HspB1 PROTECTION AGAINST AMYLOID-β TOXICITY

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By

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Abstract

Alzheimer's disease (AD) is hallmarked by the presence of neurofibrillary tangles and amyloid-β plaques. Amyloid- β plaques consist of the amyloid-β peptide (Aβ) that has been shown to induce toxic effects on neurons through the activation of stress-related signalling and neuronal loss. The small heat shock protein, HspB1 (also referred to as Hsp27 and Hsp25 in mouse), is accumulated in 15% of neocortical amyloid-β plaques in AD brains (Wilhelmus, 2006). Whether this represents a potentially protective response to stress, or is part of the disease process is unknown. We have previously reported that expression of HspB1 not only protects cortical neurons against amyloid toxicity, but also enhances total neurite growth in these neurons (King *et al.*, 2009).

The amyloid-β peptide is derived from the proteolytic processing of the Amyloid Precursor Protein (APP) by β- and γ-secretases. Mutations in APP alter secretase cleavage sites, resulting in higher production of the toxic Aβ(1-42) peptide that undergoes aggregation more readily. Since HspB1 has been shown to protect neurons against amyloid toxicity, it is conceivable that HspB1 may interact directly with Aβ or APP. Recent studies have demonstrated exogenous HspB1 binding to synthetic Aβ. We have replicated these results in an attempt to determine the role of HspB1 in the inclusion of Aβ aggregates. We hypothesize that HspB1 interacts with Aβ, or its precursor APP, to either alter the distribution of Aβ/APP within the cell, or its release from the cell.

In order to test our hypothesis, we incubated His-tagged HspB1 with synthetic Aβ(1-42), at physiological temperature 37°C overnight. Immunoprecipitation, using agarose protein A/G beads incubated with the monoclonal anti-His primary antibody, was used for our primary analysis of interaction. Western blotting of the nitrocellulose membrane, using the 6E10 β-amyloid (1-16) mouse monoclonal antibody, demonstrates that Aβ is immunoprecipitated with His-HspB1. These results point to a direct interaction between HspB1 and Aβ(1-42). We investigated the interaction of HspB1 with APP in HEK293 cell line expressing wild-type APP (APP-wt) or APP-swedish mutation (APP-swe) that predominately yields Aβ(1-42) through immunoprecipitation. His-tagged HspB1 was incubated with the conditioned media from the APP-wt and APP-swe cells overnight at 37°C. Immunoprecipitation was performed using magnetic protein A/G beads incubated with either 6E10 β-amyloid (1-16) mouse monoclonal antibody, or anti-HspB1 human (SPA-803) rabbit polyclonal antibody. Subsequent western blotting of the nitrocellulose membrane using the 6E10 and the 803 antibodies demonstrate that APP is immunoprecipitated with His-HspB1.

These data suggest HspB1, perhaps via its chaperone activity, may be altering production of APP and/or Aβ within the cell preventing secretion into the extracellular environments.

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For Dad

Your guiding hand on my shoulder will remain with me forever.

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List of Abbreviations

AD	Alzheimer's disease
ADAM10	alpha secretase enzyme
ADDL	amyloid derived diffusible ligand
AICD	APP intracellular domain
Akt	protein kinase B
APOE-ε_4	E4 type of apolipoprotein
APP	amyloid precursor protein
Ask1	apoptosis signal-regulating kinase 1
ATP	adenosine tri-phosphate
Aβ	amyloid beta
BACE1	beta secretase enzyme
BBB	blood brain barrier
Bcl-2	B cell lymphoma-2
Bip/GRP78	glucose Regulatory Protein
BME	beta mercaptoethanol
BSA	bovine serum albumin
cAMP	cyclic-adenosine monophosphate
Cdk-5	cyclin-dependent protein kinase 5
CM	conditioned media
CNS	central nervous system
CSF	cerebral spinal fluid

CTFα	C- terminal fragment alpha
CTFβ	C-terminal fragment beta
DAPI	nucleic acid stain
DDT	dithithreitol
DLB	dementia with lewy bodies
DMEM	dulbecco's modified eagle's medium
DRG	dorsal root ganglion
EC	entorhinal cortex
ECL	enzymatic chemiluminescence
EGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbant assay
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinases
EV	empty vector
FAD	familial alzheimer's disease
Fas	apoptosis antigen 1/CD95/TNFRFS6
FCS	fetal cow serum
FTDP	fronto-temporal disease with parkinsonism
G418	geneticin disulfate
GSK-3β	glycogen synthase kinase 3 beta
HEK	human embryonic kidney cells
HRP	horse radish peroxidase

HS	heat shock
Hsf-1	heat shock factor 1
Hsp	heat shock protein
ICC	immunocytochemistry
IDE	insulin degrading enzyme
IL-1/IL-6	interleukin-1/6
IP	immunoprecipitation
JNK	jun-N-terminal kinase
kDa	kilo dalton
LTD	long term depression
LTP	long term potentiation
MAPKAPK2	mitogen activated protein kinase-activated protein
MBD	microtubule binding domain
MMP2/9	matrix metalloproteinases 2/9
NF-kB	nuclear factor K-beta
NFT	neurofibrillary tangles
NMDA	*N*-methyl-*D*-aspartate
NSAIDS	non-steroidal anti-inflammatory drugs
NTFα	N-terminal fragment alpha
NTFβ	N-terminal fragment beta
P38/MAPK	p38-mitogen activated protein kinase
PBS	phosphate buffered saline

PF	protofibril
PKA	protein kinase A
PrP	prion protein
PS-1	presenilin 1 secretase enzyme
PS-2	presenilin 2 secretase enzyme
SAD	sporadic alzheimer's disease
sAPPα	soluble amyloid precursor protein alpha
sAPPβ-swe	soluble amyloid precursor protein beta swedish mutation
sAPPβ-wt	soluble amyloid precursor protein beta wild type
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sHsp	small heat shock protein
SP	senile plaques
SWE	swedish mutation
TBS	tris buffered saline
TBST	tris buffered saline 0.5% tween
Tg	transgenic mice
TGN	trans golgi network
Tip60	histone acetyltransferase
TNF-α	tumor necrosis factor alpha
WT	wild type

Chapter 1

Introduction

1.1 Familial Alzheimer's Disease

In 25 years, the now 500,000 Canadians affected by AD will double as the first "baby boomer" turns 65 this year. Healthcare costs will increase tenfold from $15 million per annum to $153 million. While the well-known sporadic form is more common, 4-5% of the reported AD cases are under age 65 presenting with the familial form of AD. Diagnosis in these genetically predisposed individuals can occur as young as 30 years of age (www.alzheimer.ca). Early – onset Familial AD (FAD) symptomatically presents indistinguishably from sporadic or late-onset Alzheimer's disease (SAD). Clinically, both are characterized by progressive neuronal deterioration resulting in loss of memory, spatial orientation, language deficits, mood as well as personality changes (Morris et al., 1989). Physiologically however, there exist differences in disease origin between AD types. FAD is an autosomal dominant genetic form of AD, occurring through mutations that usually result in increased deposition of the cytotoxic Amyloid beta 1-42 (Aβ 1-42) fragment (A. Eckert et al., 2003; A. Eckert, Marques, Keil, Schussel, & Muller, 2003; Citron et al., 1992; Weidemann et al., 1997). Three well studied mutations identified in FAD are: 1) presenilin-1 on chromosome 14 (Alzheimer's Disease Collaborative Group, 1995), 2) presenilin-2 on chromosome 1 (Levy-Lahad et al., 1995) and 3) Amyloid Precursor Protein (APP) on chromosome 21 (R. E. Tanzi et al., 1987) accounting for 2%

of all FAD cases (Querfurth et al., 1995). Trisomy-21 cases are shown to exhibit FAD pathology, associating an extra copy of the APP gene with development of FAD (Querfurth, Wijsman, St George-Hyslop, & Selkoe, 1995).

A major factor in FAD common to all mutations is an alteration in Aβ generation or clearance (Goate et al., 1991). Of the 21 APP allelic mutations identified, all occur within or immediately bordering on the Aβ coding region (Goate et al., 1991) suggesting that Aβ plays a central role in AD pathogenesis (A. Eckert et al., 2003). Mutations within the Aβ sequence influence aggregation of Aβ by disturbing the structure of the accumulated peptide (Mori,C. 2002). Mutations bordering on the Aβ coding region increase Aβ production by affecting APP processing, favouring the amyloidogenic β pathway (See Figure 1.3) (Cai, Golde, & Younkin, 1993; Citron et al., 1996; Mori et al., 2002). Certain populations appear to be exclusive for individual mutations. For example, in my research I have specifically focused on the so-called Swedish mutation. The Swedish double mutation consists of substitutions of the Lys^{670} and Met^{671} residues with Asn and Leu preceding the NH_2 terminus of Aβ, yielding a 5-8 fold increase in aggregate prone Aβ (1-42) production through enhanced APP vulnerability to β-secretase cleavage (Citron et al., 1992; Scheuner, Eckman, Jensen, Song, Citron, Suzuki, Bird, Hardy, Hutton, Kukull, Larson et al., 1996; M. Mullan et al., 1992a). The propensity of Aβ(1-42) to aggregate and form plaques that degenerate neurons is the focal point of the amyloid hypothesis, one of the proposed prerequisites of AD.

1.2 Current Hypotheses

Three hypotheses are suggested to account for the pathogenesis of AD with each being extensively studied as the primary insult that initiates the disease. Currently, it appears Aβ accumulation is present initially, followed by tau hyperphosphorylation, in conjunction with inflammatory responses that create a continuum of insults, resulting in a disease state.

1.2.1 Amyloid Cascade Hypothesis

The Amyloid Cascade Hypothesis (Figure 1.1) was introduced in 1992 soon after the Aβ peptide was found to be the primary component of senile plaques (SPs) believed to be one of the pathogenic lesions found in AD brains (Masters et al., 1985). This proposed that Aβ accumulation was the crucial measure of AD pathology (J. A. Hardy & Higgins, 1992). The discovery that Aβ was a product of APP metabolism (Selkoe, 2006a) and that mutations within APP altered Aβ deposition (Haass et al., 1992; Seubert et al., 1992; Haass,C. 1992), further strengthened the case for Aβ being at the core of AD.

Advances in the sequence of events in dementia pathology have offered support to The Amyloid Cascade Hypothesis. Firstly, an alternate form of dementia, Frontotemporal dementia with Parkinsonism (FTDP), is characterized by tau neurofibrillary tangles similar to those found in AD, yet Aβ plaques are absent (Goedert, Crowther, & Spillantini, 1998). This suggests that severe tau misfolding is not sufficient to trigger the formation of SPs found in AD. Secondly, transgenic mice overexpressing both mutant

APP and mutant tau exhibit escalated neurofibrillary tangles in comparison to mice overexpressing mutant tau alone (J. Lewis et al., 2001) suggesting APP mutations as an enhancer of further neuro-toxic insults. Thirdly, recent evidence has shown Aβ dimers generate tau hyperposphorylation causing neurite degeneration (Jin, Shepardson, Yang, Chen, Walsh, & Selkoe, 2011a). Lastly, a buildup of Aβ is observed in the brain before symptoms arise (D. M. Walsh, Klyubin, Fadeeva, Rowan, & Selkoe, 2002) suggesting that Aβ accumulation precedes clinical signs of AD.

However, the Amyloid Hypothesis has fallen under criticism as the initiator of AD since clinical trials found no improvement in cognitive function in patients immunized against Aβ, despite evidence of plaque clearance in the post-mortem brains (Golde, Petrucelli, & Lewis, 2010; Holmes et al., 2008). While amyloid load appears to poorly correlate with severity of cognitive decline in AD, SPs are present in symptomatic AD. Furthermore, the discrepancy in amyloid load and degree of cognitive decline is controversial because a definite toxic Aβ species has yet to be determined *in vivo* (discussed further under "Amyloid-β Production and Toxicity").

1.2.2 Hyperphosphorylated Tau Hypothesis

The other major hypothesis surrounding AD onset and progression is the disruption of microtubule assembly and function due to hyperphosphorylated, insoluble, filamentous Tau protein. Alteration of tau phosphorylation is the principle cause of NFTs thought to play a key role in AD onset (Hernandez, Gomez de Barreda, Fuster-Matanzo,

Lucas, & Avila, 2010). Tau is expressed in the central and peripheral nervous system and, to a lesser extent in kidney, lung and testis (Gu, Oyama, & Ihara, 1996). It is most abundant in neuronal axons (Lee & Trojanowski, 2001a), but can also be found in somatodendritic compartments (Tashiro, Hasegawa, Ihara, & Iwatsubo, 1997) and oligodendrocytes (C. Klein et al., 2002).

Tau consists of an N-terminal projection region, a proline-rich domain, a C-terminal region and a microtubule binding domain (MBD) through which tau protein binds to and thus stabilizes microtubule assembly (Mandelkow et al., 1996) (Kar, Fan, Smith, Goedert, & Amos, 2003; Santarella et al., 2004). The tandem repeat sequences within the MBD are thought to directly bind microtubules through their positive net charge, which interacts with negatively charged residues in tubulin (Jho, Zhulina, Kim, & Pincus, 2010; Kar et al., 2003). Phosphorylation of tau directly influences its ability to regulate microtubule assembly by neutralizing the positive charge and altering conformation of the MBD, detaching tau from the microtubules (Fischer et al., 2009). The detached tau can accumulate in neuronal cell bodies and neurites, forming insoluble filaments and NFTs (Lee & Trojanowski, 2001b; von Bergen, Barghorn, Biernat, Mandelkow, & Mandelkow, 2005; von Bergen, Li, & Mandelkow, 2005). In addition, the MBD of tau contains critical sequences that can assume the β-sheet structures required for tau aggregation and formation of pathological inclusions (von Bergen et al., 2001; von Bergen et al., 2005).

Similar to Aβ, tau mutations have been identified which heighten production of one isoform of tau over another. Mutations leading to changes in the ratio of three-repeat to four-repeat isoforms may result in an excess of tau in relation to microtubule binding sites, allowing for excess tau availability for filament assembly (Hutton et al., 1998; Spillantini et al., 1998; Spillantini, Crowther, Kamphorst, Heutink, & van Swieten, 1998). Missense mutations that affect all isoforms of tau result in reduced microtubule assembly (Hasegawa, Smith, & Goedert, 1998), thus causing microtubule destabilization and detrimental effects on axonal transport.

In addition, since tau activity is regulated by the phosphorylation state, hyperphosphorylation of tau as seen in AD depresses the ability of tau to bind to microtubules (Lindwall & Cole, 1984; Hampel et al., 2010). Enquiry into the mechanism behind tau hyperphosphorylation has lead to the discovery of many tau kinases including cyclin-dependent protein kinase 5 (cdk-5), cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and the most therapeutically promising glycogen synthase kinase-3β (GSK-3β) (Mendes et al., 2009; Schaffer et al., 2008). Dysregulation of these kinases and phosphatases is thought to result in tau hyperphosphorylation. Aβ is thought to promote GSK-3β activation as it is up-regulated in AD brains and its activity is shown to increase with aging (Schaffer et al., 2008). Trials implementing the GSK-3β inhibitor, lithium, failed to show alterations in tau, phospho tau, Aβ (1-42) levels or cognitive performance in mild AD patients (Hampel et al., 2010; Mendes et al., 2009), however, this may be owing to lithium's non-specificity as an inhibitor. Interestingly, HspB1 has been shown to bind hyperphosphorylated tau and promote its

dephosphorylation and degradation, thereby promoting cellular survival (Shimura, Miura-Shimura, & Kosik, 2004).

The use of transgenic mice has demonstrated that reduction of both soluble Aβ and tau is needed to ameliorate cognitive deficits seen in triple transgenics that have SPs as well as NTFs (Oddo, Caccamo, Smith, Green, & LaFerla, 2006) concluding that AD pathogenesis cannot be resolved without considering both proteins.

1.2.3 Inflammation and Oxidative Stress in AD

Studies from the 1990's followed inflammation as a causative factor in AD, yet today it remains unclear whether inflammation represents a cause or a consequence of AD. Clinical evidence in support of a role for inflammation includes elevated cytokines, inflammatory agents found in acute inflammatory responses, and activated microglia in post-mortem AD brain compared to age-matched controls (Fillit et al., 1991; Frank-Cannon, Alto, McAlpine, & Tansey, 2009). Retrospective studies of individuals under long-term treatment with NSAIDs (Non-steroidal anti-inflammatory drugs) have demonstrated delayed onset and reduced severity of AD symptoms, although recent trials have failed to confirm this (Szekely & Zandi, 2010).

Activated microglia cells surround Aβ plaques in both human AD and transgenic mouse AD brains, however their exact contribution to plaque formation is unknown (Johnston, Boutin, & Allan, 2011). Overall, it appears that moderate activation may be beneficial through an increase in Aβ clearance (Boissonneault et al., 2009; K. Chen et al.,

2006), whereas strong activation might slow down the ability to clear Aβ, increase production of pro-inflammatory cytokines and accelerate neuronal damage (Arnaud, Robakis, & Figueiredo-Pereira, 2006). Microglial cells immunoreactive for interleukin-1 were increased in tissue sections of AD patients (Griffin et al., 1989) and in APP/PS1 transgenic mice (Hickman, Allison, & El Khoury, 2008). Interleukin-6 immunoreactivity in SPs and neurons in AD brains initially was thought to be increased (Bauer et al., 1992; Heyser, Masliah, Samimi, Campbell, & Gold, 1997), yet recent papers show a protective role of interleukin-6 in plaque clearance (Chakrabarty et al., 2010). Interestingly, interleukin-6 increases APP synthesis in neuronal cells (Altstiel & Sperber, 1991) and interleukin-1 stimulates APP synthesis in endothelial cells (Goldgaber et al., 1989). Increase in an alternate cytokine, TNFα, resulted in increased tau hyperphosphorylation, intra-neuronal Aβ deposits and also neuronal death in triple transgenic AD mice (Janelsins et al., 2008). One theory behind the role of inflammation in AD is that a local insult in the brain perhaps amyloid aggregation will stimulate production of cytokines and while initiating Aβ clearance, prolonged activation (coupled with prolonged cytokine release) increases production of APP. This combined with altered APP processing, whether from genetic mutations or otherwise, brings about a cyclic pattern of immunoreactivity (Figure 1.2).

Astrogliosis is found in AD brains as well (Akiyama et al., 2000). Previous studies have revealed Aβ present in astrocyte processes (Kurt, Davies, & Kidd, 1999) and lyses of Aβ loaded astrocytes resulted in the formation of SPs (Nagele, D'Andrea, Lee, Venkataraman, & Wang, 2003). Studies have shown evidence of cellular Aβ

internalization from degenerating synapses and dendrites opening the possibility of external uptake of Aβ by astrocytes via endocytosis (Nagele et al., 2003). Also, extracellular soluble oligomeric forms of Aβ may stimulate Aβ production within astrocytes (J. L. Perez et al., 2010)(DaRocha-Souto et al., 2011). Extracellular Aβ is thought to form pores or ion channels in the cellular membrane thereby allowing influx of excess Ca^{2+} subsequently activating detrimental signalling cascades such as caspase and calpain activation (Kawahara & Kuroda, 2000; J. L. Perez et al., 2010). Treatment with Aβ can activate both JNK and p38MAPK signalling pathways thus activating apoptotic signalling (A. Eckert et al., 2001).

Taken together, it appears accumulation of Aβ into oligomers, although yet unknown if through internal or external production, can trigger astrocyte activation, possibly through increased calcium influx, in turn synthesizing and secreting toxic substances contributing to AD neurotoxicity (DaRocha-Souto et al., 2011).

1.3 Amyloid Precursor Protein

Amyloid Precursor Protein (APP) is a type-1 transmembrane protein, with a large extracellular glycosylated N-terminus and a shorter cytoplasmic C-terminus, ubiquitously expressed in many cell types and highly conserved across species (Figure 1.3) (Kang et al., 1987). The Aβ portion of APP is located at the cell surface with part of the peptide embedded in the membrane (Mattson, 2004). Alternative splicing creates three isoforms of APP in human: 770, 751 and 695 (R. E. Tanzi et al., 1988), with the 695 isoform preferentially expressed in neurons (Sisodia, Koo, Hoffman, Perry, & Price, 1993). APP

has been shown to be concentrated in the synapses of neurons, and increases in the ratio of the neuronal APP^{695} isoform to others, is associated with AD (Matsui et al., 2007). APP undergoes post-translational modifications including glycosylation, tyrosine sulfation and phosphorylation. Mutations of APP (including the Swedish mutation) are not thought to be loss-of-function mutations meaning such mutations do not interfere with APP's biological function (M. Mullan et al., 1992a). Hence, Aβ is thought to be a by-product of APP metabolism and appears to have no direct association with APP's biological role (Citron et al., 1992; Citron, Teplow, & Selkoe, 1995; Citron et al., 1996; Goate et al., 1991; Selkoe et al., 1996).

APP is thought to have numerous biological functions. As a cell surface receptor (Ho & Sudhof, 2004; Kang et al., 1987), APP may have the ability to induce cell signalling (P. R. Turner, O'Connor, Tate, & Abraham, 2003), functioning as a receptor-like modulatory protein in neuronal processes (Ashley, Packard, Ataman, & Budnik, 2005). Influencing Wnt signalling, APP is suggested to mediate β-catenin downregulation in primary neurons by specific phosphorylation of β-catenin residues (Y. Chen & Bodles, 2007). In addition, APP's large extracellular domain is capable of binding to several extracellular matrix proteins, alluding to a role in cell-cell and cell-matrix adhesion (Mattson, 1997). In neurons, APP is present in growth cones of neurites and both presynaptic and postsynaptic sites (Ferreira, Caceres, & Kosik, 1993; Sabo, Ikin, Buxbaum, & Greengard, 2003). APP is shown to increase synaptic formation and repair after injury (Priller et al., 2006), neurite outgrowth (R. G. Perez, Squazzo, & Koo, 1996) and plays a role in cell migration (Reinhard, Hebert, & De Strooper, 2005).

Knock-down of APP through *in utero* electroporation in rodent developing cortex, revealed a dependency on APP for correct migration of neuronal precursor cells into the cortical plate (Young-Pearse et al., 2007). Whereas knock-down of APP inhibited cortical plate entry, over-expression of APP caused accelerated migration of cells past the cortical plate boundary, confirming the requirement of normal APP levels for correct neuronal migration (Young-Pearse et al., 2007).

1.3.1 Proteolytic processing of Amyloid Precursor Protein

APP undergoes two types of proteolytic processing by secretase enzymes differing in whether Aβ peptide is produced (Figure 1.3). Non-amyloidogenic processing begins with the release of the extracellular N-terminus of APP by α-secretase or ADAM10 between residues $A\beta^{16}$ and $A\beta^{17}$ within the Aβ region thereby precluding Aβ production (Sisodia, 1992). The released N-terminus of APP from the cell surface produced by α-secretase is denoted as soluble APP alpha or sAPPα. This leaves an 83 amino acid cytoplasmic membrane-bound fragment (C83/CTFα), which constitutes the middle p3 fragment (extracellularly released) and APP intracellular domain (AICD) (intracellular). The CTF is cleaved by γ-secretase, generating the non-aggregating p3 fragment which substitutes for the toxic Aβ (Frigerio et al., 2010; S. A. Small & Gandy, 2006). All APP fragments generated by APP processing have been identified in one or more biological processes, yet details on the specific functions of the fragments are

unknown since investigation has focused primarily on the Aβ peptide in its relation to AD (Summarized in Table 1).

Activation of muscarinic acetylcholine receptors increases production of sAPPα, suggesting that non-amyloidogenic processing is favoured by neuronal activity (Buckner, Andrews-Hanna, & Schacter, 2008). Activity dependence on APP processing is not a novel finding (Cirrito et al., 2005; Kamenetz et al., 2003; Nitsch, Farber, Growdon, & Wurtman, 1993; Selkoe, 2006b), however, why certain neurons are robustly affected by Aβ accumulation while others appear unsusceptible, is a fairly new focus. Support is growing for a link between Aβ deposition, vulnerable heteromodal association areas and cortical dysfunction in AD (Buckner et al., 2005; Buckner et al., 2009; Klunk et al., 2004). Cortical regions implicated in AD have been identified as connectional "hubs" or areas that act as communication stations for information processing connecting otherwise segregated brain systems across task states (Sporns, Tononi, & Edelman, 2000; Sporns & Kotter, 2004; Sporns, Honey, & Kotter, 2007). Moreover, in resting humans, increased activity in the default network (Mazoyer et al., 2001), an area which corresponds to identified hubs (medial prefrontal cortex, medial temporal lobe, posterior cingulate cortex), positively correlates with SP load (Buckner et al., 2005).

