-3. Detection of KP and Aβ in normal pons sections. Normal pons sections were double labelled with anti-KP 45-54 (A) and BAM-10 anti- Aβ antibodies (B) to detect co-localization of KP and Aβ (C). Incubating the slides with secondary antibody alone was used to identify background staining (D,E,F). KP appears green and Aβ appears red. The overlap of KP and Aβ appears yellow. Bars = 50 µm.

4.2.3 Detection of KP and Aβ in an AD pons section

Pons sections from a 72-yearold male with AD (Cat No: ab4586; Lot No: B506287) were double labelled with anti-KP 45-54 (Figure 4-4A) and BAM-10 (Figure 4-4B) anti-Aβ antibodies. Results showed that the anti-KP antibody co-localized with anti-Aβ antibodies in AD pons sections (Figure 4-4C). Pre-absorption of the antibody with NPFF (10 µg/ml) (Figure 4-4D) (Iijima *et al.,* 2011) did not reduce the KP signal and co-localization with anti-Aβ antibody was still observed (Figure 4-4F). The co-localization of KP and Aβ was only observed in plaque like deposits rather than throughout the tissue. Incubation of the slides with secondary antibodies alone (Figure 4-4 G, H and I) did not give any background staining, suggesting that the staining observed is specific for KP and Aβ antibodies.

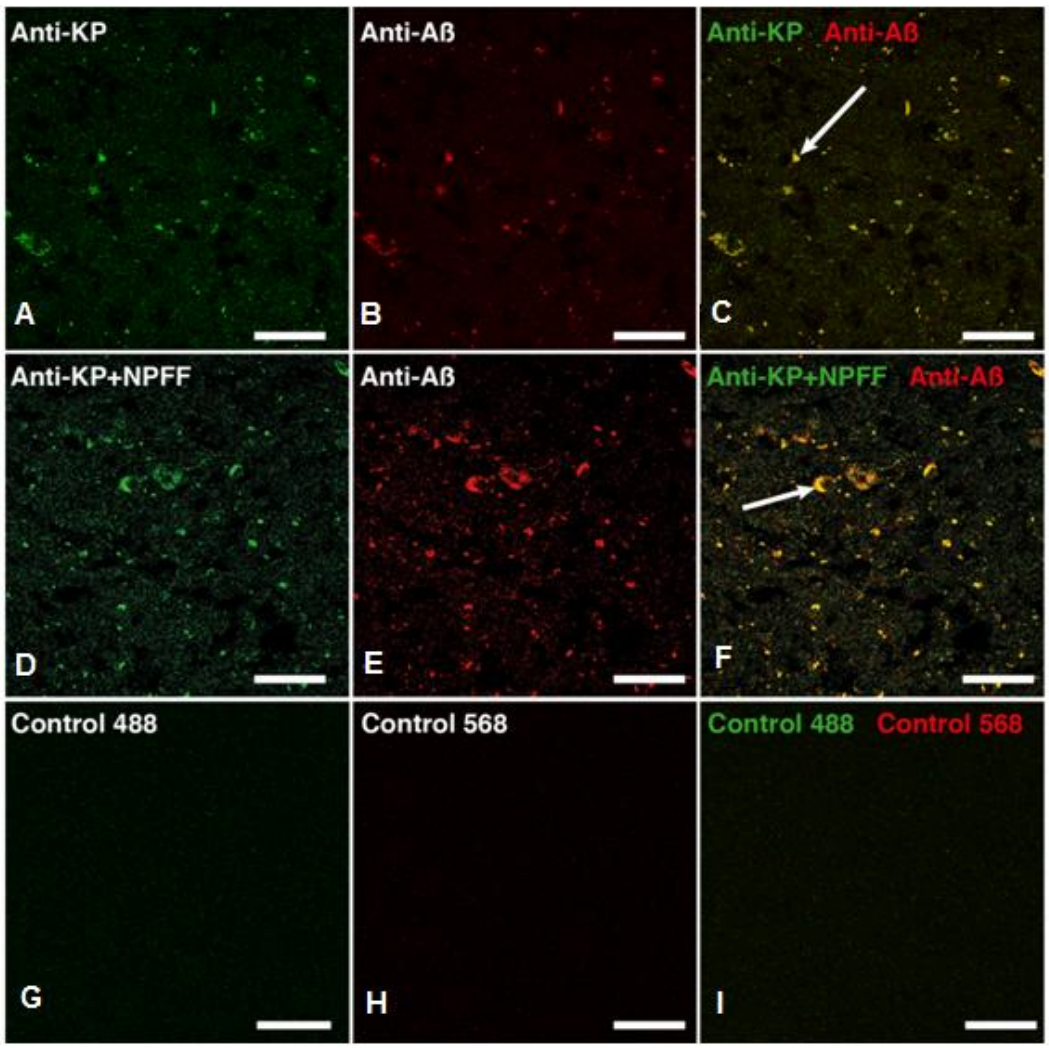

Figure 4-4. Dectection of KP and Aβ in AD pons sections. The pons sections from an AD patient were double labelled with anti-KP 45-54 (A) and BAM-10 anti-Aβ antibodies (B) to detect co-localization of KP and Aβ (C). Pre-absorption (D) of the anti-KP antibody with NPFF (10 µg/ml for 24 hrs) prior to double labelled with BAM-10 anti-Aβ antibodies (E) was used to confirm co-localization of specific KP binding and Aβ (F). Incubating the slides with secondary antibody alone was used to identify background staining (G,H,I). KP appears green and Aβ appears red. The overlap of KP and Aβ appears yellow and examples are labelled with arrows in (C) and (F). Bars = 50 µm.

4.2.4 Detection of catalase in amyloid like plaques in AD pons sections

Previous studies have shown the presence of catalase in AD amyloid plaques (Pappolla *et al.,* 1992). This observation was confirmed by double label immunohistochemistry with a CAT-505 monoclonal anti-catalase antibody (Figure 4-5A) and polyclonal anti-Aβ 21-32 antibody (Figure 4-5B) in AD pons sections. Results showed the label with both antibodies and co-localisation of catalase in amyloid like plaque deposits (Figure 4-5C). Incubation of the slides with the secondary antibodies alone did not show any background staining (Figure 4-5 D, E and F), suggesting that the staining was specific. This confirms the presence of immunoreactive catalase in plaque like deposits in AD pons section.

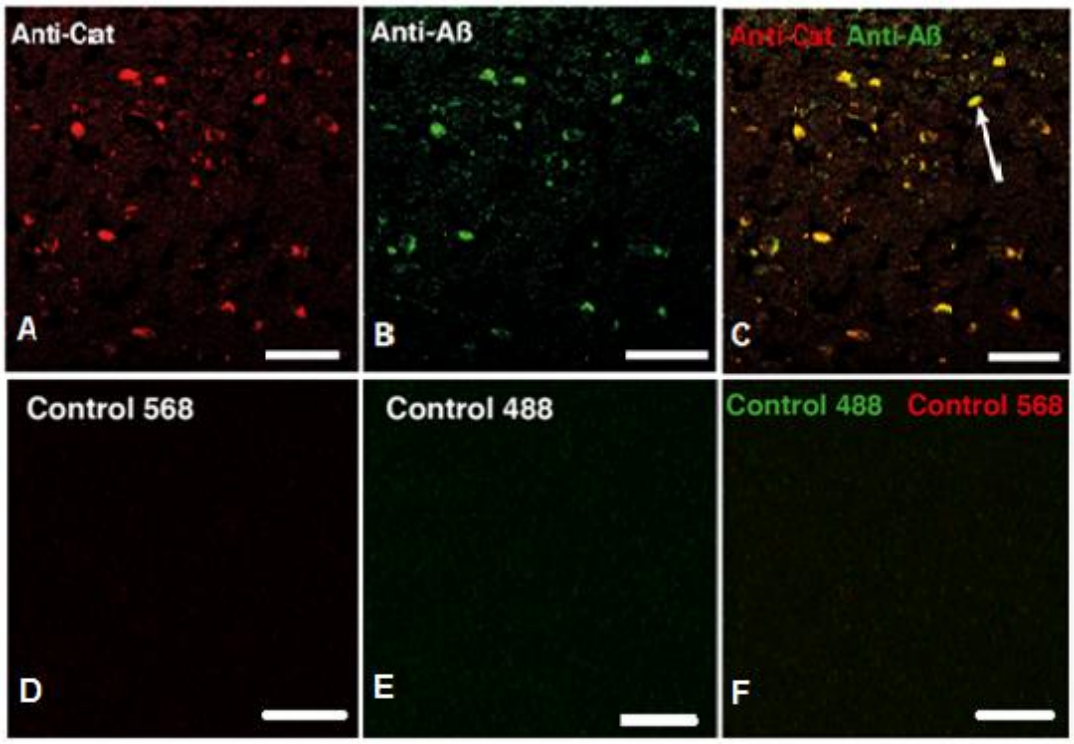

Figure 4-5. Dectection of catalase and Aβ in AD pons sections. Pons sections from AD patient were stained with CAT-505 monoclonal anti-catalase antibody (A) and polyclonal anti-Aβ 21-32 antibody (B) to detect co-localization of catalase and Aβ (C). Incubating the slides with secondary antibody alone was used to identify background staining (D,E,F). Catalase appears red and Aβ appears green. The overlap of catalase and Aβ appears yellow and an example is labelled with an arrow in (C). Bars = 50 µm.

4.2.5 Double label immunohistochemistry for KP and catalase.

The pons sections from an AD patient were stained with anti-KP antibody, pre-absorbed with NPFF (10 µg/ml) for 24 hrs (Figure 4-6A) and CAT-505 monoclonal anti-catalase antibody (Figure 4-6B). Results showed that the amyloid like plaque deposits were stained with both the catalase and KP antibodies and both the antibodies co-localized in the pons section (Figure 4-6C). At higher magnification, it was observed that catalase and KP occupied different sites, with only a small proportion of co-localization within the amyloid plaque like deposits (Figure 4-6 C, D and E). This suggests the presence of multiple binding sites on Aβ, which allows the binding of both catalase and KP, sometimes the binding sites could be next to each other allowing co-localization of catalase and KP. Amyloid plaques like deposits with only catalase or KP were not observed. Incubation of the slides with the secondary antibodies alone did not show any background staining (Figure 4-6 G, H and I).

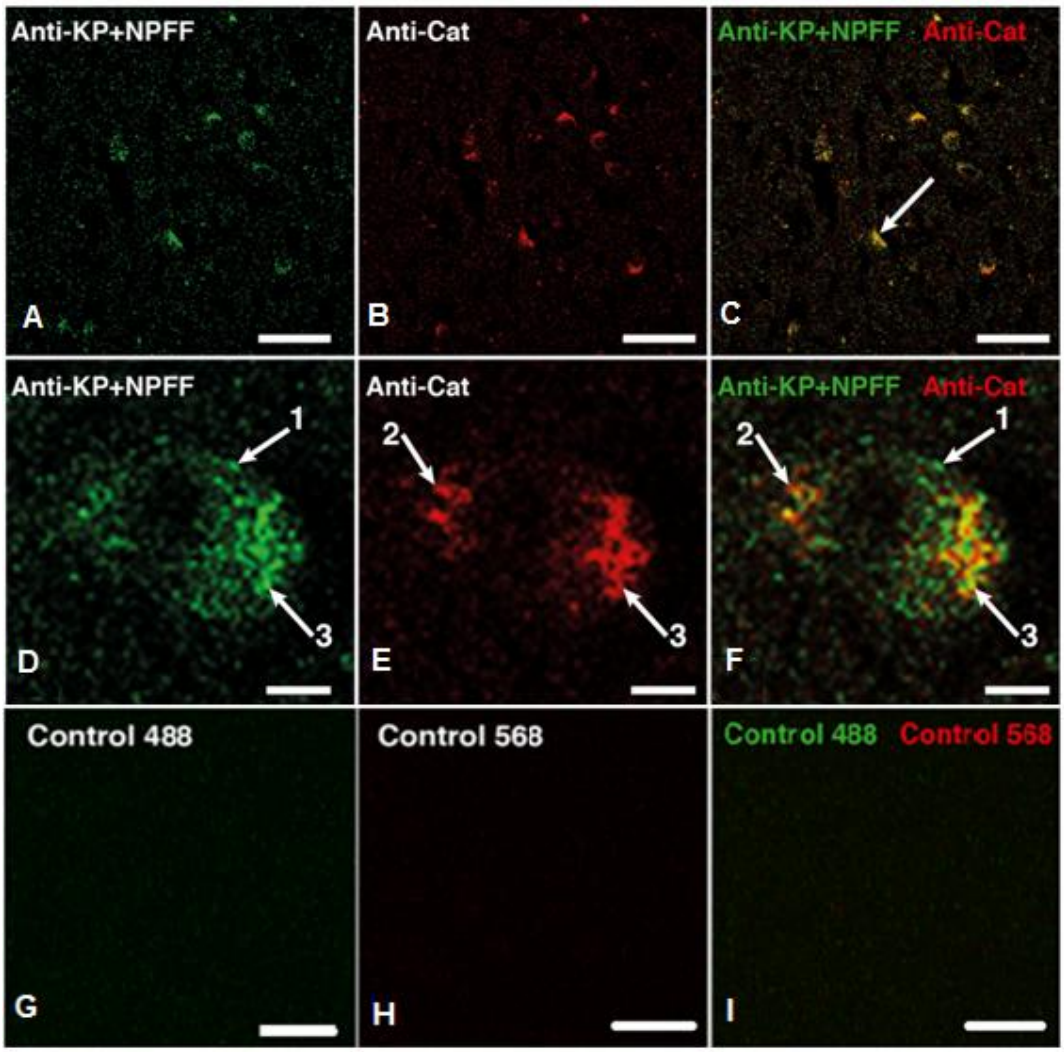

Figure 4-6. Double labeling immunohistochemistry for KP and catalase. The pons sections from an AD patient were incubated with pre-absorbed (with NPFF 10 µg/ml) anti-KP antibody (A,D) and CAT-505 monoclonal anti-catalase antibody (B,E) to detect co-localization of KP and catalase (C,F). Incubating the slides with secondary antibody alone was used to identify background staining (G,H,I). Catalase appears red, KP appears green and co-localization appears yellow. The arrows labelled 1 correspond to an example of KP only label in (D) and (F); arrows labelled 2 correspond to an example of catalase only label in (E) and (F); and arrows labelled 3 correspond to an example of KP and catalase co-localizing in (D), (E) and (F). Bars = 50 µm (A-C) and (G-I) and 5 µm (D) to (F).

4.2.6 Detection of CRH in amyloid like plaques in AD pons sections

A previous study has shown the presence of CRH in AD amyloid plaques (Powers *et al.,* 1987). To confirm the presence of CRH in pons sections of an AD patient, the sections were incubated with KCHMB001 monoclonal anti-CRH antibody (Figure 4-7A) and polyclonal anti- Aβ 21-32 antibody (Figure 4-7B). Results showed label with both antibodies and co-localization of the Aβ and CRH in plaque like deposits (Figure 4-5C). The polyclonal Aβ antibody showed a similar pattern of labeling, as seen with the BAM-10 anti- Aβ monoclonal antibody. Incubation of the slides with the secondary antibodies alone did not show any background staining (Figure 4-5D, E and F), suggesting that the staining observed was specific for the primary antibodies used.

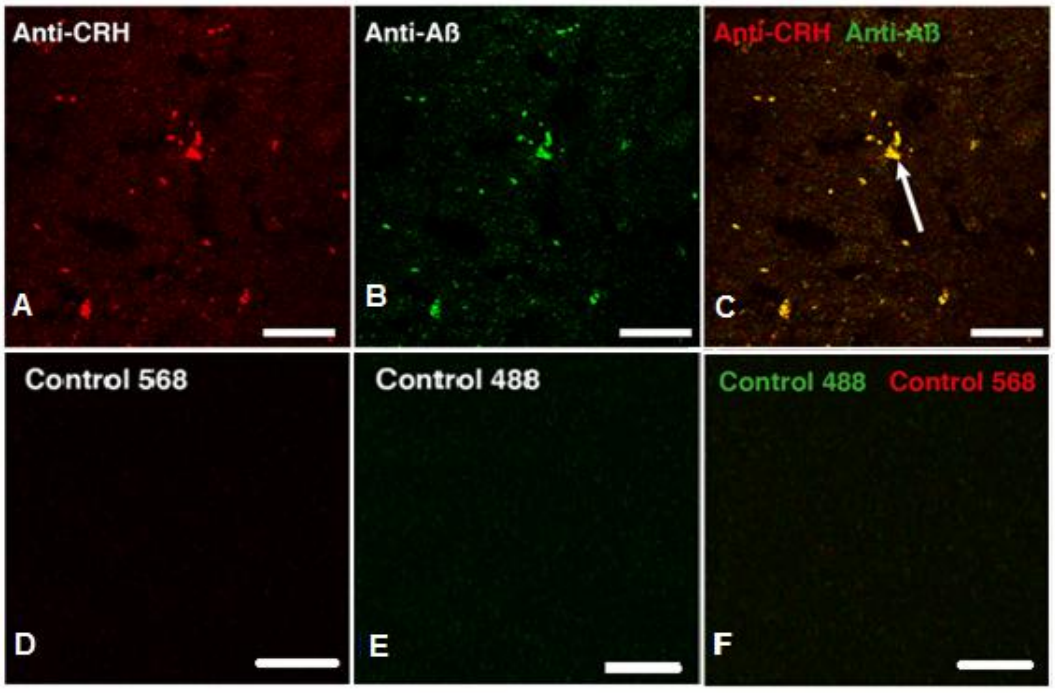

Figure 4-7. Detection of CRH and Aβ in AD pons sections. Pons sections from AD patient were stained with KCHMB001 monoclonal anti-CRH antibody (A) and polyclonal anti-Aβ 21-32 antibody (B) to detect co-localization of CRH and Aβ (C). Incubating the slides with secondary antibody alone was used to identify background staining (D,E,F). CRH appears red and Aβ appears green. The overlap of catalase and Aβ appears yellow and an example is labelled with an arrow in (C). Bars = 50 µm.

4.3 Discussion

In AD the event of deposition of amyloid plaques in the cerebellum and pons region of the brain seems to occur at the end, i.e. after deposition of Aβ plaques in all the other regions of the brain (Thal *et al.,* 2002). This suggests that endogenous neuroprotective processes may play a role in delaying Aβ deposition in these brain regions. The double label immunohistochemistry shows the co-localization of KP and Aβ in plaque like deposits. The presence of KP in amyloid plaque like deposits has never been shown before, this is a novel observation (section 4.2.3). The anti-KP antibody is known to cross react with NPFF, to eliminate the possibility of cross-reactivity, the anti-KP antibody was pre-absorbed with NPFF (10 µg/ml) for 24 hrs. The pre-absorption did not reduce the signal that was previously observed with anti-KP antibody, suggesting that the anti-KP antibody is not cross reacting with NPFF and the antibody signal observed is specific for KP (section 4.2.3). The co-localization of catalase with Aβ (section 4.2.4) and CRH with Aβ (section 4.2.6) in pons sections of AD patients, confirms previous observations (Powers *et al.,* 1987; Pappolla *et al.,* 1992).

Staining the pons section of the AD brain with anti-catalase and anti-KP antibodies showed that both KP and catalase occupied specific sites on the plaque like deposits. Co-localization of immunoreactive catalase and KP was also observed, this could be due to the presence of more than one binding site in close vicinity in the plaque like deposits (section 4.2.5).The binding of catalase (section 3.2.2) and KP (section 3.2.1) to Aβ confirmed previous studies (Milton 1999; Milton 2011). The CRH peptide does not bind Aβ (Milton 1999) and from the antibody characterization no complexes were detected (section 4.2.1). This suggests that mere the presence of KP, catalase and CRH in the plaque like deposits does not confirm binding of KP, catalase or CRH to Aβ. The peptides or proteins could be trapped in the plaques or could be present in close vicinity to Aβ or it could be that the peptides or proteins bind to some other component of the plaque.

The direct interaction between KP and Aβ (section 3.2.1) combined with the immunohistochemistry results suggest that KP and Aβ might interact in an AD

brain. The presence of both catalase and KP in the pons section of the AD brain may contribute to neuroprotection against Aβ toxicity and could delay neurodegeneration. Further experimental investigations are required to confirm these findings.

4.4 Conclusion

The results presented in this chapter confirm the co-localization of catalase with Aβ deposits in the AD brain, in agreement with previous studies (Pappolla *et al.,* 1992). The co-localization of KP with Aβ deposits in the AD brain is the first demonstration of this novel observation. The co-localization of CRH with Aβ deposits in the AD brain confirms a previous study (Powers *et al.,* 1987). In view of the failure of the anti-CRH antibody to detect CRH bound to the Aβ peptide *in vitro*, these results suggest that co-localization is not necessarily due to specific binding interactions. The use of sections from a single patient clearly points to the need for further studies to confirm the interactions observed. The results for KP and catalase co-localization with Aβ deposits in the pons region of an AD patient support the suggestion that these compounds may be protective against Aβ toxicity. The results provide further support for the rational of investigating catalase and KP overexpression in neuronal cells as a neuroprotective mechanism and suggest that such studies may be relevant to AD.

Chapter 5

Catalase neuroprotection against H_2O_2 toxicity

5.1 Introduction

The OS response can damage critical biological molecules and initiate a cascade of events leading to impaired cellular function or cell death (Halliwell 1992). It has also been suggested that the senile plaque dense regions in AD may represent an environment of elevated OS (Hensley *et al.,* 1995). The brain is more vulnerable to OS, compared to other organs due to its high lipid content, high oxygen metabolism and low levels of anti-oxidants defences (Reddy 2006). Before the onset of AD pathology, mitochondrial OS occurs leading to AD progression (Manczak *et al.,* 2006). Mitochondria are cellular organelles that perform several cellular functions, including production of cellular ATP. Mitochondria are responsible for generating reactive oxygen species mostly during the electron transport chain (Muirhead *et al.,* 2010). In a murine experimental knock-in model of AD with APP and PS1 mutations, production of excessive Aβ resulted in mitochondrial dysfunction and antioxidant enzyme impairment (Anantharaman *et al.,* 2006). Increased ROS induced by mitochondrial dysfunction causes oxidation of DNA, the hydroxyl radicals react with both the purine and pyrimidines bases of DNA (Halliwell and Cross, 1994). This leads to permanent modification of the genetic material, which is the first step involved in mutagenesis, aging and age related diseases, including cancer and AD (Mao and Reddy, 2011). Mitochondrial dysfunction is proposed to link neuronal synaptic loss and amyloid deposition (Du *et al.,* 2008).

One of the most studied effects of Aβ is to induce and be induced by OS (Borghi *et al.,* 2007). The Aβ has been suggested to be a prooxidant (Hensley *et al.,* 1996). The Aβ induces the production of H_2O_2 (Behl *et al.,* 1994) which could act as fuel to increase OS load. The accumulation of Aβ could also be influenced by OS. The APP expression is increased by the oxidative products and oxidant agents (Cheng and Trombetta, 2004; Patil *et al.,* 2006). In SH-SY5Y human neuroblastoma cells the OS induced by H_2O_2 causes an increase in the levels and intracellular accumulation of Aβ (Misonou *et al.,* 2000). It has been shown that oxidatively damaged membrane proteins promote aggregation and misfolding of Aβ proteins into fibrils, which promotes oxidative damage in synthetic lipid membranes (Murray *et al.,* 2007). Thus the vicious cycle of OS

and Aβ continues throughout AD, both increasing simultaneously in response to one another.

One of the mechanisms of Aβ induced ROS is the binding of Aβ to cellular enzymes that maintain low physiological levels of ROS (Yan *et al.,* 1997; Milton 1999). This binding interaction could potentially result in increased production of ROS or reduced degradation of ROS. The major ROS in cells are superoxides and hydroxyl radicals derived from H_2O_2 (Halliwell 1992). Mitochondria are the major source for the production of both superoxide and H_2O_2 (Cadenas and Davies, 2000). The Aβ peptide has been shown to accumulate in mitochondria (Devi *et al.,* 2006) and therefore Aβ could interact with the mitochondrial proteins. Catalase and glutathione peroxidase are the primary enzymes to breakdown H_2O_2 both inside and outside mitochondria (Halliwell and Gutteridge, 2007). Cells resistant to Aβ toxicity had elevated levels of catalase and glutathione peroxidase (Sagara *et al.,* 1996) and the activity of both the enzymes was reduced in rat brains exposed to Aβ. Overexpression of glutathione peroxidase made the neuronal cells (PC12 cells) resistant to Aβ toxicity (Barkats *et al.,* 2000). Extracellular addition of catalase was found to be protective against Aβ toxicity (Behl *et al.,* 1992). PC12 cells genetically engineered to express higher levels of both catalase and glutathione peroxidase were found to be resistant to Aβ toxicity (Sagara *et al.,* 1996). The Aβ in a cell free assay binds catalase (figure 3-2) (Milton 1999) but not glutathione peroxidase (Habib *et al.,* 2010). The catalase-amyloid binding interaction deactives the H_2O_2 degrading activity of catalase (Milton 1999) and this interaction could increase OS load.

In the present study the role of catalase overexpression in neuronal cells against OS was investigated. For this study human neuroblastoma SH-SY5Y cells were used. The SH-SY5Y cells have neuroblastic morphological characteristics, they resemble human fetal sympathetic neurons grown in primary culture (Lukas *et al.,* 1993). The SH-SY5Y cells possess many properties of dopamenergic neurons and are used as a neuronal model for Parkinson's disease (Xie *et al.,* 2010) as well as AD (Agholme *et al.,* 2010). The SH-SY5Y cells express the catalase gene (Habib *et al.,* 2010) which suggest that the cells possess the machinery to make active catalase, by folding the

protein into a tetramer. SH-SY5Y cells can grow by mitosis or by extending neurites to the surrounding area. SH-SY5Y cells were transfected with the human catalase gene to overexpress catalase protein. The catalase gene overexpression will be tested against H_2O_2 and $CoCl_2$ toxicity. The $CoCl_2$ is a mimic of hypoxia and it induces the generation of ROS (Kotake-Nara and Saida, 2007). The activity of catalase can be inhibited by 3-AT (Milton 2001), the importance of catalase activity in neuroprotection against H_2O_2 toxicity will be tested in cells overexpressing catalase gene.

5.1.1 Tetracycline inducible system

In the T-REx™ system the expression of the gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline (Yao *et al.,* 1998). The major component of the T-REx™ sytem is the inducible expression plasmid (pcDNA™4/TO/myc-His). The expression of the gene of interest (in this case the catalase gene) is controlled by the strong CMV promoter (Boshart *et al*., 1985) into which two copies of the tet operator 2 ($TetO_2$) have been inserted in tandem. Each $TetO_2$ consists of the 19 Nucleotide sequences 5´-TCCCTATCAGTGATAGAGA-3´, which serves as a binding site for two tet repressors (TetR). The second major component of the T-REx™ sytem is the pcDNA™6/TR regulatory vector which expresses high levels of TetR gene (Postle *et al.,* 1984). Both the vectors can be transfected into mammalian host cells by standard transfection methods.

In the absence of tetracycline, the Tet repressor forms a homodimer, which binds to the $TetO_2$ sequence in the promoter of the pcDNA™4/TO/myc-His vector (Hillen and Berens, 1994). The two $TetO_2$ sites in the promoter of the pcDNA™4/TO/myc-His vector serve as the binding site for four molecules of TetR. Binding of TetR to the $TetO_2$ sequence represses the transcription of the catalase gene. When tetracycline is added, it binds to the TetR homodimers with a 1:1 stoichiometry that results in a conformational change in the repressor. After the conformational change, the TetR can no longer bind to the Tet operator, which resumes the overexpression of the catalase gene (Figure 5-1). The tetracycline inducible system will be used to confirm the expression of the catalase gene in the PCat cells. The expression of the catalase gene can then

be regulated, which can be used to test the significance of catalase gene overexpression in neuroprotection against Aβ toxicity.

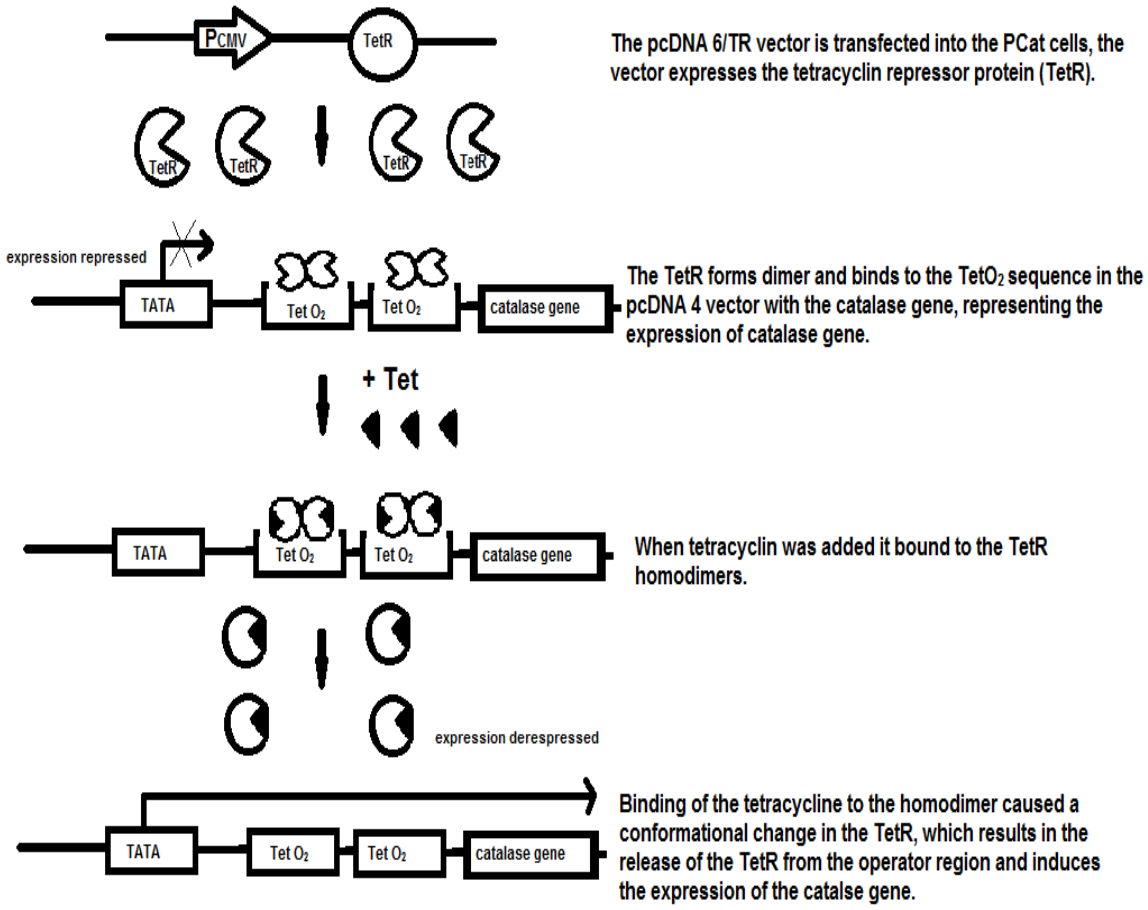

Figure 5-1. The mechanism of action of the tetracycline inducible system. (The figure was adapted from Invitrogen manual)

5.1.2 Aim

- To create and characterize a human SH-SY5Y cell line overexpressing the human catalase gene.

5.1.3 Objectives

- Amplification of the catalase gene using PCR.
- Analyzing the restriction digest of the pcDNA™4/TO/myc-His expression vector containing the catalase gene.
- Create PCat cell line stably overexpressing human catalase.
- Create PCatTR6 cell line stabley expressing a tetracycline inducible catalase expression system.
- Estimation of catalase gene mRNA expression in PCat cells.
- Analyzing the expression of the catalase gene in the presence and absence of tetracycline using the TRex inducible system (Tet On and Off System).
- Analyzing the overexpression of the catalase gene in PCat cells using Immunocytochemistry.
- Analyzing the overexpression of the catalase gene in PCat cells using western blotting.
- Investigating the effect of H_2O_2 on PCat cells.
- Measurement of catalase activity in PCat.
- The effect of cobalt chloride on PCat cells.

5.1.4 Hypothesis

Catalase is an antioxidant and could relieve from the OS seen in AD. Increasing the level of antioxidant defences could improve cell survival by protecting the cells against oxidants. The antioxidant enzyme catalase was overexpressed in SH-SY5Y cells to understand whether increased catalase mediates protection against H_2O_2 induced cell death.

5.2 Results

5.2.1 Characterization of catalase (PCat) gene overexpressing SH-SY5Y human neuroblastoma cells

For the overexpression of catalase, the human catalase gene was cloned into the pcDNA™4/TO/myc-His expression vector and transfected into naïve SH-SY5Y neuroblasoma cells.

5.2.1.1 Amplification of the catalase gene using PCR.

To investigate the role of catalase gene overexpression against Aβ and OS, the pcDNA™4/TO/myc-His expression vector (Origene) was used to clone the catalase gene, which would be later transfected into SH-SY5Y cells. Using PCR the catalase gene was amplified from the pCMV6-XL5 vector containing human catalase gene (Origene). The PCR conditions used for the amplification are mentioned in the materials and methods (2.2.2). The PCR product was run on a 1% agarose gel, where the catalase gene band was found to be 1.5 Kbp (Figure 5-2).

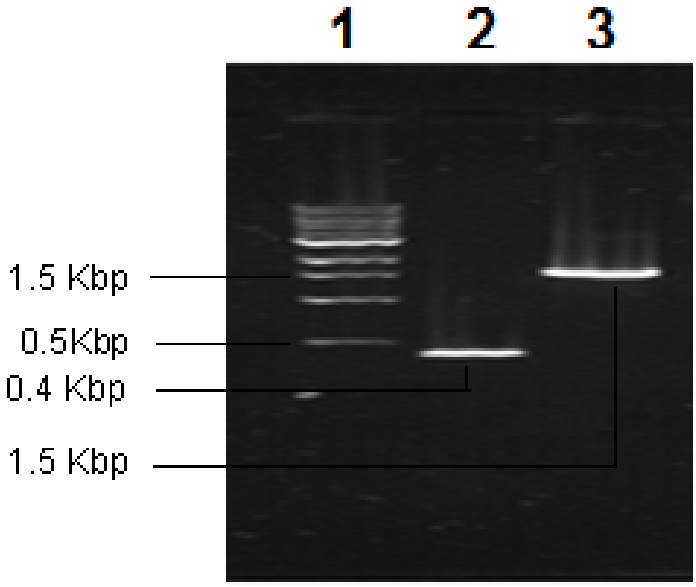

Figure 5-2. PCR amplification of the catalase gene. PCR products were seperated on a 1 % agarose gel. Lane 1 - 1kb ladder, lane 2 - PCR product of the KiSS-1 gene, lane 3 - PCR product of the catalase gene. The PCR products

were amplified from the pCMV6-XL5 vector containing human KiSS-1 gene or the human catalase gene (Origene).

5.2.1.2 Analyzing the restriction digestion of the pcDNA™4/TO/myc-His expression vector containing the catalase gene

The catalase gene was cloned in the pcDNA™4/TO/myc-His expression vector, to confirm the cloning the vectors were digested using the restriction enzymes that would release catalase from the vector (*Hind* III and *Not* I). Figure 5-3 represents the restriction enzyme digestion of the vector containing the catalase gene. The linearised empty restriction digested vector was used as a control which was found to be 5.1 Kbp whereas the cloned catalase gene was found to generate two bands, the linearised vector band 5.1 Kbp and the cloned band of 1.5 Kbp. The DNA sequence of the cloned catalase gene was further confirmed by DNA sequencing (See Appendix 10.1), the DNA sequence of the gene was found to be correct without any mutations (NM_001752.2).

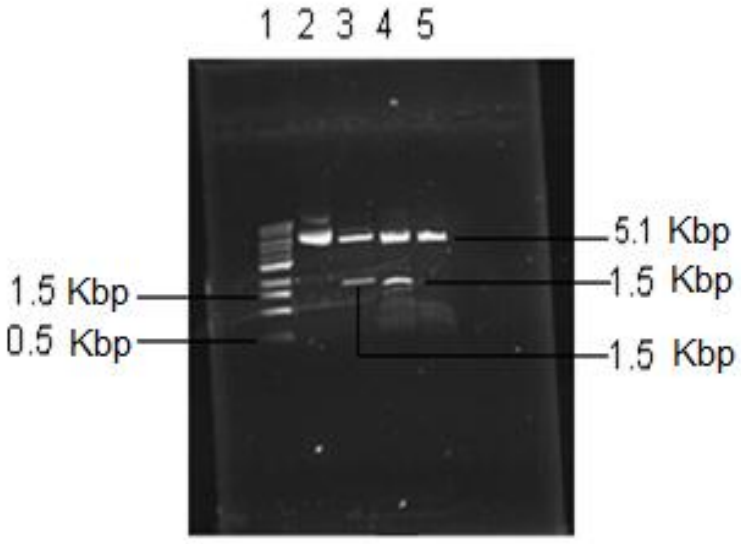

Figure 5-3. The restriction digest analysis of the pcDNA™4/TO/myc-His expression vector containing the catalase gene. The 1% agarose gel picture shows the restriction digestion of the catalase gene cloned into the pcDNA™4/TO/myc-His vector. Lane 1 - 1 kbp ladder, lanes 2 and 5 - linearised

pcDNA™4/TO/myc-His empty vector, lanes 3 and 4 - catalase gene restriction digested (1.5 Kbp) from the pcDNA™4/TO/myc-His vector (5.1 Kbp).

5.2.1.3 Transfection of catalase gene and selection of stably expressing PCat cell line

The pcDNA™4/TO/myc-His vector containing the catalase gene was transfected into the naïve SH-SY5Y cells. The cells were cultured in the presence of zeocin to select pcDNA™4/TO/myc-His vector expressing cells. After culture for 4 weeks in zeocin the stable PCat cell line was subsequently cultured under standard cell culture conditions.

A control cell line was created by transfecting the pcDNA™4/TO/myc-His vector into the naïve SH-SY5Y cells. The cells were cultured in the presence of zeocin to select pcDNA™4/TO/myc-His vector expressing cells. After culturing for a number of weeks in zeocin the stable PVect cell line was subsequently cultured under standard cell culture conditions.

To create tetracycline inducible catalase expressing cell lines PCat cells were transfected with pcDNA™6/TR and stable cell lines selected with zeocin and blasticidin for 4 weeks. The resultant PCatTR6 cell line was subsequently cultured under standard cell culture conditions.

5.2.1.4 Estimation of catalase gene mRNA expression levels in PCat cells

The total RNA extracted from the the PCat cells was used to perform RT- PCR using a one step RT-PCR kit (Qiagen). Figure 5-4 represents the RT-PCR analysis of the PCat and PVect cells. The gel picture shows an increased mRNA expression of the catalase gene band of 1.5 Kbps in the PCat cells (lane 3) compared to PVect cells (lane 5). The negative control did not show any bands (lane 4) (water + PCR master mix) which suggests that the PCR amplication was specific. From the RT-PCR analysis, which is considered a

semi-quantitative assay, it could be suggested that the catalase mRNA expression levels were higher in PCat cells compared to PVect cells.

To ensure that equal amount of the PCat and PVect cDNA were used in the PCR reaction shown in Figure 5-4, human actin primers were used to amplify the same amount of cDNA used for the RT- PCR of the PCat and PVect . The product of the RT-PCR was run on a gel and the band obtained show equal intensity indicating equal loading of the sample in each lane while performing the RT-PCR. The size of the actin bands obtained was found to be 100 bp. This indicated that the differences in PCat expression levels in Figure 5-4 were a true reflection of higher levels of expression of catalase in PCat versus PVect cells and that equal amount of sample was loaded throughout.

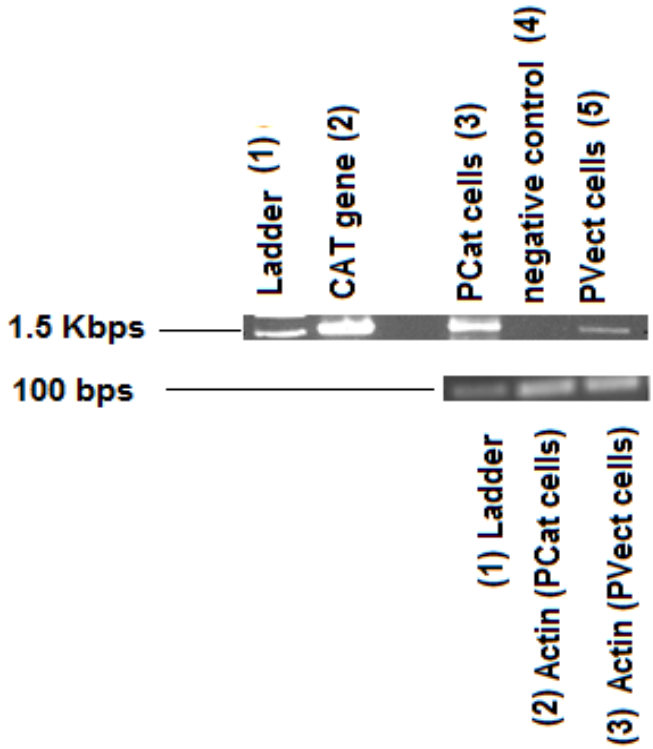

Figure 5-4. Estimation of catalase gene mRNA expression in PCat cells. The RT-PCR gel picture shows the mRNA expression levels of the catalase in the PCat and PVect cell lines. Upper panel, lane 1- 1 Kb ladder, lane 2- catalase gene (positive control amplified from pCMV6-XL5 containing Human catalase gene, Origene), lane 3- catalase mRNA expression in the PCat cells, lane 4- negative control (water+PCR master

mix), lane 5- catalase mRNA expression in the PVect cells. The lower panel shows lane 1- 100 bp ladder, lane 2 and 3- mRNA expression levels of actin in PCat and PVect cells.

5.2.1.5 Analyzing the expression of the catalase gene in the presence and absence of tetracycline using the TRex inducible system (Tet On and Off System)

The tetracycline inducible system was used to switch on and off the overexpression of the catalase gene in the PCat cells. RT-PCR was used to check the induction and repression of the catalase gene under 1 µg/ml tetracyline final concentration. Figure 5-5 shows the difference between catalase mRNA expression levels of the induced and uninduced catalase gene in the presence and absence of tetracycline. The mRNA expression levels of catalase in both the PCat and PCatTR6 induced cells was found to be similar and higher compared to PCatTR6 uninduced cells. From the gel picture it can be suggested that the tetracyline inducible system is working and could be used to switch on and off the expression of the catalase gene using tetracycline. To ensure equal loading of the samples actin primers were used to amplify actin in all the three cell types.

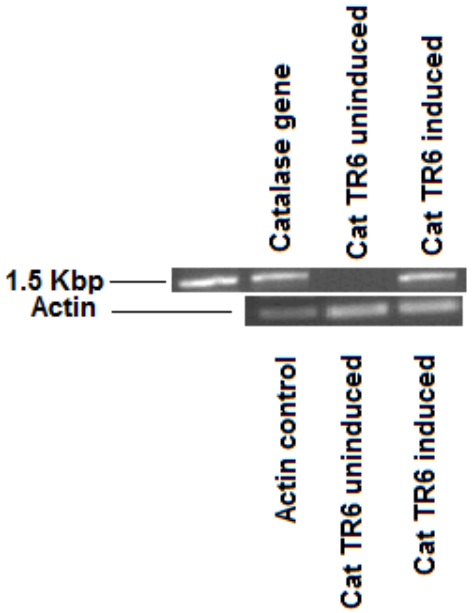

Figure 5-5. Tet inducible system and confirmation of uniform loading of the samples during RT-PCR using actin primers. The gel image shows the

induction and repression of the catalase gene in the PCatTR6 cells using tetracyline (1 µg/ml final concentration). Lane 1- 1.5 Kbps band, lane 2- mRNA expression levels of catalase in PCat cells, lane 3- mRNA expression levels of catalase in TR6 PCat uninduced cells, lane 4- mRNA expression levels of catalase in TR6 PCat induced cells (upper panel). Amplification of actin primers was used to ensure equal loading of samples during RT-PCR (lower panel).

5.2.1.6 Analyzing the overexpression of the catalase gene in PCat cells using Immunocytochemistry

Immunocytochemistry was used to confirm the overexpression of the catalase protein in the cytoplasm of the PCat cells compared to PVect cells. The CAT-505 mouse anti-catalase antibody (1:1000, 1 µg/ml final concentration) was used as the primary antibody, the goat anti-mouse IgG- Alexa-Fluor 568 was used as the secondary antibody (1:500) to stain the immunoreactive catalase present in the cytoplasm of the cells. The nucleus was stained using TO-PRO®-3 Iodide (642/661; Invitrogen).

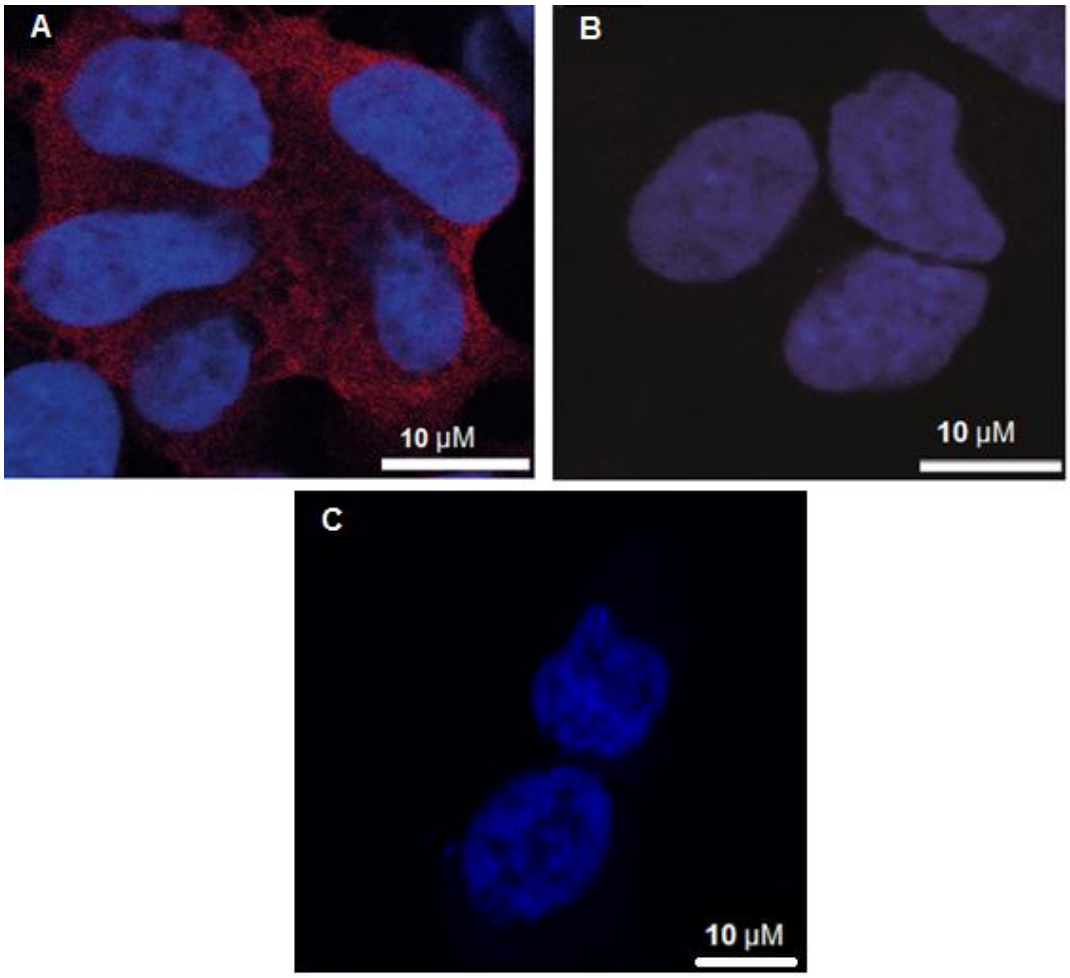

Figure 5-6. Analyzing the overexpression of catalase gene in PCat cells using immunocytochemistry. Immunocytochemistry was carried out using the CAT-505 monoclonal anti-catalase antibody to stain PCat and PVect cells. Immunoreactive catalase (stained red) and TO-PRO®-3 Iodide (stained blue) was detected by confocal microscopy in the PCat (A) and PVect (B) cells. Figure C shows the staining of the PCat cells with the secondary antibody alone, to check for nonspecific staining.

The immunocytochemistry showed a remarkable difference between the PCat cells compared to PVect cells. Figure 5-6A shows the immunoreactive catalase (stained red) in the cytoplasm of the PCat cells, which was higher compared to the level of immunoreactive catalase found in the cytoplasm of the PVect cells 5-6B. There was no catalase found in the nucleus (stained blue). The immunocytochemistry clearly shows the overexpression of catalase in cells with the catalase gene compared to the cells with vector alone. Figure 5-6C shows

the staining of the PCat cells with the secondary antibody alone, which did not show any nonspecific staining.

5.2.1.7 Analyzing the overexpression of the catalase gene in PCat cells using Western blotting

The overexpression of the catalase gene in SH-SY5Y cells was also confirmed by western blotting, this was carried out to see if the cloned catalase gene resulted in overexpression of catalase protein in SH-SY5Y cells. Cell lysates from the PCat and PVect cells were used for the western blotting. The CAT-505 monoclonal mouse anti-catalase antibody was used as the primary antibody and anti- mouse antibody was used as the secondary antibody. A band size of ~60 kDa was observed on the western blot which is approximately the right size for the catalase protein (Figure 5-7). From the western blotting it could be concluded that the catalase gene was overexpressed in the PCat cells compared to PVect cells. To confirm equal loading of the samples, the nitrocellulose membrane was stripped using the stripping buffer and it was reprobed with anti-actin antibody. The ~42 kDa band represents actin stained with anti-actin antibody shows uniform loading of the samples during SDS page.

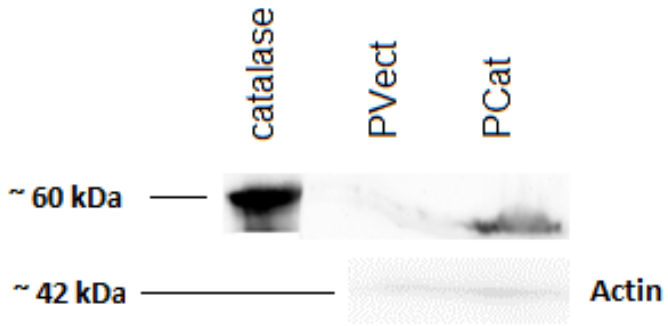

Figure 5-7. Analyzing the overexpression of catalase gene in PCat cells using Western blotting. Lane 1- synthetic catalase (Abcam), lane 2- cell lysate from PVect cells, lane 3- cell lysate from PCat cells. To confirm equal loading of the samples actin was stained on the same blot using the anti-actin antibody which stained a band size of ~42 kDa.

5.2.2 The effect of H_2O_2 on PCat cells

The PCat and PVect cells were exposed to different concentrations of H_2O_2 ranging from 0-1000 µM. The cells were exposed to H_2O_2 for 2 hrs, after which MTT assay was perfomed to determine cell viability. The PCat cells showed a significant increase in the percentage of viable cells compared to PVect cells upon exposure to increasing concentrations of H_2O_2 (Figure 5-8A). This could be due to overexpression of the catalase, which efficiently breaks down H_2O_2 and prevents the cells from experiencing OS which could lead to cell death. To confirm that the protection against H_2O_2 was due to catalase overexpression and not because of any other antioxidant enzyme, the activity of catalase was inhibited by an irreversible inhibitor of catalase activity 3-AT. The PCat and PVect cells were pretreated with different concentration of 3-AT (0-50 mM) for an hour before adding 500 µM of H_2O_2 . It was observed that the percentage of PCat viable cells significantly reduced with an increase in 3-AT concentration (Figure 5-8B). At higher concentrations of 3-AT the percentage of PCat viable cells became similar to the percentage of PVect viable cells. This observation suggests that catalase activity is important for protection against H_2O_2 , and cells overexpressing catalase were found to be resistant to H_2O_2 toxicity.

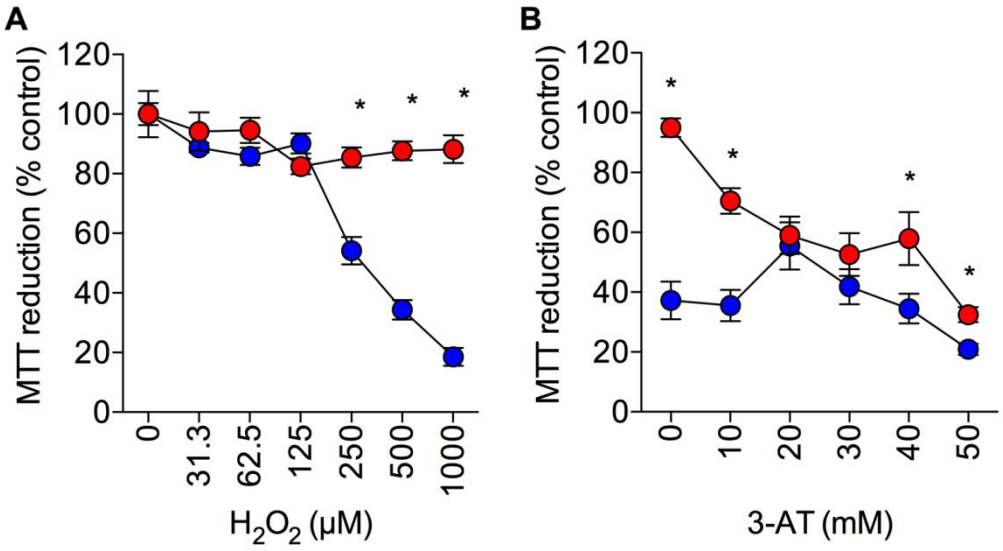

Figure 5-8. The effect of H_2O_2 on PCat cells. (A)The PCat (red circles) and PVect (blue circles) cells were exposed to a range of H_2O_2 concentrations (0 - 1000 µM) and viability determined by MTT reduction. (B) The PCat (red circles) and PVect (blue circles) cells were exposed to 500 µM H_2O_2 plus different doses of 3-AT (0 - 50 mM) and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs PVect (one-way ANOVA).

5.2.3 Measurement of catalase activity in PCat and PVect cells

The activity of the catalase produced by PCat cells was tested by measuring its ability to convert H_2O_2 to water and oxygen (Chapter 2; section 2.4). Cell lysates from PCat and PVect cells were extracted and used for the activity assay. The PCat cells with the catalase gene showed more than a 6 fold increase in the activity of catalase compared to the cells with vector alone. The activity of the catalase in the cell lysate from the PCat cells can be inhibited using 50 µM 3-AT indicating that the measured activity was due to catalase rather than other endogenous peroxidases expressed in the cells (Figure 5-9).