Conversely, Aβ production from APP processing occurs through the amyloidogenic pathway requiring the BACE enzyme or β-secretase (Vassar et al., 1999). BACE cleaves APP at the N-terminal end of the Aβ sequence thus enabling Aβ production and secretion of soluble APP beta (sAPPβ). Cleavage by γ-secretase cleaves

the membrane cytoplasmic terminal fragment (C99/CTFβ) at position $A\beta^{40/42}$ liberating the C-terminus of Aβ, allowing entry into the extracellular milieu. The remaining AICD can translocate to the nucleus where it regulates gene expression including induction of apoptotic genes through binding with Fe65 and Tip60 (Bao et al., 2007; Cao & Sudhof, 2001; D. M. Walsh et al., 2002). Amyloidogenic processing can be modulated by altering APP's susceptibility to BACE cleavage through cellular localization.

1.3.2 Intracellular Trafficking of the Amyloid Precursor Protein

Nascent APP, transcribed in the nucleus, is trafficked through the endoplasmic reticulum (ER) to the golgi apparatus, where post-translational modifications, N-glycoslylation and O-glycoslyation occurs, and continues through the trans golgi network (TGN) to yield mature APP protein (Figure 1.4). APP travels through the secretory pathway to the plasma membrane where ADAM10 (α-secretase) initiates non-amyloidogenic APP processing. A pool of APP does not undergo immediate processing, and as a consequence is internalized by the cell (Haass et al., 1992; Haass, Koo, Mellon, Hung, & Selkoe, 1992). APP then either recycles back to the cell surface or is targeted to the late endosomes/lysosomes (Marquez-Sterling, Lo, Sisodia, & Koo, 1997; Pasternak, Callahan, & Mahuran, 2004; Yamazaki, Koo, & Selkoe, 1996). Here APP encounters transmembraneous BACE in the endosomes of the early endocytic pathway, the first step in Aβ production (Haass et al., 1992; Lorenzen et al., 2010). Identified sorting motifs in APP (N-P-X-Y) and BACE (D-X-X-L-L) cytoplasmic tails target both proteins for

transport to endosomes via clathrin coated vesicles (BACE from TGN-endosomes; APP from cell surface-endosomes) (W. J. Chen, Goldstein, & Brown, 1990; He, Li, Chang, & Tang, 2005). Finalizing the production of Aβ, γ-secretase (a multimeric protein complex consisting of PS-1 and PS-2 and the site for FAD presenilin mutations), has been found enriched in the late endosomes/lysosomes (Pasternak et al., 2004). Evidence for the endosomal/lysosomal localization of the amyloidogenic secretases is provided by reduced Aβ production when blocking the internalization of cell surface APP (Cirrito et al., 2005; Koo & Squazzo, 1994), and the acidification of the endosomal-lysosomal pathway (Kaether, Haass, & Steiner, 2006; Schrader-Fischer & Paganetti, 1996; Vingtdeux et al., 2007). Reporter system studies coupling γ-secretase to fluorescence have indicated the presence of γ-secretase in the endocytic pathway, suggesting the endosomes as the major sites for Aβ production (Kaether et al., 2006).

Theories exist that mutations in APP result in a greater pool of endocytosed APP, resulting in increased availability of APP for BACE cleavage within the endocytic pathway, thus explaining the increased Aβ production observed (Hartmann et al., 1997). There is also evidence for BACE and soluble Aβ co-localization to the TGN, giving the opportunity for amyloidogenic cleavage of APP to occur before it encounters α-secretase at the plasma membrane (Greenfield et al., 1999). The Swedish mutation increases the rate of β-cleavage by 5-10 fold (Sinha & Lieberburg, 1999) and is proposed to alter the trafficking of APP (APPswe). This mutation partly disrupts sorting of secreted APP (De Strooper et al., 1995) and appears to undergo β-cleavage during transit to the cell surface (Haass, Hung, Schlossmacher, Teplow, & Selkoe, 1993; Haass, Hung, Selkoe, & Teplow,

1994; Thinakaran, Teplow, Siman, Greenberg, & Sisodia, 1996). Amyloidogenically cleaved APP/Aβ is then trafficked through the endocytic pathway to the lysosomes where the γ-secretase complex finalizes Aβ production. Since the site for processing is thought to differ in relation to the type of APP (wt versus mutated), the exact details of the transportation process back to the cell surface for release of the soluble APP forms and Aβ are unclear at this time.

A novel pathway in APP re-uptake by cells directly from cell surface into the lysosomes, thereby bypassing the endosomal compartments, has been shown by Lorenzen and colleagues. Fluorescently labelled APPwt is shown enriched in the lysosomes, and the direct pathway from cell surface to lysosome appears to be blocked by the Swedish mutation (Lorenzen et al., 2010). A possible parallel to this trafficking pathway may be the prion protein (PrP) which normally traffics through the endocytic compartments, except when pathologically misfolded, appears to traffic directly to the lysosomes (Magalhaes et al., 2002). Given that the Swedish mutation is not believed to alter the folding of the APP protein (rather the Aβ production), APPswe may be normally trafficked through the endocytic compartments evading direct entry into lysomsomes from the cell surface. If the pathway chosen has an influence on the type of APP processing (i.e. amyloidogenic versus non-amyloidogenic), endocytosis through the endocytic compartment may lead to more efficient APP cleavage by the secretase enzymes and thus higher Aβ production, whereas rapid transport to the lysosomes may

lead to degradation by lysosomal proteases (Lorenzen et al., 2010). This highlights the complexity yet to be resolved in APP trafficking and processing of both normal and mutated APP.

1.4 Amyloid-β Production and Toxicity

Amyloid is defined as a heterogenous class of tissue protein precipitates which share a common β-sheet secondary structure (Gandy, 2002). Amyloid can be produced throughout various areas of the body (systemic amyloid) or be confined to a particular area such as renal amyloid or cerebral amyloid as seen in AD (Gandy & Petanceska, 2000). Increases in either total Aβ or the relative concentration of both Aβ (1-40) (more concentrated in cerebrovascular plaques) and Aβ (1-42) (more concentrated in neuritic plaques) have been implicated in FAD and SAD (Lue et al., 1999). Multiple disease associations exist with amyloid deposition including inclusion body myositis (muscle disease) and cerebral amyloid angiopathy (Revesz, Holton, & Lashley, 2002). Although Aβ is acknowledged as a toxic species of AD, Aβ does not exist specifically to cause AD (Lahiri & Maloney, 2010). Aβ has been shown to have a multitude of normal biological processes such as activation of kinase enzymes (Bogoyevitch, Boehm, Oakley, Ketterman, & Barr, 2004; Tabaton, Zhu, Perry, Smith, & Giliberto, 2010), regulation of cholesterol transport (Igbavboa, Sun, Weisman, He, & Wood, 2009; Yao & Papadopoulos, 2002), functioning as a transcription factor (Bailey et al., 2011; B. Maloney & Lahiri, 2011), and anti-microbial activity which may be associated with Aβ's

pro-inflammatory activity (Soscia et al., 2010). Hence, Aβ(1-40) and (1-42) are produced under normal circumstances in aging brains without dementia, but are believed to be in lower ratio and altered conformation than observed in AD brains.

Abnormal accumulation of amyloid may lead to amyloidosis resulting in toxicity. Aβ toxicity induces actin stress fibre formation and is proposed to inhibit actin dynamics, triggering actin polymerization and disorganization of actin filaments in neuronal dendrites (Mendoza-Naranjo, Gonzalez-Billault, & Maccioni, 2007; Song, Perides, Wang, & Liu, 2002). Axonal transport in cultured neurons is inhibited by Aβ through its effects upon the polymerization and aggregation of intracellular actin (Hiruma, Katakura, Takahashi, Ichikawa, & Kawakami, 2003). Intracellular aggregation of actin has been described previously in neurodegenerative diseases as rod-like structures formed in hippocampal neurons in response to stress such as Aβ treatment (M. T. Maloney, Minamide, Kinley, Boyle, & Bamburg, 2005). Disruption of the actin cytoskeleton can lead to growth cone collapse and degeneration of neuronal dendrites (Meberg & Bamburg, 2000). Initially, investigation into the toxic nature of Aβ focused on the insoluble fibrillar form found in the senile plaques present in AD brains. These plaques are thought to be generated through hydrophobic interactions between Aβ fragments which associate into rod-like structures, so-called fibrils. Due to its more hydrophobic nature, Aβ(1-42) is the more aggregate prone form of the peptide (Ott et al., 2011). While SP's are the diagnostic lesions of AD neuronal damage, the insoluble fibrillar Aβ associated has been proposed as a reservoir for small oligomers (eg. dimers) within the plaque core. Neuronal toxicity has recently been associated with the conversion of non-

toxic Aβ monomers to toxic oligomers (dimers, trimers, etc.), in addition to the fibrillar form found in SPs (Selkoe, 2007; D. M. Walsh & Selkoe, 2004), (J. Hardy & Selkoe, 2002). These small oligomers can diffuse to neighbouring neurons causing synaptic injury (Shankar et al., 2008). Dendritic spine loss of ~60% is observed in hippocampal pyramidal neurons after 15 days of exposure to sub-nanomolar concentrations of Aβ small oligomers, reflecting a loss of excitatory synapses. Spine density returned to almost normal levels with cessation of Aβ treatment (Shankar et al., 2007).

Aβ dimers extracted from human CSF have been shown to disrupt synaptic plasticity (Klyubin et al., 2008). Long term potentiation (LTP) is inhibited by soluble Aβ oligomers through excessive activation of NMDA-receptors similar to inhibition of glutamate re-uptake (W. L. Klein, Krafft, & Finch, 2001; Li et al., 2011; D. M. Walsh et al., 2002). These results suggest that Aβ oligomers shift NMDAR-dependant signalling cascades toward pathways involved in the induction of LTD (long term depression) in contrast to LTP (Selkoe, 2008). Chronic activation of these pathways by soluble Aβ oligomers may underlie the synapse loss in hippocampus that occurs early in the disease process in brains of AD patients (Masliah et al., 2001; Selkoe, 2008).

Recently, subnanomolar concentrations (0.5 nM) of soluble Aβ dimers isolated from AD cortex have been shown to directly induce tau hyperphosphorylation in hippocampal neurons disrupting the microtubule cytoskeleton and resulting in neuritic degeneration (Jin, Shepardson, Yang, Chen, Walsh, & Selkoe, 2011b). This finding is significant as it describes effects by endogenous levels of Aβ on neuronal cells found

within the AD brain, in comparison to exogenously increased levels and presents evidence of direct influence on tau function by Aβ. Moreover, the isolated dimers were principally composed of Aβ (1-42) (Jin, Shepardson, Yang, Chen, Walsh, & Selkoe, 2011b). Finally, transgenic mice that express only oligomers but not plaques (APP^{E693Q}) develop AD symptoms similar to mice engineered to convert oligomers into plaques (APP^{E693Q} X PS1ΔE9) (Gandy et al., 2010). Even though a growing amount of evidence is emerging in regards to soluble Aβ oligomers, this does not discount the role of plaques in neuron degeneration. There is evidence that peri-plaque Aβ assemblies are associated with neuritic dystrophy (Knowles et al., 1999), and local dendritic spine loss ((Koffie et al., 2009), therefore placing soluble oligomers and insoluble fibrils as co-contributors to Aβ neuronal toxicity (Selkoe, 2011).

Aβ can be degraded by different systems, which include enzymatic degradation or receptor-mediated clearance (R. E. Tanzi, Moir, & Wagner, 2004). Several peptidases have been described to possess the ability to degrade Aβ including insulin degrading enzyme (IDE) (Qiu et al., 1998; Vekrellis et al., 2000) and neprylisin (Sastre & Gentleman, 2010; A. J. Turner, Isaac, & Coates, 2001).

In addition, Aβ can be cleared through phagocytosis by activated microglia and via transport through the blood brain barrier (BBB) which can be enhanced by binding to chaperones such as ApoE and α-macroglobulin (Suzuki & Nakaya, 2008).

1.5 Heat Shock Protein beta-1 (HspB1) in the Brain

Heat shock proteins are up-regulated in response to cellular stress including elevated temperatures, exposure to heavy metals, ethanol and anoxia (Lindquist, 1986). Induction of Hsps is regulated by heat-shock factors and a heat shock element present at the promoter region of the heat shock gene (Morimoto, Kline, Bimston, & Cotto, 1997). Heat shock proteins can be divided into two families based on size and function: classic Hsps such as Hsp100, Hsp90, Hsp70, Hsp60, and the small heat shock family: αB-crystallin, HspB1, Hsp20, HspB2 and HspB3 (Kappe et al., 2003; Wilhelmus et al., 2006). Hsps with a molecular weight of 60 kDa or more possess an ATP-binding site and are actively involved in the process of refolding misfolded proteins (Gusev et al., 2002). Small Hsps, with a molecular weight of 40 kDa or less, lack this ATP-binding domain and assist the larger Hsps in their refolding function (MacRae, 2000).

HspB1 (also referred to as Hsp27/25) is highly expressed in muscle (Wilhelmus et al., 2006) and glial cells, specifically reactive astrocytes (Renkawek, Voorter, Bosman, van Workum, & de Jong, 1994; Shinohara, Inaguma, Goto, Inagaki, & Kato, 1993). In the nervous system, HspB1 expression is seen in spinal cord motor neurons and peripheral sensory neurons, where it plays a key role in promoting neuronal survival and axonal growth, particularly in adult neurons (Costigan et al., 1998; Dodge et al., 2006; S. E. Lewis et al., 1999; Williams, Rahimtula, & Mearow, 2005; Williams, Rahimtula, & Mearow, 2006). While HspB1 is expressed in some areas of the brain such as cranial nerve nuclei and hypothalamus (Armstrong, Krueger-Naug, Currie, & Hawkes, 2001;

Plumier, Hopkins, Robertson, & Currie, 1997), it is not expressed in cortical neurons (M. King, Nafar, Clarke, & Mearow, 2009). HspB1 has been co-localized to post-synaptic structures in rat cerebellum and peri-synaptic glial processes following hyperthermia and this increase was not seen in unstressed animals (Bechtold & Brown, 2000). Hsps may be secreted by astrocytes and may be taken up by neighbouring neurons thus possibly providing a compensatory mechanism for lack of cortical expression (Ouyang, Xu, Sun, & Giffard, 2006; Taylor, Robinson, Gifondorwa, Tytell, & Milligan, 2007). The common functions of HspB1 are chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development and cell differentiation, and interaction with cytoskeletal elements in cell growth (Arrigo, 2005; Arrigo, 2007; Charette & Landry, 2000a; Parcellier et al., 2003; Vargas-Roig, Fanelli, Lopez, Gago, Tello, Aznar, & Ciocca, 1997b).

1.5.1 HspB1 as a chaperone protein

Heat shock proteins (Hsp) are the cell's defence mechanism in times of stress and are thus termed "chaperones". Chaperones can be defined as proteins that: 1) have a role in the intracellular management of misfolded proteins, 2) induce conformational changes of proteins, 3) act as a transporter of proteins (Hendrick & Hartl, 1993; Wilhelmus, de Waal, & Verbeek, 2007). Hsps can bind unfolded proteins and keep them in their native state (Walter & Buchner, 2002), or recognize misfolded proteins and transport them to the proteosome for degradation (Wilhelmus et al., 2006; Wilhelmus et al., 2007).

Molecular chaperones work by recognizing and binding to hydrophobic amino acids exposed to the surface of the substrate protein thereby preventing unproductive aggregation (Hartl, 1996).

HspB1 contains a highly conserved amino acid sequence, the α-crystallin domain at the C-terminus, which forms β-sheets important for the formation of stable dimers (Van Montfort, Slingsby, & Vierling, 2001). The N-terminus is essential for the development of high molecular oligomers (Haslbeck, 2002; Theriault et al., 2004), which exclusively have chaperone function *in vitro*. These oligomers consist of stable dimers of neighboring monomers (Gusev et al., 2002). Oligomerization of HspB1 depends on exposure to stress and its phosphorylation state. Stress induces an increase of expression (after hours) and phosphorylation (after several minutes) of HspB1. HspB1 in unstressed cells exists as large oligomers, while upon phosphorylation HspB1 dissociates into smaller species, including dimers and monomers (Lambert, Charette, Bernier, Guimond, & Landry, 1999; Rouse et al., 1994; Williams et al., 2005). Stimulation of the p38 MAP kinase cascade leads to the activation of MAPKAPK2 which is reported to directly phosphorylate HspB1 (Kyriakis & Avruch, 1996; Rogalla et al., 1999). HspB1 is phosphorylated on 3 serine residues in the human HspB1 (S15, S78, S82) and 2 in the rodent HspB1 (S15 and S86 in mouse or S90 in hamster HspB1). Oligomerization appears to be connected to chaperone activity; aggregates of large oligomers have high chaperone activity, whereas dimers have no chaperone activity (Gusev et al., 2002).

Hsps have been involved in rescue of aggregate prone proteins in many instances. In Dementia with Lewy Bodies (DLB), α-synuclein protein inclusions adopt a β-sheet structure leading to aggregation such as oligomeric assembly in cortical and limbic areas (Spillantini & Goedert, 2000), until it reaches a stable fibrillar form (Conway et al., 2000) similar to Aβ in AD. Previously, HspB1 and αB-crystallin have been shown to protect against α-synuclein-induced toxicity and aggregation (McLean et al., 2002; Rekas et al., 2004; Waudby et al., 2010). This has been further indicated as evidence of recombinant sHsps, including HspB1, were shown to bind (though through a weak and transient interaction) to α-synuclein thus inhibiting mature α-synuclein fibril formation and aggregation (Bruinsma et al., 2011). Moreover, mutant ataxin-1 transfected HeLa cells display large nuclear aggregates that co-localize with endogenous Hsp70, ubiquitin and 20S proteosome, suggesting that the cell's endogenous protein degradation system is insufficient to suppress these aggregates. Over-expression of Hsp70 resulted in a significant reduction of nuclear aggregates (Cummings et al., 1998), showing that an exogenous over-expression of Hsp was better able to protect than an endogenous one. Bip/GRP78, part of the Hsp70 family found in the ER, binds directly to APPswe impairing maturation and decreasing sAPP, Aβ (1-40) and Aβ (1-42) recovery in conditioned medium of human embryonic kidney 293 cells (HEK 293) (Y. Yang, Turner, & Gaut, 1998). The mechanism behind the reduction in APP metabolites in conditioned medium may be retention of APP in the ER thus weakening the chance of β/γ secretase cleavage and/or substrate depletion.

HspB1 may act to inhibit apoptosis through interacting with the outer mitochondrial membrane and interfering with the activation of the cytochrome c/Apaf-1/dATP complex inhibiting the activation of procaspase-9 (Bruey et al., 2000; Concannon, Orrenius, & Samali, 2001; Samali et al., 2001; Sarto, Binz, & Mocarelli, 2000). The phosphorylated form of HspB1 has been seen to inhibit Daxx apoptotic protein and prevent the association of Daxx with Fas and Ask1 (Charette & Landry, 2000b; Charette, Lavoie, Lambert, & Landry, 2000). Apoptosis may also be stopped by activation of Akt or ERK and pro-survival transcription factor, nuclear factor-κB (NF-κB) (Kennedy, Kandel, Cross, & Hay, 1999; Levresse, Butterfield, Zentrich, & Heasley, 2000), all of which HspB1 has been shown to enhance the activation of, thus acting in a pro-survival nature (Parcellier, Gurbuxani, Schmitt, Solary, & Garrido, 2003) .

Aβ toxicity induces actin stress fibre formation leading to inhibition of axonal transport, growth cone collapse and degeneration of neuronal neuronal dendrites. Actin stabilization by sHsps has been shown through their ability to act as an actin capping protein thus promoting survival (Guay et al., 1997; Gusev, Bogatcheva, & Marston, 2002).

The pro-survival and chaperone functions of HspB1 may be potential mechanisms in which HspB1 can effect APP processing or distribution within the cell and influence the production of Aβ thereby impeding Aβ oxidative damage.

1.6 Alzheimer's Disease and HspB1

HspB1 has been recognized in 15% of SPs in neocortical areas of AD patients brains (Wilhelmus et al., 2006; Wilhelmus et al., 2007). Whether this localization is an attempt by HspB1 to ameliorate the detrimental effects of Aβ on cells or part of the disease process is unknown, however previous work from our lab has observed increased neuronal survival in cells exposed to Aβ (1-42) when transfected with HspB1 (M. King et al., 2009) pointing to a possible protective mechanism. In support of this, HspB1 can decrease the level of phosphorylated tau and rescue tau-mediated cell death (Shimura et al., 2004) and has been implicated in neurite growth and cytoskeletal stability in DRG neurons (Williams et al., 2006; Williams & Mearow, 2011).

Direct interaction between αB-crystallin and HspB1 with Aβ has been demonstrated in vitro (Kudva, Hiddinga, Butler, Mueske, & Eberhardt, 1997; Liang, 2000). Wilhelmus et al., 2006 investigated the roles of sHsps on Aβ cytotoxicity. They found HspB1, Hsp20 and αB-crystallin, but not HspB2B3, bind to Aβ (1-42) and decrease its aggregation by reducing the propensity of Aβ to form β-sheet conformation. Furthermore, HspB1, Hsp20 and αB-crystallin inhibited cerebrovascular Aβ toxicity, probably by reducing the accumulation of Aβ at the cell surface (Wilhelmus et al., 2006). A more recent study found HspB1, APP and Bcl-2 form a complex within mitochondria of serum starved N2a cells which is associated with apoptosis. This effect was increased in cells containing the APPswe mutation and APP over-expression increased HspB1

presence in mitochondria. It is important to note that APP over-expression alone did not induce apoptosis instead a second insult in the form of serum starvation was needed (T. T. Yang, Hsu, & Kuo, 2009). .

Perhaps HspB1 is recruited to the mitochondria under stressful conditions to provide chaperone activity and activates apoptosis through mitochondrial dysfunction or conversely, the sequestering of HspB1 inside the mitochondria impair it from binding to the cytosolic cytochrome c, thus inhibiting HspB1 from providing its anti-apoptotic effects.

While HspB1 has been implicated in neuronal protection and reduction of protein aggregation, the question remains whether HspB1 localized in SPs of AD brains is a failed attempt to counteract Aβ aggregation or part of the disease process.

1.7 Proposed Therapies

Researchers in AD have identified various areas as possible interventions against amyloid aggregation given evidence for Aβ toxicity (Citron, 2004):

1.) β-secretase inhibitors (e.g. Memapsin 2). These block the first amyloidogenic cleavage of APP, inhibiting the formation of Aβ N-terminus (Chang et al., 2004) however these have been proven ineffective in clinical trials.

2.) γ – secretase inhibitors (e.g. semagacestat). These block the second cleavage of APP in the cell membrane that would stop the formation of Aβ C-terminus. These have not been successful to date as γ-secretase binds ligands other than APP such as Notch, hence inhibition has other negative effects (Basi et al., 2010; Imbimbo & Peretto, 2009; Shih & Wang, 2007).

3.) Selective Aβ (1-42) lowering agents (e.g. tarenflurbil). These modulate γ-secretase to reduce Aβ (1-42) production in favour of shorter Aβ versions. Tarenflurbil unfortunately was found to have no beneficially effects on AD patients upon completion of the Phase III clinical trial (Green et al., 2009).

4.) Immunotherapies. These stimulate the host immune system to recognize and attack Aβ or provide antibodies that prevent plaque deposition or enhance clearance of plaques. Unfortunately, Aβ immunotherapies can cause micro-haemorrhages due to activation of matrix metalloproteinases, MMP2 and MMP9 (Wilcock et al., 2011).

5.) Anti-aggregation agents (e.g. apomorphine). These prevent Aβ fragments from aggregating or clear aggregates once they are formed (Lashuel et al., 2002; Parker et al., 2002). HspB1 may fall under this category as an endogenous anti-aggregation agent through its chaperone activity.

1.8 Summary

FAD is the genetic form of AD accounting for 4-5% of AD cases. Mutations, such as the Swedish mutation in the APP gene have been shown to increase Aβ toxicity

through favouring the amyloidogenic pathway, hastening production of aggregate prone Aβ(1-42) (Citron et al., 1992; B. Maloney & Lahiri, 2011). Aβ can also assemble into oligomers which appear to be the focus of Aβ toxicity recently. These oligomers increase apoptosis, disrupt cell signalling and inhibit LTP and synaptic plasticity (D. M. Walsh & Selkoe, 2004). This has brought the focus of Aβ research away from clearing SPs to disrupting amyloid oligomerization.

A possible mechanism for disruption of misfolded or aggregated proteins is through the work of molecular chaperones. Exogenous HspB1 protects neurons from cell death caused by Aβ treatment (M. King et al., 2009). HspB1 though not found endogenously in cortical neurons, is secreted by neighbouring glial cells and may be taken up by adjacent neurons to elicit its protective effects. Through its chaperone function, HspB1 may sequester mutated or misfolded APP tagging it for degradation before processing occurs or sequester APP within the cell inhibiting its secretion into the extracellular environment. Since both Aβ and HspB1 are involved in the cell death pathway, HspB1 could inhibit Aβ activation of apoptosis. The possibility also exists for HspB1 to exert changes on cytoskeletal elements thus interfering with tau misfolding and inhibiting production of NFTs.