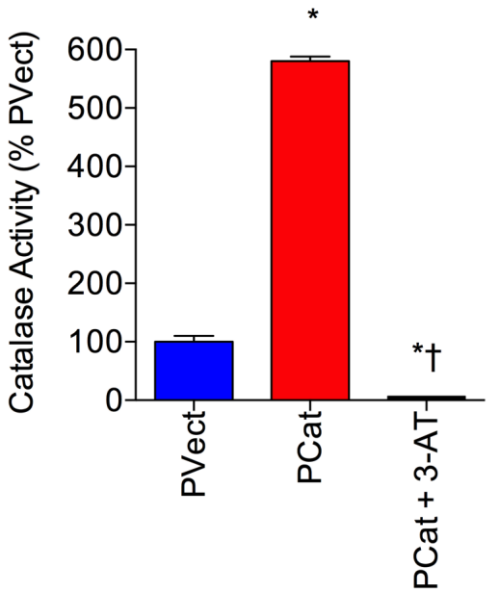

Figure 5-9. Measurement of catalase activity in PCat and PVect cells. The graph shows catalase activity of cell lysates of PVect, PCat and PCat treated with 3-AT (50 µM), with results expressed as % of the PVect cell lysate activity. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs PVect extracts; † = P < 0.05 vs PCat extracts (one-way ANOVA).

5.2.4 The effect of cobalt chloride on PCat cells

The PCat cells were found to be resistant to H_2O_2 toxicity, the PCat and the PVect cells were tested against a rage (0-500 µM) of $CoCl_2$ (which is a mimic of hypoxia) concentrations. The cells were incubated with $CoCl_2$ overnight and the next day MTT assay was performed. The dose response curve (Figure 5-10) shows that there was no significant difference between the toxicity found in the PCat and the PVect cells at doses above 10 µM. However, at the doses below 10 µM there was significant protection by catalase overexpression in the PCat cells. The $CoCl_2$ is known to activate ROS production (Jung *et al.,* 2008) and the biphasic dose response curve seen in PVect cells suggests that the toxicity may have multiple components. The protection against low dose toxicity in the PCat cells suggests that at low doses of $CoCl_2$ there may be H_2O_2 component to the toxicity. This indicates that both the cells lines were equally susceptible to $CoCl_2$ toxicity at concentrations above 10 µM, and suggests that PCat and PVect cells would react the same way for any cytotoxin added in other experiments. This also shows that the resistance of PCat cells towards H_2O_2 toxicity is quite specific, and does not extend to completely $CoCl_2$ toxicity, as the $CoCl_2$ toxicity is not primarily mediated via H_2O_2 (Kotake-Nara *et al.,* 2007).

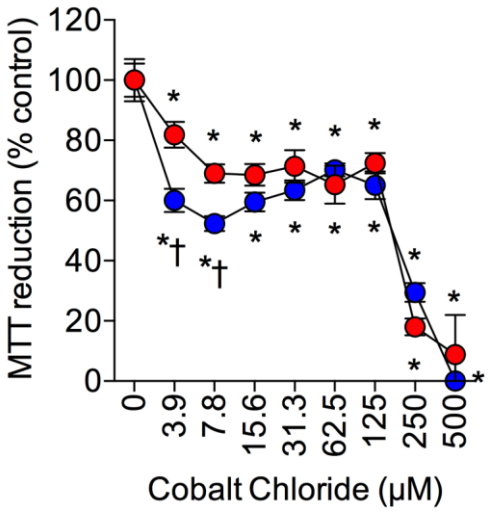

Figure 5-10. The effect of cobalt chloride on PCat cells. Represents the dose reseponse curve of $CoCl_2$ on PCat and PVect cells, both the cell lines

were treated with different doses of $CoCl_2$ ranging from 0 - 500 µM overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (0 µM $CoCl_2$); † = P < 0.05 PCat vs PVect (one-way ANOVA).

5.2.5 Tetracycline inducible system

The tetracycline inducible system was used to regulate the catalase gene overexpression in PCatTR6 cells. In the absence of tetracycline the expression of catalase gene is repressed (uninduced cells). The PCatTR6 cells were treated with 1000 µM H_2O_2 for 2 hrs (Figure 5-11), a significant decrease in the percentage of viable cells compared to control cells was observed. This susceptibility of the PCatTR6 cells to H_2O_2 was observed due to repression of the catalase gene. The PCatTR6 cells were induced by tetracycline (1 µg/ml final concentration) overnight. When the induced PCatTR6 cells were treated with 1000 µM H_2O_2 for 2 hrs, a significant increase in the percentage of viable cells compared to the uninduced PCatTR6 cells was observed. This suggests that the neuroprotection against H_2O_2 is due to catalase gene overexpression, which could be regulated by using the tetracycline inducible system.

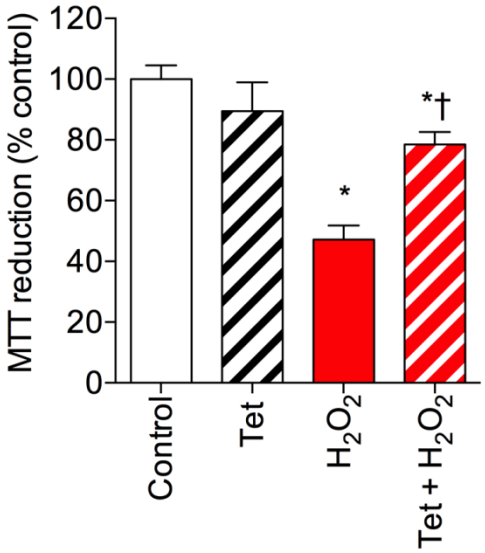

Figure 5-11. Effect of H_2O_2 on PCatTR6 cells in the presence and absence of tetracycline. The PCatTR6 cells were preincubated with or without

tetracycline (1 µg/ml) overnight prior to exposure to 1000 µM H_2O_2 for 2 hrs and viability determination by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control; † = $P < 0.05$ vs H_2O_2 alone (one-way ANOVA).

5.2.6 The conditioned media from PCat cells was neuroprotective against H_2O_2 toxicity

Fresh media was added to the PCat and PVect cells, the media was collected after 24 hrs and was used to test if the conditioned media from the PCat and PVect cells would protect against H_2O_2 toxicity. The naïve SH-SY5Y cells were treated with different doses of H_2O_2 (0-1000 µM) and conditioned media from the PCat and PVect cells for 2 hrs. Results showed that the percentage of viabile cells was significantly higher in the naïve cells treated with conditioned media from the PCat cells compared to cells treated with conditioned media from PVect cells (Figure 5-12A). When SH-SY5Y cells were treated with H_2O_2 (1000 µM) plus 50 mM 3-AT together with PCat or PVect conditioned media on SH-SY5Y cells, the PCat and PVect cells were equally susceptible to H_2O_2 toxicity (Figure 5-12B).

The SH-SY5Y cells treated with PCat conditioned media were resistant to H_2O_2 toxicity unlike SH-SY5Y cells treated with PVect conditioned media. This could be due to the release of catalase into the media, which converts H_2O_2 to water and oxygen and protects the SH-SY5Y cells against ROS. This neuroprotection by the PCat conditioned media can be inhibited by 3-AT, confirming that the effect was not mediated by other peroxidase enzymes released into the media.

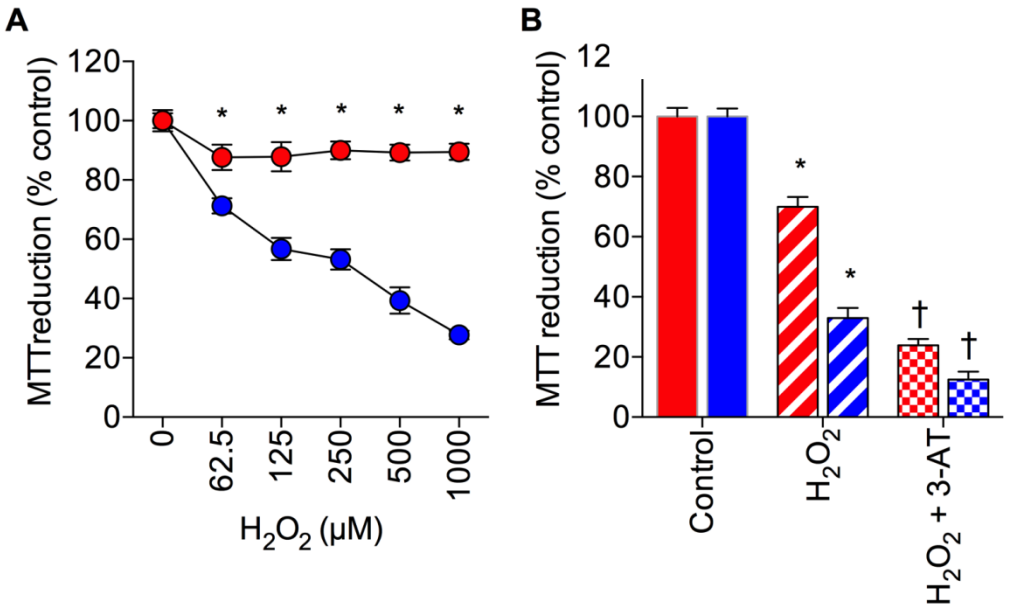

Figure 5-12. Effect of conditioned media from PCat and PVect cells on H_2O_2 toxicity. (A) Conditioned media from PCat (red circles) and PVect cells (blue circles) added to naïve SH-SY5Y cells in the presence of H_2O_2 (0-1000 µM) and viability determined by MTT reduction. (B) Naïve SH-SY5Y cells were treated with conditioned media from PCat or PVect cells plus H_2O_2 (1000 µM) and 50 µM 3-AT and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs PVect conditioned media; † = $P < 0.05$ vs H_2O_2 alone (one-way ANOVA).

5.2.7 His tag detection in PCat and PVect cells

To detect the His-tagged catalase protein the PCat and PVect cells were double labelled with either HIS-1 monoclonal anti-poly-His antibody, to stain poly-His positive proteins, or CAT-505 monoclonal anti-catalase antibody, to detect overexpressed His-tagged catalase plus the nucleus was stained with TO-PRO®-3 Iodide. The immunocytochemistry results showed label with TO-PRO®-3 Iodide (blue), HIS-1 monoclonal anti-poly-His antibody (red) in both PVect (Figures 5-13) and PCat (Figure 5-14) cells. The pattern of staining by anti-poly-His antibody was similar in both PVect and PCat cells, the staining was observed in the nucleus and anti-poly-His antibody co-localized with TO-PRO®-3 Iodide staining, the co-localization appeared purple, suggesting that the His-tagged catalase was in the nucleus (Figure 5-14). The staining with anti-

catalase antibody was not in the nucleus and did not co-localize with TO-PRO®-3 Iodide staining (Figure 5-14), the staining was in the cytoplasm as found in previous studies (Habib *et al.,* 2010). This suggests that the HIS-1 monoclonal anti-poly-His antibody did not detect His-tagged catalase, but the anti-poly-His antibody stained endogenous poly-His containing proteins within the nucleus. The cross-reactivity observed and the failure of the anti-poly-His antibody to stain the overexpressed His-tagged catalase suggests that this antibody is not suitable to discriminate between the endogenous catalase and His-tagged catalase in the SH-SY5Y neuroblastoma cell line.

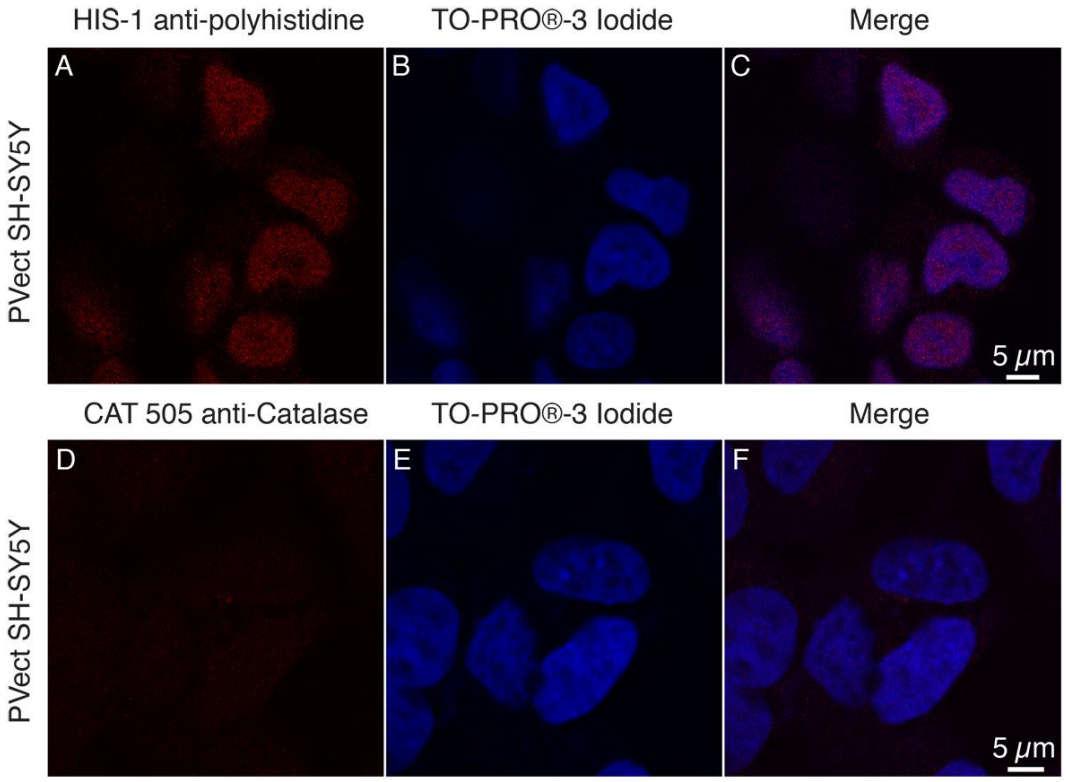

Figure 5-13. Poly-histidine and catalase immunocytochemical staining in PVect cells. The PVect cells were double labelled with HIS-1 monoclonal anti-polyhistidine (A) and TO-PRO®-3 Iodide DNA staining (B) to detect co-localization of polyhistidine containing proteins and DNA (C). The PVect cells were double labelled with CAT-505 monoclonal anti-catalase (D) and TO-PRO®-3 Iodide (E) to detect co-localization of polyhistidine containing proteins and DNA (F). Bars = 5 µm (A-F).

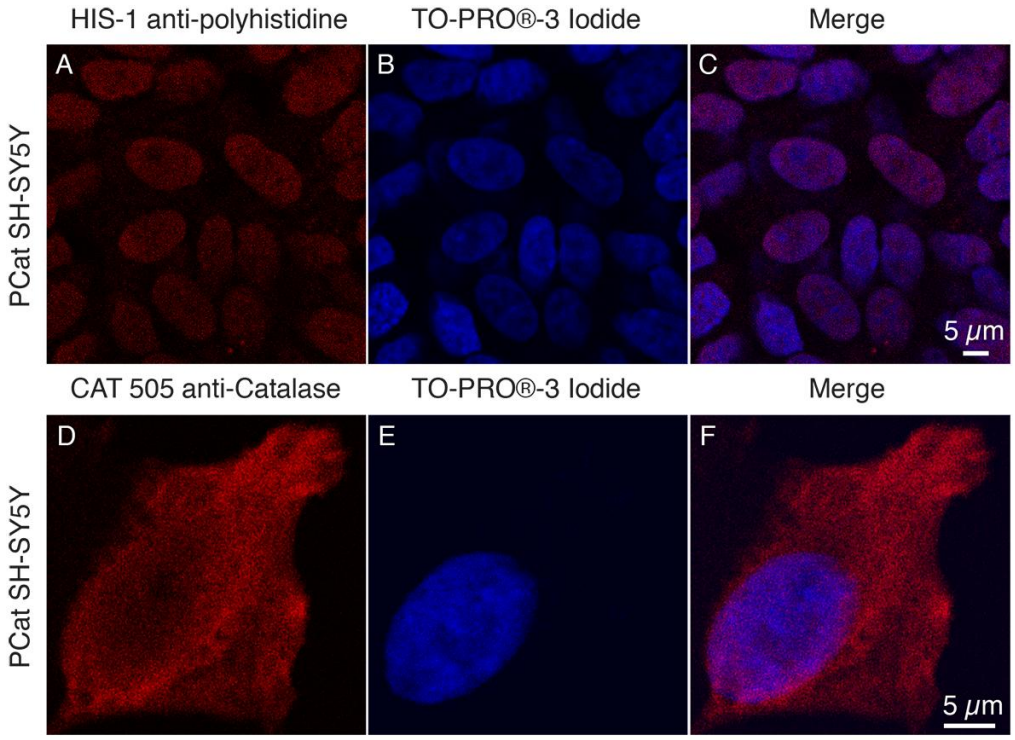

Figure 5-14. Poly-histidine and catalase immunocytochemical staining in PCat cells. The PCat cells were double labelled with HIS-1 monoclonal anti-polyhistidine (A) and TO-PRO®-3 Iodide DNA staining (B) to detect co-localization of polyhistidine containing proteins and DNA (C). The PCat cells were double labelled with CAT-505 monoclonal anti-catalase (D) and TO-PRO®-3 Iodide (E) to detect co-localization of polyhistidine containing proteins and DNA (F). Bars = 5 µm (A-F).

5.3 Discussion

To investigate the effect of catalase overexpression on H_2O_2 toxicity in the human neuroblastoma SH-SY5Y cells, an SH-SY5Y cell line that overexpressed catalase gene was created by cloning the catalase gene into pcDNA™4/TO/myc-His vector (section 5.2.1.1 and 5.2.1.1) and stably transfecting the vector into SH-SY5Y cells. The overexpression of catalase gene in transfected SH-SY5Y cells was confirmed by RT-PCR (section 5.2.1.4), immunocytochemistry (section 5.2.1.6) and western blotting (section 5.2.1.7). Furthermore, an activity assay (Sagara *et al.,* 1996) was used to compare the catalase activity between the PCat and PVect cell lysates, by measuring the ability of the cell lysates to breakdown H_2O_2. The PCat cell lysate showed 6 fold increase in catalase activity compared to PVect cell lysate (section 5.2.3). Taken together, these results confirmed that PCat expressed significantly higher levels of active catalase than PVect cells.

Overexpression of the catalase gene in SH-SY5Y cells was tested against H_2O_2 toxicity. Results demonstrated that the enhanced level of the catalase gene expression in SH-SY5Y neurons is neuroprotective against H_2O_2 toxicity (section 5.2.2). The catalase neuroprotection against H_2O_2 toxicity could be inhibited by 3-AT (section 5.2.1). The 3-AT is an irreversible inhibitor of catalase activity (Milton 2001). This suggests that catalase overexpression can protect SH-SY5Y cells against H_2O_2 toxicity and the mechanism of neuroprotection requires active catalase. Inhibition of catalase activity by 3-AT makes the PCat cells susceptible to H_2O_2 toxicity.

The PCat and PVect cells were not resistant to $CoCl_2$ toxicity (a mimic of hypoxia) (section 5.2.4). This suggests that the neuroprotection by catalase overexpression is specific for H_2O_2 toxicity. To confirm that the catalase neuroprotection was by overexpression of the catalase gene in PCat cells, the tetracycline inducible system was used to regulate the catalase gene expression. The PCat cells were transfected with pcDNA6/TR vector, resulting in PCatTR6 cells, which could be induced using tetracycline (1 µg/ml). The working of the tetracycline inducible system was confirmed by RT-PCR (section 5.2.1.5). In the absence of tetracycline, PCatTR6 cells (uninduced) were

significantly susceptible to H_2O_2 toxicity (section 5.2.5). In the presence of tetracycline, the gene expression was resumed which made the PCatTR6 (induced) cells resistant to H_2O_2 toxicity. This observation suggests that the neuroprotection observed against H_2O_2 toxicity was due to catalase gene overexpression. The conditioned media harvested from PCat cells was neuroprotective against H_2O_2 toxicity (section 5.2.6). The conditioned media from PCat or PVect cells was added to the naïve SH-SY5Y cells together with H_2O_2 and 3-AT, both the naïve cells treated with PCat or PVect conditioned media were found to be susceptible to H_2O_2 toxicity in the presence of 3-AT. This suggests that conditioned media from PCat cells is neuroprotective against H_2O_2 toxicity, and this neuroprotection could be inhibited by 3-AT (section 5.2.6). This could be due to the presence of neuroprotective components in PCat cells conditioned media, which was absent in the PVect cells conditioned media.

The immunocytochemical studies with the HIS-1 anti-poly-His antibody suggest that the antibody cross reacts with other poly-histidine proteins in the nucleus and failed to detect his-tagged catalase in PCat cells (section 5.2.7). It has been suggested that a poly-His sequence specifically directs proteins to the nucleus (Paraguison *et al.,* 2005; Salichs *et al.,* 2009). The observations in the present study suggest that using HIS-1 anti-poly-His antibody may contribute to misinterpretation of results.

5.4 Conclusion

The results presented in this chapter illustrate the creation of the catalase gene overexpressing SH-SY5Y cell line. The PCat cells were found to be resistant to H_2O_2 mediated cell death and this resistance to H_2O_2 could be inhibited by 3AT. This suggests that catalase activity is the key to protection against H_2O_2 mediated toxicity. The expression of catalase could be regulated by the tetracycline inducible system which provides a further model system for investigation of catalase neuroprotection.

Chapter 6

Catalase neuroprotection against Amyloid-β toxicity

6.1 Introduction

The Aβ peptide plays a pivotal role in the development of AD, amyloid plaques are the hallmarks used to diagnose AD in brain tissues (Selkoe, 1994; LaFerla *et al.,* 2007). The data presented by Hansson Peterson *et al.,* 2008 demonstrated the transport of Aβ into rat mitochondria via the translocase of the outer membrane and Aβ was found to localize within mitochondrial cristae. Mitochondrial mislocalisation of Aβ leads to neuronal dysfunction in a drosophila model of AD (Iijima-Ando *et al.,* 2009). Accumulation of Aβ in mitochondria disrupts mitochondrial function resulting in increased production of ROS (Mao and Reddy 2011). The production of ROS induced by Aβ can lead to DNA damage and lipid peroxidation (Behl *et al.,*1994). Interestingly, there is increasing data that shows that antioxidant enzymes can significantly reduce ROS production, eventually improve cell survival and cellular function *in vitro* and *in vivo* (Wadsworth *et al.,* 2008; Manczak *et al.,* 2010; Van Raamsdonk and Hekimi 2010; Ma *et al.,* 2011).

An APP transgenic mouse was crossed with a mitochondrial targeted catalase mouse, to produce a double transgenic mouse. In the double transgenic mouse there was not only significantly reduced DNA oxidative damage but also inhibition of Aβ depositions, eventually improving the AD mouse lifespan (Mao *et al.,* 2012). Cell line (PC12) genetically engineered to express higher levels of the antioxidant enzymes was found to be resistant to Aβ toxicity (Sagara *et al.,* 1996). Exogenous addition of catalase or antioxidants protects cultured neurons from Aβ toxicity (Behl *et al.,* 1992; Behl *et al.,* 1994). Catalase is an antioxidant enzyme which can efficiently break down H_2O_2 to water and oxygen (Kirkman and Gaetani 2007). Catalase protects cells against increased levels of H_2O_2 induced by Aβ toxicity (Behl *et al.,* 1994). In AD patients the blood levels of catalase protein are unchanged, but a decrease in the activity has been observed (Kharrazi *et al.,* 2008; Puertas *et al.,* 2012). This could be due to the interaction of catalase and Aβ within the erythrocytes, suggesting that the interaction could take place even in minimal cell devoid of nucleus (Clementi *et al.,* 2004). The binding of catalase to Aβ possibly occurs between the CAβBD contained in the wrapping loop of the catalase 400-409 region and the 25-35

region of the Aβ peptide (Milton *et al.,* 2001). A previous study has shown that only Aβ 29-40 fibrils but not Aβ 1-28 or Aβ 33-42 fibrils can bind to catalase (Milton and Harris, 2009). This suggests that amino acids 29-32 (Gly-Ala-Ile-Ile) of Aβ are key for the catalase-amyloid interaction. The 31-35 fragment of Aβ is the smallest fragment that could inhibit the activity of catalase (Milton 1999) and also inhibits catalase binding to Aβ fibrils (Milton and Harris, 2009). In a previous study (Sagara *et al.,*1996) the PC12 cells that were selected for Aβ resistance, were less susceptible to H_2O_2 toxicity. A higher expression and activity of the antioxdant enzymes was observed in the Aβ resistant PC12 cells. This study also demonstrated that cell lines engineered to express higher levels of antioxidant enzymes such as catalase and glutathione peroxidase were resistant to Aβ toxicity (Sagara *et al.,* 1996). In the present study, the catalase gene has been overexpressed in PCat SH-SY5Y cells to determine the role of catalase gene overexpression in protection against Aβ toxicity.

Catalase not only binds Aβ, but has been shown to bind other amyloid peptides such as the IAPP and PrP (Milton and Harris, 2010; Milton and Harris, 2013). This could be due to the sequence similarity shared by all the amyloid peptides, containing a potential catalase-binding domain (Figure 1-3). The amyloid deposits found in T2DM consists of IAPP (Janson *et al.,* 1996; Höppener *et al.,* 2008).

Compounds that specifically disrupt catalase-amyloid interactions were found to be neuroprotective against Aβ induced OS and toxicity (Habib *et al.,* 2010). The KP peptide is one of the peptides that can bind Aβ and inhibit catalase-amyloid interaction (Figure 3-3). Both KP and catalase have been found together in amyloid plaque like deposits in the pons section of the AD brain (Figure 4-6). Binding studies have shown that BTA-EG_4 can disrupt catalase-amyloid binding interaction (Figure 3-3). The tetra-ethylene glycol derivatives of BTA surround the aggregated Aβ with a bioresistive coating, preventing the association of aggregated Aβ with cellular proteins (Inbar *et al.,* 2006). BTA derivatives can also cross the blood brain barrier and show high affinity for aggregated Aβ (Inbar *et al.,* 2006). This suggests that disrupting the interaction between catalase and Aβ could be neuroprotective. The BTA derivatives can bind a

range of amyloid peptides (Inbar *et al.,* 2006; Habib *et al.,* 2010; Capule *et al.,* 2012) suggesting they could also be used to disrupt catalase interactions with IAPP and PrP (Milton and Harris, 2010; Milton and Harris, 2013).

The inhibitors of catalase activity such as 3-AT or homocysteine (Milton 2008) were shown to significantly enhance Aβ toxicity. This suggests that catalase activity is required for protection against Aβ induced OS and toxicity. The 3-AT does not play any role in disruption of catalase-amyloid interaction (Figure 3-4). The 3-AT inhibits activity of catalase, but inactive catalase could still bind Aβ (Zang *et al.,* 1996), which could be neuroprotective.

6.1.1 Aim

- To investigate the mechanism of neuroprotection of catalase gene overexpression against Aβ toxicity.

6.1.2 Objectives

- To investigate the effect of Aβ toxicity on PCat cells
- To test the tetracycline inducible system against Aβ toxicity
- To test the effect of PCat conditioned media on SH-SY5Y cells against Aβ toxicity.
- To test the effect of different amyloid peptides on PCat cells
- To determine the effect of BTA-EG_4 on catalase neuroprotection against Aβ toxicity in PCat cells
- To investigate the effect of H_2O_2 on PCat cells treated with BTA-EG_4, KP 45-50 and 3-AT

6.1.3 Hypothesis

Catalase shares sequence similarity with Aβ, it has also been found associated with amyloid plaque like deposits. The binding interaction between catalase and Aβ could be neuroprotective or it could lead to further cell death. By inhibiting the binding interaction using BTA-EG_4, the effect of catalase amyloid interaction in SH-SY5Y cells overexpressing catalase gene can be investigated.

6.2 Results

6.2.1 The effect of Aβ toxicity on PCat cells

The PCat and PVect cells were treated with Aβ 25-35 to determine the role of catalase gene overexpression in neuroprotection against Aβ toxicity. The PCat and PVect cells were exposed to different Aβ 25-35 doses (0 - 50 µM) overnight (Figure 6-1A) prior to MTT assay of cell vialibity. The dose response curve shows a significant increase in the percentage of PCat viable cells compared to PVect cells. Even at higher doses (50 µM) the PCat cells were more resistant to Aβ toxicity. The neuroprotection by catalase gene overexpression against Aβ has been shown here for the first time.

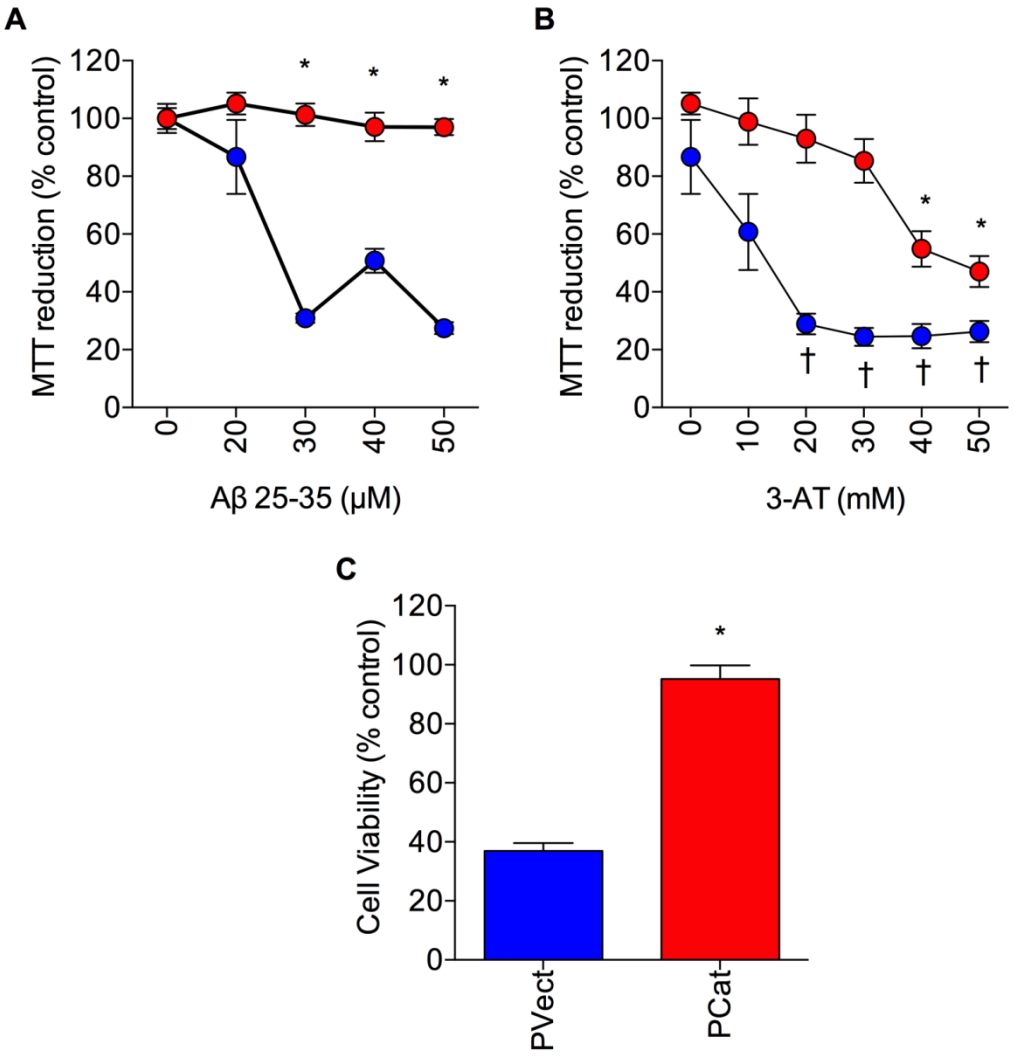

Figure 6-1. The effect of Aβ toxicity on PCat cells. (A) The PCat and PVect cells were exposed to different doses of Aβ 25-35 (0-50 µM) and viability determined by MTT assay. (B) The PCat and PVect cells were treated with different doses of 3-AT (0-50 mM) and 10 µM Aβ 25-35 and viability determined by MTT reduction. (C) The effects of Aβ (10 µM) in PCat and PVect cells determined by trypan blue assay (C). Results are mean ± S.E.M (n = 8); * = P < 0.05 vs PVect plus (A) Aβ, (B) Aβ and 3-AT, (C) Aβ; † = P < 0.05 vs PVect plus Aβ; one-way ANOVA (A,B) or students t-test (C).

To determine if catalase activity is required for neuroprotection against Aβ 25-35, the PCat and PVect cells were pretreated with different concentrations of 3-AT (0-50 mM) (Figure 6-1B) for an hour prior to being treated with 10 µM Aβ 25-35 plus different concentrations of 3-AT. The viability of the cells was measured by MTT cell viability assay. Unlike the PVect cells, the PCat cells were still resistant to Aβ 25-35 toxicity, even at higher doses of 3-AT. The viability of the PCat cells did not reach the low level cell viability levels of the PVect cells, this suggests that the neuroprotection provided by catalase is partially but not entirely dependent on its activity.The other possible mechanism of neuroprotection could be a catalase-amyloid interaction, as Aβ can still bind inactive catalase (Zang *et al.,* 1996) and this binding interaction could prevent Aβ toxicity.

The neuroprotection by catalase overexpression against Aβ toxicity was also confirmed by trypan blue assay. The PCat cells were treated with 10 µM Aβ 25-35 overnight, the trypan blue assay was used to count the number of viable cells (Figure 6-1C). Results obtained were consistent with the MTT assay result (Figure 6-1A), where the PCat cells were more resistant to amyloid toxicity compared to PVect cells.

6.2.2 Tetracycline inducible system

The tet inducible system was used to regulate levels of catalase gene expression, to determine the neuroprotection by catalase gene overexpression against Aβ (31-35) toxicity. The working of the tetracycline inducible system was confirmed by RT-PCR (Figure 5-5). In the absence of tet, the PCatTR6 cells

were treated with 50 µM Aβ (31-35) overnight. The PCatTR6 cells were found to be significantly susceptible to Aβ (31-35) toxicity in the absence of tet. However following the treatment of the PCatTR6 cells with tet (1 µg/ml) and 50 µM Aβ (31-35) overnight, the PCatTR6 cells were resistant to Aβ (31-35) toxicity (Figure 6-2). Tet alone did not have any effect on PCatTR6 cell viability. This clearly demonstrates that catalase gene overexpression is key for neuroprotection against Aβ (31-35) toxicity.

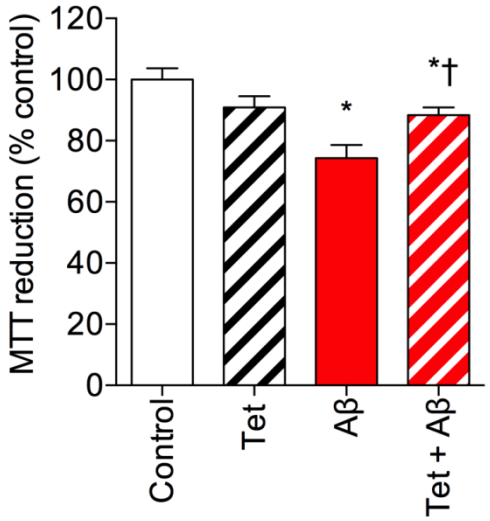

Figure 6-2. Effect of Aβ on PCatTR6 cells in the presence and absence of tetracycline. The PCatTR6 cells were preincubated with or without tetracycline (1 µg/ml) overnight prior to exposure to 50 µM Aβ (31-35) overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control; † = $P < 0.05$ vs Aβ alone (one-way ANOVA).

6.2.3 The effect of PCat conditioned media on SH-SY5Y cells against Aβ toxicity.

The conditioned media from PCat cells was harvested, to test if it was neuroprotective against Aβ toxicity on SH-SY5Y cells. The SH-SY5Y cells were treated with 25 µM Aβ (25-35) plus conditioned media from PCat or PVect cells overnight. Results showed that the SH-SY5Y cells treated with PCat conditioned media were resistant to Aβ toxicity (Figure 6-3). The SH-SY5Y cells treated with PVect conditioned media were significantly susceptible to Aβ

toxicity. Next, the SH-SY5Y cells were treated with conditioned media from PCat or Pvect cells plus 25 µM Aβ (25-35) and 50 µM 3-AT overnight. Results showed that the SH-SY5Y cells treated with PCat conditioned media were not affected by addition of 3-AT, but the toxicity of Aβ was still significant in the SH-SY5Y cells treated with PVect conditioned media, 3-AT and Aβ. This suggests that the PCat conditioned media is neuroprotective against Aβ toxicity, and this neuroprotective component in the PCat conditioned media was not effected by 3-AT (results consistent with previous study by Zhang *et al.,* 1996).

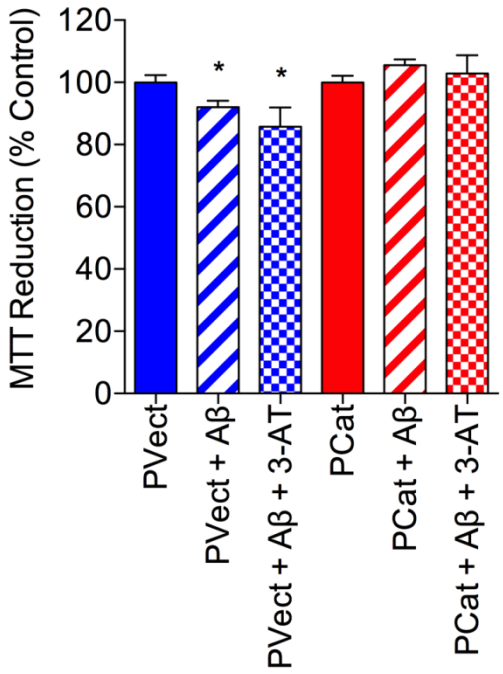

Figure 6-3. Effect of conditioned media from PCat and PVect cells on Aβ toxicity. Naïve SH-SY5Y cells were treated with conditioned media from PCat or PVect cells plus 25 µM Aβ (25-35) and 50 µM 3-AT overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs PVect conditioned media (one-way ANOVA).

6.2.4 The effect of different amyloids on PCat cells

The sequence similarity between the different amyloid peptides suggest that they could interact with catalase and overexpression of the catalase gene could be neuroprotective against Aβ, ABri, ADan, IAPP and PrP toxicity. To investigate the role of catalase in neuroprotection, the PCat and PVect cells were exposed to 25 μM Aβ (25-35), ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) toxicity overnight. The results (Figure 6-4A) showed that the PCat cells were significantly resistant to Aβ, ABri, ADan, IAPP and PrP toxicity compared to PVect cells. This is the first demonstration of catalase gene overexpression being neuroprotective against Aβ, ABri, ADan, IAPP and PrP toxicity.

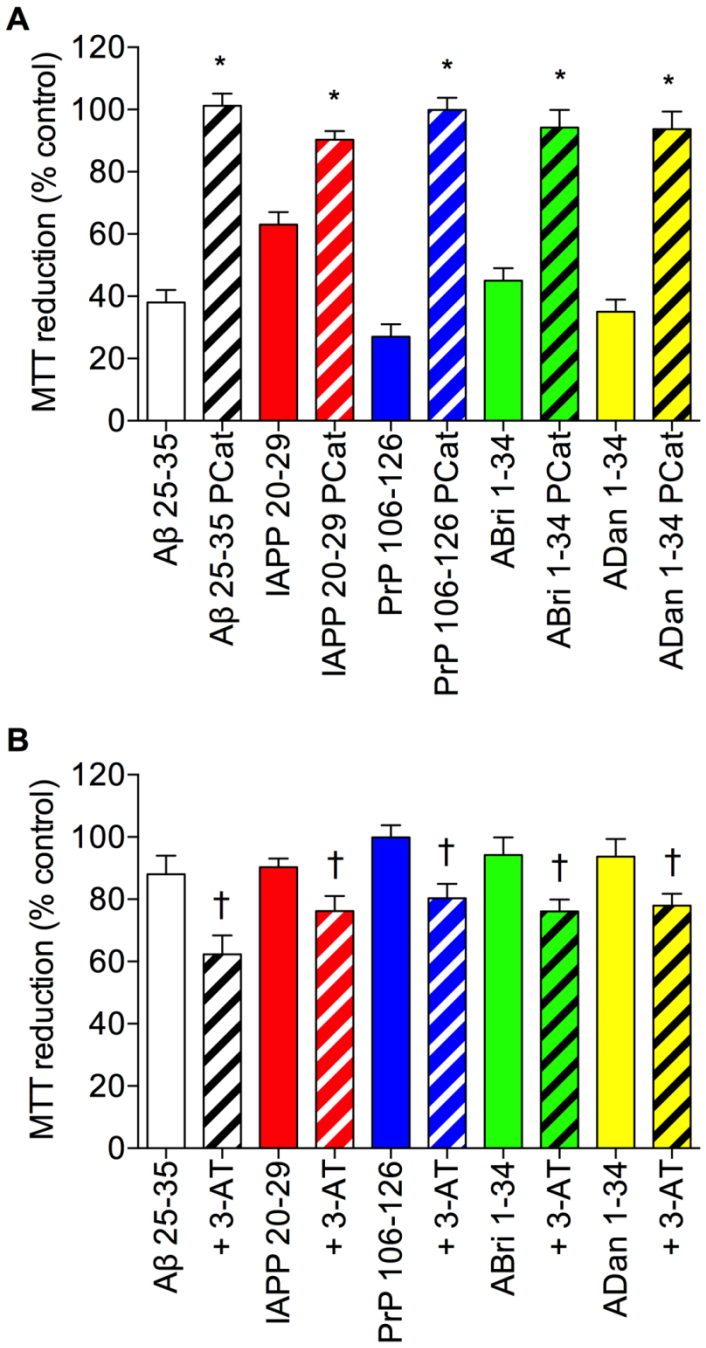

Figure 6-4. The effect of different amyloid peptides on PCat cells. (A) The PCat and PVect cells were exposed to 25 µM Aβ, ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) overnight and viability determined by MTT reduction. (B) The PCat cells were exposed to either 25 µM Aβ, ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) or together with 3-AT (50 mM) overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs PVect; † = $P < 0.05$ vs amyloid peptide alone (one-way ANOVA).

The activity of catalase in the PCat cells was inhibited by 3-AT. The cells were pretreated with 50 mM 3-AT for an hour prior to the addition of 25 μM of Aβ, ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) overnight (Figure 6-4B). The 3-AT significantly enhanced the toxicity of various amyloid peptides, which decreased the percentage of viable cells compared to the cells treated with amyloid peptides alone. This suggests that catalase activity is required for neuroprotection against amyloid peptides. The possible mechanism of neuroprotection being, catalase antioxidant activity, which breaks down H_2O_2 induced by amyloid peptides.

6.2.5 The effect of BTA-EG_4 on catalase neuroprotection against Aβ toxicity in PCat cells

To understand the importance of the catalase-amyloid interaction in catalase neuroprotection, BTA-EG_4 was used to block the catalase-amyloid interaction. The BTA-EG_4 has a relatively low molecular weight (416.18 Da) and it has been shown to be taken up by SH-SY5Y neurons and to disrupt catalase-amyloid interactions (Habib *et al.,* 2010). The BTA-EG_4 was not toxic to SH-SY5Y neurons and was previously shown to be neuroprotective against Aβ toxicity at concentrations of 20 μM or higher (Habib *et al.,* 2010). This was observed in the previous study done by Habib *et al.,* 2010 where 25 μM Aβ was pre-incubated with different concentrations of BTA-EG_4 for 12 hrs before adding the mixture to the naïve cells to block catalase-amyloid interaction.

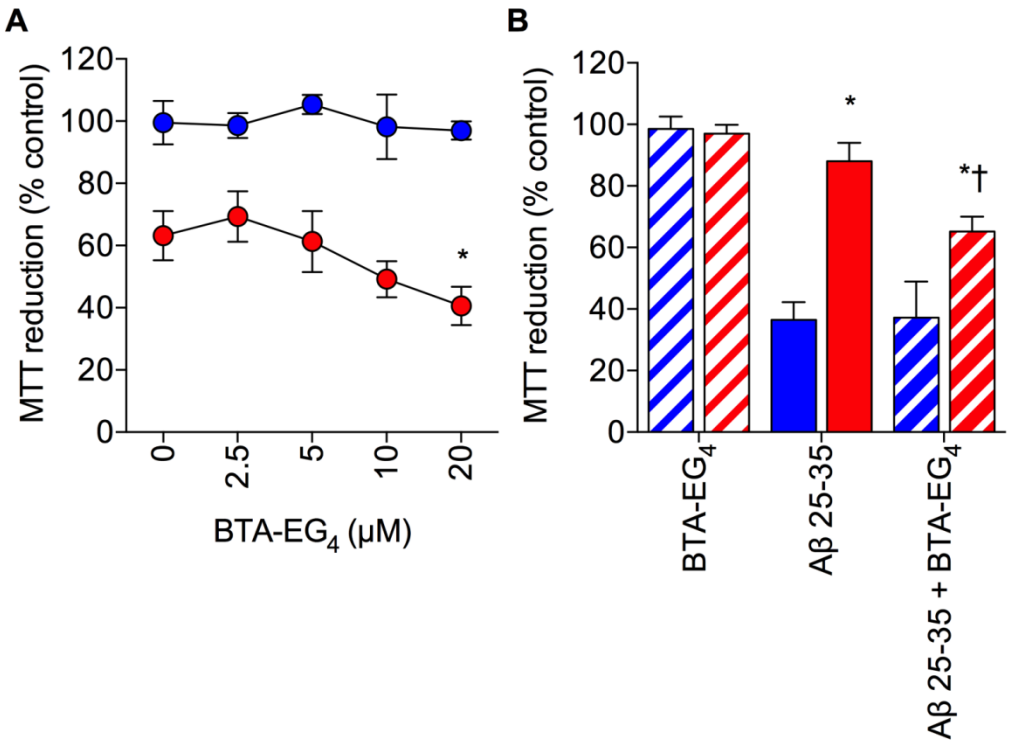

Figure 6-5. The effect of BTA-EG$_4$ on catalase neuroprotection against Aβ toxicity in PCat cells. (A) PCat cells were incubated with 0 - 20 μM BTA-EG$_4$ alone (blue circles) or BTA-EG$_4$ plus 50 μM Aβ (red circles) overnight and viability determined by MTT reduction. (B) PVect cells (blue columns) or PCat cells (red columns) were incubated with 20 μM BTA-EG$_4$ alone (striped open columns), 25 μM, Aβ (filled columns) or BTA-EG$_4$ plus 25 μM Aβ (striped filled columns) overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); (A) * = $P < 0.05$ vs 50 μM Aβ alone; (B) * = $P < 0.05$ vs PVect; † = $P < 0.05$ vs 25 μM Aβ alone (one-way ANOVA).

In the present study, contrasting results were found to previously published findings (Habib *et al.,* 2010); this could be due to the difference in the experimental procedure. The PCat cells were pretreated with different concentration of BTA-EG$_4$ (0 - 20 μM) overnight, to allow the uptake of BTA-EG$_4$ into the cells before adding Aβ to the cells. The next day 50 μM Aβ (25-35) was added together with BTA-EG$_4$ to the PCat cells and was further incubated for 24 hrs prior to determination of cell viability. There was no toxicity observed when PCat cells were treated with different concentrations of (0-20 μM) BTA-EG$_4$ alone (Figure 6-5A). But, when the PCat cells were treated with different

concentrations of BTA-EG_4 (0-20 μM) and 50 μM Aβ, at 20 μM the BTA-EG_4 significantly increased the toxicity of 50 μM Aβ. The increased toxicity of 50 μM Aβ suggests that a dose of 20 μM BTA-EG_4 is sufficient to disrupt catalase-amyloid interaction and to allow Aβ to exert a toxic effect on PCat cells. This suggests that blocking the catalase-amyloid interaction could increase the toxicity of Aβ, which could lead to cell death. When the PCat and the PVect cells were treated with 20 μM BTA-EG_4 plus 25 μM Aβ, the BTA-EG_4 increased the toxicity of Aβ in the PCat cells (Figure 6-5B). The 20 μM BTA-EG_4 alone was not toxic to the PCat or PVect cells. This suggests that BTA-EG_4 enhances the toxicity of Aβ by inhibiting catalase-amyloid interaction, which could be the reason for increased Aβ toxicity only in PCat cells and not in PVect cells.

6.2.6 The effect of KP 45-50 against Aβ toxicity in PCat

To eliminate the possibility that any protein binding interaction with Aβ in PCat cells will enhance the toxicity of Aβ, the binding interaction between KP and Aβ was examined. The experimental conditions used were same as used for BTA-EG_4, were the PCat cells were pretreated with different concentrations of KP 45-50 (0 - 20 μM) overnight prior to treatment with KP (0- 20 μM) alone or 50 μM Aβ (25-35) plus KP 45-50 (0 - 20 μM) for 24 hrs.

Results showed PCat cells treated with KP alone or Aβ plus KP did not enhance the toxicity of Aβ (Figure 6-6). The higher doses of KP (10 and 20 μM) were found to be neuroprotective against Aβ toxicity. This suggests that the enhancement of Aβ neurotoxicity in PCat cells by BTA-EG_4 was specific, and that any endogenous Aβ binding compound such as KP may not have a similar effect.

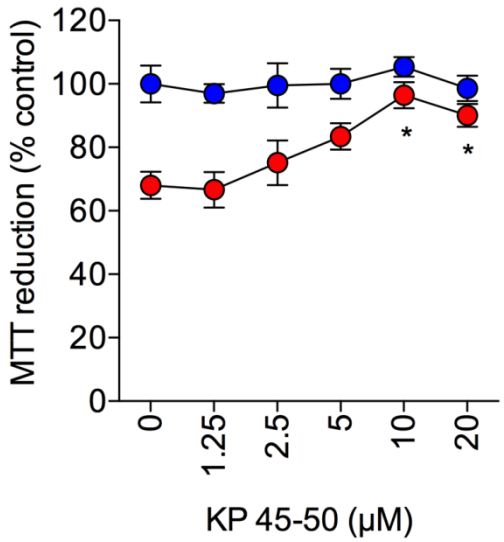

Figure 6-6. The effect of KP 45-50 against Aβ toxicity in PCat cells. The PCat cells were pretreated with 20 μM KP 45-50 overnight, prior to the treatment with 20 μM KP alone (blue circles) or 20 μM KP + 50 μM Aβ (red circles) and incubated overnight with viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs 50 μM Aβ alone (one-way ANOVA).

6.2.7 The effect of BTA-EG_4 on various catalase-amyloid (Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29 and PrP 106-126) interactions in PCat cells

The Aβ and catalase interaction was inhibited by BTA-EG_4; different amyloid peptides were tested to see if BTA-EG_4 would disrupt catalase-amyloid (Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29 and PrP 106-126) interactions and induce cell toxicity. The PCat cells were pretreated with 20 μM BTA-EG_4 for 24 hrs prior to the treatment with different amyloid peptides.

The cells were treated with 25 μM amyloid peptides; Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29, PrP 106-126 and 20 μM BTA-EG_4 for 24 hrs. Results showed that BTA-EG_4 increased the toxicity of all the amyloid peptides, compared to the cells treated with the peptide alone (Figure 6-7). This suggests that a binding interaction between catalase and the amyloid peptides is the key for

neuroprotection, inhibiting the catalase-amyloid interaction increases the toxicity of the amyloid peptides.

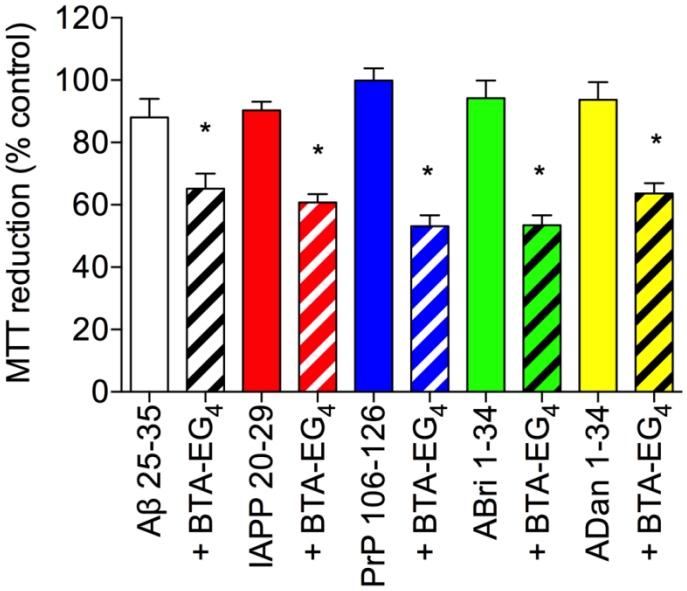

Figure 6-7. The effect of BTA-EG$_4$ on amyloid toxicity in PCat cells. The effect of 20 µM BTA-EG$_4$ pre-treatment prior to addition of 25 µM amyloid peptides; Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29, PrP 106-126 plus 20 µM BTA-EG$_4$ was tested on PCat cells incubated overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs amyloid peptide alone (one-way ANOVA).

6.2.8 The effect of H_2O_2 on PCat cells treated with BTA-EG$_4$, KP 45-50 and 3-AT

To understand the affect of BTA-EG$_4$, KP and 3-AT on PCat cells treated with H_2O_2, the PCat cells were pretreated with 20 µM BTA-EG$_4$, 10 µM KP 45-50 and 50 mM 3-AT overnight. Next day, 500 µM of H_2O_2 plus 20 µM BTA-EG$_4$ or 10 µM KP 45-50 or 50 mM 3-AT was added to the cells for 2 hrs prior to the MTT assay. Results showed that BTA-EG$_4$ and KP did not enhance the toxicity of H_2O_2 unlike 3-AT, which significantly enhanced the toxicity of H_2O_2 (Figure 6-8). This suggests that the enhancement of Aβ toxicity in catalase gene overexpressing cells by BTA-EG$_4$ is specific for amyloid peptides and more

likely be due to inhibition of catalase-amyloid binding interaction, BTA-EG_4 and KP did not the affect the ability of catalase to breakdown H_2O_2.

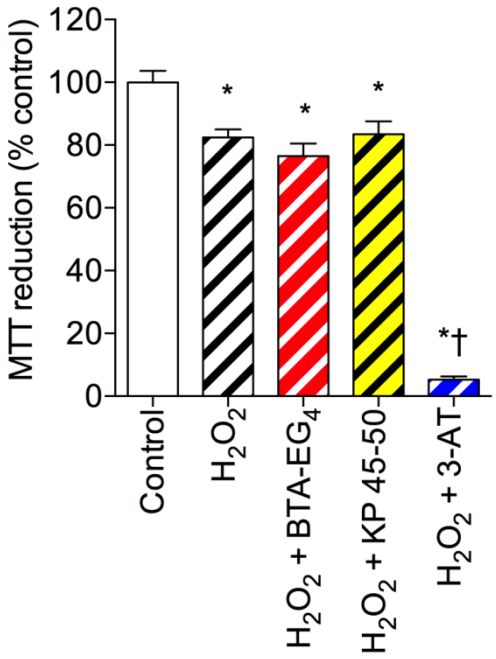

Figure 6-8. The effect of H_2O_2 on PCat cells treated with BTA-EG_4, KP 45-50 and 3-AT. The PCat cells were pretreated with either 20 µM BTA-EG_4, 10 µM KP 45-50 or 50 mM 3-AT prior to the treatment with 500 µM H_2O_2 for 2 hrs and viability determination by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cell treated with media only), † = $P < 0.05$ vs cells treated with H_2O_2 only, (one-way ANOVA).