HspB1 has many therapeutic possibilities in neurodegenerative diseases such as AD. My focus on interactions between HspB1 and APP/Aβ explore the potential effects of the small heat shock protein on APP processing and distribution within the cell. As

mentioned previously, location of APP within the cell plays a role in the type of processing that will occur and thus the production of Aβ. To further my investigation, I compare the wild type APP against the Swedish mutated APP in regards to distribution and release of proteolytic products (including Aβ1-40 and Aβ1-42) to determine possible modifications in the presence of HspB1.

1.9 Hypothesis

HspB1 interacts with Aβ or its precursor APP and through this interaction is able to alter APP processing and hence the distribution/release of toxic Aβ peptide.

1.10 Objective

Investigate the correlation between HspB1 and APP/Aβ

HspB1 and Aβ (1-42)

Objective 1: Is there an interaction between Aβ and HspB1?

Objective 2: Can we manipulate the relationship between HspB1 and Aβ to decrease the amount of released Aβ?

HspB1 and APP

Objective 3: Is there an interaction between HspB1 and APP?

Objective 4: Can we manipulate the relationship between HspB1 and APP to decrease the amount of Aβ production?

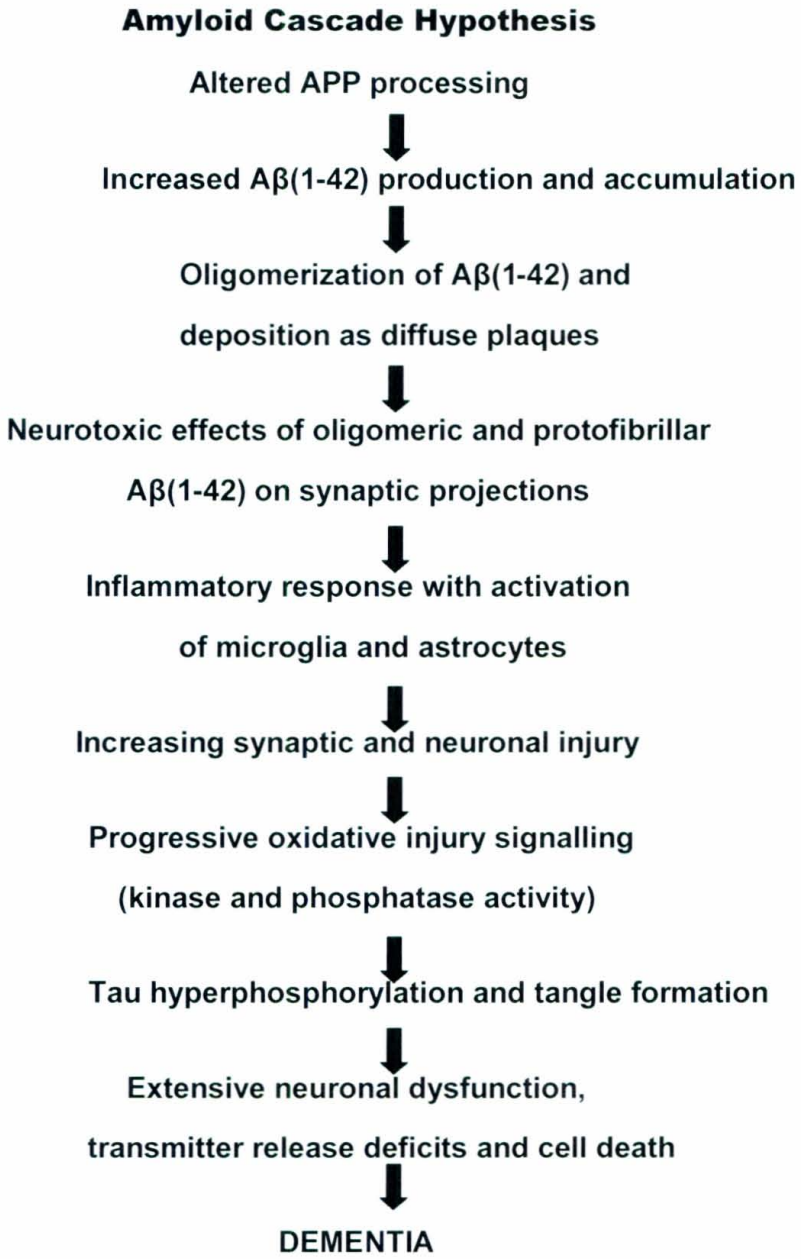


Figure 1.1 Proposed Amyloid Cascade Hypothesis (From (J. Hardy & Selkoe, 2002). Reprinted with permission from AAAS.

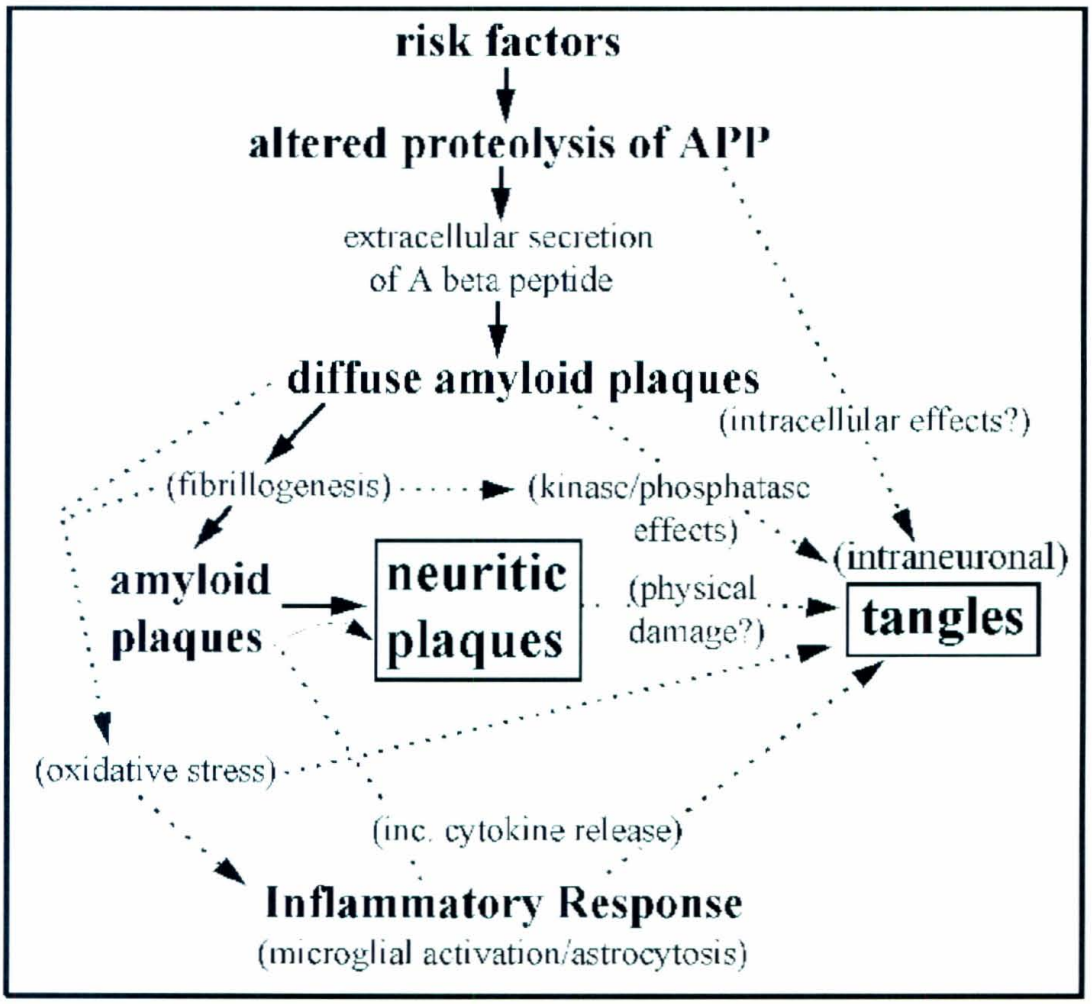


Figure 1.2 Schematic of the main hypotheses implicated in AD pathology and their interactions or dependence on each component for AD development.

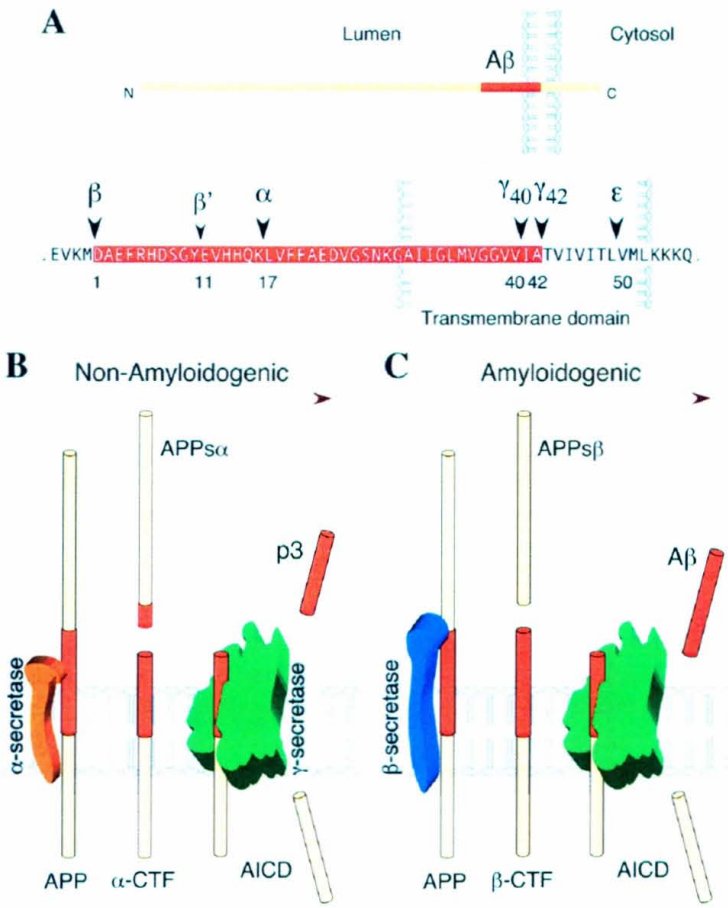


Figure 1.3 Schematic of APP showing the (A) Aβ peptide within the transmembrane region along with the secretase enzyme cleavage sites. Notice the altered γ-secretase cleavage site used to produce Aβ (1-42) which is up-regulated in the APP-swe mutation. APP processing occurs through (B) the non-amyloidogenic (α-secretase) pathway eluding the formation of Aβ or (C) the amyloidogenic (β-secretase) pathway favouring the formation of the Aβ peptide. From (Thinakaran & Koo, 2008). Reprinted with permission from ASBMB.

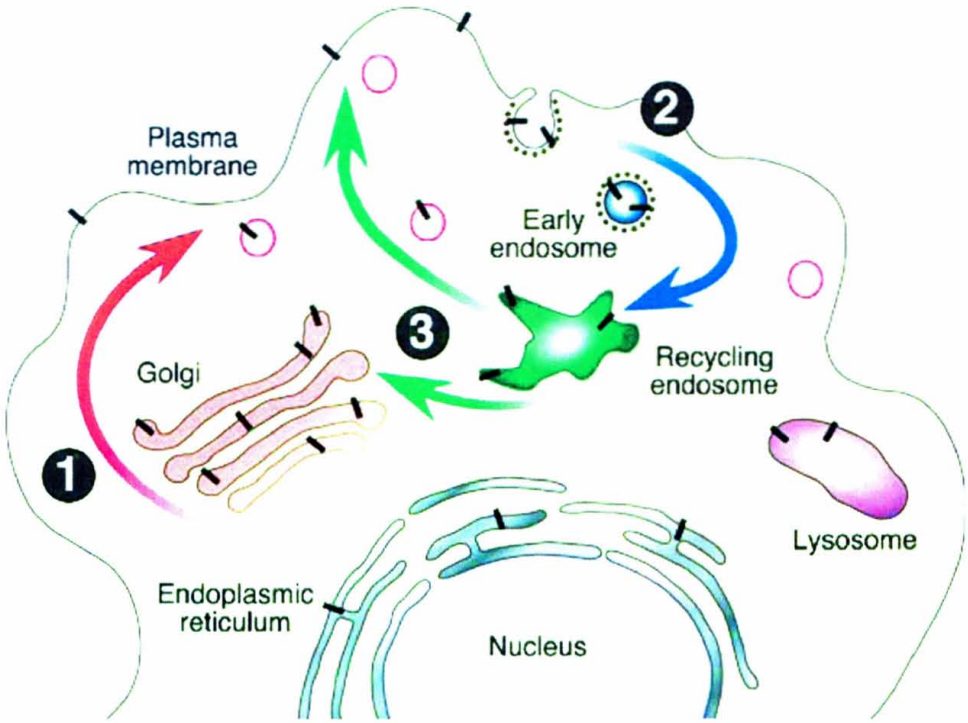


Figure 1.4 Schematic of cellular trafficking of APP from maturation within the golgi to transport to the plasma membrane via the secretory pathway and continuing through recycling into the endocytic/lysosomal pathway for degradation. From (Thinakaran & Koo, 2008). Reprinted with permission from ASBMB.

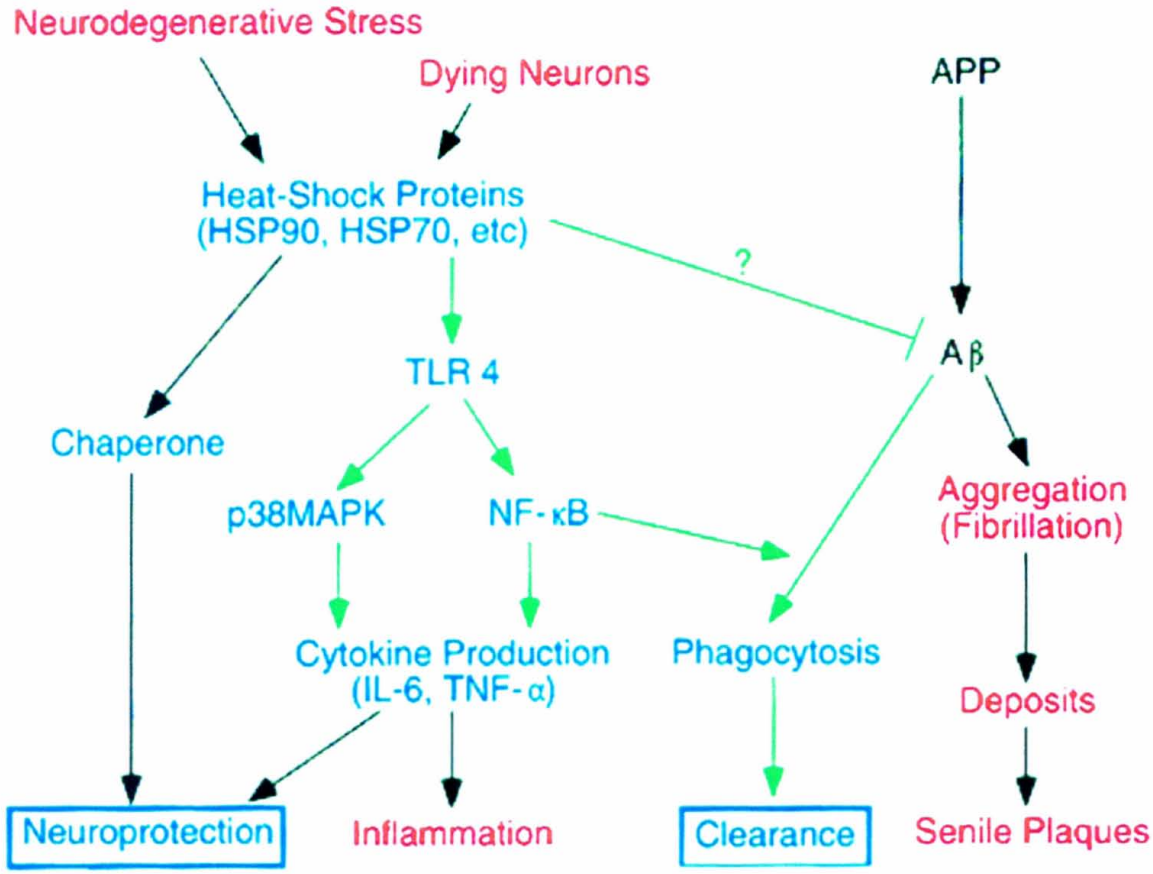


Figure 1.5 Schematic of possible Hsp effects on neurodegenerative disease.

Table 1. Identified functions of APP products

Product	Function	Binding Partners	References
sAPP	Neurite outgrowth Synaptogenesis Synaptic plasticity Neuronal excitability Regulator of neuronal stem cell division	Undefined	(Mattson, 1997) (S. A. Small & Gandy, 2006; D. H. Small et al., 1999) (Caille, Allinquant, Dupont, Bouillot, Langer, Muller, & Prochiantz, 2004b; P. R. Turner et al., 2003)
Aβ	Surface receptor Cell adhesion Neurite outgrowth Regulator of C2+ homeostasis	ApoE ECM Undefined	(LaFerla, 2002; Mattson, 1997; D. H. Small et al., 1999; P. R. Turner et al., 2003)
AICD	Axonal transport cargo receptor Kinase mediated signalling Gene transcription	Kinesin, JIP Shc/Grb2, JIP Fe65, JIP, Numb	(Bao et al., 2007; G. D. King & Scott, 2004; Lazarov et al., 2005; Tarr, Roncarati, Pelicci, Pelicci, & D'Adamio, 2002; Taru et al., 2002)
CTF	Pre-mRNA splicing	snRNP	(Muresan & Muresan, 2004)

Chapter 2

Materials and Methods

2.1 Cell Culture

Naïve Human Embryonic Kidney 293 (HEK293) and stable cell lines of wild-type Amyloid Precursor Protein (APPwt) and mutated swedish APP (APPswe) (a kind gift from Dr. Paul Fraser – University of Toronto) were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 1% pen-strep/L-glutamine (P/S/G), 10% Fetal Bovine Serum (FCS) and 400 µg Geneticin Disulfate (G418, Gibco, Invitrogen, CA), to 80% confluency in 10ml petri dishes. G418 supplementation was used only for stable cell lines and stopped upon initiation of experimental procedures. Cells were incubated at 37°C with 5% CO_2 supplementation.

2.2 Plasmids and Transfection

As shown in Fig. 2.3 Experimental model, I performed experiments as follows: i) HEK 293 naïve cells co-transfected with APPwt or APPswe construct, pmaxGFP (positive control vector with enhanced green fluorescent protein) (VSC-1001, Lonza, Basel
Switzerland) and pEGFP-C2-HaHspB1-WT fusion protein which has been previously shown to be functional in PC12 cells (Mearow et al., 2002) or the control empty vector (EVC2) and ii) APPwt and APPswe HEK 293 stable cell lines transfected with pCIG-HaHspB1-WT-IRES-EGFP construct. These experimental models were initially used

interchangeably, however, upon experimentation it was noted the large EGFP tag fused to the HspB1-Wt protein may cause interference in the normal metabolism of the HspB1 protein thus affecting the results obtained. In addition, interference in results may occur from the stress of over-expressing two proteins (HspB1-Wt and APP) by transfection within the cell. Therefore, to address these discrepancies, the APPwt/swe HEK 293 stable cell lines transfected with the smaller pCIG-HaHspB1-WT-IRES-EGFP construct was used for the majority of the experimentation unless otherwise noted.

Confluent HEK293 cells, APPwt and APPswe stable cell lines were transiently transfected in low serum media using Lipofectamine 2000^{TM} (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocol. Briefly, 20 µl Lipofectamine 2000 reagent was added to an aliquot of 500 µl Opti-Mem Reduced Serum media (Gibco Invitrogen, Carlsbad, CA, USA) while 8.0 µg DNA was added to a second aliquot containing 500 µl Opti-Mem Reduced Serum media and incubated for 5 min. Since the APPwt and APPswe constructs are not tagged proteins, it is impossible to visualize the transfection efficiency of these proteins in comparison to the tagged HspB1-Wt that was transfected. To overcome this issue, I added pmaxGFP to the APP co-transfection reaction in a ratio of 1:4 since the pmaxGFP is transfected at a much lower concentration than APP. Therefore, if 2 µl of pmaxGFP yield, 50% transfection efficiency then I can assume 8 µl of APP will give at least 50% transfection efficiency. The Lipofectamine solution was added to the DNA slowly and incubated for 20 min upon which the solution was added to the HEK293 cells. Media was changed 4-6 hrs post transfection from the reduced serum media to media containing DMEM with 1% P/S/G, 0.5% FCS, 0.5 X B-27 (Gibco,

Invitrogen, Carlsbad, CA, USA) supplement and the Aβ degradation inhibitor 0.1 % phosphoramidon disodium salt (17.7 mM, Sigma-Aldrich, Oakville, Ont., Canada).

2.3 Protein and Conditioned Media Collection

Cell lysate and cellular conditioned medium were collected 24 and 48 hrs post medium change. Cellular conditioned medium was collected on ice with addition of protease inhibitor cocktail tablet (Roche Diagnostics, Laval, Quebec, Canada) immediately upon collection. Media was centrifuged at 14,000 g for 5 min at 4°C to discard any cell debris. HEK293 cells were collected by adding 1 ml ice cold TBS with 200 mM sodium vanadate and scraping cells off the plate with a rubber policeman. Cells were pelleted for 5 min at 4000 g at 4°C and re-suspended in ice cold protein lysis buffer (1% NP40, 10% glycerol, O-β-thioglucopyranoside, protease inhibitor tablet, 200 mM sodium vanadate, sodium fluoride and magnesium chloride in TBS) and stored at -20°C until experimentation.

2.4 Western Blot Analysis

Western blot analysis was performed with samples of total cellular lysate and cellular conditioned medium. Samples were thawed on ice and centrifuged at 14,000 g for 10 min at 4°C and supernatants were used to determine protein concentrations using a BSA protein assay kit (Pierce Chemicals, Rockford, IL, USA). Laemmli sample buffer (10% SDS, glycerol, 1M Tris pH 6.8, dH_20, 0.01% Bromophenol blue) containing fresh β-mercaptoethanol (BME) was added to 50 µg of either cellular lysate or cellular

conditioned media protein, boiled and separated on an pre-cast 4-20% gradient Tris-glycine gel using the X-Cell Surelock System (Invitrogen, Carlsbad, CA, USA) at 120V, 300 A for ~1.5 hr. Protein from gel was then transferred to a nitrocellulose membrane at 100V for 1 hr at 4°C using a transfer tank and transfer buffer (1M Tris base, glycine, dH_2O, MeOH). Western blots were stained with Ponceau Red for assessment of equivalent protein loading. Blots were washed with TBS-T (1M Tris base, 2.5M NaCl, 50% Tween) to remove Ponceau Red stain and blocked with either 3% milk or BSA depending on the primary antibody dilution conditions for 1 hr to prevent non-specific binding. Once blocked, blots were incubated with the following antibodies overnight at 4°C on a shaking platform: anti-APP 22C11 mouse monoclonal directed against the N-terminus regions of APP (Millipore, Billerica, MA, USA); 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA); C-terminal anti-APP mouse monoclonal (Zymed, Carlsbad, CA, USA); anti-Hsp25 rodent (SPA-801) rabbit polyclonal (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA); anti-sAPPβ-swe mouse monoclonal (IBL, Gunma, Japan); anti-actin rabbit polyclonal (Sigma-Aldrich, Saint Louis, MI, USA); all antibodies were used at a concentration of 1:1000. Blots were subsequently washed with TBS-T 3x15 min for a total of 45 min on shaking platform. Protein was detected using horseradish peroxidase (HRP) labelled secondary antibodies (1:5000 - 1:10000 in 3% milk) and Super Signal West Pico chemiluminescence substrate (ECL; Thermo Scientific, Rockford, IL, USA) for 5 min and exposed to X-ray film. Densitometry analysis was performed using ImageJ software and images prepared with Adobe Photoshop graphics software.