6.3 Discussion

The catalase gene was overexpressed in SH-SY5Y cells to determine the effects of catalase gene overexpression against Aβ toxicity. The catalase gene overexpression was found to be neuroprotective against Aβ toxicity (section 6.2.1). This is consistant with a previous study where increased expression of catalase and glutathione peroxidase made cells resistant to amyloid toxicity (Sagara *et al.,* 1996). Catalase neuroprotection against Aβ toxicity was partly inhibited by 3-AT (section 6.2.1). As 3-AT only inhibits catalase activity (section 5.2.2) and does not interfere with the catalase-amyloid interaction (section 3.2.3) this suggests that 3-AT could decrease catalase neuroprotection by inhibiting catalase activity. This also shows that although 3-AT inhibits the activity of catalase, inactive catalase could still bind Aβ (Zang *et al.,* 1996), which could be neuroprotective. The catalase neuroprotection against Aβ toxicity was confirmed by both MTT (section 6.2.1) and trypan blue assay (section 6.2.1).

The tetracycline inducible system was used to regulate catalase gene expression. Results showed that catalase gene expression was key for neuroprotection against Aβ toxicity, repressing the expression of the catalase gene made the PCatTR6 cells susceptible to Aβ toxicity (section 6.2.2). The conditioned media from the PCat cells was also found to be neuroprotective against Aβ toxicity (section 6.2.1), where addition of 3-AT did not seem to alter the neuroprotective effect of the PCat conditioned media. This suggests that the neuroprotective component of the PCat conditioned media was unaltered by 3-AT.

The catalase gene overexpression was found to be neuroprotective against Aβ toxicity; the PCat cells were used to test neuroprotection against other amyloid peptides such as Aβ (25-35), ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126). Results showed that catalase was resistant to Aβ (25-35), ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) toxicity (section 6.2.4). The toxicity of the amyloid peptides was significantly enhanced by 3-AT in PCat cells (section 6.2.4), which suggests that the neuroprotection was due to activity of catalase.

To further understand the mechanism of neuroprotection by catalase binding to Aβ, the catalase-amyloid interaction was disrupted by BTA-EG_4. The BTA-EG_4 surrounds Aβ aggregates and does not allow Aβ to interact with cellular proteins (Habib *et al.,* 2010; Capule *et al.,* 2012). The inhibition of the catalase-amyloid interaction by BTA-EG_4 has been confirmed (section 3.2.3). The BTA-EG_4 by itself was not toxic to PCat cells, but when added together with Aβ, it enhanced the toxicity of Aβ at a dose of 20 µM BTA-EG_4 (section 6.2.5) in PCat cells. The BTA-EG_4 did not have any effect on Aβ toxicity in PVect cells (section 6.2.1). This suggests that disruption of catalase-amyloid interaction by BTA-EG_4 could enhance Aβ toxicity in PCat cells.

To confirm that any compound that can bind Aβ will not enhance Aβ toxicity in PCat cells, the effect of KP 45-50 was tested on PCat cells against Aβ toxicity. The KP 45-50 can bind Aβ and could inhibit the interaction catalase-amyloid interaction (section 3.2.3). Unlike the BTA-EG_4 the KP did not enhance the toxicity of Aβ in PCat cells, but was found to be neuroprotective against Aβ toxicity (section 6.2.6). This suggests that the role of BTA-EG_4 is specific and that the increased toxicity observed was due to disruption of catalase-amyloid interaction. It also shows that not all the compounds that bind Aβ will necessarily enhance the toxicity of Aβ in PCat cells.

The effect of treatment of PCat cells with BTA-EG_4 and different amyloid peptides; Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29 and PrP 106-126 were investigated. The BTA-EG_4 enhanced the toxicity of all the amyloid peptides (section 6.2.7). This suggests that BTA-EG_4 enhances the toxicity of all the amyloid peptides by disrupting catalase-amyloid interaction. The effects of BTA-EG_4, KP and 3-AT on the toxicity of H_2O_2 were also investigated in the PCat cells (section 6.2.8). Results showed that BTA-EG_4 and KP did not enhance the toxicity of H_2O_2, unlike 3-AT in PCat cells. This indicates that BTA-EG_4 does not affect the activity of catalase and could enhance the toxicity of Aβ by disrupting catalase-amyloid interaction (section 6.2.8).

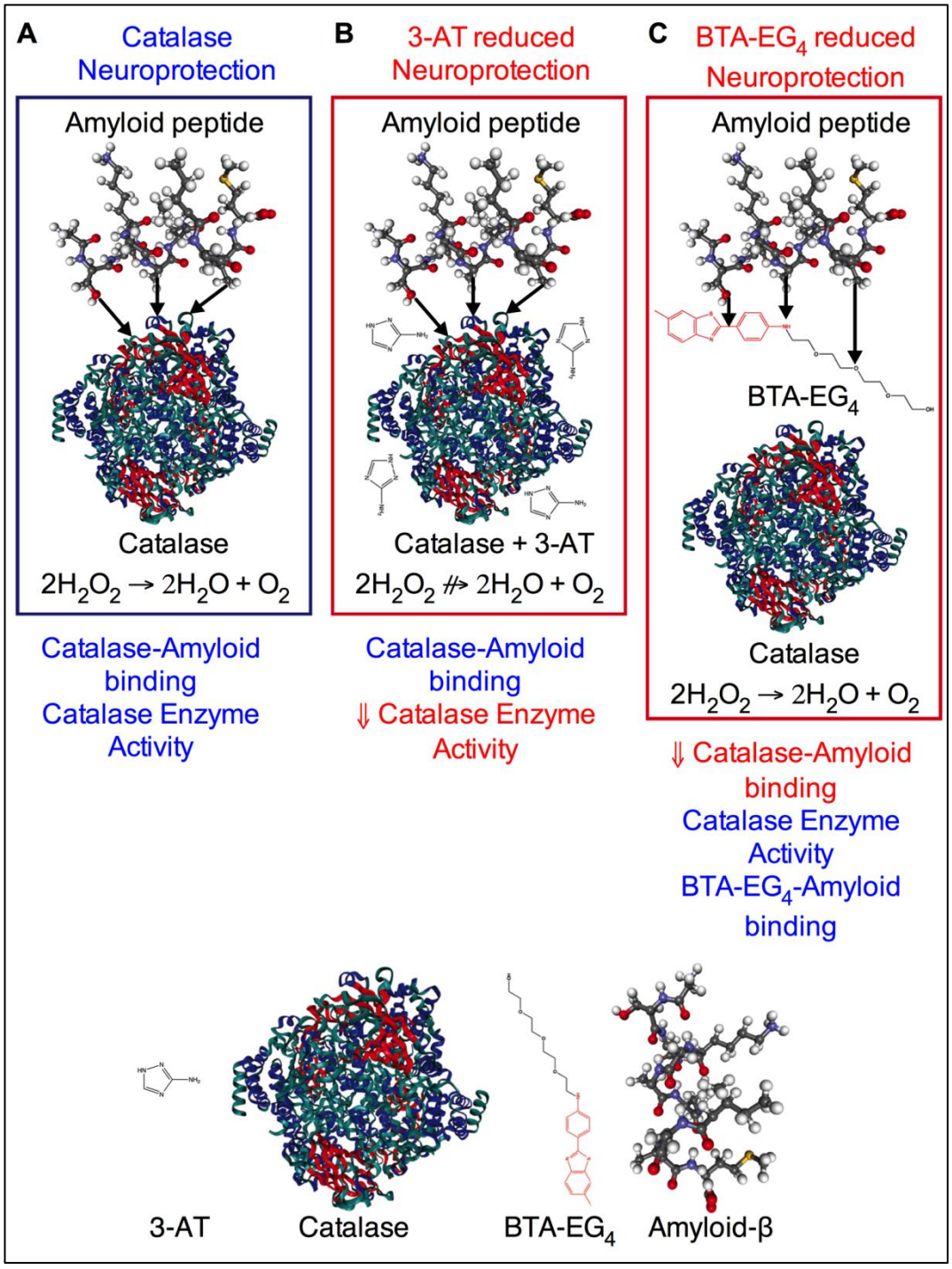

Figure 6-9.The proposed model of catalase-amyloid interaction. The diagram represents the proposed model of catalase-amyloid interaction (A). The 3-AT could bind to catalase (B) resulting in decreased catalase activity (can no longer breakdown H_2O_2) and increased amyloid toxicity.The catalase-amyloid interaction could be blocked by BTA-EG_4 (C), which leaves catalase unaffected (can efficiently breakdown H_2O_2) by Aβ.

6.4 Conclusions

The results of this chapter represent the mechanism of catalase neuroprotection against Aβ toxicity. Catalase was found to be neuroprotective against not only Aβ but also other amyloid peptides such as IAPP and PrP. The mechanism of neuroprotection was found to have both an activity of catalase and catalase-amyloid binding interaction components. This neuroprotection could be inhibited by either 3-AT or BTA-EG_4. The compounds that inhibit catalase-amyloid interaction have been found to be neuroprotective (Habib *et al.,* 2010; Capule *et al.,* 2012). The results obtained in the present study contradict the previous reports, as the inhibition of catalase-amyloid interaction was found to have decreased the viability of the PCat cells. It could be possible that BTA-EG_4 enhances the toxicity of Aβ only in PCat cells, since no increase in toxicity was observed in PVect cells treated with BTA-EG_4 and Aβ.

Chapter 7

KiSS-1 neuroprotection against Amyloid-β toxicity

7.1 Introduction

The KP peptides are major regulators of the HPG axis, which act on GnRH neurons to activate GnRH release, which in turn stimulates LH and FSH (gonadotrophins) (Navarro and Tena-Sempere, 2011). The KP peptide was discovered as a product of a human melanoma KiSS-1 metastasis suppressor gene (Kotani *et al.,* 2001). Overexpression of KiSS-1 gene in human melanoma cell lines suppressed metastasis in athymic nude mice (Lee *et al.,* 1996). The KP peptides are products of the KiSS-1 gene, the full length pre-pro-protein gets processed to yield KP-54, and shorter derivatives of KP peptide comprising of the C-terminal KP-14 (41-54), KP-13 (42-54) and KP-10 (45-54) (Kotani *et al.,* 2001). The KP-10 peptide is the smallest form, which is biologically active, to stimulate the GPR-54 receptor (Kotani *et al.,* 2001; Bilban *et al.,* 2004). Binding of KP to GPR-54 leads to GnRH secretion, failure of GPR-54 stimulation by KP leads to hypogondotropic hypogonadism (De Roux *et al.,* 2003). Recent studies have shown that KP-13, KP-10 and KSO can stimulate the NPFF receptors and therefore some KP actions could also be mediated by the NPFF receptors (Lyubimov *et al.,* 2010; Oishi *et al.,* 2010; Milton 2012).

It has been demonstrated that catalase residues 400-409 contain the Aβ binding domain (Milton 2001), which was found to be neuroprotective and that a peptide containing these residues, CAβBD, can inhibit interactions between catalase and Aβ fibrils (Milton and Harris, 2009). A comparison between human catalase and KiSS-1 preproprotein sequences has shown that the KiSS-1 preproprotein contains a CAβBD-like sequence (Figure 1-8).

The sequence similarity as shown in Figure 1-8 raises the possibility of KP binding to Aβ, IAPP and PrP, and this suggests that it could be neuroprotective against Aβ toxicity (Milton 2011; Milton 2013). The SH-SY5Y neuroblastoma cells express the KiSS-1 gene (Poomthavorn *et al.,* 2009) and contain the necessary secretary vesicle machinery to release neuroendocrine hormones (Godall *et al.,* 1997). This suggests that the SH-SY5Y cells can process the KiSS-1 preproprotein to smaller kisspeptins. In the present study, the KiSS-1 gene was cloned in a pcDNA™4/TO/myc-His vector and the SH-SY5Y cells

were transfected with the KiSS-1 gene (PKiSS cells). The SH-SY5Y cells transfected with empty vector were used as control (PVect cells). The overexpression of the KiSS-1 gene was confirmed by RT-PCR and immunocytochemistry. The PKiSS and PVect cells were exposed to Aβ, IAPP and PrP to investigate the role of KP in neuroprotection against Aβ, IAPP and PrP. To understand the mechanism of neuroprotection the anti-KP 45-50 antibody (Iijima *et al.,* 2011), GPR-54 and NPFF receptor antagonists KP234 (Roseweir *et al.,* 2009) and RF9 (Simonin *et al.,* 2006) were also tested in these experiments.

Previous studies have shown a relationship between gonadotrophins and OS (Muthuvel *et al.,* 2006; Murugesan *et al.,* 2007). Increases in OS lead to a decrease in LH and FSH, which is induced by hypothalamic malfunction. It has also been shown that production of LH is reduced in the anterior pituitary due to OS, and that the normal levels could be restored by treatment with an antioxidant, vitamin C (Muthuvel *et al.,* 2006; Murugesan *et al.,* 2007). A previous study has also shown that serum LH and FSH increase in parallel with catalase and SOD in post-varicocelectomy patients (Hurtado *et al.,* 2007). In a study carried out by Aydin *et al.,* 2010, the KP was shown to have an indirect antioxidant effect. In this study high level of SOD and catalase were found in young male rats treated with KP. In the present study, the indirect role of KP as an antioxidant was investigated in PKiSS cells. High levels of KP should increase the expression level of catalase protein (Aydin *et al.,* 2010), if so the PKiSS cells should be resistant to H_2O_2 toxicity similar to the PCat cells. The expression level of catalase in PKiSS cells will be determined by RT-PCR and the indirect antioxidant effect of KP will be tested by exposing the PKiSS cells to H_2O_2.

7.1.1 Aim

- To determine the effect of KiSS-1 gene overexpression in SH-SY5Y cells against Aβ toxicity.

7.1.2 Objectives

- Amplification of the KiSS-1 genes using PCR.
- Estimation of KiSS-1 gene mRNA expression in PKiSS cells.
- To investigate whether the overexpression of the KiSS-1 gene protects against Aβ toxicity.

7.1.3 Hypothesis

The KP peptide shares sequence similarity and binds to Aβ, it has been found associated with amyloid plaque like deposits in the AD patient pons brain sections. As peptides that bind Aβ could be neuroprotective, the overexpression of KiSS-1 gene could also show neuroprotection either via binding interaction with Aβ or through binding to its receptor. The effect of KiSS-1 gene overexpression in SH-SY5Y cells against Aβ toxicity was investigated in this chapter.

7.2 Results

7.2.1 KP peptide neuroprotection against different amyloid peptides (Aβ, IAPP, PrP, ABri and ADan).

The KP 45-50 peptide has recently been identified as an amyloid binding peptide, which binds to the same region of Aβ, IAPP and PrP as catalase and inhibits their toxicity (Milton 2011; Milton 2013). The effect of pretreatment of naïve SH-SY5Y cells with 1 µM and 10 µM of KP 45-50 for 24 hrs prior to the treatment with 25 µM amyloid peptides (Aβ, IAPP, PrP, ABri and ADan) was tested. Results show that 1 µM KP does not have any significant effect on the toxicity of Aβ 25−35, IAPP 20−29, PrP 106−126, ABri 1−34, or ADan 1−34 (Figure 7-1A). Treatment with 10 µM KP 45-50 significantly reduced the toxicity of the Aβ 25−35, IAPP 20−29, and PrP 106−126, but had no effect on the toxicity of ABri 1−34 or ADan 1−34 (Figure 7-1B).

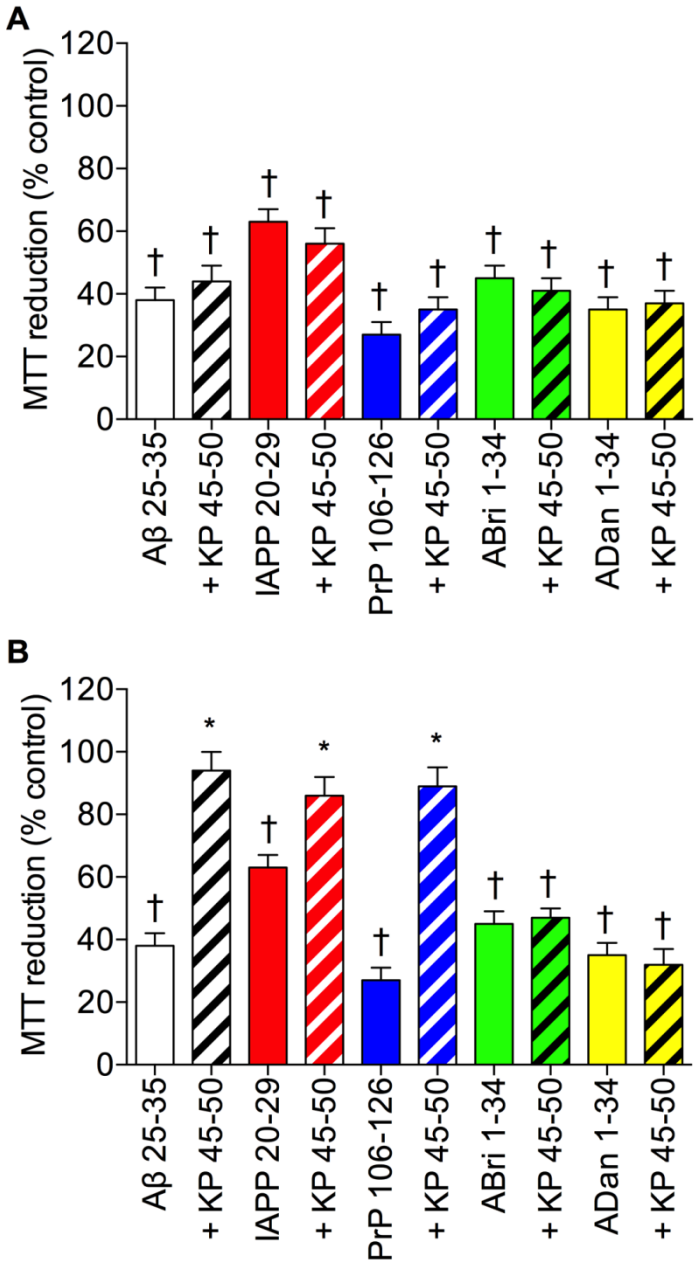

Figure 7-1. KP 45-50 neuroprotection against different amyloid peptides. Naïve SH-SY5Y cells were pretreated with 1 µM (A) and 10 µM (B) of KP 45-50 for 24 hrs prior to the treatment with 25 µM Aβ 25−35, IAPP 20−29, PrP 106−126, ABri 1−34, or ADan 1−34 alone or together with KP 45-50, after overnight incubation the viability was determined by MTT reduction. Results are mean ± S.E.M (n = 8); † = $P < 0.05$ vs control (cell treated with media only were considered as 100 percent), * = $P < 0.05$ vs cells treated with amyloid peptide only, (one-way ANOVA).

This experiment suggests that KP is neuroprotective against amyloid peptides and overexpression of KP in SH-SY5Y cells could protect the cells against amyloid insults.

7.2.2 Characterization of PKiSS human KiSS-1 gene overexpressing SH-SY5Y human neuroblastoma cells

For the overexpression of KiSS-1, the human KiSS-1 gene was cloned into the pcDNA™4/TO/myc-His expression vector and transfected into naïve SH-SY5Y neuroblasoma cells.

7.2.2.1 Amplification of the KiSS-1 gene using PCR

The KiSS-1 gene was cloned into the pcDNA™4/TO/myc-His vector (Invitrogen) and this was later transfected into SH-SY5Y cells, to investigate the role of KiSS-1 gene overexpression against Aβ toxicity. The KiSS-1 genes was amplified from pCMV6-XL5 vector containing human KiSS-1 gene (Origene) using PCR, the PCR conditions used for the amplification are mentioned in the materials and methods (2.2.2). After the PCR, the PCR product was run on 1% agarose gel, where the KiSS-1 gene band was found to be 0.47 Kbps (Figure 7-2).

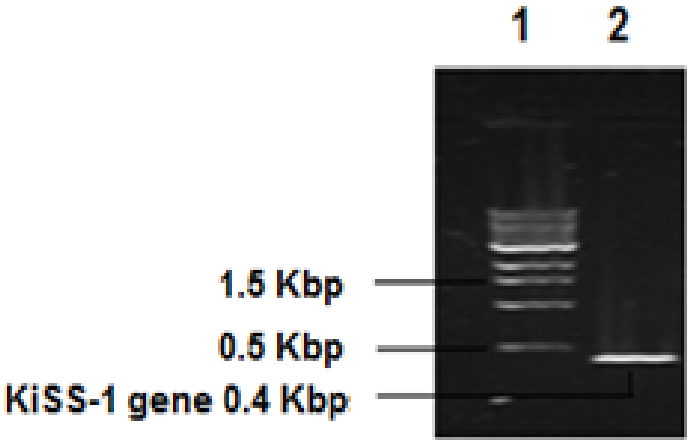

Figure 7-2. Amplification of the KiSS-1 genes using PCR. The 1 % agarose gel picture shows the PCR amplification of the KiSS-1 gene from the pCMV6-XL5 vector containing human KiSS-1 gene (Origene). Lane 1- 1kb ladder, lane 2- PCR product of the KiSS-1 gene.

7.2.2.2 Analyzing the restriction digestion of the pcDNA™4/TO/myc-His expression vector containing the KiSS-1 gene

The KiSS-1 gene was cloned in the pcDNA™4/TO/myc-His expression vector. To confirm the cloning the vector was restriction enzyme digested using the restriction enzymes - KiSS-1 (*Bam* HI and *Xhol*). Figure 7-3 represents the restriction digestion of the vector containing the KiSS-1 gene. The cloned KiSS-1 gene generated two bands upon restriction enzyme digestion, the linearized pcDNA™4/TO/myc-His vector band 5.1 Kbps and the KiSS-1 band of 0.47 Kbps, which is the right size for the KiSS-1 gene. The sequence of the cloned KiSS-1 gene was further confirmed by DNA sequencing (UCL DNA sequencing service, see Appendix 10.2); the DNA sequence of the gene was found to be correct without any mutations.

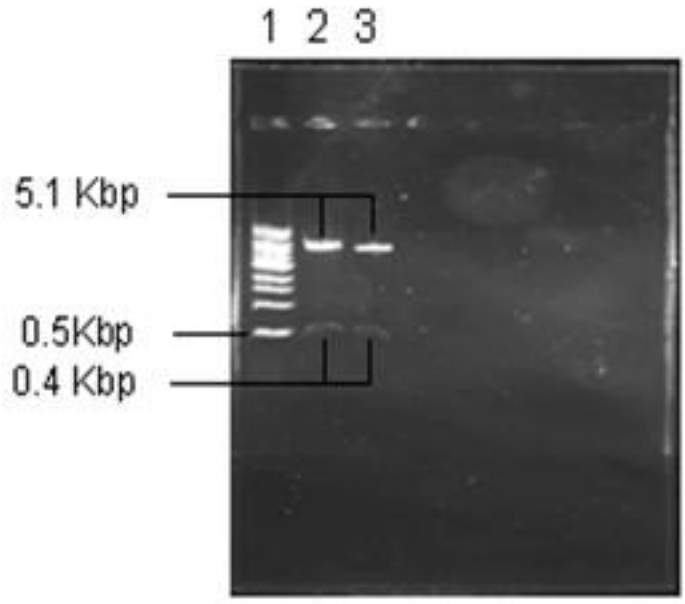

Figure 7-3. Analysis of the restriction digest of the pcDNA™4/TO/myc-His expression vector containing the KiSS-1 gene. Restriction enzyme digestion of the KiSS-1 gene cloned into the pcDNA™4/TO/myc-His vector, the size of the vector was found to be 5.1 Kbps and the KiSS-1 gene 0.47 Kbps (lane 2 and 3), which is the predicted size for the KiSS-1 gene, lane 1- 1kb ladder.

7.2.2.3 Transfection of KiSS-1 gene and selection of stably expresing PKiSS cell line

The pcDNA™4/TO/myc-His vector containing the KiSS-1 gene was transfected into the naïve SH-SY5Y cells. The cells were cultured in the presence of zeocin to select pcDNA™4/TO/myc-His vector expressing cells. After culture for 4 weeks in zeocin the stable PKiSS cell line was subsequently cultured under standard cell culture conditions.

The PVect cell line (Chapter 5: Section 5.1.2.3) was used as a control.

7.2.2.4 Estimation of KiSS-1 gene mRNA expression levels in PKiSS cells

The overexpression of the KiSS-1 gene was confirmed by RT-PCR. The gel image (Figure 7-4) of the KiSS -1 gene RT-PCR represents a comparison of expression levels of KP mRNA in PKiSS, PCat and PVect cells. The mRNA expression of KP is higher in the PKiSS cells (lane 5) compared to PVect cells (lane 4), this suggests that KP is overexpressed in PKiSS cells compared to PVect cells or PCat cells.

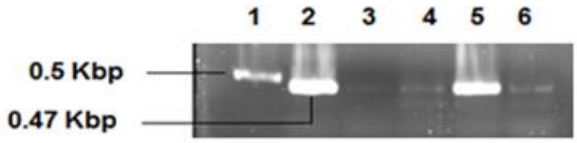

Figure 7-4. Estimation of KiSS-1 gene mRNA levels expression in PKiSS cells. The gel picture represents the KP mRNA expression levels in the PVect, PCat and PKiSS cells. Lane 1 - 1 Kb ladder, lane 2 - KiSS-1 gene (positive control amplified from vector (invitrogen)) , lane 3 - negative control (water), lane 4 - KP mRNA expression levels in the PVect cells, lane 5 - KP mRNA expression levels in the PKiSS cells, lane 6 - mRNA expression levels of KP in PCat cells.

7.2.2.5 The mRNA expression levels of the catalase gene in PKiSS cells, and confirmation of uniform loading of the samples during RT-PCR using actin primers

To investigate the role of KP as an indirect antioxidant, which may act by increasing catalase levels, RT-PCR was performed using PKiSS, PCat and PVect cells. If KP was acting indirectly as an antioxidant by increasing catalase levels in cells, then the mRNA expression levels of catalase could be higher in PKiSS cells compared to PVect cells. Results showed that increased catalase mRNA expression was only observed in PCat cells but not in PKiSS or PVect cells (Figure 7-5). This suggests that KP does not increase catalase levels in PKiSS cells and therefore probably does not act as an indirect antioxidant by activating catalase (as seen in liver by Aydin *et al.,* 2010).

To ensure that equal amounts of the sample were loaded into each lane of the gel shown in Figure 7-5, actin was used as a control. Human actin primers were used to amplify the same amount of cDNA used for the RT-PCR of PKiSS and PVect cells. The product of the RT-PCR was run on a gel, the bands obtained show that equal amount of each sample were loaded into each lane while performing the RT-PCR. The size of the actin bands obtained was found to be 100 bps which is the expected band size.