2.5 Co-Immunoprecipitation

Synthetic Aβ (1-42) peptide (1 µg, r-peptide, Bogart, GA, USA) was incubated with recombinant human his-tagged HspB1 (2 µg, Stressmarq Biosciences Inc., Victoria, BC, Canada) in 500 µl of media (DMEM, 1% P/S/G, 0.5% FCS, 0.5 X B27 supplement, phosphoramidon) on a nutator at 37°C overnight. The following day, protein A/G PLUS-agarose beads (40 µl per sample, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were equilibrated with the above described media and 20 µl were added to his-HspB1/ Aβ (1-42) sample on rotation for 1 hr at 4°C. Sample was centrifuged at 3000g for 5 min to pellet beads and supernatant was placed in a fresh centrifuge tube. 3 µl of anti-HspB1 human (SPA-803) rabbit polyclonal (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) was then added to supernatant to detect the human recombinant his-HspB1 protein on rotation at 4°C for 1 hr followed by addition of 20 µl beads overnight to co-immunoprecipitate any Aβ(1-42) and his-HspB1 complexes that formed. The above protocol was repeated adding the 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA) antibody to the supernatant to detect the synthetic Aβ (1-42) peptide portion of co-immunoprecipitated Aβ(1-42) and his-HspB1 complexes. Immunprecipitation of human recombinant his-HspB1 using the anti-HspB1 human (SPA-803) rabbit polyclonal antibody (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) and synthetic Aβ (1-42) using 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA) antibody were used as positive controls. All samples were centrifuged at 3000 g for 5 min to separate co-IP

(A/G beads, antibody and any protein complexes attached) from supernatant with co-IP samples subsequently washed twice with DMEM to rid the sample of non specific binding. The co-IP sample was resuspended with 40 µl of 2X laemmli sample buffer with fresh DTT and electrophoresed as per western blot protocol. Blots were probed with 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA) and anti-HspB1 human (SPA-803) rabbit polyclonal (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) primary antibodies.

Alternate co-IP experimentation involving cellular conditioned media were performed similarly to the above protocol with the exception of the sample preparation steps. Initially, samples were thawed and centrifuged at 14,000 g for 10 min. Protein concentration was determined and 200 µg of protein was used for co-IP experimentation. Magnetic A/G beads were used to replace the agarose A/G beads due to magnetic beads being easier to use and thus more efficient. Three samples were electrophoresed by western blot including a crude sample "C" consisting of the beads from the initial equilibration incubation which contain any non-specific binding to the beads before antibodies were added to the reaction, a supernatant "S" sample consisting of the discarded sample left after centrifugation of the IP and finally the co-IP sample "IP" consisting of the beads and bound his-HspB1/sAPP complexes as denoted in Figure 3.10. Remaining steps were as per above protocol.

2.6 ELISA

Invitrogen Human Aβ40 and Aβ42 ELISA kits were used to quantify the amounts of Human Aβ40 and Human Aβ42 in cellular lysate and cellular conditioned medium of APPwt and APPswe, transfected with or without pCIG-HaHspB1-WT-IRES-EGFP. Cellular lysate and cellular conditioned medium was collected 24 and 48 hrs post transfection with pCIG-HaHspB1-WT-IRES-EGFP on ice. Cellular lysate was collected and resuspended with lysis buffer (1% NP40, 10% glycerol, O-β thioglucopyranoside, protease inhibitor tablet, 200 mM sodium vanadate, sodium fluoride and magnesium chloride in TBS) and cellular conditioned media was collected with protease inhibitor cocktail tablet (Roche Diagnostics, Laval, Quebec, Canada) added immediately upon collection as described in section 2.3. Media was centrifuged at 14,000 g for 5 min at 4°C to discard any cell debris. Collected cellular lysate and cellular conditioned media was subjected to protein concentrations using a BSA protein assay kit (Pierce Chemicals, Rockford, IL, USA). Since 5 ml of cellular conditioned media was collected per 100 ml dish yet only 110 µl per well was required for the ELISA kit, collected media was concentrated via a 3 kDa nanosep centrifugal device (Sigma-Aldrich, Oakville, Ont., Canada). Through the nanosep omega filter, the sample is concentrated by centrifugal force allowing biomolecules larger than the cutoff of the membrane (>3 kDa) to be retained in the sample reservoir. Solvent and lower molecular weight molecules (<3 kDa) pass through into filtrate. This allowed a 500 µl sample of cellular conditioned media to be concentrated to 50 µl without loss of Aβ peptide (4 kDa). Samples were diluted in the dilution buffer provided with the ELISA kit in a 70:30 dilution as per

manufacturer's protocol. Media containing DMEM with 1% P/S/G, 0.5% FCS, 0.5X B-27 supplement (Gibco, Invitrogen, Carlsbad, CA, USA) and the Aβ degradation inhibitor 0.1 % phosphoramidon disodium salt (17.7 mM, Sigma-Aldrich, Oakville, Ont., Canada) was diluted in a 70:30 ratio with the provided dilution buffer and used as the negative control for the conditioned media while lysis buffer diluted 70:30 with the provided dilution buffer was used for the cellular lysate negative control. Aβ (1-40) and Aβ (1-42) standards were provided with the kit and diluted with 70:30 media/dilution buffer in serial dilutions from 100,000 pg/ml - 7.81 pg/ml. Standards of 7.81pg/ml – 250 pg/ml were measured with up to 125 pg/ml used to construct the standard curve. 1mM of the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) was added to each standard and sample as recommended by the manufacturer due to the ability of serine proteases to degrade Aβ peptides. ELISA's were performed as per manufacturer's protocol through colometric assay. Upon 3 hr incubation with a Human Aβ (1-40/1-42) detection antibody followed by detection using the supplied anti-rabbit IgG HRP antibody, addition of stabilized chromogen coloured the samples blue reflective of the amount of Aβ present. A reaction stop solution changed the colometric blue to yellow to signify the cessation of the assay. Absorbance of each well was read at 450nm. ELISA Aβ concentrations were lower than reported results (Figure 3.8). An explanation for this discrepancy in values may be attributed to the masking of Aβ by BSA found in the FCS present in the media as select protocols call for formic acid extraction to measure Aβ amounts (Sweeney, Darker, Neville, Humphries, & Camilleri, 1993). Aβ extraction was not carried out in my experimentation however FCS was decreased as much as possible

to allow for cell viability and concentrated media Aβ values were similar to Aβ values in non-concentrated media. Although the amount of BSA in the media was lowered as much as possible to still ensure cell viability, presence may have altered the Aβ results negatively. Alternatively, minimal amounts of monomeric Aβ present in the media may have passed through the nanosep into the discarded filtrate. Given the high volume of media that was collected per sample plate to the expected low percentage of Aβ secreted by the cells in comparison to total secreted protein, a sample of 500 µl may not be an appropriate sampling to obtain a high concentration of Aβ since theoretically only 10% of secreted Aβ would be collected by this method. Therefore using smaller surface area plates such as 6-well plates would allow for a smaller sample of cellular conditioned media to be collected yet a more Aβ concentrated sample.

2.7 Immunocytochemistry

HEK293 cells were plated at 1.5 x 10^6 on poly-lysine coated 16-well Lab-Tek® chamber slides (Lab-Tek®). Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, washed with PBS and permeabilized with 0.1% Triton X and blocked with 10% Horse Serum for 1 hr. Primary antibodies added included anti-APP rabbit polyclonal (1:100, Abcam 15272, Cambridge, MA, USA); 6E10 β-amyloid (1-16) mouse monoclonal (1:200, Covance, Emeryville, CA, USA); C-terminal anti-APP mouse monoclonal (1:100, Zymed, Invitrogen, Carlsbad, CA, USA); anti-Golgi mouse monoclonal (1:100, 27043 Abcam, Cambridge, MA, USA); Rab 5 rabbit polyclonal (early endosome, 1:100, 21435 Cell Signalling, New England, USA); Rab 9 rabbit

polyclonal (late endosome, 1:100, 51185 Cell Signalling, New England, USA); anti-LAMP-1 (H4A3) mouse monoclonal (1:100, Hybridoma Cell Bank) anti-Hsp25 (SPA-801) rabbit polyclonal (1:100, Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA); anti-HspB1 goat polyclonal (1:100, Santa Cruz Biotechnology, Santa Cruz CA, USA) . Cells were incubated in primary antibodies overnight (~ 20 hr) at 4°C, rinsed with PBS and incubated with secondary antibodies for 1 hr in the dark. Secondary antibodies included: Alexa Fluor®555 goat anti-mouse IgG (H&L) (1:250, Invitrogen, Carlsbad, CA, USA), Dyelight™ 549-conjugated AffiniPure donkey anti-mouse IgG and Dyelight 649-conjugate AffiniPure donkey anti-rabbit (1:250, Jackson Laboratories, West Grove, PA, USA); Cy 2-conjugate bovine anti-goat (1:250, Jackson Laboratories, West Grove, PA, USA). Cells were rinsed with TBS-½T (Tris-buffered saline with 0.25% Tween) and nuclear stained with DAPI (Sigma–Aldrich, Steinheim,CH) in TBS for 5 min. Cells were again washed with TBS-½T and cover-slipped using polyvinyl alcohol mounting medium with DABCO® (Sigma–Aldrich, Steinheim,CH). Images were obtained in four channels (405, 488, 549, 647 nm) using confocal scanning microscopy with sequential Z-stage scanning (Olympus Fluoview 1000 microscope).

2.8 Co-localization Analysis

Co-localization analysis was performed on confocal optical z-stacked sections using Imaris 6.1.5 with Imaris Co-localization module (Bitplane) to examine the co-localization of the brightest 2% of pixels in each channel (protein of interest) by using the

computer generated automatic thresholding option. This allows an unbiased threshold to be set for each protein channel ruling out problems caused by variance between cell-to-cell expression and image brightness/exposure thus allowing direct comparison between cells/experiments. When analysing intracellular locality, the masking option was used to select only those pixels corresponding to the intracellular compartment channel. For example, the Golgi channel was masked to identify APP isolated within the golgi compartment therefore excluding the remainder of the cell. To further this, co-localization analysis was calculated on a user determined specific region of interest (ROI). The ROI was used to isolate each cell individually for co-localization measurement. Together, this allows the user to zoom in and isolate a specific region such as the golgi (through masking) within a specific cell (through ROI) and measure only the staining found localized within that region. An average of 5 images composed of 4 cells in each totalling 20 cells per experiment were analysed for co-localization. Values represent co-localization in the set ROI. Graphing and statistical analysis was performed using GraphPad Prism 4.0 using two-way ANOVA with Bonferroni post-test. Statistical significance was determined as * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$. The range of co-localization was determined using the Pearson's coefficient reference guide which set the limits as follows: None < 0.09; Small 0.1-0.3; Medium 0.3-0.5; Large >0.5.

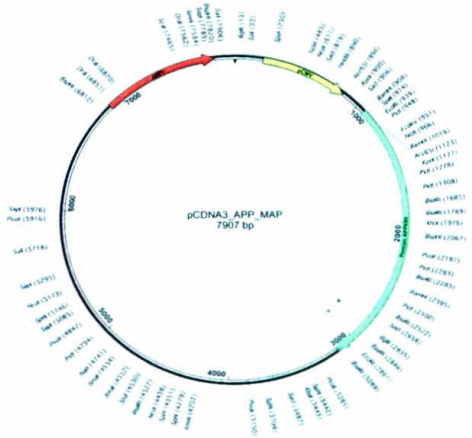
2.9 Statistical analysis

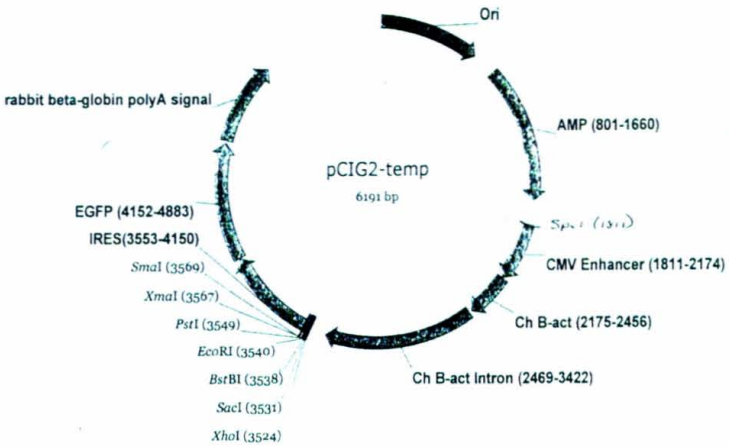
Statistical analysis was performed in GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA). Figures are shown with mean values $\pm$ SEM with

significance determined by either one-way ANOVA testing followed by Tukey post-hoc tests, two-way ANOVA followed by Bonferroni post tests or t-tests to compare two groups, for example +/- HspB1. Significance was determined when $p < 0.05$ unless otherwise stated.

3.0 Constructs

Constructs employed for transfection of HEK293 were: pEGFP-C2-HaHspB1-WT with subsequent EVC2 (empty vector) used as a control (Mearow et al., 2002; Williams et al., 2006) or pCIG-HaHspB1-Wt-IRES-EGFP and APPwt or APPswe in combination with pmaxGFP.




Ori
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CMV Enhancer (1811-2174)
Ch B-act (2175-2456)
Ch B-act Intron (2469-3422)
rabbit beta-globin polyA signal
EGFP (4152-4883)
IRES(3553-4150)
SmaI (3569)
XmaI (3567)
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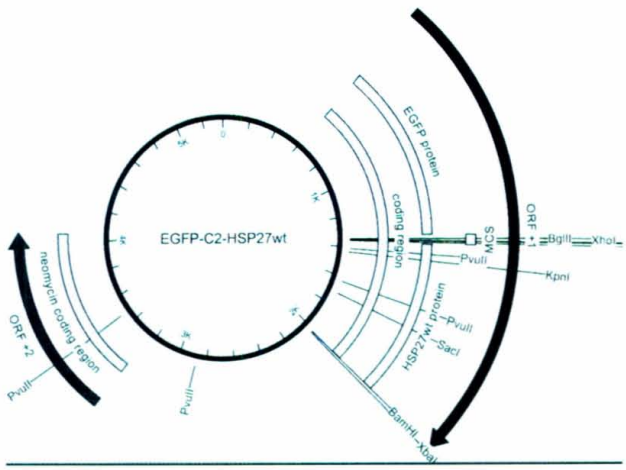
BstBI (3538)
SacI (3531)
XhoI (3524)
pCIG2-temp
6191 bp


EGFP-C2-HSP27wt
EGFP protein coding region
HSP27wt protein
neomycin coding region
ORF +1
ORF +2
SV40 pA
BglII
XhoI
PvuII
KpnI
SacI
BamHI
XbaI

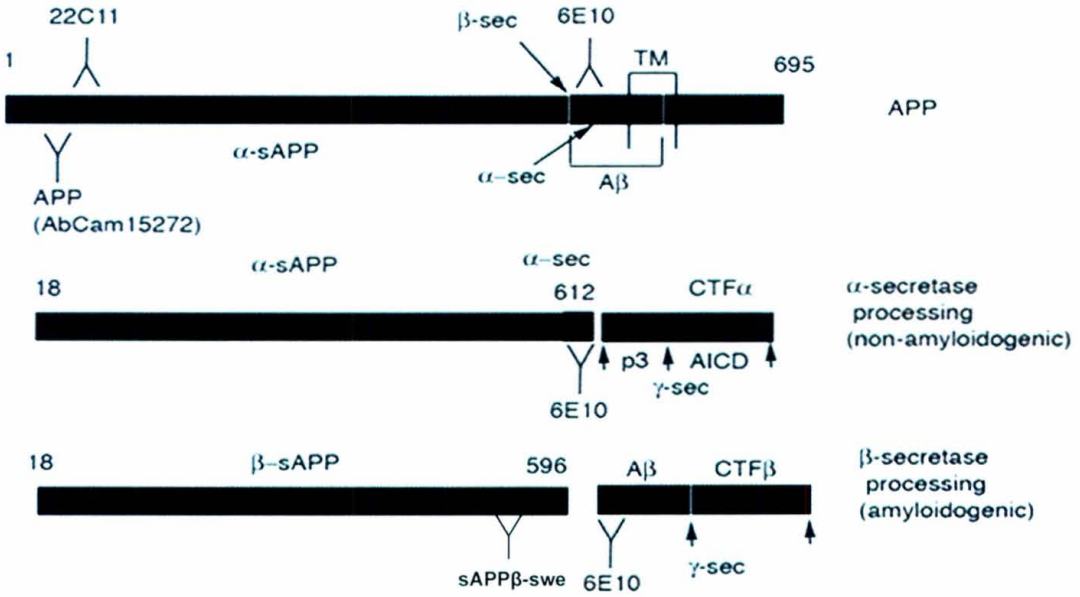


Figure 2.2 Schematic of APP antibody recognition sites. Standard APP antibodies were employed including the anti-APP rabbit polyclonal (Abcam 15272, Cambridge, MA, USA) recognizing the N-terminus of the APP protein, 22C11 mouse monoclonal (Millipore, Billerica, MA, USA) antibody directed against the N-terminus of APP and thus recognizing the soluble forms of APP as well, the 6E10 β-amyloid (1-16) mouse monoclonal antibody (Covance, Emeryville, CA, USA) which will also detect full length APP and soluble αAPP and finally the C-terminal anti-APP mouse monoclonal (Zymed, Invitrogen, Carlsbad, CA, USA) antibody which will detect the C-terminal fragment along with full length APP. I also employed the anti-sAPPβ-swe (6A1) mouse monoclonal (IBL, Gunma, Japan) which specifically detects the soluble βAPP-swe fragment.

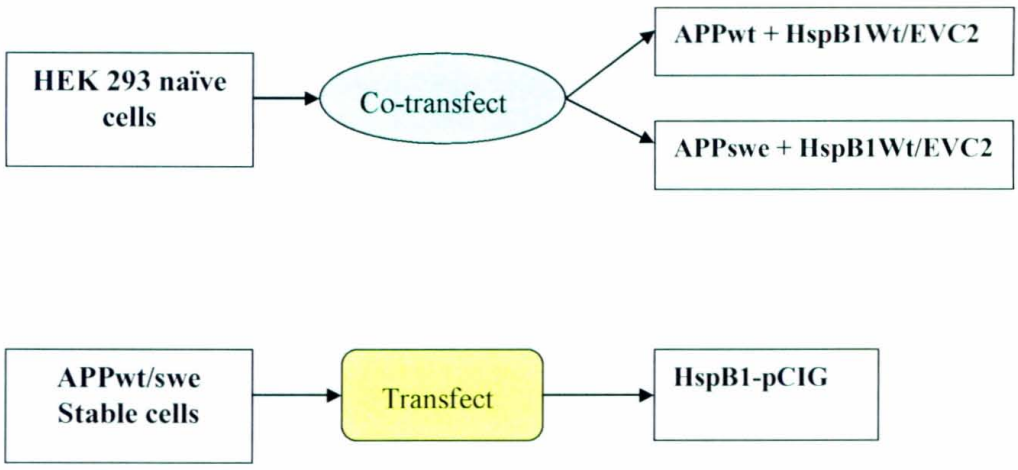


Figure 2.3 Experimental Models. HEK 293 naïve cells were co-transfected with APPwt or APPswe plasmid along with HspB1-Wt or Empty vector (EVC2). Alternatively, APPwt and APPswe HEK 293 stable cell lines were transfected with the smaller HspB1-pCIG construct.

Chapter 3

Results

HspB1 has been localized in 15% of the Aβ senile plaques associated with AD (Wilhelmus et al., 2006). In addition, work from our lab has shown HspB1 exogenously expressed in cortical neurons ameliorates cell death caused by Aβ (1-42) (M. King et al., 2009). More widely studied members of the heat shock protein family, HSP70 and 90 have also been shown to inhibit early stages of Aβ (1-42) aggregation and this effect was thought to be chaperone based (Evans, Wisen, & Gestwicki, 2006).

Previous research has shown that while APP is constitutively expressed in cell types, HspB1 may not operate under detectable endogenous levels in all cell types including cortical neurons (M. King et al., 2009). HEK 293 cells are a human cell line that expresses human HspB1 although in relatively low levels in non-stressed conditions. Due to the availability of the HEK 293 APPwt and APPswe stable cell lines and their ease of transfection yielding an efficiency of 70-80%, I opted to use the HEK 293 cells as my experimental model. HEK 293 cells proved to be a reliable model as they allowed for the analysis of transfected rodent HspB1 without endogenous human HspB1 interference. HEK 293 cells have been used in APP processing and Aβ studies previously (Belyaev, Kellett, Beckett, Makova, Revett, Nalivaeva, Hooper, & Turner, 2010b; Citron et al., 1992; Henriques et al., 2009; Steinhilb, Turner, & Gaut, 2001; Steinhilb, Turner, & Gaut, 2002; Sullivan et al., 2011). Consequently, my first aim was to investigate both the

endogenous and exogenous expression of HspB1 and APP/Aβ in naive, stable and transfected HEK 293 cells.

3.1 Endogenous/exogenous expression of HspB1, APPwt and APPswe in HEK 293 naïve cells

To examine the expression of HspB1 and APP in HEK 293 cells in culture, naive and APP stable cell lines with and without HspB1 transfection were plated on collagen coated 100 ml dishes and grown to 80% confluency. Protein lysates were extracted for Western blot analysis. Figure 3.1 shows the transfection efficiency of co-transfected APPwt, APPswe and EGFP-HspB1-Wt in naïve HEK 293 cells. HEK 293 cells were co-transfected with either APPwt or APPswe and HspB1-Wt fusion protein or EVC2 (empty vector). A transfection efficiency of 70-80% was observed when APP or HspB1 were singly or co-transfected. Column 1 displays HEK 293 cells under transmitted light (TL) to show the total number of plated cells. Column 2 displays the transfected cells (green) under the fluorescence filter (FL). EGFP-HspB1-Wt appears green due to the fused EGFP tag. APP transfection is shown indirectly by observing the green of the pmaxGFP construct which was added to the transfection in a 1:4 ratio to the APP construct. Column 3 is a merged image showing the high ratio of transfected to non-transfected cells (Merge). Expression of co-transfected APPwt, APPswe and HspB1-Wt fusion protein in naïve HEK 293 cells is shown in Figure 3.2 through western blot. Transfection of EVC2 alone was used as a negative control while single transfections of each HspB1-

Wt, APPwt and APPswe were used as positive controls (lanes 2-4). APPwt, APPswe and HspB1-Wt are expressed well when co-transfected. Endogenous levels of HspB1 are undetectable as HspB1 is only observed in HEK 293 cells transfected with the rodent HspB1 construct and probed with the rodent selective anti-HspB1 (801). The EGFP tag has a molecular weight of 29 kDa similar to the HspB1 protein (27 kDa) causing the fused protein to appear at the 56 kDa molecular weight. To further confirm the ability to co-transfect and thus exogenously express both the APPwt/swe constructs and HspB1-Wt, I employed immunocytochemistry (Figure 3.3). Cells were stained with the anti-Aβ 6E10 (1-16) antibody shown in red which is a standard antibody used for detection of not only Aβ but also souble APP alpha and full-length APP. HspB1 expression is evident in green due to the fused EGFP tag on the transfected HspB1 construct. A merged column is shown to identify the cells expressing both proteins. Cells expressing both APP and HspB1 proteins (white arrows) do not appear to show increased APP staining (red) in the presence of HspB1 (green) in comparison to cells not transfected with HspB1 (arrow heads).

3.2 Soluble APP (sAPP) is secreted into the cellular conditioned media of co-transfected HEK 293 cells.

The detrimental effects of Aβ and SPs on neuronal cells is believed to occur extracellularly therefore it was pertinent to determine if detectable levels of sAPP were being released into the media of HEK 293 cells. Cellular lysate and cellular conditioned media from HEK 293 cells transfected with the APPwt and APPswe constructs were

collected over 12-72 hrs and analysed through Western blotting (Figure 3.4). Immediately following transfer, membranes were stained with ponceau red as a protein loading control (bottom panels). An increase in both expression (A-cellular lysate) of APPwt and APPswe and secretion of sAPPwt and sAPPswe (B-conditioned media) was observed when probed with anti-Aβ 6E10 (1-16) antibody. Detection with rodent anti-HspB1 801 antibody reveals undetectable endogenous HspB1 levels as a negative control (Fig. 3.4).

3.3 Endogenous/exogenous expression of HspB1 in HEK 293 APPwt and APPswe stable cells

Transfection of pCIG-HspB1 in APPwt and APPswe stable cell lines is shown in Figure 3.5. Endogenous levels of APP are detected in control lanes 1-2 since the anti-Aβ 6E10 (1-16) antibody recognizes human APP from the human cell line however a clear over-expression of APP is evident in the stable cell lines (lanes 3-6). pCIG-HspB1 is only detectable in transfected samples (lanes 2,4,6) due to the specificity of the anti-HspB1 antibody 801 for rodent HspB1. Ponceau red staining used as an indicator of protein loading displays equal amounts of protein across samples confirming that differences observed on the western blots are due to differences in protein expression and not discrepancies in protein loading. Expression of HEK 293 APPwt and APPswe stable cell lines transfected with pCIG-HspB1 was further confirmed by immunocytochemistry (Figure 3.6). Unlike the fused EGFP-HspB1-Wt protein, positive staining for EGFP in cells transfected with pCIG-HspB1 does not necessarily correlate to positive HspB1

expression therefore staining for HspB1 using the rodent anti-HspB1 801 antibody was required. As evident in the EGFP (green) and HspB1 (red) images, cells were positive for both signifying a successful transfection and working construct. APP staining was performed using the anti-Aβ 6E10 (1-16) antibody (cyan). Cells expressing both APP and HspB1 (white arrows) may show slightly increased APP staining (6E10-cyan) in the presence of HspB1 (red) in comparison to cells not transfected with HspB1 (arrow heads) however this is difficult to determine from the low magnification and densely populated cells.

APP stable cells transfected with pCIG-HspB1 appear to express higher levels of APP than the APPwt and APPswe stable cells alone (Fig. 3.5). This is not seen in the 293 + APPwt/APPswe + HspB1-Wt co-transfected samples (Fig. 3.2) leading to the question whether the large EGFP tag on the HspB1-Wt fusion protein is interfering in normal cellular protein metabolism.

3.4 Absence of Aβ expression in cellular lysate of APPwt and APPswe stable cell lines

Figure 3.7 Western blot of cellular lysate shows APP expression in the APPwt and APPswe stable cells in comparison to naive HEK293 cells. As expected, APPwt and APPswe stable cell lines expressed much higher levels of APP compared to HEK 293 cells as seen in Figure 3.5. Synthetic Aβ (1-42) peptide was used as a positive control for Aβ however Aβ levels were undetectable through Western blot in the cellular lysate. While failure to detect Aβ in the cellular lysate may seem to question the reliability of the HEK 293 cells as a model, it should be noted that much controversy exists in the Aβ field

as to whether the Aβ fragment exists intracellularly or only as a secreted peptide. Many investigators believe previous intracellular staining by 6E10 of what was thought to be Aβ may actually be the Aβ fragment within the uncleaved APP protein and not free Aβ as our technology for visualization of cellular components advances {{294 Winton, M.J. 2011}}. If this is the case, a high presence of the 4kDa Aβ peptide would not be expected in the cellular lysate.

3.5 Increased secretion of Aβ (1-42) in cellular conditioned media after 48 hrs is decreased in the presence of HspB1

Due to undetectable levels of Aβ in cellular lysate via Western blot and the present controversy surrounding its presence intracellularly, I turned my attention to the more sensitive ELISA. ELISA kits specific for each Aβ (1-40) and Aβ (1-42) were purchased from Invitrogen. ELISA of Aβ (1-42) was first carried out on 24 and 48 hr cell lysate of stable APPwt and APPswe cell lines transfected with HspB1-pCIG. Aβ (1-42) was undetectable in cellular lysate samples. This experiment was carried out once in duplicate therefore further experimentation is needed to conclude the absence of Aβ within cellular lysate however coupled with the negative western blot data I decided against additional lysate testing for Aβ. Next, cellular conditioned media from 24 and 48 hr stable APPwt and APPswe samples transfected with HspB1-pCIG was measured for secreted Aβ (1-40) and (1-42) levels. Results from three biological samples in duplicate were statistically analysed as shown in Figure 3.8. APPwt cell media displayed increased levels of Aβ (1-40) over Aβ (1-42) suggesting that the APPwt cells are undergoing non-

amyloidogenic processing more readily than amyloidogenic processing as expected. Aβ (1-40) levels remained fairly constant over 48 hrs in both APPwt and APPswe cell media with and without HspB1 expression suggesting that HspB1 does not have an effect on the non-amyloidogenic Aβ (1-40). In contrast, Aβ (1-42) protein levels show a significant increase after 48 hrs in the APPswe cell media as expected, yet this increase is not observed when HspB1 is present. These results indicate HspB1 may have an effect on the levels of Aβ (1-42) being released into the media, thus I explored a plausible relationship between HspB1 and Aβ (1-42). Statistical analysis was performed using a two-way Anova with Bonferroni post-hoc test, n=3; f=0.04 and p=0.9870 (Aβ (1-40)) and n=3; f=4.49; p=0.0181 (Aβ (1-42)).

3.6 Synthetic Aβ (1-42) peptide and recombinant His-HspB1 peptide co-immunoprecipitate.

To investigate a relationship between HspB1 and Aβ (1-42), I employed a co-immunoprecipitation technique. Through incubation of human recombinant his-HspB1 protein and synthetic Aβ (1-42) peptide with antibody-bound magnetic A/G beads, I was able to demonstrate that human recombinant his-HspB1 and synthetic Aβ (1-42) co-immunoprecipitate, suggesting a direct interaction. The anti-Aβ 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA) was used for the IP and the blot probed with the anti-HspB1 human (SPA-803) rabbit polyclonal (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) and vice versa (Figure

3.9) through Western blot analysis to confirm an interaction. If HspB1 is able to directly bind to Aβ (1-42), it may be able to bind to APP. This led me to consider a potential relationship with HspB1 and the full length APP.

3.7 Recombinant His-HspB1 co-immunoprecipitates with soluble APP alpha

I repeated the co-immunoprecipitation technique this time using 48 hr sample of cellular conditioned media from stable APPwt and APPswe cell lines incubated with human recombinant his-HspB1. Similar to my findings with Aβ, these data suggest a direct interaction between his-HspB1 with secreted sAPPα of APPwt and APPswe indicated by the high molecular weight band (APP~110 kDa) when immunoprecipitated with anti-Aβ 6E10 β-amyloid (1-16) mouse monoclonal (Covance, Emeryville, CA, USA) and probed with anti-HspB1 human (SPA-803) rabbit polyclonal (Assay Designs, Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA) (Figure 3.10). Since HspB1 co-immunoprecipitates both APPwt and APPswe soluble alpha fragments, it is plausible that HspB1 is interacting with the Aβ sequence within sAPPα; however this is speculation at this point.

Figure 3.1 Transfection efficiency of APPwt, APPswe, EGFP-HspB1-Wt and EGFP-EVC2 in naive HEK 293 cells. A transfection efficiency of 70-80% was observed when APP and HspB1 were singly or co-transfected. Column 1 consists of plated HEK 293 cells under transmitted light (TL) microscopy without the flourescence filter. Column 2 displays the transfected cells under the fluorescence filter (FL). EFGP-HspB1-Wt appears green due to the fused EGFP tag. APP tranfection is shown indirectly by observing the green of the pmax-GFP construct which was added to the transfection in a 1:4 ratio to the APP construct. Therefore, if 2 µl of pmax-GFP yield 50% transfection efficiency then I can assume 8 µl of APP will give at least 50% transfection efficiency. Column 3 is a merged image showing the high ratio of transfected to non-transfected cells (Merge).

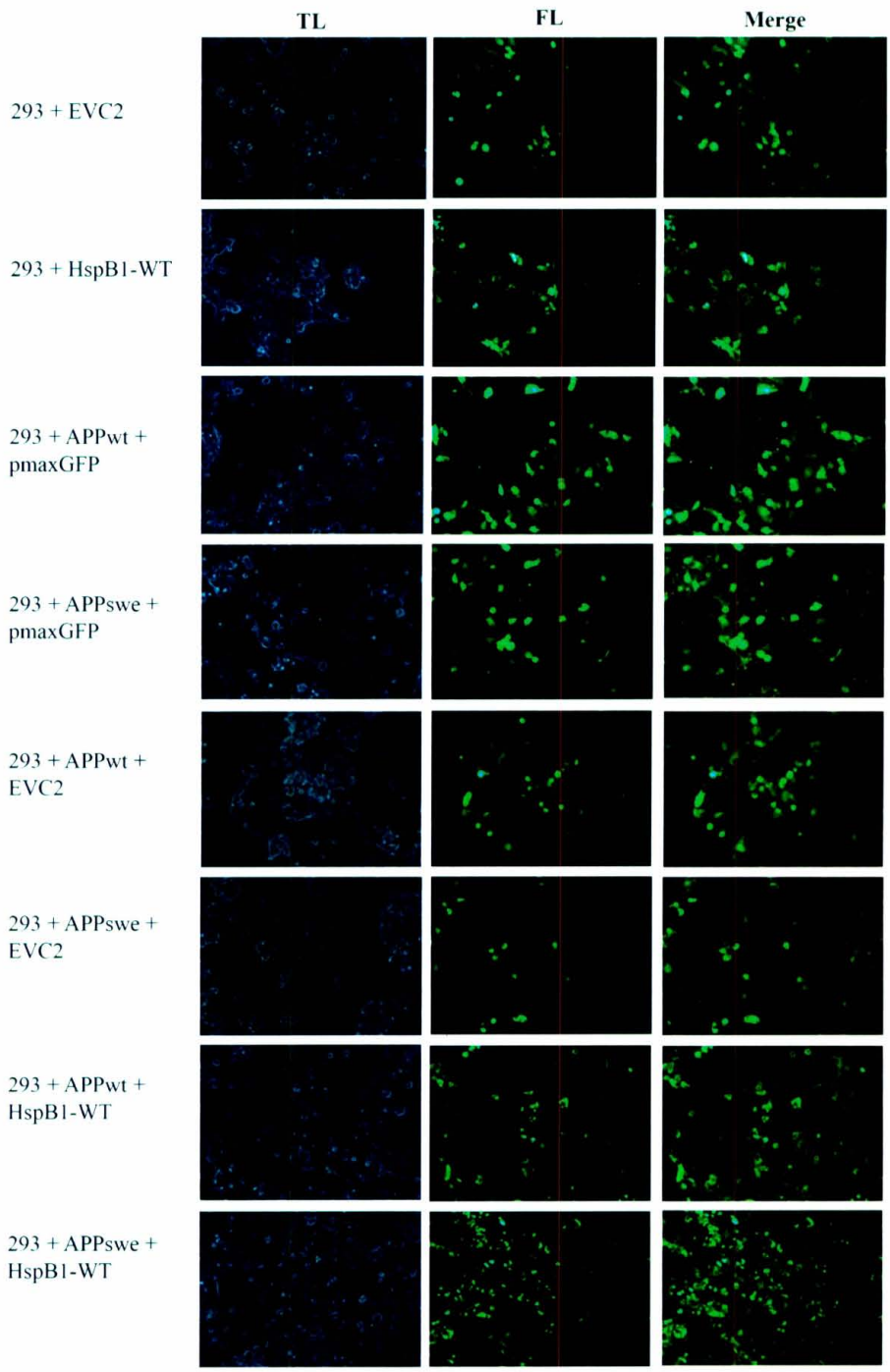

TL
FL
Merge
293 + EVC2
293 + HspB1-WT
293 + APPwt + pmaxGFP
293 + APPswe + pmaxGFP
293 + APPwt + EVC2
293 + APPswe + EVC2
293 + APPwt + HspB1-WT
293 + APPswe + HspB1-WT

Figure 3.2. Expression of co-transfected APPwt, APPswe and EGFP-HspB1-Wt fusion protein in naïve HEK 293 cells. HEK 293 cells were co-transfected with either APPwt or APPswe and EGFP-HspB1-Wt fusion protein or EGFP-EVC2 (empty vector). Transfection of EGFP-EVC2 alone was used as a negative control while single transfections of each EGFP-HspB1-Wt, APPwt and APPswe were used as positive controls (lanes 2-4). APPwt, APPswe and EGFP-HspB1-Wt are expressed well when co-transfected (lanes 7-8). Endogenous levels of HspB1 are undetectable as HspB1 is only observed in HEK 293 cells transfected with the rodent EGFP-HspB1-Wt construct and probed with the rodent HspB1 detectable antibody 801. The EGFP tag has a molecular weight of 29 kDa similar to the HspB1 protein (27 kDa) causing the fused protein to appear at the 56 kDa molecular weight.

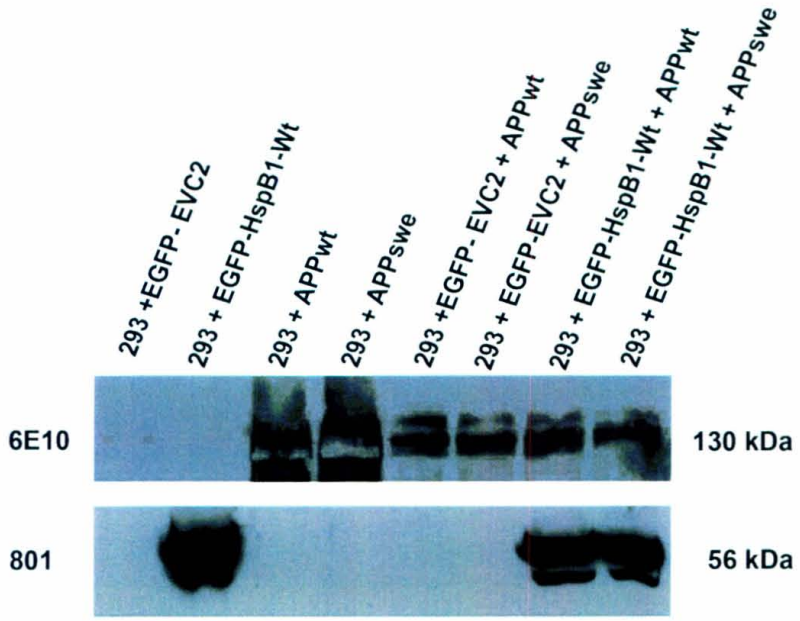

293 +EGFP- EVC2
293 + EGFP-HspB1-Wt
293 + APPwt
293 + APPswe
293 +EGFP- EVC2 + APPwt
293 + EGFP-EVC2 + APPswe
293 + EGFP-HspB1-Wt + APPwt
293 + EGFP-HspB1-Wt + APPswe
6E10
130 kDa
801
56 kDa

Figure 3.3 Expression of co-transfection of naive HEK 293 cells with EGFP-HspB1-Wt and APPwt or APPswe constructs by immunocytochemistry. HEK 293 naive cells were co-transfected with either APPwt or APPswe and pEGFP-C2-HspB1-Wt fusion protein. Cells were stained with the anti-Aβ 6E10 (1-16) antibody shown in red which is a standard antibody used for detection of not only Aβ but also souble APP alpha and most importantly for this work, full-length APP. HspB1 expression is evident in green due to the fused EGFP tag on the transfected HspB1 construct. A merged column is shown to identify the cells expressing both proteins. Cells expressing both APP and HspB1 constructs (white arrows) do not appear to show increased APP staining (6E10-red) in the presence of HspB1 (green) in comparison to cells not transfected with HspB1 (arrow heads).

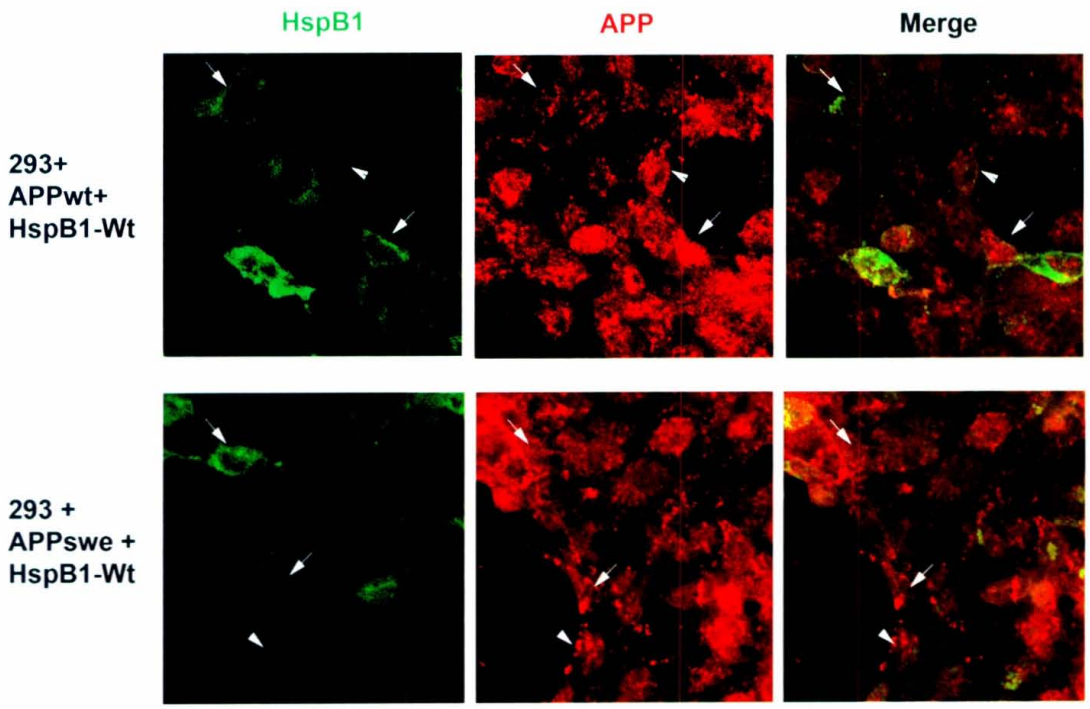

HspB1
APP
Merge
293+
APPwt+
HspB1-Wt
293 +
APPswe +
HspB1-Wt

Figure 3.4 Levels of APP expression in cellular lysate and conditioned media of naive HEK293 cells transfected with APPwt and APPswe construct over 72hrs. Cellular lysate and cellular conditioned media from HEK 293 cells transfected with the APPwt and APPswe constructs were collected over 12-72 hrs and analysed through Western blotting. Immediately following transfer, membranes were stained with ponceau red as a protein loading control (bottom panels). An increase in both expression (A-cellular lysate) and secretion (B-conditioned media) of APPwt and APPswe was observed when probed with anti-Aβ 6E10 (1-16) antibody. Detection with rodent anti-HspB1 801 antibody reveals undetectable endogenous HspB1 levels as a negative control.

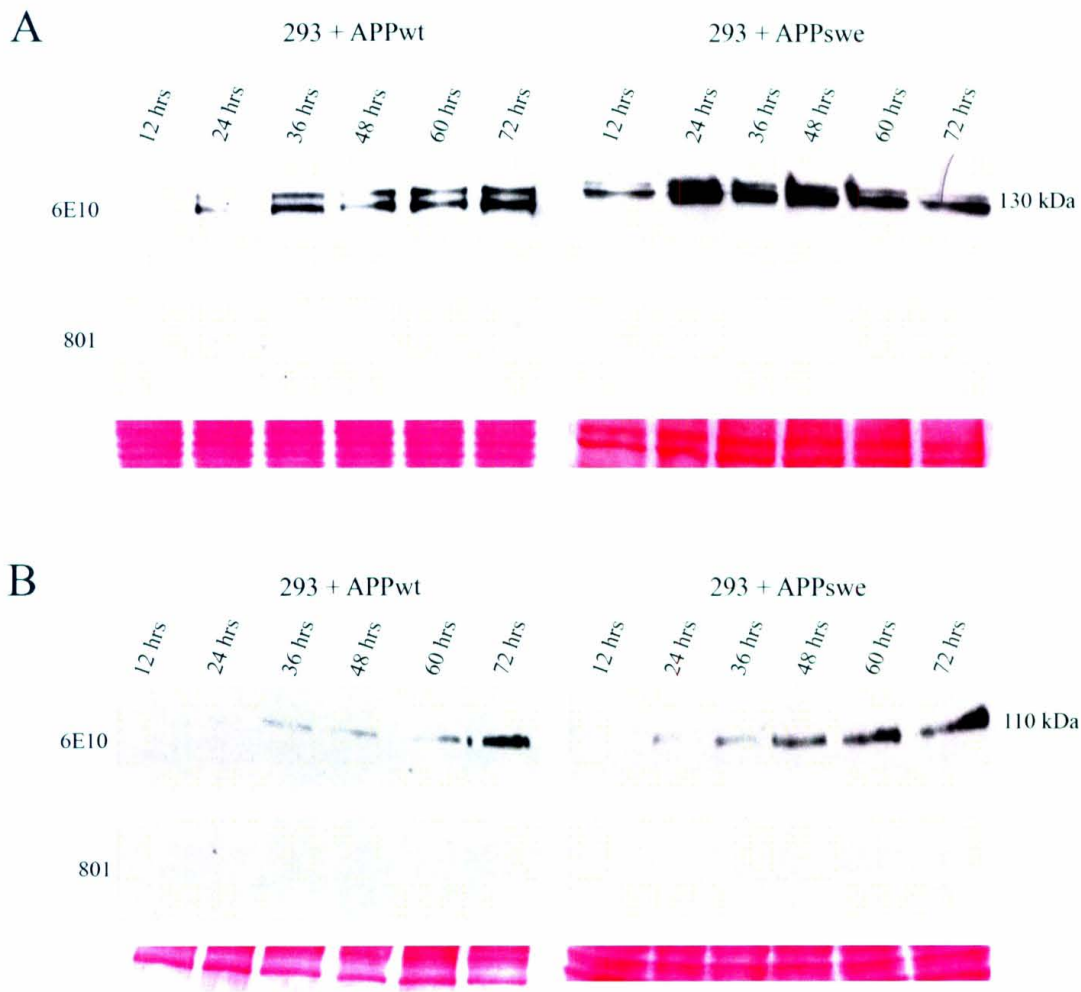

A
293 + APPwt
293 + APPswe
12 hrs
24 hrs
36 hrs
48 hrs
60 hrs
72 hrs
6E10
801
130 kDa
B
293 + APPwt
293 + APPswe
110 kDa

Figure 3.5. Expression of transfected pCIG-HspB1 in APPwt and APPswe stable cell lines. Endogenous levels of APP are detected in control lanes 1-2 since the anti-Aβ 6E10 (1-16) antibody recognizes human APP from the human cell line however a clear over-expression of APP is evident in the stable cell lines (lanes 3-6). pCIG-HspB1 is only detectable in transfected samples (lanes 2,4,6) due to the specificity of the anti-HspB1 antibody 801 for rodent HspB1. APP stable cells transfected with pCIG-HspB1 appear to express higher levels of APP than the APPwt and APPswe stable cells alone. This is not seen in the 293 + APPwt/APPswe + HspB1-Wt co-transfected samples lending the question as to whether the large EGFP tag on the HspB1-Wt fusion protein is interfering in the normal cellular metabolism. Ponceau red staining used as an indicator of protein loading displays equal amounts of protein across samples confirming that differences observed on the western blots are due to differences in protein expression and not discrepancies in protein loading.

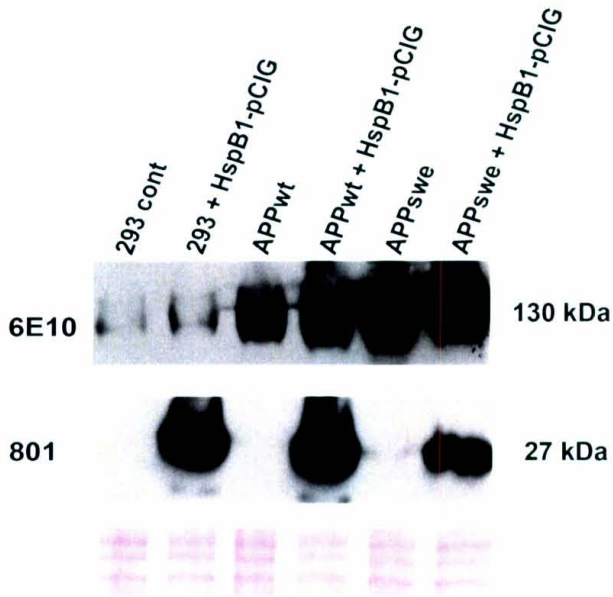
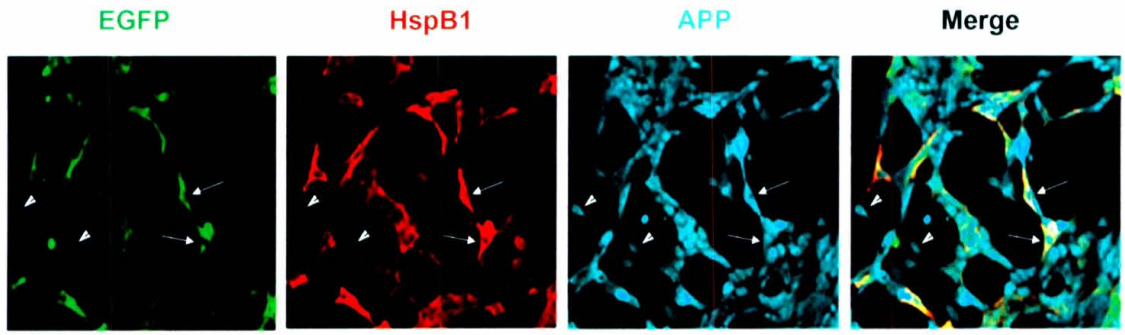

293 cont
293 + HspB1-pCIG
APPwt
APPwt + HspB1-pCIG
APPswe
APPswe + HspB1-pCIG
6E10
130 kDa
801
27 kDa

Figure 3.6 Expression of HEK 293 stable APPwt and APPswe stable cell lines transfected with pCIG-HaHspB1-WT-IRES-EGFP by immunocytochemistry. HEK 293 stable cell lines of APPwt and APPswe were transfected with the pCIG-HspB1 construct and subjected to immunocytochemistry. Cells expressing both APP and HspB1 (arrows) may show slightly increased APP staining (6E10-cyan) in the presence of HspB1 (red) in comparison to cells not transfected with HspB1 (arrow heads) however this is difficult to determine from the low magnification and densely populated cells. Since HspB1 is not fused to EGFP (green) in the pCIG construct as it is in the EGFP-HspB1Wt, staining for HspB1 using the rodent anti-HspB1 801 was required (red). As evident in the EGFP (green) and HspB1 (red) images, cells were positive for both signifying a successful ttransfection and working construct.