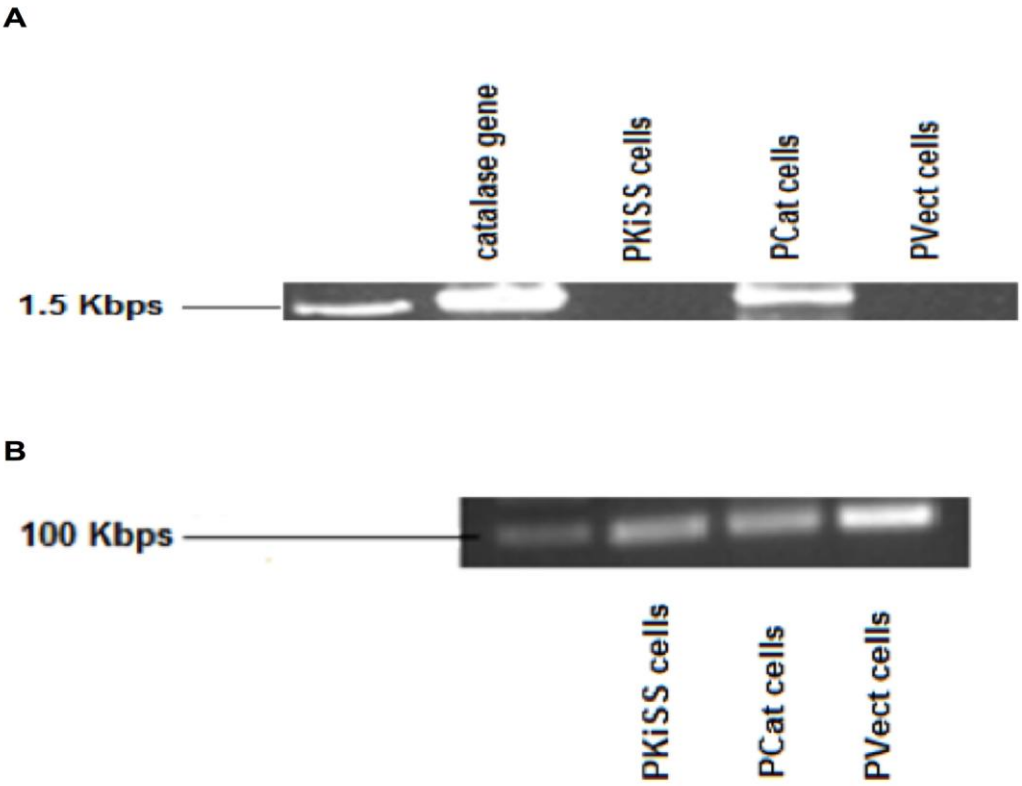

Figure 7-5. The mRNA expression levels of the catalase in PKiSS cells. The catalase gene expression in PKiSS, PCat and PVect cells was determined by RT-PCR. The β-actin in PKiSS, PCat and PVect cells were determined as a control.

7.2.2.6 Analyzing the overexpression of the KiSS-1 gene in PKiSS cells using Immunocytochemistry.

Immunocytochemistry was used to confirm the overexpression of the KiSS-1 gene in PKiSS cells and also to observe the localisation of kisspeptin within the cells. The SH-SY5Y cells were plated at a density of $1.2x10^6$ in the 6-well plate, were first blocked with 10% bovine serum albumin. The primary antibody anti-KP 45-54 (1:1000) was made in the blocking solution to detect the presence of KP in SH-SY5Y cells. The secondary antibody used was goat anti-rabbit IgG-Alexafuor 488 (Abcam PLC, Cambridge; 1:500). The cells were visualised using confocal microscopy.

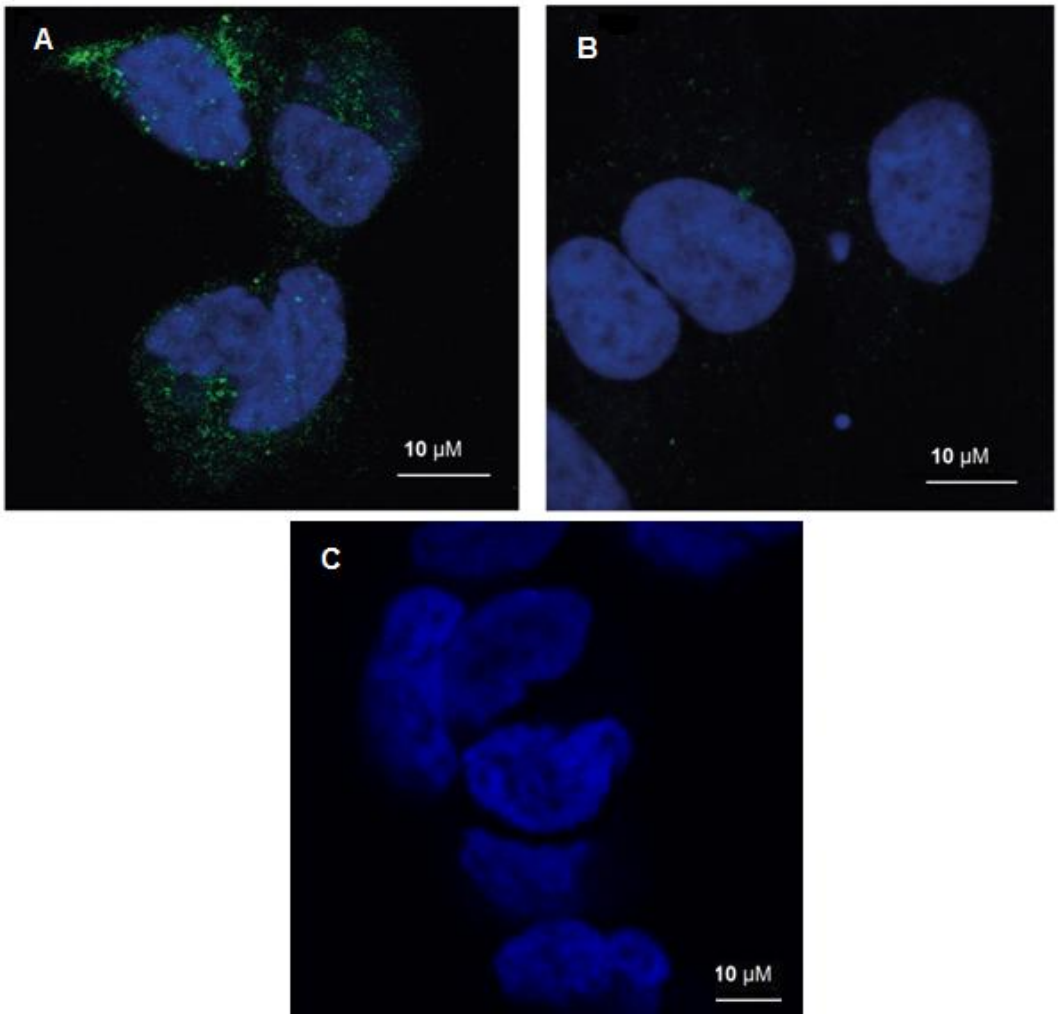

Figure 7-6. Analyzing the overexpression of the KiSS-1 gene in PKiSS cells using immunocytochemistry. PKiSS cells (A) and PVect cells (B) were double labelled with rabbit anti-KP 45-54 antibody (stained green) and TO-PRO®-3 Iodide DNA staining (stained blue). PKiSS cells (C) were staining with the secondary antibody alone. (Bar scale = 10 µM).

The immunocytochemistry results showed the presence of immunoreactive KP in both the PKiSS and PVect cells. The Figures 7-6A and 7B show an increase in the level of KP expression in PKiSS cells (Figure 7-6A) compared to PVect cells (Figure 7-6B),. The KP (stained green) seems to be located in primarily in the cytoplasm of the SH-SY5Y cells. Figure 7-6C shows the staining of the PKiSS cells with the secondary antibody alone, which did not give any nonspecific staining.

7.2.3 Overexpression of the KiSS-1 gene was neuroprotective against Aβ toxicity

The KP neuroprotection against Aβ toxicity was investigated by exposing the PKiSS cells to 25 µM Aβ (25-35) toxicity overnight. The PKiSS and PVect cells were treated with different doses of Aβ (25-35) (0-50 µM) overnight. The PKiSS cells were more resistant to Aβ (25-35) toxicity compared to PVect cells, even at higher doses of Aβ. The neuroprotection of PKiSS cells against Aβ (25-35) toxicity was determined using an MTT assay (Figure 7-7A). Trypan blue cell viability assay was also used to determine the percentage of viable cells. Results showed that PKiSS cells had higher resistance to Aβ (25-35) toxicity compared to PVect cells (Figure 7-7B). This suggests that PKiSS cells were neuroprotective against Aβ toxicity compared to PVect cells.

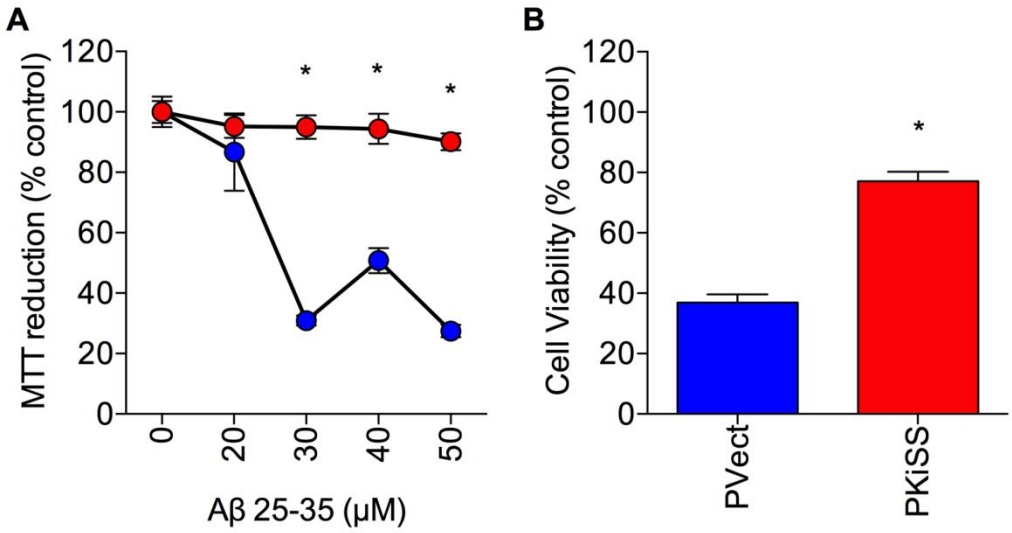

Figure 7-7. Effect of Aβ on the viability of PKiSS cells. The PKiSS and PVect cells were exposed to (A) different doses of Aβ (25-35) (0-50 µM) overnight and viability determined by MTT reduction or (B) 25 µM Aβ (25-35) overnight and viability determined by Trypan Blue dye exclusion. Results are mean ± S.E.M (n = 8); (A) * = $P < 0.05$ vs PVect plus Aβ, one-way ANOVA. (B) * = $P < 0.05$ vs PVect plus Aβ students t-test.

7.2.4 KiSS-1 overexpression neuroprotection against different amyloid peptides

As KiSS-1 gene overexpression was found to be neuroprotective against Aβ toxicity, the PKiSS and PVect cells were treated with different amyloid- Aβ 1-42, Aβ 25-35, PrP 106-126 or IAPP 1-37 peptides 25 µM each overnight, to test if PKiSS cells were resistant to other amyloid peptides. Cell viability was determined by MTT assay, results showed that PKiSS cells were resistant to Aβ, PrP and IAPP toxicity compared to PVect cells (Figure 7-8). This suggests that KiSS-1 overexpression neuroprotection is not restricted to Aβ; it is also neuroprotective against PrP and IAPP.

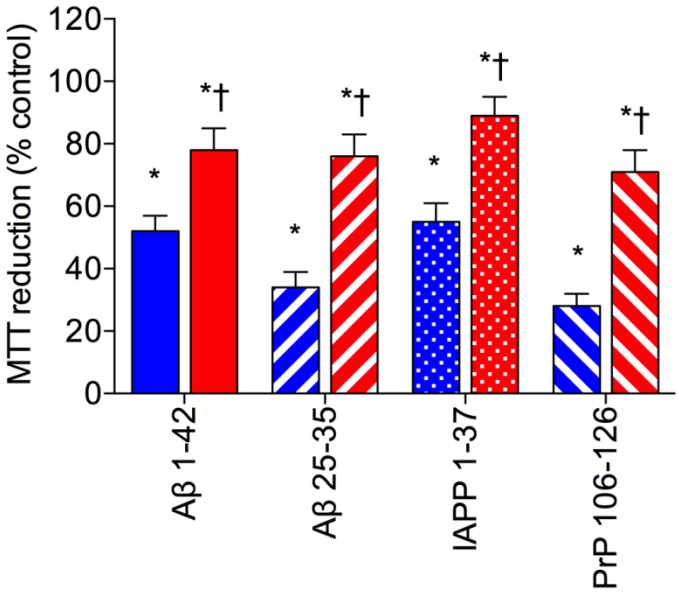

Figure 7-8. Effect of amyloid peptides on the viability of PKiSS cells. The PKiSS (red columns) and PVect (blue columns) cells were exposed to 25 µM Aβ 1-42, Aβ 25-35, PrP 106-126 or IAPP 1-37 overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cell treated with media only), † = $P < 0.05$ vs PVect cells treated with amyloid peptide, (one-way ANOVA).

7.2.5 Possible mechanism of KiSS-1 overexpression neuroprotection

To understand the mechanism of KiSS-1 overexpression neuroprotection against Aβ toxicity, the PKiSS cells were treated with 5 µM Aβ (1-42) and anti-KP 45-50 antibody (10 µg/ml), GPR-54 receptor antagonist KP234 (10 µM) or NPFF receptor antagonist RF9 (10 µM) overnight, the cell viability was determined using MTT assay. Results showed that the anti-KP 45-50 antibody enhanced the toxicity of Aβ in PKiSS cells (Figure 7-9). This observation suggests that an extracellular binding interaction could be involved in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity, as the extracellular binding of KP to Aβ can be inhibited by the anti-KP antibody (Milton 2011; Milton 2013). Blocking the binding interaction using anti-KP 45-50 antibody increased the toxicity of Aβ in PKiSS cells. The KP or NPFF receptor antagonists did not have any effect on PKiSS cells. This suggests that the KiSS-1 overexpression neuroprotection is not mediated via actions of KP peptides on GPR-54 or NPFF receptors.

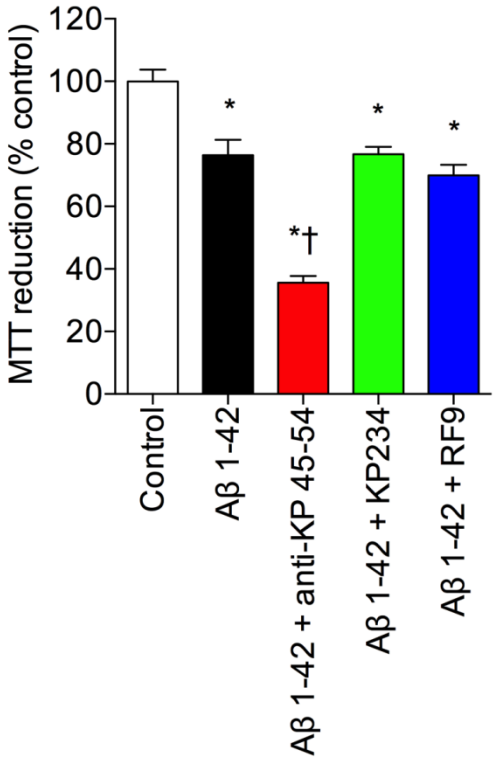

Figure 7-9. Possible mechanism of KiSS-1 overexpression neuroprotection. The PKiSS cells were exposed to 5 µM Aβ plus anti-KP 45-50 antibody (10 µg/ml) or GPR-54 receptor antagonist KP234 (10 µM) or NPFF receptor antagonist RF9 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (cells treated with media alone); † = P < 0.05 vs Aβ 1- 42; one-way ANOVA.

7.2.6 KiSS-1 overexpressing cells conditioned media protects against Aβ toxicity

To investigate the secretion of KP into the cullture media and to determine the neuroprotection of secreted KP against Aβ toxicity, the conditioned media from PKiSS, PVect and SH-SY5Y cells was collected after overnight incubation with the cells. The conditioned media from PKiSS, PVect and SH-SY5Y cells were added to the naïve cells together with Aβ 25-35 (10 µM) overnight. MTT assay results showed that the naïve cells treated with PKiSS media plus Aβ showed higher viability compared to the cells treated with PVect media plus Aβ (Figure 7-10A).

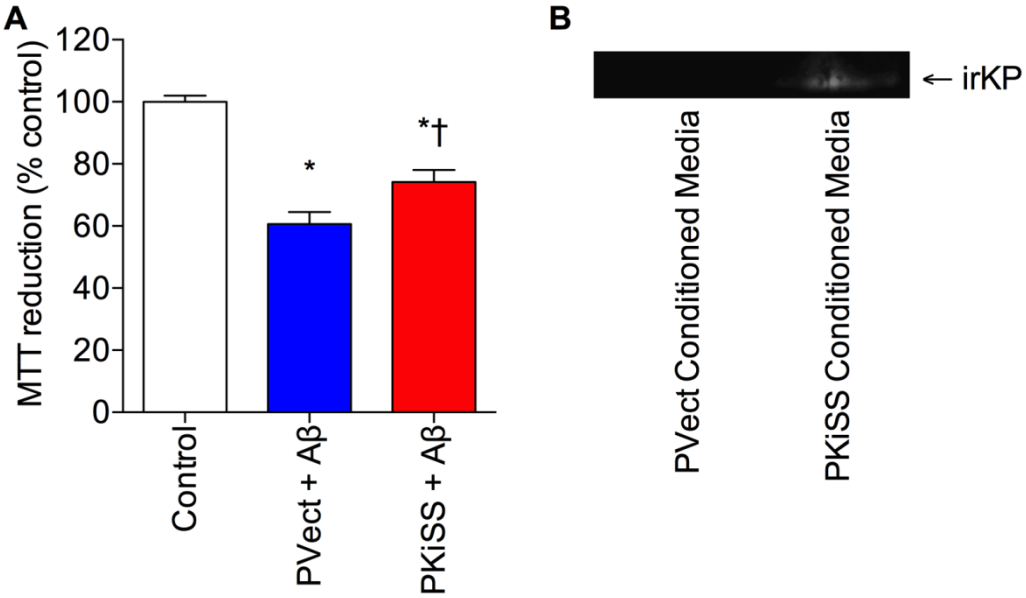

Figure 7-10. Effect of conditioned media from PKiSS and PVect cells on Aβ toxicity. (A) Naïve SH-SY5Y cells were treated with conditioned media from PCat or PVect cells plus 25 µM Aβ (25-35) overnight and viability determined by

MTT reduction. (B) The western blotting analysis immunoreactive KP in the conditioned media from PKiSS and PVect cells. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control; † = P < 0.05 vs PVect conditioned media (one-way ANOVA).

To determine the presence of KP release into the media from the PKiSS and PVect cells, the proteins in 6 ml of conditioned media were concentrated using Amicon system (Merck Millipore, UK). The concentrated media was loaded onto the SDS page without subjecting the media to denaturation; the proteins from the gel were transferred onto a nitrocellulose membrane. The KP was detected using anti-KP 45-54 primary antibody and horse peroxidase-conjugated goat anti-rabbit antibody. The western blot showed a band of less than 10 kDa, which represents immunoreactive KP in the conditioned media (Figure 7-10B). This suggests that KP is released into the media when the KiSS-1 gene is overexpressed in the SH-SY5Y cells.

7.2.7 Testing the viability of KiSS-1 overexpressing cells against H_2O_2 toxicity

To investigate the effect of H_2O_2 PKiSS and PVect cells were exposed to a range of H_2O_2 (0 - 1 mM) concentrations for 2 hrs prior to the MTT assay. Cell viability was determined by MTT assay, results showed that PKiSS and PVect cells were equally susceptible to H_2O_2 toxicity, there was no significant difference between the two cell types (Figure 7-11). This suggests that PKiSS neuroprotection is limited to Aβ, PrP and IAPP toxicity, KP does not act as an indirect antioxidant in PKiSS cells and does not protect cells against H_2O_2 toxicity.

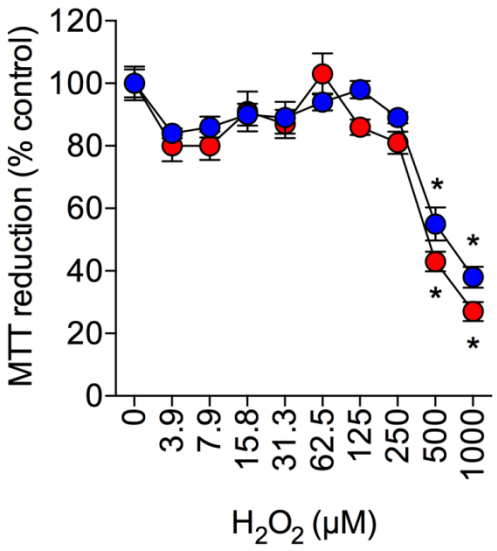

Figure 7-11. Effect of H_2O_2 on PKiSS and PVect viability. Represents the dose reseponse curve of H_2O_2 on PCat and PVect cells, both the cell lines were treated with different doses of H_2O_2 ranging from 0 - 1000 µM overnight and viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (0 µM H_2O_2); (one-way ANOVA).

7.3 Discussion

A sequence similarity was found between catalase Aβ binding domain (400-409) and KP residues 42-51 (Figure 1-8) (Milton 2011; Milton 2013). This sequence similarity allows KP to bind Aβ and this binding interaction was found to be neuroprotective against Aβ toxicity. Exposure of naïve cells to amyloid peptides in the presence of 10 µM KP 45-50 was found to be neuroprotective, compared to 1 µM KP 45-50 which had no significant effect on amyloid peptides (section 7.2.1) toxicity. Overexpression of the KiSS-1 gene in human neuroblastoma SH-SY5Y cells was found to be neuroprotective against Aβ (section 7.2.3), PrP and IAPP toxicity (section 7.2.4). The overexpression of the KiSS-1 gene was confirmed by RT-PCR (section 7.2.2.4) and immunocytochemistry (section 7.2.2.6).

This property of KP peptide neuroprotection against different amyloids is similar to the neuroprotection by catalase against Aβ, IAPP and PrP (Milton 2011; Milton 2013). This suggests that the sequence similarity between catalase and KP plays a key role in neuroprotection against different amyloids. To further understand the mechanism of neuroprotection, the PKiSS and PVect cells were treated with Aβ (1-42) and anti-KP 45-50 antibody or GPR-54 receptor antagonist KP234 or NPFF receptor antagonist RF9 (section 7.2.5). Only anti-KP 45-50 antibody increased the toxicity of Aβ, whereas the KP234 and R9 did not alter the toxicity of Aβ in both PVect and PKiSS cells. This further strengthens the concept that extracellular KP-amyloid interaction is the key for neuroprotection, as anti-KP 45-50 antibody can inhibit KP and amyloid peptide interaction extracellularly. Inhibition of KP-amyloid interaction by anti-KP antibody increases Aβ toxicity. This also suggests that KP neuroprotection is not receptor (KP or NPFF) mediated or it acts via other receptors.

Results obtained from RT-PCR (section 7.2.2.5) and MTT assay, obtained by exposing the PKiSS cells to H_2O_2 toxicity (section 7.2.7) suggest that the catalase levels were not increased in PKiSS cells. KP therefore does not act as an antioxidant directly or indirectly and does not play a role in protecting the cells against H_2O_2 toxicity.

The effects of KP on hormone secretion has already been studied in humans, KP could be used for stimulating reproductive activity (Chan 2013) or for the treatment of hypothalmic amenorrhoea (Jayasena *et al.,* 2014). The expression of KP could be regulated by sex steriods *in vivo* (Matthew *et al.,* 2013), suggesting that KP could be a potential therapy for protecting the brain against Aβ toxicity. The increased expression of catalase in AD brain could be beneficial compared to increased expression of KP, as catalase provides dual protection against the oxidative stress and Aβ toxicity. It could be difficult to increase catalase expression in the brain, whereas sex steroids could regulate KP.

7.4 Conclusion

The results presented in this chapter illustrate the creation of the PKiSS human kisspeptin overexpressing neuronal cell line, derived from the SH-SY5Y neuroblastoma. The KiSS-1 overexpression was found to be neuroprotective against Aβ, IAPP and PrP. The mechanism of KiSS-1 overexpression neuroprotection resembles catalase overexpression neuroprotection (Chapter 6). In both cases an Aβ binding interaction with either extracellular KP or catalase appears to play a significant role. This could be due to the sequence similarity between catalase and KP (Milton 2011; Milton 2013). The neuroprotection was not GPR-54 receptor mediated, as the GPR-54 receptor antagonist KP234 did not block KiSS-1 overexpression neuroprotection against Aβ. Unlike catalase, KiSS-1 overexpression could not protect the PKiSS cells against H_2O_2 toxicity. The conditioned media from PKiSS cells was protective againt Aβ toxicity and immunoreactive KP was detective in the condition media of the PKiSS cells, suggesting that the released KP products played a role.

Chapter 8

The role of neurotransmitters in KiSS-1 neuroprotection against Amyloid-β toxicity

8.1 Introduction

The KP peptides are ligands for the GPR-54 (Kotani *et al.,* 2001; Kirby *et al.,* 2010) and NPFF receptors (Lyubimov *et al.,* 2010; Oishi *et al.,* 2010). The KSO peptide, a shorter derivative of KP peptide (828.88 Da), activates NPFF like receptors but not GPR-54 receptor (Milton 2012). The KP peptide plays a central role in the HPG axis by acting on GnRH neurons to stimulate GnRH release (Navarro and Tena-Sempere, 2012). Overexpression of the KiSS-1 gene in human neuroblastoma SH-SY5Y cells was found to be neuroprotective against Aβ toxicity (Figure 7-7). The mechanism of KP neuroprotection was not receptor mediated (GPR-54 receptor) (Figure 7-9), a binding interaction between KP and Aβ was suggested to be the possible mechanism for neuroprotection.

Recent studies have shown the effect of KP on passive avoidance learning and potential involvement of adrenergic, serotonergic, dopaminergic, cholinergic, gabaergic, opiate and nitric oxide receptors in mice. Results have shown that KP may partly act via all these receptors to facilitate passive avoidance learning in mice (Telegdy and Adamik, 2013). Another study has shown that KP has anti-depressant like effects in mouse, which could involve adrenergic and serotonergic receptors (Tanaka *et al.,* 2013). This suggests that KP may act via other receptors and therefore the present study was conducted to characterize a model of KiSS-1 gene overexpression (PKiSS cells) neuroprotection against Aβ toxicity in SH-SY5Y neurons, and to determine the role of neurotransmitter systems in the neuroprotection. The KP peptide also plays a role in thermoregulation (Csabafi *et al.,* 2013) and may therefore invoke prostaglandin responses (Brenneis *et al.,* 2006), The effects of antagonists of KP, NPFF, opioids, oxytocin, estrogen, adrenergic, cholinergic, dopaminergic, serotonergic and γ- aminobutyric acid (GABA) receptors were tested (Table 3). Inhibitors of catalase, cyclooxygenase, nitric oxide synthase and the mitogen activated protein kinase cascade were also tested (Table 3). The PKiSS cells were pretreated with individual antagonists or antibody or inhibitors, 2 hrs prior to treatment with Aβ toxicity, cell viability was determined by MTT assay.

Table 3 Mechanism of KiSS-1 neuroprotection.

Toxin		**Dose**
Aβ 1-40		10 µM
Drug Target	**Chemical**	**Dose**
Aβ-Catalase interaction	ASCAT peptide	100 µM
Aβ-Protein interactions	BTA-EG4 hydrate	10 µM
Receptor Target	**Antagonist**	**Dose**
Adrenergic	Phenoxybenzamine hydrochloride (PHB)	10 µM
	Prazosin hydrochloride (PR)	250 nM
	Propranolol hydrochloride (PROP)	50 nM
	Yohimbine hydrochloride (YO)	50 nM
Cholinergic	Atropine sulfate (ATR)	10 µM
	Mecamylamine hydrochloride (MEC)	10 µM
Serotonergic	Methysergide maleate (MET)	1 µM
	Cyproheptadine hydrochloride (CYPR)	10 nM
GABA-A	1(S),9(R)-(−)-Bicuculline methiodide (BIC)	50 µM
Estrogen	Tamoxifen (TAMOX)	10 µM
GPR-54	KP234	10 µM
NPFF	RF9	10 µM
Opioid	Naloxone (NAL)	1 µM
	Naltrexone (NALTR)	1 µM
Dopaminergic	Haloperidol (HAL)	10 µM
Oxytocin	Atosiban (ATO)	1 µM
Enzyme Target	**Drug**	**Dose**
MAPK	PD98059	50 µM
Cyclooxygenase	SC-560	1 µM
Nitric oxide synthase	N^G-Methyl-L-arginine acetate salt (LNMA)	1 mM
Catalase	3-Amino-1,2,4-triazole (3-AT)	50 mM

Table 3 shows the various chemicals used to block different receptors, enzymes and the Aβ-protein interactions to understand the mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.