APPwt + pCIG-HspB1



APPswe + pCIG-HspB1

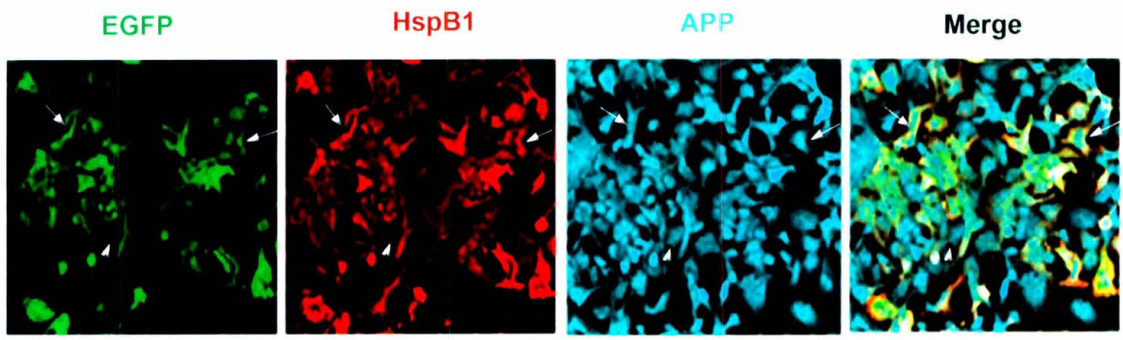


Figure 3.7 Absence of Aβ expression in cellular lysate of APPwt and APPswe stable cell lines.
Western blot of cellular lysate shows APP expression in the APPwt and APPswe stable cells in comparison to naive HEK293 cells. As expected, APPwt and APPswe stable cell lines expressed much higher levels of APP compared to HEK 293 cells as seen in Figure 3.5. Synthetic Aβ (1-42) peptide was used as a positive control for Aβ however Aβ levels were undetectable through Western blot in the cellular lysate. This may be due to lower than detectable amounts of Aβ through western blot or Aβ being absent from cellular lysate and only present in the secreted form found in the extracellular mileu.

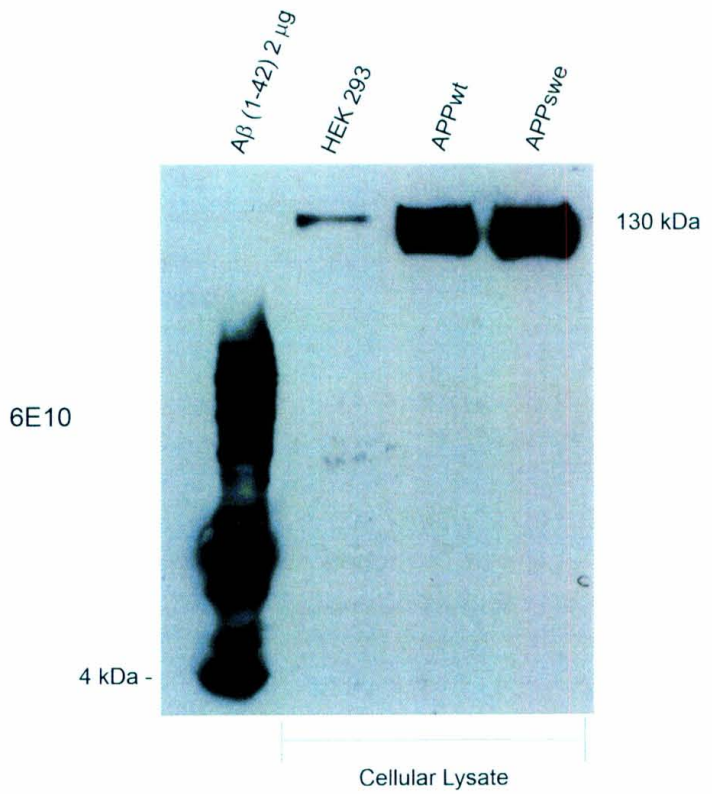
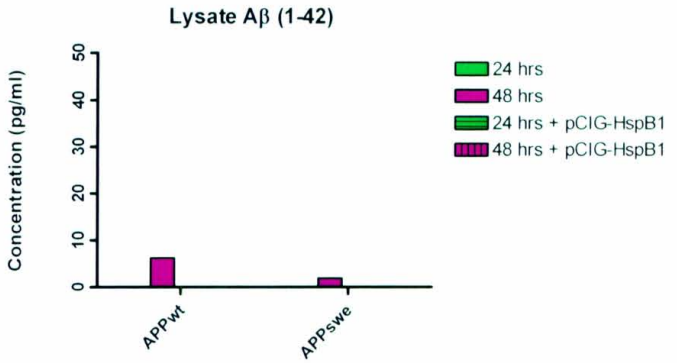
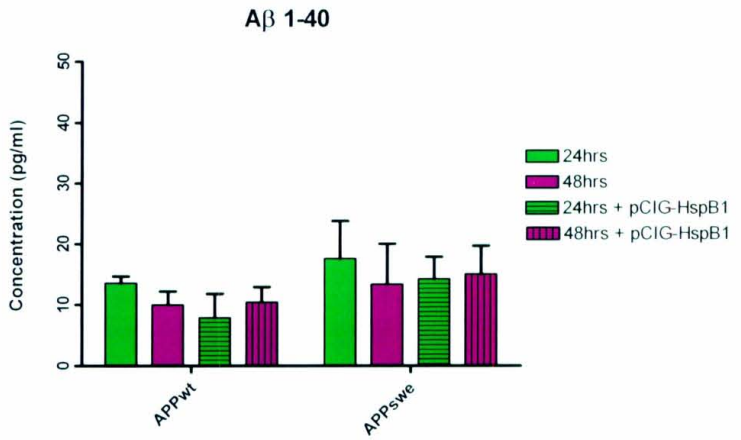

Aβ (1-42) 2 μg
HEK 293
APPwt
APPswe
130 kDa
6E10
4 kDa -
Cellular Lysate

Figure 3.8 Increased protein expression of Aβ (1-42) after 48 hrs is not seen in the presence of HspB1. Due to the undetectable levels of Aβ in the cellular lysate via western blot, the more sensitive ELISA was used. **A**: Aβ (1-42) was first carried out on 24 and 48 hr cellular lysate of stable APPwt and APPswe cells transfected with pCIG-HspB1. Aβ (1-42) was dectable within the cellular lysate through ELISA but does not appear to vary significantly between APPwt and APPswe with pCIG-HspB1 transfection however this is an N=2 therefore further testing is needed to confirm values. **B**: Cellular conditioned media from 24 and 48 hr stable APPwt and APPswe samples transfected with pCIG-HspB1 was measured for secreted Aβ (1-40) and (1-42) levels. APPwt cells displayed increased levels of Aβ (1-40) over Aβ (1-42) suggesting that APPwt cells are undergoing non-amyloidogenic processing more readily than amyloidogenic as expected. Ab (1-40) levels remain fairly constant over 48 hrs in both the APPwt and APPswe cells with and without HspB1 expression suggesting that HspB1 does not affect the non-amyloidogenic Aβ (1-40). In contrast, Aβ (1-42) protein level is significantly increased in the APPswe cell line at 48 hrs compared to all other conditions. Interestingly, this increase is not detectable in the APPswe cells transfected with HspB1. *$p<0.05$

A


Lysate Aβ (1-42)
Concentration (pg/ml)
0
10
20
30
40
50
APPwt
APPswe
24 hrs
48 hrs
24 hrs + pCIG-HspB1
48 hrs + pCIG-HspB1

B


Aβ 1-40
Concentration (pg/ml)
0
10
20
30
40
50
APPwt
APPswe
24hrs
48hrs
24hrs + pCIG-HspB1
48hrs + pCIG-HspB1

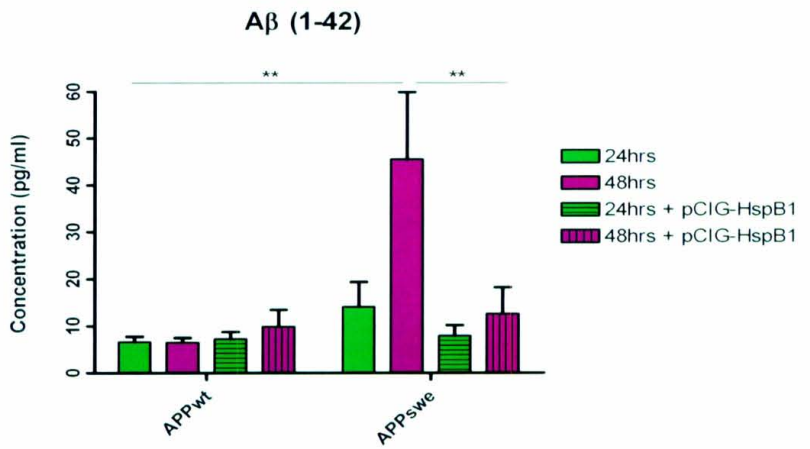

Aβ (1-42)
**
**
Concentration (pg/ml)
0
10
20
30
40
50
60
APPwt
APPswe
24hrs
48hrs
24hrs + pCIG-HspB1
48hrs + pCIG-HspB1

Figure 3.9 Synthetic Aβ (1-42) peptide is co-immunoprecipitated with recombinant human his-tagged HspB1 in vitro. Synthetic Aβ (1-42) and recombinant human his-HspB1 were incubated overnight at 37° in PBS. The following day, coorelative reverse IP's were performed using anti-human HspB1 antibody 803 or anti-Aβ 6E10 (1-16) with magnetic dynabeads. Western is probed with 803 and 6E10. Synthetic Aβ (1-42) and recombinant his-HspB1 were run as positive controls as well as immunoprecipitated his-HspB1 and Aβ (1-42). Presence of both proteins when co-ip'ed with 803 or 6E10 suggests a direct interaction between HspB1 and Aβ (1-42). The lower of the two bands in the 803 blot is believed to be non-specific binding as it appears to correspond to IgG's molecular weight.

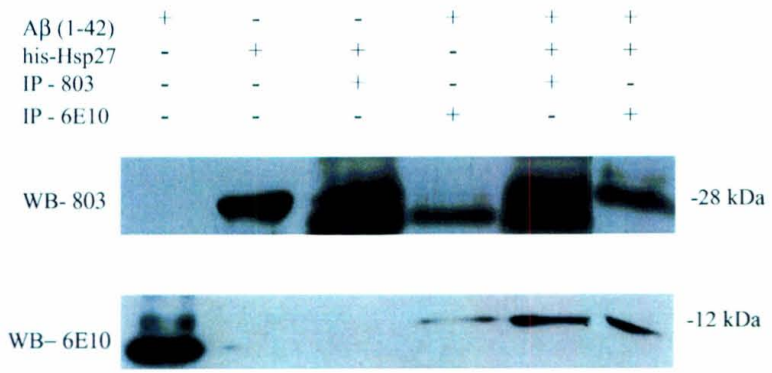
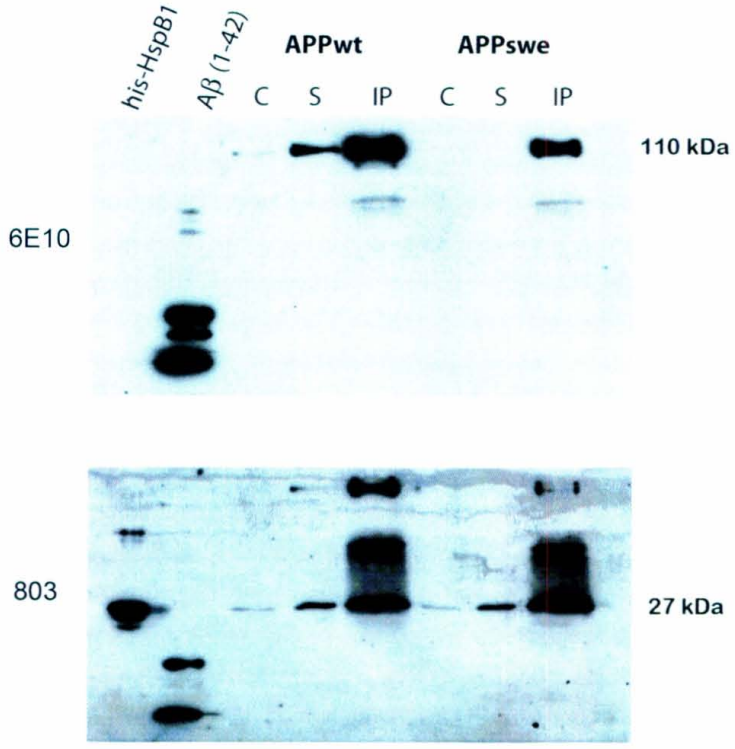

Aβ (1-42) + - - + + +
his-Hsp27 - + + - + +
IP - 803 - - + - + -
IP - 6E10 - - - + - +
WB- 803
-28 kDa
WB– 6E10
-12 kDa

Figure 3.10 HspB1 successfully co-immunoprecipitates with soluble APP in cellular conditioned media. Conditioned media from APPwt and APPswe cells was incubated with recombinant human his-HspB1 overnight at 37° degrees. 6E10 was used to immunoprecipitate sAPPα with magnetic beads. Three samples were electrophoresed by western blot including a crude sample "C" consisting of the beads from the initial equilibration incubation which would contain any non-specific binding before antibodies were added to the reaction; a supernatant sample "S" consisting of the discarded sample left after centrifugation of the co-IP and finally the co-IP sample consisting of the beads and bound HspB1/sAPPα complexes. HspB1 is co-immunoprecipitated with sAPPα as seen by the bands corresponding to 27 kDa when probed with anti-human HspB1 803 (bottom) and immunoprecipitated with 6E10. Western was probed with 6E10 (APP) and 803 (HspB1) to show the presence of both proteins. Western blot of 6E10 (top) was not stripped following probing accounting for the high molecular weight bands and Aβ control still present in 803 blot.


his-HspB1
Aβ (1-42)
APPwt
APPswe
C
S
IP
C
S
IP
110 kDa
6E10
803
27 kDa

3.8 Does HspB1 decrease the accumulation of APP products within cells?

Studies investigating the roles of familiar heat shock proteins such as Hsp70, Hsp90 and ataxin-2, focus primarily on their chaperone ability as the main effective function in inhibition of protein aggregation (Evans et al., 2006). This lead me to question whether there are differences in APP accumulation in HEK 293 APPwt and APPswe stable cells when HspB1 is present. Using immunocytochemistry, HEK 293 APPwt and APPswe stable cells transfected with pEGFP-C2-HaHspB1-WT were plated at 1.0×10^5 densities on 16-well slides. As seen in Figure 3.11, cells negative for HspB1 show accumulation of APP/Aβ staining (red-6E10) throughout the cytosol. Again, this antibody not only detects the full-length APP, but also the immature APP, N-truncated forms as well as the Aβ region as it recognizes the amino acids 1-16 of APP. HspB1 is shown in green and as expected, appears cytosolic; the cDNA construct used results in an EGFP-HspB1 fusion protein. The merged image shows HspB1 positive cells (green-EGFP) do not appear to have similar staining of APP/Aβ in the APPswe cells in comparison to APPwt. In fact, localization of the APP staining of the APPswe cells appears to change in the presence of HspB1.

3.9 Does HspB1 affect the production and release of amyloidogenic proteolytic products of APPswe?

Once I determined that HspB1 is able to bind directly to synthetic Aβ (1-42) along with sAPP, I was interested in whether HspB1 elicited any effects directly on

processing of APP or on the APP proteolytic products. Since the Aβ peptide is a product of APP proteolytic processing, it cannot be released if APP is not cleaved by enzymes BACE and γ-secretase.

3.9.1 Expression of mature and immature APP remains unchanged in the presence of HspB1 in both APPwt and APPswe cells.

The novel finding of HspB1 interacting with sAPPα, the decrease seen in Aβ amounts present in the media of APPswe cells when HspB1 is present, taken with the undectable Aβ levels intracellularly, lead me to question whether HspB1 influences the maturation process of APP thereby decreasing the availability of APP for cleavage and Aβ production. Employing western blot analysis, I characterized mature and immature APP by antibody recognition of the N-terminal region (22C11) in the cellular lysate of APPwt and APPswe cells transfected with pCIG-HspB1. Although the western blot data shown appears to show some variations in expression, upon analysis of triplicate samples no statistical differences were found in total levels of mature or immature APP whether -wt or –swe, concluding HspB1 having an influence on maturation of APP to be unlikely (Mature APP_{695} n=3, P=0.27, F=1.9; Immature APP_{695} n=3, P=0.17, F=2.8 - Figure 3.12A&C). Endogenous levels of APP were also measured which did not show any significant differences in APP levels between APPwt and APPswe stable cells (Mature $APP_{770/751}$ n=3, P=0.61, F=0.67; Immature APP770/751 n=3, P=0.98, F=0.06 - Figure 3.12B).

3.9.2 CTFβ levels remain constant when HspB1 is expressed in HEK 293 APPwt/swe stable cells.

To test whether proteolytic products of APP processing are affected by HspB1 expression, I probed the cellular lysate western blots with an antibody which detects the C-terminal fragment of APP that is detected after α or β-secretase cleavage. When I employed densitometry analysis, although a slight elevation in the CTF-β fragment or the fragment cleaved by β-secretase, was seen in the APPswe cells in the presence of HspB1, this was not significant when statistical analysis was employed. Similarly, CTF-α did not show alternating levels (CTF-β n=3, P=0.95, F=0.10; CTF-α n=3, P=0.88, F=0.22 - Figure 3.12D). HspB1-pCIG appears to have better transfection efficiency in the APPswe cells accounted by the denser band in the western blot data for Figure 3.12.

3.9.3 Is Release of soluble APP influenced by the presence of HspB1 in APPswe cells?

Next I considered HspB1 influencing the release of soluble APP from the cells into the media. Media conditioned from the cells used in the above experiment was collected in addition to the cellular lysate and assessed using western blot analysis (Figure 3.13A). Secretion levels of sAPP were unchanged in the presence of HspB1 and although slight variation in total sAPP protein was observed, statistical analysis showed no difference between triplicate matched timepoint samples with and without HspB1. Steady sAPPα levels were seen in APPswe media samples and again variation observed was insignificant when triplicate experimentation was analysed. Similar to CTFβ levels, sAPPβ-swe levels remained constant in the presence of HspB1. Overall, results from the

cellular conditioned media coincide with the corresponding cellular lysate data of APP proteolytic products concluding HspB1 does not appear to have an effect on the maturation or processing of APPwt or APPswe in vitro as evident by the steady levels of APP and APP proteolytic products observed (Total sAPP n=3, P=0.88, F=0.22; sAPPα n=3, P=0.57, F=0.75; sAPPβ-swe n=3, P=0.90, F=0.19 - Figure 3.13B).

3.10 Cellular localization of APP and HspB1

Investigation into the effects of HspB1 on APP/Aβ accumulation required further investigation into the cellular localization of the two proteins thus providing insight into possible areas of interaction. 24 and 48 hr samples of cellular lysate were taken from APPwt and APPswe cell lines plated on 5cm collagen coated cell culture dishes transfected with the HspB1-pCIG construct. Protein was run and western blot analysis was subjected to antibodies against early endosomes (Rab5 rabbit polyclonal 21435 Cell Signalling), late endosomes (Rab 9 rabbit polyclonal 51185 Cell Signalling) and lysosomes (LAMP-1 H4A3 mouse monoclonal Hybridoma Cell Bank) to determine if there were changes in the intracellular compartments where APP is known to undergo processing. Levels of all three cellular compartments were similar in samples with or without HspB1 present (Figure 3.14). This signifies that the presence of HspB1 does not affect the concentration of the intracellular compartments yet did not answer my question of whether HspB1 and APP were interacting within these compartments or whether distribution of APP proteolytic products between these compartments was altered by the

presence of HspB1. To explore these questions I employed immunocytochemistry along with computer software generated 3D-rendering and co-localization analysis to visually conceptualize possible areas of interaction and distribution changes between APP products and HspB1 within specific intracellular compartments. Pairing of the antibodies was chosen based on the recognized APP product and hypothesized intracellular location as well as the antibody species to ensure visualization of all antibodies used. APP is known to mature within the golgi compartment where it becomes glycosylated therefore the anti-golgi mouse monoclonal antibody was used with the anti-APP rabbit polyclonal 15272 Abcam antibody. Likewise, early and late endosomes are both thought to be sites of secretase cleavage and thus the 6E10 β-amyloid (1-16) mouse monoclonal was employed with both the early and late endosomal markers Rab 5 and Rab 9 rabbit polyclonal to not only recognize full length APP but also Aβ and soluble APP. Finally, due to the recent evidence that APPswe undergoes trafficking to the lysosomes, the C-terminal anti-APP mouse monoclonal (Zymed) was paired with the lysosomal marker anti-LAMP-1 H4A3 mouse monoclonal (Hybridoma) to identify APP and the C-terminal end product of γ-secretase cleavage to determine if this change in trafficking would be evident in the Swedish mutated APP cells. Figure 3.15 displays both immunocytochemistry as well as images generated through the co-localization software of a representative cell from the APPswe and APPswe + pCIG-HspB1 groups to visually represent the collection of data. Panel 1, 2, and 3 show immunocytochemistry of APP using the anti-APP rabbit polyclonal 15272 Abcam antibody (red), golgi staining using the anti-golgi mouse monoclonal 27043 Abcam antibody (cyan) and a merged image also

displaying HspB1 transfection using the anti-HspB1 goat polyclonal Santa Cruz antibody respectively. Panel 4 is a 3-D surface rendering image of the thresholds set by the brightest pixels of staining cancelling out the background. Additionally, the region of interest (ROI) is shown as a white outlined box surrounding the cell to be measured. Finally, panel 5 is an image of the co-localization shown in violet. This represents only the pixels containing both the red (APP) and cyan (Golgi) after co-localization calculation has been performed. This procedure was repeated for 20 cells of each group and the generated co-localization average values were plotted in Figure 3.16. Figure 3.16A displays co-localization of APPwt and APPswe with HspB1 in specific cellular compartments to address the question of which cellular area has the highest co-localization of APP and HspB1. Data displayed in 3.16B answers the question of whether changes in distribution of APP products occur when HspB1 is present. This looks particularly at localization of APP in cellular compartments with and without the presence of HspB1. Neither APPwt nor APPswe were strongly co-localized with HspB1 given the Pearson's coefficient values for co-localization in any of the intracellular compartments. This is not surprising since HspB1 is a cytosolic protein and therefore any binding action would be expected extracellularly as seen with Aβ. Interesting results emerged when the distribution of APP was measured in the cellular compartments with and without HspB1. APPswe is significantly decreased in all compartments when measured in the presence of HspB1. This corresponds to earlier ICC data where APP accumulation appeared to decrease in the presence of HspB1 in the APPswe cells (Fig. 3.11).

Figure 3.11 Immunocytochemistry displaying differences in cellular accumulation of APP in APPswe stable cells transfected with pEGFP-C2-HspB1-Wt.
Using immunocytochemistry, HEK 293 APPwt and APPswe stable cells transfected with pEGFP-C2-HaHspB1-WT were plated at 1.0 x 105 densities on 16-well slides. HspB1 is shown in green and as expected, appears cytosolic; the cDNA construct used results in an EGFP-HspB1 fusion protein. Cells negative for HspB1 (without green) show accumulation of APP/Aβ staining (red-6E10) throughout the cytosol in the APPswe cells. The merged image shows HspB1 positive cells (green-EGFP) do not appear to have similar accumulation of APP/Aβ in the APPswe cells in comparison to cells without HspB1 (arrows).