8.1.1 Aim

- To investigate the role of neurotransmitters, enzyme pathways and amyloid binding interactions in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.

8.1.2 Objectives

- To use different amyloid binding compounds to understand the mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.
- To use different neurotransmitter antagonists to understand the mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.
- To use different enzyme inhibitors to understand the mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.

8.1.3 Hypothesis

Recently KP has been shown to play a role in passive avoidance learning and memory consolidation by acting via the neurotransmitters; it has also been shown to have an anti-depressant like effect. The KP mediated neuroprotection against Aβ could be via the neurotransmitters. In the present study various neurotransmitter antagonists will be used to understand the role of neurotransmitter in KP mediated neuroprotection against Aβ toxicity.

8.2 Results

8.2.1 KP - Aβ interaction inhibited by ASCAT and BTA-EG_4 in PKiSS cells

The ASCAT peptide contains an Aβ like sequence (Milton *et al.,* 2001). The ASCAT inhibits IAPP and PrP binding to KP but not Aβ binding (Milton 2011; Milton 2013), and could compete with KP to bind Aβ. Therefore, PKiSS cells were treated with ASCAT (100 µM) and Aβ (1-40) (10 µM) overnight. The dose of ASCAT used was previously shown to prevent Aβ inhibition of catalase, without having a neuroprotective effect (Milton *et al.,* 2001). The ASCAT peptide did not enhance the toxicity of Aβ (1-40) in PKiSS cells (Figure 8-1). The BTA-EG_4 compound binds Aβ and prevents the association of Aβ with other proteins (Inbar *et al.,* 2006). Treatment of PKiSS cells with BTA-EG_4 (10 µM) and Aβ (1-40) (10 µM) overnight did not enhance the toxicity of Aβ (Figure 8-1). The dose of BTA-EG_4 used was previously shown to displace Aβ binding to catalase, and was not neuroprotective by itself (Habib *et al.,* 2010). Results showed that blocking the interaction between KP and Aβ by ASCAT and BTA-EG_4 did not increase Aβ toxicity, suggesting that KiSS-1 overexpression neuroprotection is not entirely mediated via a binding interaction or that it could be due to the compounds targetting a different part of Aβ to the KP products and therefore the compounds tested are not effective in this system.

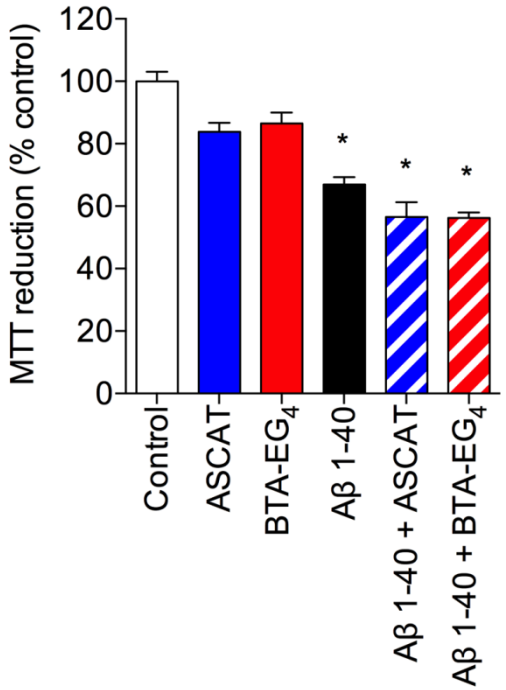

Figure 8-1. Effect of Aβ binding compounds on Aβ toxicity in PKiSS cells. The PKiSS cells were treated with Aβ (1-40) (10 µM) and ASCAT (100 µM) or BTA-EG_4 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cells treated with media alone); one-way ANOVA.

8.2.2 The effect of opioid receptor antagonist, naloxone and naltrexone on KP mediated neuroprotection against Aβ toxicity in PKiSS cells

Opioids were found to be neuroprotective against Aβ toxicity (Szegedi *et al.,* 2006; Cui *et al.,* 2011), they were found to be involved in KP activation of GnRH (Lehman *et al.,* 2010; Mostari *et al.,* 2013). The effect of opioid antagonist NAL and NALTR (Sirohi *et al.,* 2009) on KiSS-1 overexpression mediated neuroprotection was therefore tested. A dose of 1 µM of either NAL or NALTR effectively blocked the actions of opioid in cell culture models (Szegedi *et al.,* 2006; Cui *et al.,* 2011). The PKiSS cells were treated with NAL (1 µM) or NALTR (1 µM) for 2 hrs prior to Aβ (10 µM) treatment overnight. The MTT results showed that NAL (Figure 8-2) and NALTR (Figure 8-2) significantly

enhanced KiSS-1 overexpression mediated neuroprotection against Aβ toxicity. The NAL alone did not have any effect of PKiSS cells, whereas NALTR had a proliferative effect on PKiSS neurons resulting in enhanced MTT reduction.

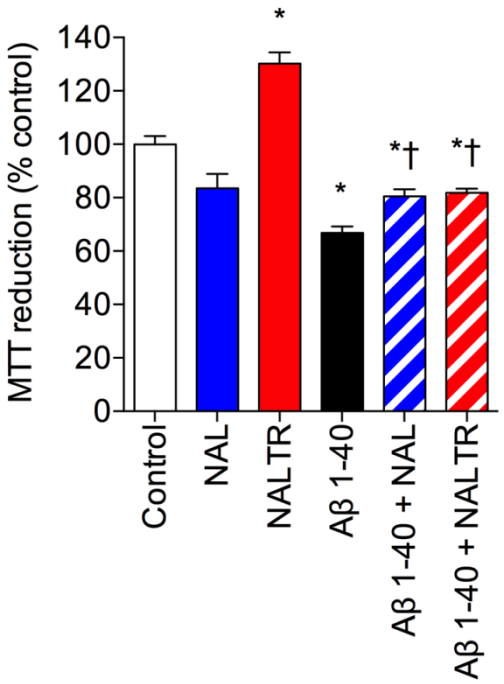

Figure 8-2. Effect of opioid receptor antagonists on Aβ toxicity in PKiSS cells. The PKiSS cells were treated with naloxone (NAL) and naltrexone (NALTR) (1 µM each) for 2 hrs prior to the treatement with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cells treated with media alone); † = $P < 0.05$ vs Aβ 1- 40; one-way ANOVA.

8.2.3 The role of oxytocin receptor activation in KP mediated neuroprotection in PKiSS cells

The KP peptide stimulates the release of oxytocin in rats (Scott and Brown, *et al.,* 2013). The SH-SY5Y cells express oxytocin receptors (Cassoni *et al.,* 1998) and oxytocin has a proliferative effect on SH-SY5Y neurons (Bakos *et al.,* 2012). The KP peptide activates both oxytocin and vasopressin neurons (Kotani *et al.,* 2001; Han *et al.,* 2010; Scott and Brown, 2011). The ATO peptide derivative is an oxytocin antagonist, which also has affinity for vasopressin

receptors (Manning *et al.,* 1995). Therefore the effect of oxytocin antagonist, ATO (1 µM) (Bakos *et al.,* 2013) on KP mediated neuroprotection against Aβ (10 µM) was tested in PKiSS cells. The PKiSS cells were pretreated with ATO for 2 hrs prior to the treatment with Aβ overnight. The MTT viability results showed that ATO significantly enhanced the Aβ toxicity in PKiSS cells. The PkiSS cells treated with ATO alone were uneffected (Figure 8-3). This suggests that oxytocin receptor system plays a role in KP mediated neuroprotection against Aβ toxicity.

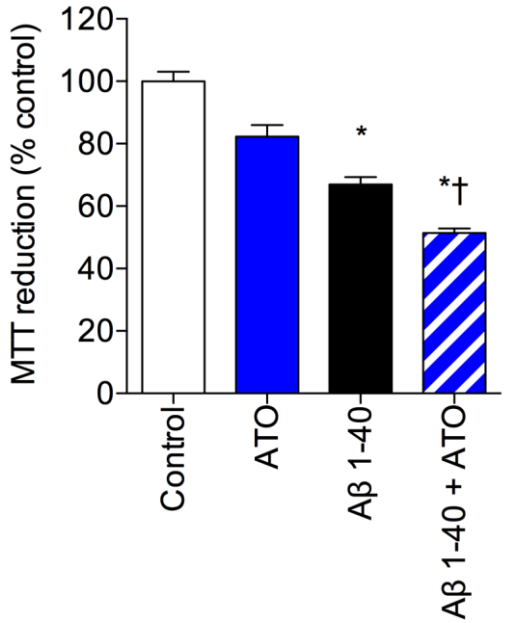

Figure 8-3. Effect of an oxytocin receptor antagonist on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with atosiban (ATO) (10 µM) for 2 hrs prior to the treatement with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (cells treated with media alone); † = P < 0.05 vs Aβ 1- 40; one-way ANOVA.

8.2.4 The role of adrenergic receptor activation on KP mediated neuroprotection against Aβ toxicity in PKiSS cells

A previous study has shown that KP-13 facilitates passive avoidance learning and memory consolidation in mice (Telegdy and Adamik, 2013), which could be inhibited by the α adrenergic receptor antagonist PHB, the α1/α2β adrenergic receptor antagonist PR, the α2-adrenergic receptor antagonist YO and the β-adrenergic receptor antagonist PROP. The KP peptide was also shown to have anti-depressant effect in mice, which could be inhibited by the α2-adrenergic receptor antagonist YO. The effects of these α and β adrenergic receptor antagonists were tested against KP mediated neuroprotection against Aβ toxicity. The doses of PHB (10 µM) (Figure 8-4) (Bodenstein *et al.,* 2005), PR (250 nM) (Liu *et al.,* 2011), YO (50 nM) and PROP (50 nM) have previously been demonstrated to be effective in neuronal cell culture models.

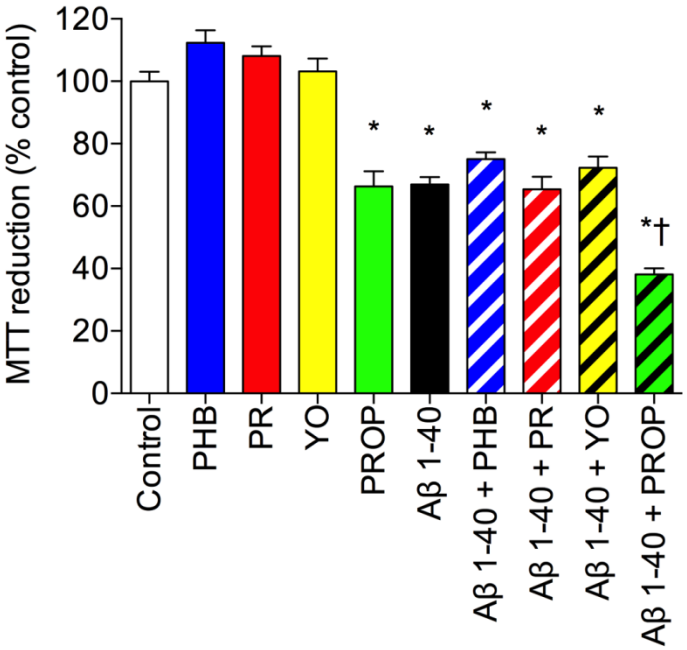

Figure 8-4. Effect of adrenergic receptor antagonists on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with Phenoxybenzamine hydrochloride (PBH) (10 µM) or yohimbine hydrochloride (YO) (50 nM) or prazosin hydrochloride (PR) (250 nM) or propranolol hydrochloride (PROP) (50 nM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$

vs control (cells treated with media alone); † = P < 0.05 vs Aβ 1- 40; one-way ANOVA.

The PKiSS cells were pretreated with PBH or YO or PR or PROP for 2 hrs, prior to treatment with Aβ (10 µM) toxicity overnight. MTT results showed that PHB, YO and PR (Figure 8-4) did not enhance the toxicity of Aβ in PKiSS cells. PHB, YO and PR alone did not have any effect on the cells. PROP alone was significantly toxic to the cells, and it also further enhanced the toxicity of Aβ in PKiSS cells. This suggests that the effect of PROP was additive to Aβ toxicity.

8.2.5 The role of cholinergic receptor activation in KP mediated neuroprotection against Aβ toxicity in PKiSS cells

A previous study has shown that KP peptide facilitates passive avoidance learning and memory consolidation in mice, which could be inhibited by muscarinic (ATR) but not nicotinic (MEC) antagonists in mice (Telegdy and Adamik, 2013). The effect of ATR and MEC was tested on PKiSS cells. The doses of ATR (10 µM) and MEC (10 µM) have previously been demonstrated to be effective in neuronal cell culture models. The PKiSS cells were pretreated with ATR and MEC, prior to treatment with (10 µM) Aβ toxicity. Results showed that ATR (Figure 8-5) and MEC did not alter Aβ toxicity. This suggests that KP mediated neuroprotection against Aβ toxicity is not mediated via muscarinic or nicotinic cholinergic receptors.

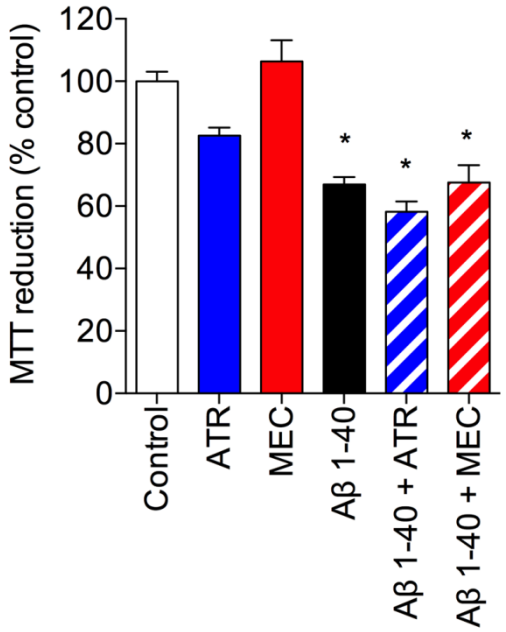

Figure 8-5. Effect of cholinergic receptor antagonists on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with atropine (ATR) (10 µM) or mecamylamine (MEC) (10 µM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cells treated with media alone); one-way ANOVA.

8.2.6 The role of dopaminergic receptor activation in KP mediated neuroprotection against Aβ toxicity in PKiSS cells

Previous study has shown that KP regulates dopamine levels in adult rats (Szawka *et al.,* 2010). A sub population of KP neurons has been shown to synthesize dopamine (Clarkson and Herbison, 2011). The dopaminergic antagonist HAL has been suggested to be neuroprotective against Aβ toxicity (Yang and Lung, 2011). The human neuroblastoma SH-SY5Y cells are dopaminergic (Xie *et al.,* 2010). Therefore, the role of dopaminergic receptors in KP mediated neuroprotection against Aβ toxicity was tested using dopamine receptor antagonist HAL, on PKiSS cells. The PKiSS cells were pretreated with HAL (10 µM) for 2 hrs, prior to treatment with (10 µM) Aβ (1-40) overnight. The dose of HAL (10 µM) has previously been demonstrated to be effective in neuronal cell culture models. Results showed that HAL had no significant effect

on Aβ toxicity (Figure 8-6). The neuroprotective effect of HAL against Aβ toxicity was not seen in PKiSS cells.

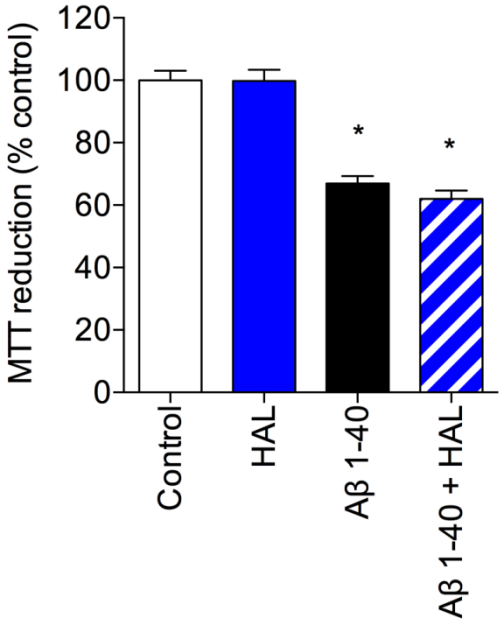

Figure 8-6. Effect of a dopaminergic receptor antagonist on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with haloperidol (HAL) (10 µM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cells treated with media alone); one-way ANOVA.

8.2.7 The role of serotonergic receptor activation in KP mediated neuroprotection against Aβ toxicity in PKiSS cells

The KP peptide was shown to have antidepressant like effect, which could be inhibited by 5-HT_2 serotonergic receptor antagonists in mice (Tanaka *et al.,* 2013). The KP peptide facilitates passive avoidance learning and memory consolidation in mice, which could also be inhibited by 5-HT_2 serotonergic antagonists (Telegdy and Adamik, 2013). The effect of MET, a mixed 5-HT_1/ 5-HT_2 serotonergic receptor antagonist, and CYPR, a nonselective 5-HT_2 serotonergic receptor antagonist, in KP mediated neuroprotection was therefore tested. The PKiSS cells were pretreated with MET (1 µM) and CYPR (10 nM)

for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight. The doses used have previously been shown to be effective in neuronal cell culture models.

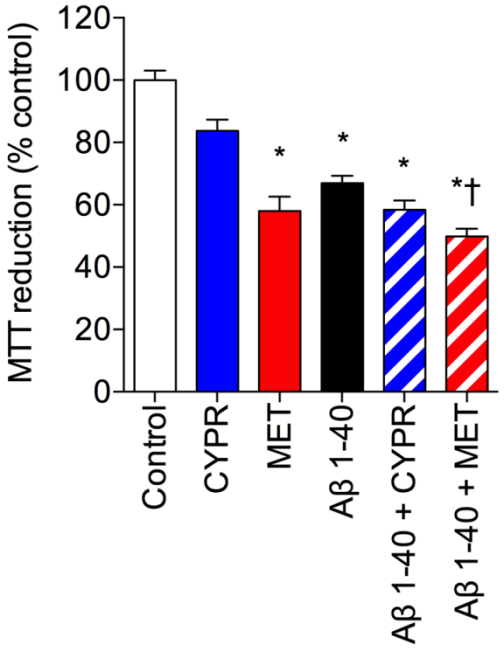

Figure 8-7. Effect of serotonergic receptor antagonists on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with Methysergide hydrochloride (MET) (1 µM) or Cyproheptadine hydrochloride (CYPR) (10 nM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (cells treated with media alone); † = P < 0.05 vs Aβ 1- 40; one-way ANOVA.

Results showed that CYPR alone was not toxic to PKiSS cells and it did not enhance Aβ toxicity (Figure 8-7). The MET alone was toxic to PKiSS cells, which significantly reduced the percentage of PKiSS viable cells, at a dose that is non-toxic to neuronal cell lines (Lambert *et al.,* 1989). The MET further enhanced the toxicity of Aβ, suggesting that the toxicity of MET was additive to the toxicity of Aβ (Figure 8-7).

8.2.8 The role of GABA-A receptor activation in KP mediated neuroprotection against Aβ toxicity in PKiSS cells

The KP peptide facilitates passive avoidance learning and memory consolidation in mice, which could be inhibited by the GABA-A antagonist, BIC (Telegdy and Adamik, 2013). Therefore the effect of BIC on KP neuroprotection against Aβ toxicity was tested in PKiSS cells. The PKiSS cells were pretreated with BIC (50 µM) for 2 hrs, prior to treatment with Aβ (10 µM) overnight. The dose used has previously been shown to be effective in neuronal cell lines. The MTT assay results showed that BIC had no significant effect on Aβ toxicity (Figure 8-8), which suggests that GABA-A receptor does not play a role in KP mediated neuroprotection against Aβ toxicity.

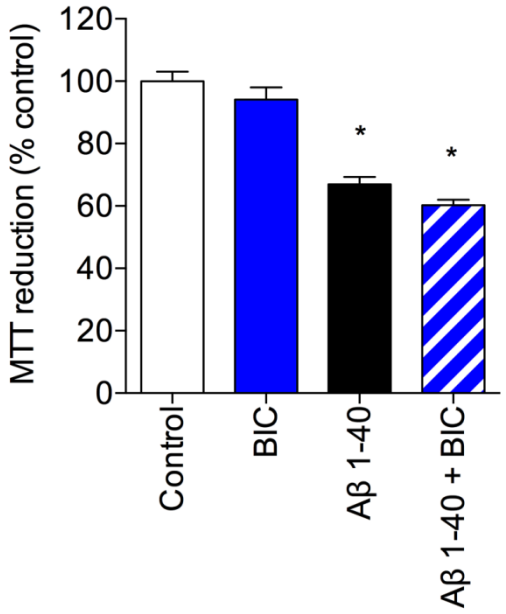

Figure 8-8. Effect of a GABA-A receptor antagonist on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with bicuculline (BIC) (50 µM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (cells treated with media alone); one-way ANOVA.

8.2.9 The role of estrogen receptor activation on KP mediated neuroprotection against Aβ toxicity in PKiSS cells

Estrogen receptors play a role in neuroprotection against Aβ toxicity (Zhang *et al.,* 2004; Cui *et al.,* 2011). Activation of estrogen receptors alters KP levels in

female mouse (Smith *et al.,* 2005; Alçin *et al.,* 2013). The effect of the estrogen receptor antagonist tamoxifen (TAMOX) on KP mediated neuroprotection against Aβ toxicity was therefore tested. The PKiSS cells were pretreated with TAMOX (10 µM) for 2 hrs, prior to treatment with Aβ (10 µM) overnight. The dose of TAMOX (10 µM) has previously been demonstrated to be effective in neuronal cell culture models. The MTT assay results showed the TAMOX had no significant effect on the toxicity of Aβ in PKiSS cells (Figure 8-9).

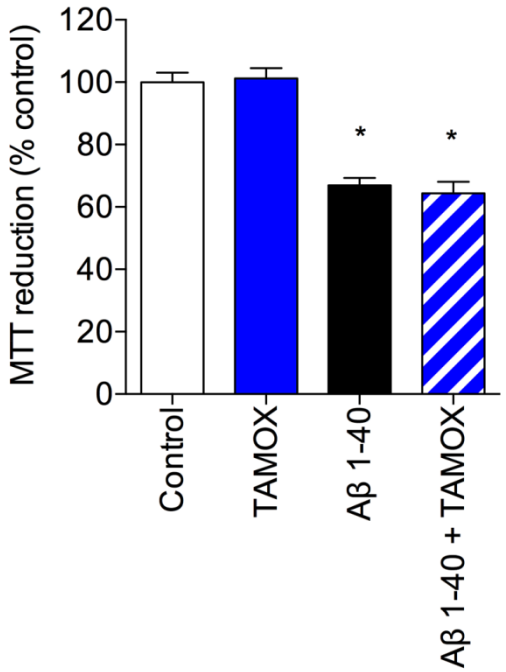

Figure 8-9. Effect of an estrogen receptor antagonist on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with tamoxifen (TAMOX) (10 µM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = P < 0.05 vs control (cells treated with media alone); one-way ANOVA.

8.2.10 The role of catalase, cyclooxygenase, nitric oxide synthase and MEK kinase enzymes in KP neuroprotection against Aβ toxicity

The KP peptide is known to increase catalase activity (Aydin *et al.,* 2010), which is also neuroprotective against Aβ toxicity (Milton 2001). The KP peptide also

has thermoregulatory effects (Csabafi *et al.,* 2013) and acts via nitric oxide in the facilitation of passive avoidance learning plus memory consolidation in mice (Telegdy and Adamik, 2013). Another possible mechanism of KP action can be via activation of MAPK pathway (Novaira *et al.,* 2009). The effects of catalase inhibition, cyclooxygenase inhibition, nitric oxide synthase inhibition and also the mitogen activated protein kinase cascade inhibitor PD98059 on KiSS-1 overexpression mediated neuroprotection against Aβ were tested to determine if these processes were involved in the observed neuroprotection (figure 8-10).

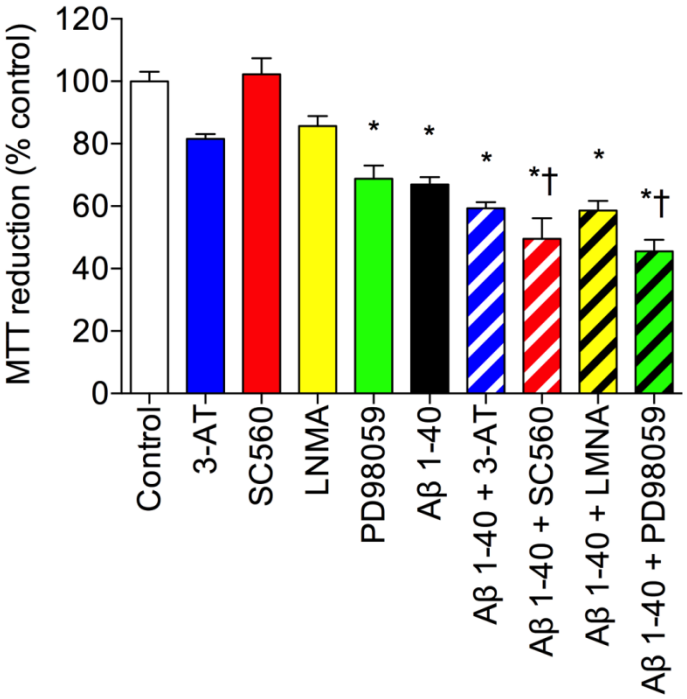

Figure 8-10. Effect of enzyme antagonists on Aβ toxicity in PKiSS cells. The PKiSS cells were pretreated with 3-Amino-1,2,4-triazole (3-AT) (50 mM); 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole SC-560 (1 µM); N^G-Methyl-L-arginine acetate salt (LMNA) (1 mM) and 2'-Amino-3'-methoxyflavone (PD98059) (50 µM) for 2 hrs, prior to treatment with Aβ 1-40 (10 µM) overnight and cell viability determined by MTT reduction. Results are mean ± S.E.M (n = 8); * = $P < 0.05$ vs control (cells treated with media alone); † = $P < 0.05$ vs Aβ 1- 40; one-way ANOVA.

The PKiSS cells were pretreated with the catalase inhibitor 3-AT (50 mM); the cyclooxygenase inhibitor SC-560 (1 µM); the nitric oxide synthase inhibitor N^G-

Methyl-L-arginine acetate (LMNA) (1 mM) and the MAPK pathway inhibitor PD98059 (50 µM) for 2 hrs, prior to treatment with (10 µM) Aβ. The doses used were previously found to be effective in neuronal cell lines. The MTT assay results showed that inhibitor of catalase activity, 3-AT did not have any effect on Aβ toxicity (Figure 8-10). The cyclooxygenase inhibitor SC-560 significantly enhanced Aβ toxicity (Figure 8-10B). The SC-560 alone was not toxic to PKiSS cells, but SC-560 increased the toxicity of Aβ in PKiSS cells, suggesting that KiSS-1 overexpression mediated neuroprotection may act via cyclooxygenase to protect against Aβ toxicity. The LMNA did not alter Aβ toxicity or have a significant effect on its own (Figure 8-10). The PD98059 alone was, however, toxic to PKiSS cells and the toxicity of PD98059 was additive to the toxicity of Aβ in PKiSS cells.