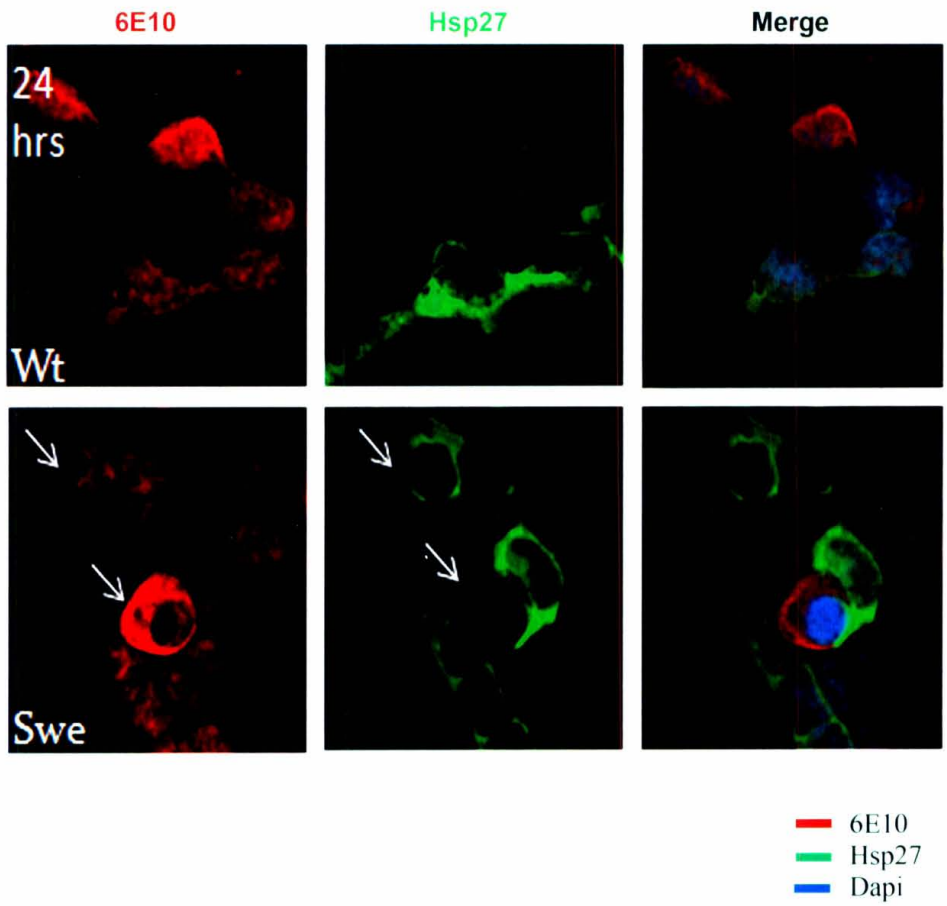
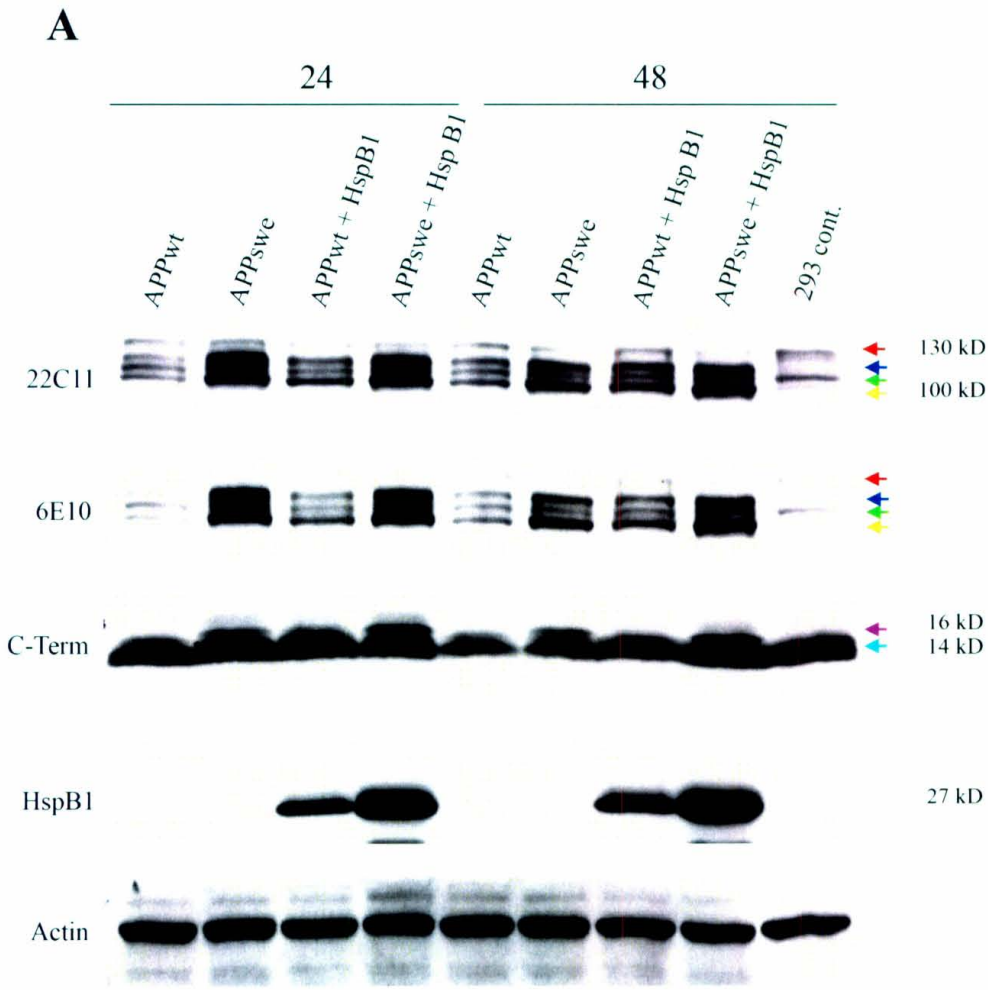
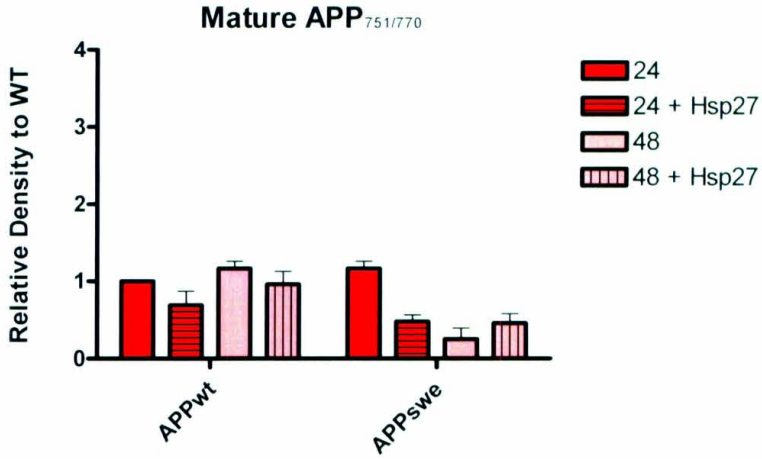
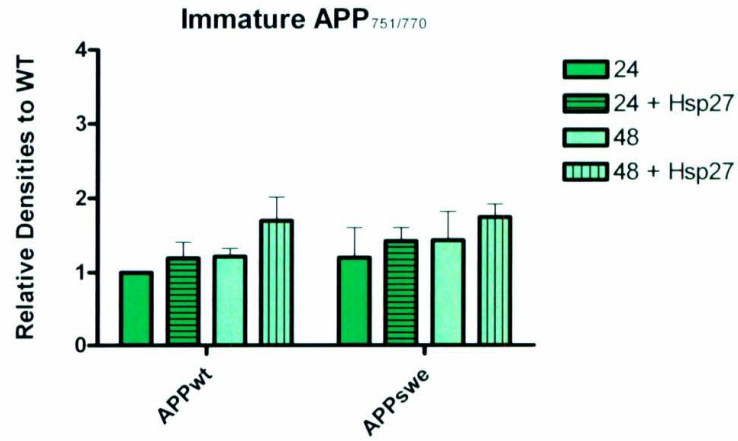

6E10
Hsp27
Merge
24
hrs
Wt
Swe
6E10
Hsp27
Dapi

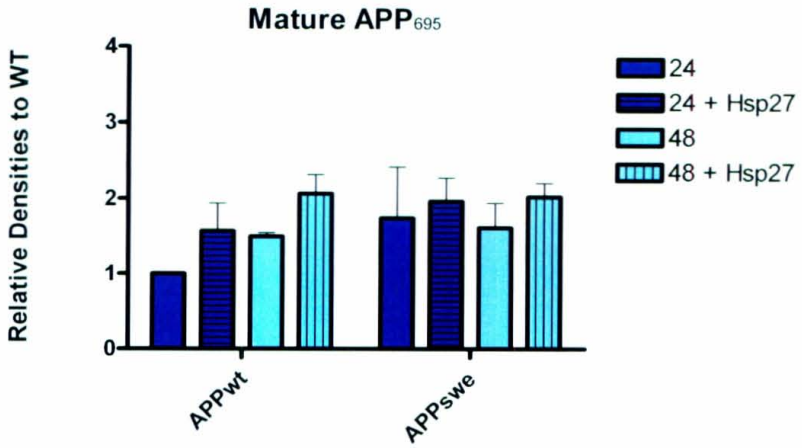
Figure 3.12 Processing of APP in HEK 293 APPwt and APPswe stable cell lines transfected with pCIG-HspB1 construct. HEK 293 APPwt and APPswe stable cell lines were grown to 80% confluency and transfected with the pCIG-HspB1 construct. Protein and conditioned media were collected 24 and 48 hrs post transfection. **A**: Employing western blot analysis, I characterized mature and immature APP in cellular lysate by antibody recognition using the N-terminal region antibody 22C11 (Millipore) and the C-terminal fragments were recognized using the APP anti-C-terminal antibody (Zymed). Cellular lysate shows variation in expression of the different forms of APP present thoughout processing with and without pCIG-HspB1 over 48 hrs. Red arrow: mature APP770/751; blue arrow: mature 695; green arrow: Immature 770/751; yellow arrow: Immature 695; lilac arrow: C-terminal β; aqua arrow: C-terminal α. **B**: Densitometry analysis on endogenous levels of APP770 forms in the presence of pCIG-HspB1. HspB1 does not appear to alter overall expression of mature or immature APP770 as there are no significant changes in levels regardless of pCIG-HspB1 expression. **C:** Although the western blot data shown appears to show some variations in expression, upon analysis of triplicate samples no statistical differences were found in total levels of exogenous mature or immature APP695 whether -wt or –swe, concluding HspB1 having an influence on maturation of APP to be unlikely. **D:** Western blot shows intracellular CTFα and CTFβ remains fairly consistant among conditions when pCIG-HspB1 is present. Differences seen in pCIG-HspB1 levels between APPwt and APPswe cells are indicative of a stronger transfection within the APPswe cells.

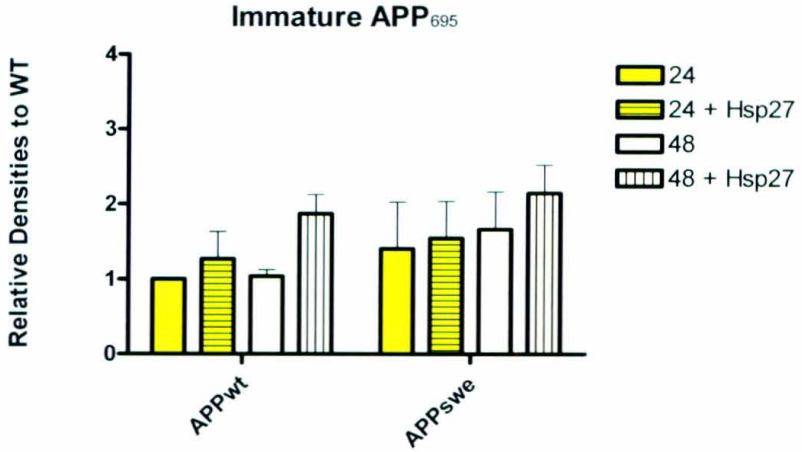
Note: APPwt and APPswe cell lines overexpress the 695 isoform; 770/751 is endogenous to HEK293 cells.

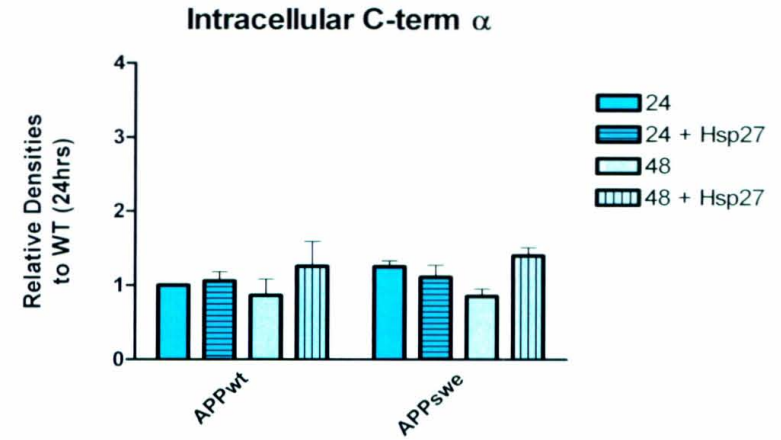

A
24
48
APPwt
APPswe
APPwt + HspB1
APPswe + Hsp B1
APPwt
APPswe
APPwt + Hsp B1
APPswe + HspB1
293 cont.
22C11
6E10
C-Term
HspB1
Actin
130 kD
100 kD
16 kD
14 kD
27 kD


Mature APP751/770
Relative Density to WT
4
3
2
1
0
24
24 + Hsp27
48
48 + Hsp27
APPwt
APPswe


Immature APP751/770
Relative Densities to WT
4
3
2
1
0
24
24 + Hsp27
48
48 + Hsp27
APPwt
APPswe


Mature APP695
Relative Densities to WT
4
3
2
1
0
APPwt
APPswe
24
24 + Hsp27
48
48 + Hsp27


Immature APP695
Relative Densities to WT
4
3
2
1
0
APPwt
APPswe
24
24 + Hsp27
48
48 + Hsp27


Intracellular C-term α
Relative Densities to WT (24hrs)
0
1
2
3
4
APPwt
APPswe
24
24 + Hsp27
48
48 + Hsp27

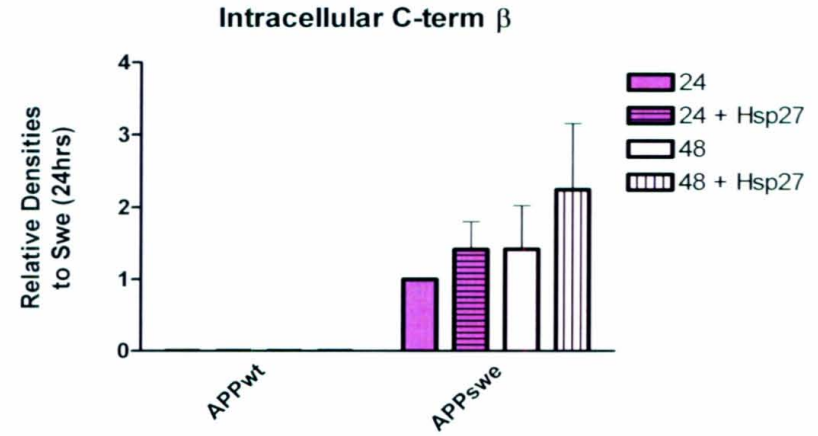
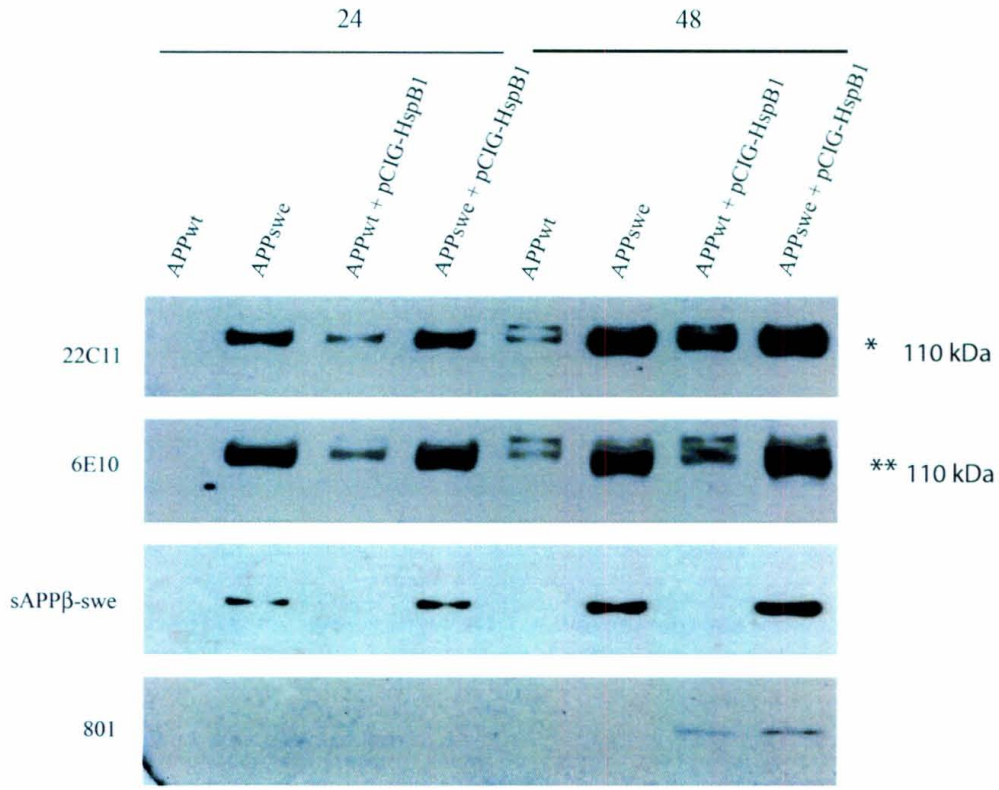

Intracellular C-term β
Relative Densities to Swe (24hrs)
0
1
2
3
4
APPwt
APPswe
24
24 + Hsp27
48
48 + Hsp27

Figure 3.13 Soluble APP levels in pCIG-HspB1 positive conditioned media. **A**:Western blot of conditioned media collected from cells shown in Figure 3.12 showing the variation of release of soluble APP forms into media with and without pCIG-HspB1: *sAPP total, **sAPPα. **B**: Densitometry analysis on release of soluble APP forms in the presence of HspB1. Secretion of sAPPα and sAPPβ-swe follows similar trend to CTFα and CTFβ levels showing little change due to HspB1 presence.

A


24
48
APPwt
APPswe
APPwt + pCIG-HspB1
APPswe + pCIG-HspB1
APPwt
APPswe
APPwt + pCIG-HspB1
APPswe + pCIG-HspB1
22C11
* 110 kDa
6E10
** 110 kDa
sAPPβ-swe
801

B

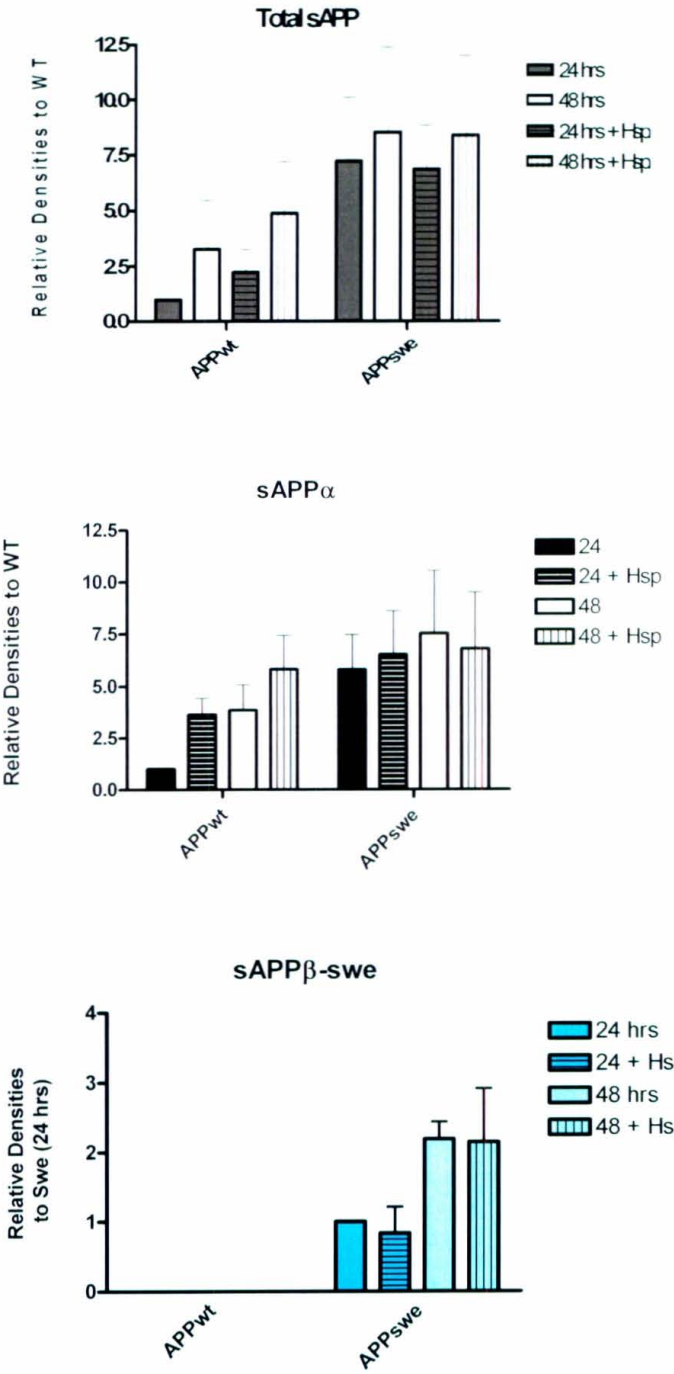

Total sAPP
Relative Densities to WT
12.5
10.0
7.5
5.0
2.5
0.0
24 hrs
48 hrs
24 hrs + Hsp
48 hrs + Hsp
APPwt
APPswe
sAPPα
Relative Densities to WT
12.5
10.0
7.5
5.0
2.5
0.0
24
24 + Hsp
48
48 + Hsp
APPwt
APPswe
sAPPβ-swe
Relative Densities to Swe (24 hrs)
4
3
2
1
0
24 hrs
24 + Hsp
48 hrs
48 + Hsp
APPwt
APPswe

Figure 3.14. Intracellular markers. Western Blot of APPwt and APPswe cell line lysate with and without pCIG-HspB1 over 48 hrs probed with intracellular markers for lysosomes (LAMP1), early endosomes (Rab5) and late endosomes (Rab9). No significant differences can be seen across samples.