8.3 Discussion

The KiSS-1 overexpression protects SH-SY5Y cells against Aβ toxicity (section 7.2.3). The KP234 and RF9 did not reduce KiSS-1 overexpression mediated neuroprotection against Aβ toxicity, which suggests that KiSS-1 overexpression neuroprotection is not mediated via an action on the GPR-54 or NPFF receptors (section 7.2.5). The KiSS-1 overexpression neuroprotection was reduced by addition of anti-KP antibody, suggesting that binding interaction between KP and Aβ could be neuroprotective. Disrupting this interaction by anti-KP antibody could enhance Aβ toxicity. The levels of KP released by SH-SY5Y cells in response to Aβ may not be sufficient to provide full neuroprotection via a binding interaction. Compounds that block KP- Aβ interactions such as ASCAT and BTA-EG_4 (Habib *et al.,* 2010) did not enhance Aβ toxicity (section 8.2.1). Therefore the mechanism of neuroprotection may involve an alternative process, which could be receptor mediated.

The opioid antagonists NAL and NALTR enhanced KiSS-1 overexpression neuroprotection against Aβ toxicity, suggesting that endogenous opioids play a role in Aβ toxicity. The NALTR caused a significant increased in the viability of PKiSS cells, which makes it difficult to interpret whether the increase in neuroprotection against Aβ was due to blocking the opioid receptors or due to the observed increase in cell proliferation (section 8.2.2). The KP anti-opioid activity has been suggested by activation of NPFF receptors (Lyubimov *et al.,* 2010; Oishi *et al.,* 2010) and the KiSS-1 derivative KSO also acts as an NPFF ligand (Milton *et al.,* 2012). The inhibitor of NPFF receptor RF9 is known to block the anti-opioid activity (Han *et al.,* 2013), but it has recently been shown to be ineffective at blocking all the actions of NPFF (Maletinska *et al.,* 2013). In PKiSS cells RF9 did not alter Aβ toxicity, suggesting that KiSS-1 overexpression mediated neuroprotection may not involve endogenous opioid action. The ATO, a receptor antagonist of oxytocin system, significantly enhanced the toxicity of Aβ in PKiSS cells. The action of ATO also involves inhibition of vasopressin receptors (Manning *et al.,* 2005). The KP peptide activates both oxytocin and vasopressin (Kotani *et al.,* 2001; Han *et al.,* 2001; Scott and Brown, 2011), suggesting that KiSS-1 overexpression mediated neuroprotection could involve either or both oxytocin and vasopressin systems.

The adrenergic, cholinergic, dopaminergic, serotonergic and GABA neurotransmitter systems plus the nitric oxide and estrogen receptor activated systems do not appear to play a role in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity. The doses of β-adrenergic antagonist PROP and the mixed 5-HT_1/5-HT_2 receptor antagonist MET used were found to be non-toxic to SH-SY5Y cells (Lambert *et al.,* 1989; Mikami *et al.,* 2008). These doses were, however, toxic to PKiSS cells and also enhanced the toxicity of Aβ. The increased toxicity of Aβ observed was due to toxicity of PROP and MET on the PKiSS cells rather than the involvement of β-adrenergic and mixed 5-HT_1/5-HT_2 receptors directly in the neuroprotection.

The MAPK inhibitor PD98059 has previously been used to understand the mechanism of Aβ toxicity (Pettifer *et al.,* 2004; Wang *et al.,* 2007; Wang *et al.,* 2010). In the present study PD98059 did not enhance Aβ toxicity, suggesting that KiSS-1 overexpression mediated neuroprotection in PKiSS cells may not be via MAPK signaling pathway. The KP peptide is known to increase catalase activity (Aydin *et al.,* 2010), but inhibiting catalase activity by 3-AT did not enhance Aβ toxicity, suggesting catalase activity may not be enhanced in PKiSS cells.

The SC-560 significantly reduced KiSS-1 overexpression neuroprotection and enhanced Aβ toxicity. This suggests that KiSS-1 overexpression mediated neuroprotection could be via a cyclooxygenase dependent pathway (figure 8-11). The SC-560 is an inhibitor of cyclooxygenase-1 that has previously been shown to reduce Aβ production in an AD mouse model (Choi *et al.,* 2013). The SC-560 is not specific for cyclooxygenase-1 and could also inhibit cyclooxygenase-2 in some cell types (Brenneis *et al.,* 2006). The KP peptide can modulate thermoregulatory responses in rats (Csabafi *et al.,* 2013), which could be regulated via cyclooxygenase inhibitors (Nakamura 2011). As such KiSS-1 overexpression could be acting via prostaglandin synthesis mediated via cyclooxygenase in this overexpression model and *in vivo*.

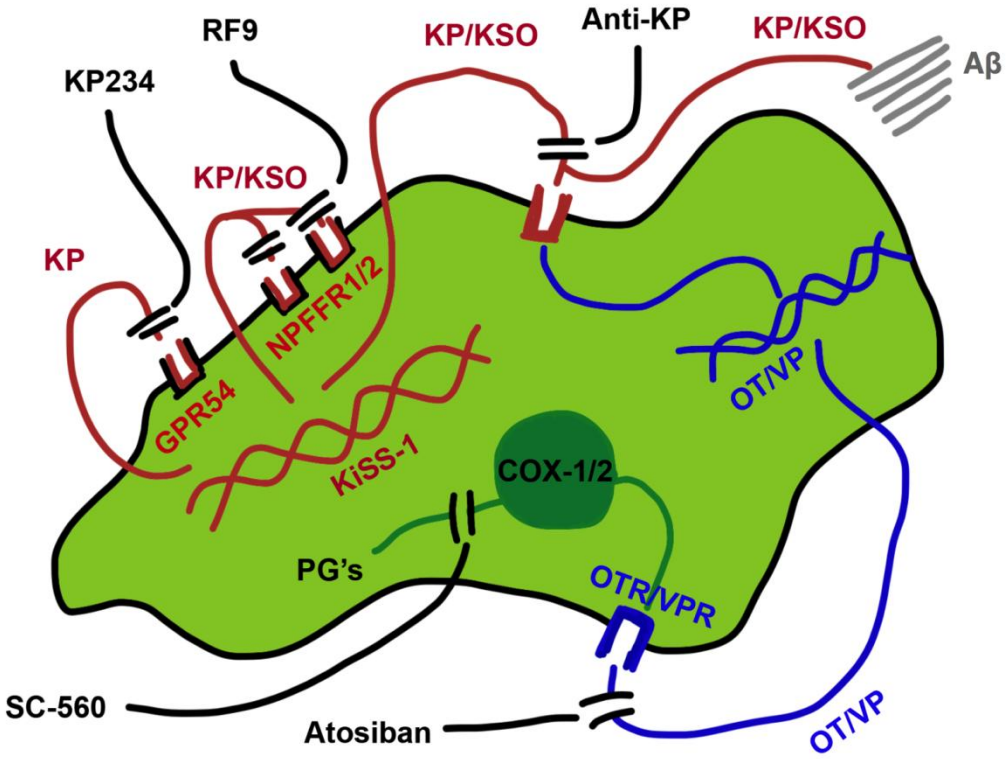

Figure 8-11. The mechanism of KiSS-1 overexpression neuroprotection against Aβ toxicity. The KP (GPR-54) and NPFF (NPFFR 1/2) receptors do not play a role in neuroprotection, as the receptor anatagonists KP234 and RF9 did not enhance Aβ toxicity. Anti-KP antibody increased the toxicity of Aβ, suggesting that a binding interaction between KP and Aβ could be neuroprotective. Overexpression of KiSS-1 neuroprotection against Aβ toxicity was observed to involve oxytocin (OT) /vasopressin (VP) receptor activation plus have a cyclooxygenase (COX-1/2) dependent component. Atosiban and SC-560 used to block OT/VP or COX1/2 pathway significantly enhanced Aβ toxicity.

The KP and NPFF receptors do not play a role in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity (Figure 8-11). The toxicity of Aβ was enhanced by anti-KP antibody, suggesting KP-Aβ interaction may provide some neuroprotection. The observations from the present study suggest that KiSS-1 overexpression mediated neuroprotection could involve oxytocin/vasopressin plus a cyclooxygenase dependent component, which could be due to endogenous oxytocin or vasopressin activating

cyclooxygenase. Both *in vivo* and *in vitro* administration of oxytocin (Gulliver *et al.,* 2013; Penrod *et al.,* 2013) and vasopressin (Milton *et al.,* 1993; Nakatani *et al.,* 2007) causes an activation of prostaglandin synthesis that is cyclooxygenase dependent. Stimulation of prostaglandin receptors was found to be neuroprotective against OS and Aβ toxicity (Echeverria *et al.,* 2005). Blocking this pathway by oxytocin/vasopressin receptor antagonist ATO and the cyclooxygenase-1 inhibitor SC-560 enhances Aβ toxicity, suggesting a link between these pathways in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity. Further experimental investigations are required to confirm the mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ toxicity.

8.4 Conclusion

The mechanism of KiSS-1 overexpression mediated neuroprotection against Aβ has been illustrated in the present chapter. A range of neurotransmitter antagonists were to used to determine the role of neurotranmistters in KiSS-1 overexpression mediated neuroprotection agasint Aβ. It was found that oxytocin/vasopressin antagonist ATO and cyclooxygenase-1 inhibitor SC-560 enhanced Aβ toxicity in PKiSS cells. The KiSS-1 overexpression could be acting via activation of oxytocin/vasopressin receptors either directly or indirectly, which could in turn activate the cyclooxygenase pathway.

Chapter 9

Discussion

It is well documented that catalase binds Aβ (Milton 1999; Habib *et al.,* 2010); the binding of catalase to Aβ was confirmed by a dose response curve (section 3.2.2). Catalase is an antioxidant enzyme and shows binding to different lengths of Aβ (section 3.2.2). Therefore, the binding studies were found to be in agreement with previous studies showing catalase-amyloid interaction. Since, KP and catalase shares sequence similarity with the catalase Aβ binding site; the binding studies have shown that KP 54, KP 13 and KP 10 also bind Aβ (section 3.2.1). This binding between Aβ and KP could be inhibited by Congo red (section 3.2.1). This suggests that Congo red and KP target similar binding sites on Aβ, resulting in decreased KP binding to Aβ in the presence of Congo red. The present study suggests that peptides sharing sequence similarity with catalase Aβ binding domain could potentially bind Aβ.

Formation of amyloid plaques is the central feature of AD; a study has shown that amyloid deposition is seen last in the cerebellum and pons region of the brain (Thal *et al.,* 2002). This resistance to Aβ deposition in pons and cerebellum is consistent with the presence of endogenous neuroprotective components that delay the deposition of Aβ in these sections of the brain. The KP peptide (Brailoiu *et al.,* 2005), CRH peptide (Austin *et al.,* 2003) and catalase (Moreno *et al.,* 1995) are all found in the pons sections of AD brain. Previous studies have shown the presence of catalase (Pappolla *et al.,* 1992) and CRH (Facci *et al.,* 2003) in amyloid plaques. In the present study, immunoreactive catalase, KP and CRH were detected in the amyloid plaque like deposits in the pons section of an AD patient. Prior to the detection of immunoreactive catalase, KP and CRH, antibody for each peptide was characterized for specific binding (section 4.2.1). Only anti KP 45-50 cross reacted with NPFF peptide, this is consistent with a previous study (Iijima *et al.,* 2011). All the other antibodies used were found to be specific for their peptides. Staining of the pons section of the AD patient with anti-catalase, KP, CRH and amyloid antibody showed the presence of immunoreactive catalase, KP and CRH in amyloid plaque like deposits (section 4.2.3, 4.2.4, 4.2.6). CRH did not bind Aβ (section 4.2.1) and the presence of immunoreactive CRH in amyloid plaques suggests that, the mere presence of immunoreactive CRH in the amyloid plaques does not confirm the binding of CRH to Aβ.

To eliminate the possibility of cross reactivity, the rabbit anti KP 45-50 antibody was pre-absorbed with NPFF overnight. The staining of the pons section of the AD brain with the pre-absorbed antibody did not decrease the immunoreactive KP signal (section 4.2.3). Staining the pons section of the AD patient with rabbit anti-KP 45-50 antibody, pre-absorbed with NPFF for 24 hrs (section 4.2.5) and CAT-505 monoclonal anti-catalase antibody (section 4.2.5) showed that catalase and KP occupied specific sites, with only a small proportion of co-localization within the amyloid plaque like deposits (section 4.2.5). This study suggests that the presence of catalase and KP in the pons section of the AD brain may contribute to the neuroprotection and the late development of amyloid plaques in the pons section. KP interacts with Aβ (section 3.2.1), and the presence of KP in the amyloid plaque like deposits in the pons section of the AD patient suggests that KP and Aβ might interact in an AD brain.

After confirmation of the presence of catalase and KP in the amyloid plaques of the AD brain section, the neuroprotective effect of KP and catalase overexpression in SH-SY5Y cells was tested. For this study, the catalase (NM_001752.2) and the KiSS-1 gene (NM_002256.2) were overexpressed in the human neuroblastoma SH-SY5Y cells.

The catalase gene was cloned in the pcDNA™4/TO/myc-His vector and the vector was stably transfected in the naïve SH-SY5Y cells. The overexpression of the catalase gene in stably transfected cells was confirmed by RT-PCR (section 5.2.1.4), immunocytochemistry (section 5.2.1.6) and western blotting (section 5.2.1.7). The PCat cells showed a 6 fold increase in the catalase activity compared to PVect cells (section 5.2.3). As catalase is an antioxidant (Chelikani *et al.,* 2003) and OS is one of the major events in the AD progression (Perry *et al.,* 2000), the overexpression of catalase gene in SH-SY5Y cells was tested against H_2O_2 toxicity. The exposure of the PCat cells to H_2O_2 toxicity was found to be neuroprotective even at higher concentrations of H_2O_2 toxicity (section 5.2.2). In the presence of 3-AT an irreversible inhibitor of catalase activity (Margoliash and Novogrodsky, 1958), the neuroprotection decreased with an increase in the 3-AT concentration (section 5.2.2). This suggests that catalase activity is important for protection against H_2O_2 toxicity. The PCat and PVect cells were also exposed to $CoCl_2$ (a mimic of hypoxia) (section 5.2.4) toxicity, both the cell lines were equally susceptible to $CoCl_2$ toxicity, suggesting

that the neuroprotection by catalase is specific for H_2O_2 toxicity. To confirm that the catalase neuroprotection was provided by overexpression of catalase gene in PCat cells, the tetracycline inducible system was used to regulate the catalase gene expression. In the absence of tetracycline PCatTR6 cells (uninduced) were significantly susceptible to H_2O_2 toxicity (section 5.2.5). In the presence of tetracycline the PCatTR6 (induced) cells were significantly resistant to H_2O_2 toxicity (section 5.2.5). The conditioned media of PCat cells also was neuroprotective against H_2O_2 toxicity, and this neuroprotection could be inhibited by 3-AT (section 5.2.6). As the neuroprotection could be inhibited by 3-AT, the neuroprotection could be due to the presence of protective components such as catalase in the PCat cells conditioned media which was absent in the PVect cells conditioned media.

The catalase gene overexpression was also found to be neuroprotective against Aβ toxicity, this was confirmed by both MTT (section 6.2.1) and trypan blue assay (section 6.2.1). This neuroprotection was partly inhibited by 3-AT (section 6.2.1). As 3-AT only inhibits catalase activity (section 5.2.2) and does interfere with catalase-amyloid interaction (section 3.2.3). This supports the fact that 3-AT only inhibits activity of catalase, but inactive catalase could still bind Aβ (Zang *et al.,* 1996), which could be neuroprotective. The conditioned media from the PCat cells was also found to be neuroprotective against Aβ toxicity (section 6.2.3). The addition of 3-AT did not seem to alter the neuroprotective affect of the PCat conditioned media, this suggests that the protection of extra-cellular added catalase could be protective via a binding action. This suggests that the neuroprotective component of the PCat conditioned media was unaltered by 3-AT against Aβ toxicity. The tetracycline inducible system also supports the hypothesis that catalase gene expression was key for the neuroprotection against Aβ toxicity, as repressing the expression of catalase gene made the PCatTR6 cells susceptible to Aβ toxicity (section 6.2.2).

Catalase not only binds Aβ, but has been shown to bind other amyloid peptides such as the IAPP, PrP (Milton and Harris, 2012), ABri and ADan. This could be due to the sequence similarity shared by all the amyloid peptides, containing potential catalase binding domain (section 1.1.11). Results showed that catalase was resistant to Aβ (25-35), ABri (1-34), ADan (1-34), IAPP (20-29) and PrP (106-126) toxicity (section 6.2.4). The toxicity of the amyloid peptides

was significantly enhanced by 3-AT in PCat cells (section 6.2.4), which suggests that the neuroprotection was due to activity of catalase.

Congo red and Thioflavin T compete for the binding sites on Aβ (Groenning 2010). Thioflavin derivative BTA-EG_4 was shown to inhibit catalase-amyloid interaction (Habib *et al.,* 2010). The effect of BTA-EG_4, catalase activity inhibitor 3-AT and KP 45-50 peptide on catalase-amyloid interaction was investigated. Results showed that BTA-EG_4 and KP 45-50 significantly inhibited catalase-amyloid interaction, whereas 3-AT did not have any effect on catalase-amyloid binding interaction (section 3.2.3). The BTA-EG_4 compound surrounds Aβ aggregates and does not allow Aβ to interact with cellular proteins (Habib *et al.,* 2010). The BTA-EG_4 by itself was not toxic to PCat cells, but when added together with Aβ, it enhanced the toxicity of Aβ at a dose of 20 µM BTA-EG_4 (section 6.2.5) in PCat cells. The BTA-EG_4 did not have any effect on Aβ toxicity in PVect cells (section 6.2.5); suggesting that inhibition of catalase-amyloid interaction by BTA-EG_4 at higher doses could enhance Aβ toxicity. To confirm that any compound that can bind Aβ will not enhance Aβ toxicity in PCat cells, the effect of KP 45-50 was tested on PCat cells against Aβ toxicity (As KP binds Aβ section 3.2.3). KP did not enhance the toxicity of Aβ in PCat cells, but was found to be neuroprotective against Aβ toxicity (section 6.2.6). This suggests that the role of BTA-EG_4 is specific and that the increased toxicity observed was due to disruption of catalase-amyloid interaction, and not all the compounds that bind Aβ will necessarily enhance the toxicity of Aβ in PCat cells.

The BTA-EG_4 also enhanced the toxicity of Aβ 25-35, ABri 1-34, ADan 1-34, IAPP 20-29 and PrP 106-126 (section 6.2.7). This suggests that BTA-EG_4 enhances the toxicity of all the amyloid peptides by disrupting catalase-amyloid interaction. The effect of BTA-EG_4, KP and 3-AT on catalase activity was also investigated in the presence of H_2O_2 toxicity in PCat cells (section 6.2.8). BTA-EG_4 and KP did not enhance the toxicity of H_2O_2, whereas 3-AT enhanced the toxicity of H_2O_2 in the PCat cells.

Catalase overexpression had dual protection, peroxisome targeted catalase can prevent or even reverse the effects of OS (Giordano *et al.,* 2014). The sequence of catalase that binds Aβ could be ideal for designing drugs that can bind to Aβ.

Appendix

A comparison between human catalase and KiSS-1 preproprotein sequences has shown that the KiSS-1 preproprotein contains a catalase like Aβ binding domain (Milton *et al.,* 2012) (section 1.2.9). The SH-SY5Y cells express the KiSS-1 gene (Poomthavorn *et al.,* 2009) and contain the necessary secretary vesicle machinery to release neuroendocrine hormones (Godell *et al.,* 1997). The sequence similarity as shown in section 1.2.9 raises the possibility of KP binding to Aβ, IAPP and PrP, and this suggests that it could be neuroprotective against Aβ toxicity. Overexpression of the KiSS-1 gene in human neuroblastoma SH-SY5Y cells was found to be neuroprotective against Aβ (section 7.2.3), PrP and IAPP toxicity (section 7.2.4). This property of KiSS-1 overexpression neuroprotection against different amyloids is similar to the neuroprotection by catalase overexpression neuroprotection against Aβ, IAPP and PrP. This suggests that the sequence similarity between catalase and KP plays a key role in neuroprotection against different amyloids. To further understand the mechanism of neuroprotection, the PKiSS and PVect cells were treated with Aβ (1-42) and anti-KP 45-50 antibody or GPR-54 receptor antagonist KP234 or NPFF receptor antagonist RF9 (section 7.2.5). Only anti-KP 45-50 antibody increased the toxicity of Aβ, whereas the KP234 and NPFF did not alter the toxicity of Aβ in both PVect and PKiSS cells. This further strengthens the concept that KP-amyloid interaction is the key for neuroprotection as anti-KP 45-50 antibody probably inhibits KP and amyloid peptide interaction. Inhibition of KP-amyloid interaction by anti-KP antibody increases Aβ toxicity. This also suggests that KP neuroprotection is not receptor (KP or NPFF) mediated or it acts via other receptors.

Recent studies have shown the effect of KP on passive avoidance learning and potential involvement of adrenergic, serotonergic, dopaminergic, cholinergic, gabaergic, opiate and nitric oxide receptors in mice. Results have shown that KP may partly acts via all these receptors to facilitate passive avoidance learning in mice (Telegdy and Adamik, 2013). Another study has shown that KP has anti-depressant like effects in mouse, which could involve adrenergic and serotonergic receptors (Tanaka *et al.,* 2013). This suggests that KP may act via other receptors and therefore the present study was conducted to characterize a model of KiSS-1 gene overexpression (PKiSS cells) neuroprotection against Aβ toxicity in SH-SY5Y neurons, and to determine the role of neurotransmitter

systems in the neuroprotection. The KP and NPFF receptors do not play a role in KiSS-1 overexpression mediated neuroprotection against Aβ toxicity (section 7.2.5). The toxicity of Aβ was enhanced by anti-KP antibody, suggesting KP-Aβ interaction may provide some neuroprotection. The observations from the present study suggest that KiSS-1 overexpression mediated neuroprotection could involve oxytocin/vasopressin plus a cyclooxygenase dependent component, which could be due to endogenous oxytocin or vasopressin activating cyclooxygenase. The SC-560 significantly reduced KiSS-1 overexpression neuroprotection and enhanced Aβ toxicity (section 8.2.10). This suggests that KiSS-1 overexpression mediated neuroprotection could be via cyclooxygenase. The SC-560 inhibitor of cyclooxygenase-1 has previously been shown to reduce Aβ production in AD mouse model (Choi *et al.,* 2013). The SC-560 is not specific for cyclooxygenase-1 and could also inhibit cyclooxygenase-2 in some cell types (Brenneis *et al.,* 2006). KP can modulate thermoregulatory responses in rats (Csabafi *et al.,* 2013), which could be regulated via cyclooxygenase inhibitors (Nakamura 2011; Morrison *et al.,* 2012). As such KiSS-1 overexpression could be acting via prostaglandin synthesis mediated via cyclooxygenase in this overexpression model.

9.1 Conclusions

The results of the present study represent the characterization of the catalase and KiSS-1 gene overexpressing SH-SY5Y cell lines. The overexpression of catalase gene in SH-SY5Y cells was found to be neuroprotective against Aβ as well as H_2O_2 toxicity. The The effect of BTA-EG_4 on PCat and PVect cells suggests that blocking catalase-amyloid interaction could be neuroprotective but may also have other effects. This study shows the presence of KP in amyloid plaque like deposits in the pons section of an AD patient. Overexpression of KiSS-1 gene in SH-SY5Y cells was found to be neuroprotective against Aβ toxicity. The observations from the present study also suggest that KiSS-1 overexpression mediated neuroprotection against Aβ could involve oxytocin/vasopressin plus a cyclooxygenase dependent component.

9.2 Limitations and future work

The Immunohistochemistry study (chapter 4) was performed using one patient sample, a larger sample size and different regions of brain sections such as hippocampus could provide further understanding of catalase and KP co-localization with Aβ in AD brain sections. The overexpression of catalase gene in SH-SY5Y cells could also be used to study the role of antioxidant catalase in other diseases such as Stroke and Parkinson's disease which share an oxidative stress component. The KiSS-1 gene could be overexpressed in a pancreatic cell line to understand the role of KP in T2DM. The neurotransmitters study (chapter 8) performed using single dose of the neurotransmitter antagonist requires further investigation. A dose response curve for each of the neurotransmitter antagonist can provide an appropriate dose for blocking the receptors in SH-SY5Y cells. The amino acids involved in catalase Aβ interaction could be mutated to understand the significance of the amino acids in the binding interaction as well as to create modified CAβBD sequences which can bind Aβ with higher affinity. The CAβBD sequence could also be used to design peptides that can bind Aβ, which could be of therapeutic use in designing drugs for treating AD.

Appendix

10.1 Catalase sequence from UCL sequencing facility

CCCGGGTGGGACATTAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCC
TATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCAGATCG
CCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGAC
CGATCCAGCCTCCGGACTCTAGCGTTTAAACTTAAGCTTATGGCTGACAGC
CGGGATCCCGCCAGCGACCAGATGCAGCACTGGAAGGAGCAGCGGGCC
GCGCAGAAAGCTGATGTCCTGACCACTGGAGCTGGTAACCCAGTAGGAGA
CAAACTTAATGTTATTACAGTAGGGCCCCGTGGGCCCCTTCTTGTTCAGGA
TGTGGTTTTCACTGATGAAATGGCTCATTTTGACCGAGAGAGAATTCCTGA
GAGAGTTGTGCATGCTAAAGGAGCAGGGGCCTTTGGCTACTTTGAGGTCA
CACATGACATTACCAAATACTCCAAGGCAAAGGTATTTGAGCATATTGGAA
AGAAGACTCCCATCGCAGTTCGGTTCTCCACTGTTGCTGGAGAATCGGGT
TCAGCTGACACAGTTCGGGACCCTCGTGGGTTTGCAGTGAAATTTTACACA
GAAGATGGTAACTGGGATCTCGTTGGAAATAACACCCCCATTTTCTTCATC
AGGGATCCCATATTGTTTCCATCTTTTATCCACAGCCAAAAGAGAAATCCTC
AGACACATCTGAAGGATCCGGACATGGTCTGGGACTTCTGGAGCCTACGT
CCTGAGTCTCTGCATCAGGTTTCTTTCTTGTTCAGTGATCGGGGGATTCCA
GATGGACATCGCCACATGAATGGATATGGATCACATACTTTCAAGCTGGTT
AATGCAAATGGGGAGGCAGTTTATTGCAAATTCCATTATAAGACTGACCAG
GGCATCAAAAACCTTTCTGTTGAAGATGCGGCGAGACTTTCCCAGGAAGAT
CCTGACTATGGCATCCGGGATCTTTTTAACGCCATTGCCACAGGAAAGTAC
CCCTCCTGACTTTTACATCCAGGTCATGACATTTAATCAGCAGAAACTTTTC
CATTTATCCATTCGATCTCACAGTTGACCTCACAGACTACCCTCTCATCCA
GTGTTAACTGATCTAACGATTCAGTTATTACTGCTGAGATGACGATGTCTC
GACAAGCAACATGCCAACTACATTGAAGTG

10.2 Kisspeptin sequence from UCL sequencing facility

CTGGTTTCGGGATGGTATCAATAGAGAGCTCTCCCTATCAGTGATAGAGAT
CTCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCA
GATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACC
GGGACCGATCCAGCCTCCGGACTCTAGCGTTTAAACTTAAGCTTGGTACC
GAGCTCGGATCCATGAACTCACTGGTTTCTTGGCAGCTACTGCTTTTCCTC
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