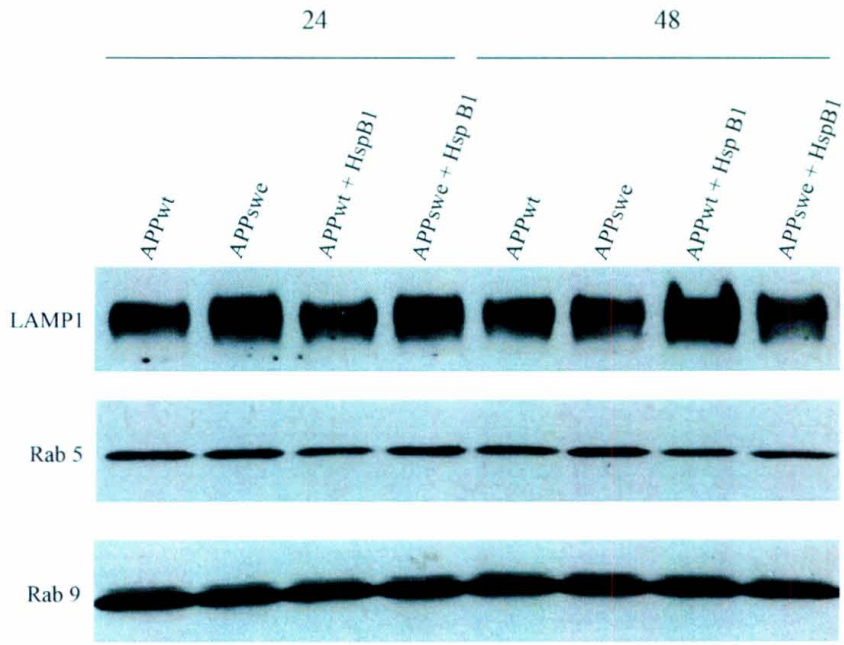
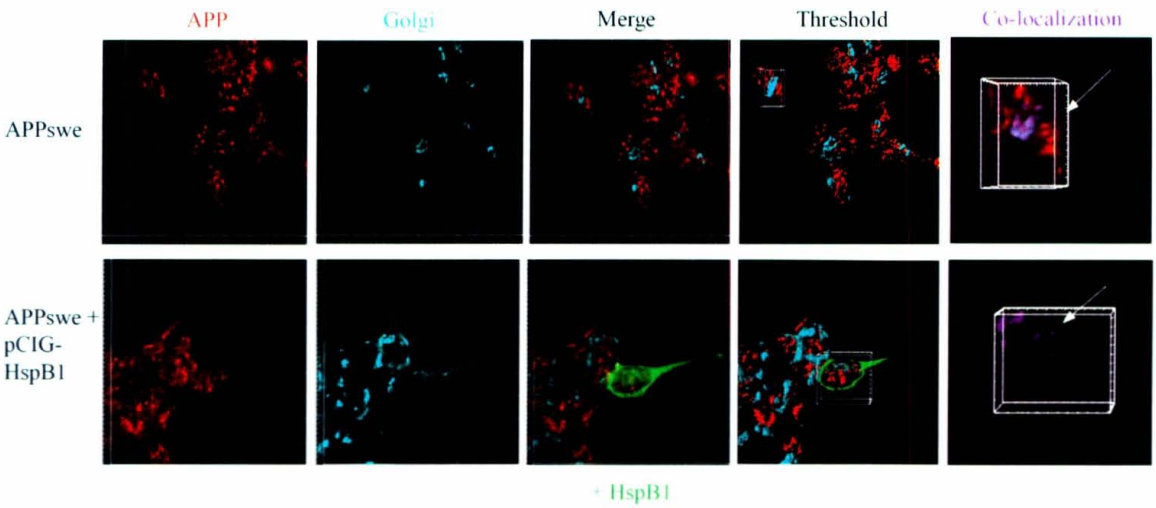
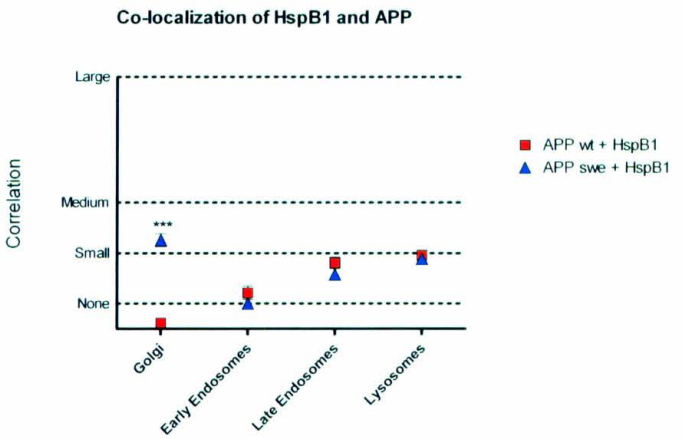

24
48
APPwt
APPswe
APPwt + HspB1
APPswe + Hsp B1
APPwt
APPswe
APPwt + Hsp B1
APPswe + HspB1
LAMP1
Rab 5
Rab 9

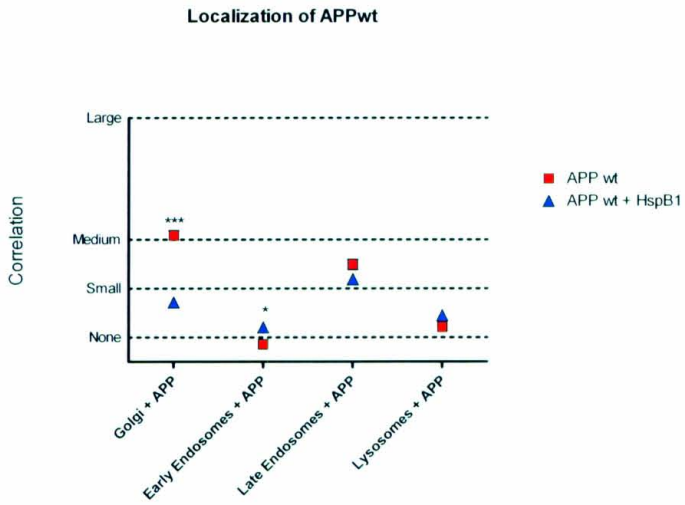
Figure 3.15 Co-Localization analyses of APPwt, APPswe and HspB1 within intracellular compartments. Co-localization analysis was applied to immunocytochemistry of APPwt, APPswe and HspB1 in various cellular compartments including the golgi apparatus, early and late endosomes and lysosomes to determine if HspB1 is co-localized to the same region as APP and if APP localization within the cell changes when HspB1 is present. A: Immunocytochemistry was applied to label cells with the various antibodies. APP (anti-Ab 6E10 (1-16) mouse monoclonal), HspB1 (anti-HspB1 goat polyclonal), and Golgi (anti-golgi mouse monoclonal) are shown as an example. Automatic thresholds were calculated for each color channel as seen in panel 4. The white box surrounding a single cell represents the selected region of interest (ROI). These thresholds and the ROI were then used to calculate the co-localization using the software provided with Imarus. Co-localization is shown in purple in panel 5. Purple co-localization is isolated to the ROI and is calculated using the pixels only selected by the thresholds excluding the background staining and remaining cells. Through selecting the specific intracellular compartment (masking), the co-localization of APP and HspB1 was measured in each compartment. A slight co-localization is seen in APPswe and HspB1 in the golgi however due to the low co-localization values throughout the compartments, this is thought to be negligible. B: Localization of APPwt and APPswe was analysed in each cellular compartment with and without HspB1 transfection and compared. HspB1 significantly decreased the APPwt localized within the golgi. Most interestingly, APPswe shows significant decrease in localization through all cellular compartments tested when HspB1 is present. Correlation values: None <0.1, Small 0.1-0.3, Medium 0.3-0.5, Large 0.5-1.0

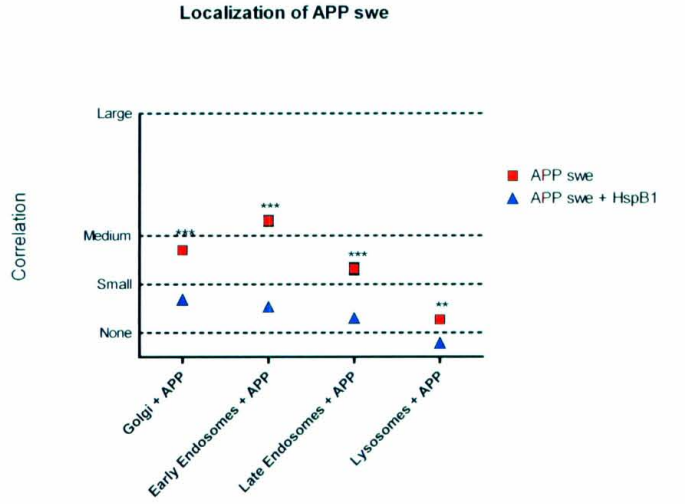
A


APP
Golgi
Merge
Threshold
Co-localization
APPswe
APPswe + pCIG-HspB1
+ HspB1

B


Co-localization of HspB1 and APP
Large
Medium
Small
None
Correlation
***
APP wt + HspB1
APP swe + HspB1
Golgi
Early Endosomes
Late Endosomes
Lysosomes


Localization of APPwt
Large
Medium
Small
None
Correlation
***
*
APP wt
APP wt + HspB1
Golgi + APP
Early Endosomes + APP
Late Endosomes + APP
Lysosomes + APP


Localization of APP swe
Large
Medium
Small
None
Correlation
***
***
***
**
APP swe
APP swe + HspB1
Golgi + APP
Early Endosomes + APP
Late Endosomes + APP
Lysosomes + APP

Chapter 4

Discussion

4.1 The Significance of this Study

Hsps are upregulated under environmental stressors such as alcohols, heavy metals, elevated temperatures (Hightower, 1991; Lindquist, 1986) and are thought to prevent cell death through protective mechanisms such as chaperone activity, thermotolerance, inhibition of apoptosis, enhancement of survival pathways and cytoskeleton stabilization (Arrigo, 2005; Charette et al., 2000; Hargis et al., 2004; Mearow, Dodge, Rahimtula, & Yegappan, 2002; Parcellier et al., 2003; Vargas-Roig, Fanelli, Lopez, Gago, Tello, Aznar, & Ciocca, 1997a).

While the well known Hsps such as Hsp90 and Hsp70 have been extensively studied in regards to the stress response (Buchner, 1996; Gusev et al., 2002) the class of small heat shock proteins is emerging as protectors in protein aggregation diseases such as AD (Kudva et al., 1997; Liang, 2000; Shimura et al., 2004; Wilhelmus et al., 2006; T. T. Yang et al., 2009). AD is a progressive, degenerative disease of the brain characterized by specific neuronal death in areas within the frontal and temporal lobes (Mattson, 2004) associated with NFTs and extracellular senile plaques (Price, Tanzi, Borchelt, & Sisodia, 1998; Song et al., 2002; R. E. Tanzi & Bertram, 2001; R. E. Tanzi et al., 2004). These amyloid containing plaques derived from the processing of the APP protein also contain various other proteins, namely HspB1 (Wilhelmus et al., 2006). Elevated HspB1 levels are often found in individuals with neurodegenerative disorders

for example Parkinson's disease, ALS, and AD (Renkawek et al., 1994; Renkawek, Stege, & Bosman, 1999; Vleminckx et al., 2002). It is probable that increased HspB1 expression is part of a protective response mounted by neuronal supportive glial cells, acting as a chaperone in an attempt to promote cell survival (D. E. Read & Gorman, 2009b; Sherman & Goldberg, 2001). Conversely, HspB1 may be released as an inflammatory response by activated microglia under cytotoxic stressors. Whether HspB1 is found in SPs as a protective mechanism or part of the disease process is unknown.

My thesis sought to investigate whether possible interactions between HspB1 and Aβ or its precursor APP affect the production and distribution of the Aβ peptide from both APPwt and the Aβ (1-42) producing APPswe cells. Firstly, I investigated the effect of HspB1 on Aβ release into the extracellular environment, secondly, the interactions between HspB1, Aβ (1-42) as well as APP, thirdly, if HspB1 affects APP processing, and finally the distribution of HspB1 and APP throughout the cellular environment and if any changes in cellular localization of APP products occur in the presence of HspB1.

4.2 HEK293 Cells Lack Endogenous HspB1

Hsps are conserved proteins whose expression is dependent on cell type (Renkawek et al., 1994; Shinohara et al., 1993; Stetler, Gao, Signore, Cao, & Chen, 2009; Wilhelmus et al., 2006). HspB1 has been found in the nervous system in spinal cord motor neurons and peripheral sensory neurons (Costigan et al., 1998; Dodge et al., 2006; D. E. Read & Gorman, 2009a; Williams et al., 2005; Williams et al., 2006) as well as

specific brain areas such as cranial nerve nuclei and hypothalamus (Armstrong et al., 2001; Plumier et al., 1997). HspB1 is not found in cortical neurons (M. King et al., 2009) however Bectold and Brown (Bechtold & Brown, 2000; Bechtold & Brown, 2003) found HspB1 localized to peri-synaptic glial processes and specifically expressed in neuronal supportive astrocytes when induced by hyperthermic stress. This localization could allow secretion of HspB1 from astrocytes into the environment surrounding neurons thereby providing a compensatory mechanism for lack of cortical expression.

While it may be suggested that primary cortical neurons may have been a better choice for this study, the use of primary cells is not ideal for the experimentation performed. Studying the effects of HspB1 required both cellular transfection along with collection of large amounts of cellular lysate for protein analysis through western blot and co-immunoprecipitation. Cortical neurons have consistently shown a transfection rate of only 30% therefore collecting HspB1 positive neurons for lysate samples would prove difficult. In order to mimic the lack of cortical endogenous expression of HspB1, it was important to choose an in vitro model which allowed for study of only exogenous HspB1 expression. My study shows that rodent HspB1 is not present in HEK293 cells except upon transfection of my constructs and this exogenous expression remains consistent when APP is overexpressed. This enabled me to study the effects of addition of rodent HspB1 without interference. In addition, I examined the collected media from my cells to ensure adequate presence of APP as it is known to be a secreted protein. This provided me with initial evidence that by expressing HspB1 I was not affecting the normal APP

secretion process from the cell. Also, access to APPwt and APPswe stable cell lines of HEK293 cells influenced my decision to use these cells as my experimental model.

4.3 Decreased Release of Aβ (1-42) from APPswe Cells with HspB1

When APP undergoes amyloidogenic enzymatic processing, the soluble N-terminal fragment and the freed Aβ peptide are transported and released into the extracellular milieu although the mechanism for release remains to be elucidated. Aβ (1-42) is perceived as the predominant toxic species produced from APP processing since it is shown to fibrillize, aggregate and generate oxidative species contributing to neuronal death (J. Hardy & Selkoe, 2002; Harper, Wong, Lieber, & Lansbury, 1999; Mattson, 2004; Price et al., 1998). While I was able to detect soluble forms of APP via western blot analysis, Aβ appeared to be released in undetectable amounts in the cellular conditioned media although this is contradictory to the published amounts of released Aβ seen previously (Cai et al., 1993; Citron et al., 1992; Wang, Sweeney, Gandy, & Sisodial, 1996; T. T. Yang et al., 2009). For this reason, I turned to the more sensitive ELISA which also enabled the distinct analysis of Aβ (1-40) and Aβ (1-42) species in both APP-wt and APP-swe cells. ELISA Aβ concentrations were undetectable in cellular lysate and lower than reported results in cellular conditioned media. An explanation for this discrepancy in values may be attributed to the masking of Aβ by BSA found in the FCS present in the media as select protocols call for formic acid extraction to measure Aβ amounts (Sweeney, Darker, Neville, Humphries, & Camilleri, 1993). Aβ extraction was

not carried out in my experimentation however FCS was decreased and concentrated media showed similar values. Although the amount of BSA in the media was lowered as much as possible to still ensure cell viability, presence may have altered the Aβ results negatively. Discrepancies in protocols between reports may also contribute to the perceived lower Aβ amounts. Though many studies have reported levels of Aβ release, few are consistent in the type of Aβ measured (synthetic versus endogenous), the procedure used (ELISA versus Mass Spectrometry) and the cell type (neuronal cell lines versus non-neuronal) (Bateman, McLaurin, & Chakrabartty, 2007; Belyaev, Kellett, Beckett, Makova, Revett, Nalivaeva, Hooper, & Turner, 2010a; Gouras et al., 1998; Sweeney et al., 1993; Wang et al., 1996). Any of these inconsistencies may contribute to the variance in Aβ release in the literature. Release of Aβ (1-40) remained steady throughout time points in both cell types independent of HspB1 expression. This result supports the literature as Aβ (1-40) is not believed to be a harmful species to cells and is readily found in tissues without detrimental effects (Bailey et al., 2011; Bogoyevitch et al., 2004; Gandy, 2002; Igbavboa et al., 2009; Lahiri & Maloney, 2010; Soscia et al., 2010). Expectedly, APPwt cells released insignificant amounts of Aβ (1-42) into the media suggesting these cells do not readily produce the toxic species. In contrast, Aβ (1-42) amounts significantly increased at 48hrs in APPswe cells. Evidently, the Swedish mutation does indeed result in a greater amount of the aggregate prone Aβ (1-42) known to correlate with toxicity (Caille, Allinquant, Dupont, Bouillot, Langer, Muller, & Prochiantz, 2004a; Caille, Allinquant, Dupont, Bouillot, Langer, Muller, & Prochiantz, 2004b; Citron et al., 1992; M. Mullan et al., 1992b; Scheuner, Eckman, Jensen, Song,

Citron, Suzuki, Bird, Hardy, Hutton, & Kukull, 1996; Sinha & Lieberburg, 1999). The high quantity of released Aβ (1-42) is lower when co-expressed with HspB1 at 24hrs and significantly decreased at 48hrs. This lead to two possibilities: 1) HspB1 is binding to Aβ (1-42) perhaps through chaperone activity and inhibiting its release into the media or 2) HspB1 is affecting the processing of APP-swe to prevent the production of Aβ (1-42). Since HspB1 and other sHsps have been shown to bind to Aβ (Clarimon, Bertranpetit, Boada, Tarraga, & Comas, 2003; Kudva et al., 1997; Muchowski & Wacker, 2005; Perrin et al., 2007; Wilhelmus et al., 2006; T. T. Yang et al., 2009; Y. Yang et al., 1998) I decided to explore the first option of HspB1 binding to Aβ (1-42).

4.4 HspB1 Directly Interacts with Aβ (1-42)

HspB1 has been reported to both interact with synthetic forms of Aβ (Clarimon et al., 2003; Goldstein et al., 2003; Liang, 2000) inhibit aggregation and toxicity of cerebral Aβ (Kudva et al., 1997; Wilhelmus et al., 2006) and rescue already aggregated protein (Perrin et al., 2007). To investigate a possible interaction between HspB1 and Aβ (1-42), I employed the co-IP technique. By selecting an antibody to a known protein in the solution (HspB1) that may be in a complex with another protein of interest (Aβ (1-42)), it is possible to pull the entire protein complex out of solution and thereby identify both proteins as interacting. Co-IP was performed with antibodies specific for human HspB1 since the recombinant human his-HspB1 protein was used as well as the correlative reverse IP using anti-Aβ (1-42) to ensure probability of an interaction. Results from co-

IP using HspB1 and Aβ (1-42) antibodies both suggested a direct interaction between the recombinant his-HspB1 protein and the synthetic Aβ (1-42) peptide. This is promising in that HspB1 is able to bind to the harmful Aβ (1-42). In theory, if inhibition of Aβ (1-42) release occurs, a greater pool of Aβ (1-42) would be found within the cellular lysate. Since I did not observe Aβ (1-42) present in the cell lysate in the presence of HspB1 either through western blot or ELISA, it appeared more likely that any effect HspB1 is having on extracellular Aβ(1-42) concentrations is upstream of release via production. Further investigation into the contents of the cellular lysate using ELISA is needed to dismiss HspB1 inhibiting the release of Aβ through direct interaction as the performed cell lysate ELISA was with one biological sample in duplicate therefore not conclusive whereas the cellular conditioned media ELISA was replicated with three biological samples. A potential direct interaction between HspB1 and Aβ (1-42) presents insight into how HspB1 may become incorporated in amyloid plaques in AD. Perhaps through its chaperone activity, HspB1 recognizes increased levels of Aβ (1-42) released into the extracellular space and interacts in an attempt to sequester the harmful protein however is unsuccessful in abolishing the plaque formation. The presence of HspB1 in SPs may be explained by the cross-talk between activated microglial and activated astrocytes. Micoroglial activation promotes phagocytosis of damaged neurons and SPs which is thought to contribute to the neuronal loss associated with AD (DeWitt, Perry, Cohen, Doller, & Silver, 1998; Gahtan & Overmier, 1999). Microglial activation in turn stimulates astrocytes which secrete HspB1 and are able to inhibit phagocytosis (Smits et al., 2001; Tichauer, Saud, & von Bernhardi, 2007; von Bernhardi & Eugenin, 2004)

leaving SPs present when both cell types are co-cultured (DeWitt et al., 1998; Liu et al., 2011).

4.5 HspB1 Directly Interacts with Soluble Amyloid Precursor Protein

Given that HspB1 interacts with Aβ it is conceivable that HspB1 may also bind to the precursor protein APP. A binding to APP would introduce the probability of HspB1 eliciting effects on APP before Aβ production. Through co-IP I again saw an interaction, this time between HspB1 and soluble APP in both –wt and –swe forms. Given that APP is constitutively expressed and holds many biological roles necessary for survival, it seems unlikely that HspB1 would be targeted to the wild type form unless it recognises the Aβ sequence within the soluble protein and is able to bind to that portion. Whether HspB1 interacts with the Aβ portion of sAPP or recognises a different sequence within the protein product is speculation at this point. To my knowledge, HspB1 directly binding to sAPP is a novel idea which warrants further examination. The ability of HspB1 to bind to sAPP presents therapeutic possibilities of sequestering APP mutated proteins before Aβ is produced through processing.

4.6 HspB1 does not Affect Processing of APPswe

Aβ results from the processing of APP, therefore changes in the release or production of Aβ may be attributable to changes in the processing of APP caused by

HspB1. Proteins that have been found to bind to APP and decrease Aβ secretion are thought to do so by blocking the maturation process (Steinhilb et al., 2001; Y. Yang et al., 1998). Mature and immature APP were classified using antibody recognition and molecular weight as cited in the literature (Frigerio et al., 2010; Henriques et al., 2009; E. Portelius et al., 2010a; E. Portelius et al., 2010b). Contrary to this view, HspB1 did not appear to influence the maturation process of APP when analysed through western blot as similar concentrations of mature and immature APP were seen across time points and conditions. Changes in processing did not occur with HspB1 in the APPwt or APPswe cells as seen by fairly constant levels of the CTF and sAPP fragments. While constant CTFβ and sAPPswe-β levels may seem contradictory to the decrease seen in Aβ (1-42), it can be explained by the amyloidogenic processing sequence of events. sAPPswe-β and CTFβ are cleaved from each other by β-secretase therefore levels of both fragments should be constant. CTFβ is composed of the Aβ portion and the AICD before γ-secretase cleavage frees both fragments. Therefore, steady CTFβ and sAPPswe-β levels may be explained by normal β-secretase activity, but a deficit in γ-secretase ability to further cleave CTFβ resulting in a decrease in Aβ production without decreasing overall CTFβ or sAPPβ-swe levels. Plausible explanations may be the binding of HspB1 to the Aβ (1-42) region of APP would inhibit γ-secretase ability to gain access to its Aβ cleavage site. Alternatively, disruption in APPswe trafficking by HspB1 may have varying effects on the proteolytic products of APPswe as it is thought to undergo processing in various compartments of the cell.

4.7 Cellular Co-Localization of HspB1 and APP Products

My hypothesis that HspB1 may alter APP processing or distribution of APP products required investigation into specific cellular compartments where APP may be found and examine possible effects due to HspB1. I proposed to answer two questions. Firstly, are HspB1 and APP found co-localized in a specific cellular area where a possible interaction between the two proteins is plausible? Secondly, does the distribution of APP within specific cellular areas change in the presence of HspB1? To explore this I employed image analysis software and a cellular colocalization module (Imaris, Bitplane Corp) to assess the co-localization of APP and HspB1 in the golgi, early and late endosomes and lysosomes of the cell. Through selecting each cellular compartment individually, I was able to analyse the APP and HspB1 present in the isolated region (ROI). APPswe and HspB1 were slightly co-localized in the glogi compartment however this value was not considered a strong co-localization given the Pearson's coefficient values for co-localization. Neither APPwt nor APPswe showed strong co-localization with HspB1 in any of the cellular compartments analysed. This may be explained by HspB1 and APP binding in the extracellular environment as seen in my earlier results and not having this binding effect intracellularly. Interesting results emerged when the distribution of APP was measured in the cellular compartments with and without HspB1. APPswe is significantly decreased in all compartments when measured in the presence of HspB1. This corresponds to earlier ICC data where APP staining appeared to decrease in the presence of HspB1 in the APPswe cells. Given all the results thus far, HspB1 appears to alter the amount of APPswe and its toxic Aβ (1-42)

product. It may be theorised that disruption in the normal amyloidogenic processing of APPswe results from the addition of HspB1 into the intracellular environment. Such a disruption in the trafficking of APPswe would coincide with changes in the processing of APPswe and thus the proteolytic products formed. Since the details regarding the normal amyloidogenic processing of mutated APP including APPswe are unknown, it is difficult to predict where HspB1 may be enforcing its effects on APPswe. However, it is evident from the decrease in Aβ (1-42) secretion, the binding to sAPP and Aβ(1-42), and finally the decrease in intracellular APP in all cellular compartments when HspB1 is present that HspB1 is eliciting an effect on the Aβ(1-42) prone APPswe.

4.8 Future Directions

This study has investigated the role of HspB1 with Aβ (1-42) and the Aβ(1-42) producing

Swedish mutated APP with respect to its effects on Aβ production and release. It has addressed the hypothesis that HspB1 interacts with Aβ and sAPP and is able to alter release of the toxic Aβ (1-42) peptide. It was shown that HspB1 is able to modify the release of Aβ (1-42) without affecting the maturation process of APP or abolishing APP processing altogether yet also appears to disturb cellular localization of APPswe. However, a number of questions remain concerning the mechanism by which the alteration of Aβ release occurs as well as the binding kinetics of HspB1 with sAPP/Aβ(1-42). Furthermore, it is unclear, although appears promising, whether HspB1 is acting through its chaperone activity and in a protective measure against Aβ toxicity.

Given the observations made here, it would be interesting to investigate the enzymatic activity of the secretases in the presence of HspB1 especially the γ-secretase to look for alterations in its ability to process APPswe. Further investigation into the trafficking of mutated APP proteins and their processing is under investigation and would highlight areas in which HspB1 may intercept. Noteworthy as well is that while HEK293 cells have been used to investigate Aβ and other aggregating proteins in vitro, a more neuronal cell line would offer greater benefits. Discrepancies most likely exist in the nature of APP processing, trafficking and Aβ accumulation between HEK293 cells and neuronal cells. HspB1 has been shown to be up-regulated in glial cells exposed to stress (Bidmon et al., 2005; King et al., 2009) and our lab has shown robust HspB1 presence in the media of cultured astrocytes (King et al., 2009). Thus, having an environment more similar to an in vivo scenario where astrocytes are the main generator of HspB1 in the extracellular milieu and measuring the effect on Aβ would further validate these results.

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