The neuroprotective role of somatostatin against beta amyloid induced toxicity in *in vitro* models of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a chronic neurodegenerative disease affecting more than 60 million people worldwide. This debilitating disease harbors toxic environment to the brain causing neuronal cell death and causes general impairment of the cognitive function. In our laboratory, we have been studying the effect of somatostatin (SST) in serving neuroprotective role against various disease models including hyperinflammation, Huntington's disease and AD. In the present study, we aim to study the mechanisms involved in SST mediated neuroprotection against beta amyloid induced toxicity in blood brain barrier and in neurons.

In AD, the impaired clearance of β-amyloid peptide (Aβ) due to disrupted tight junction and transporter proteins is the prominent cause of disease progression. We demonstrate that SST prevents Aβ induced blood brain barrier permeability by regulating low density lipoprotein receptor-related protein and receptor for advanced glycation end products expression and improving the disrupted tight junction proteins. Furthermore, SST abrogates Aβ induced c-JUN NH2-terminal kinase phosphorylation and expression of matrix metalloproteinase. Next, as the neurites are often the initial point of damage upon accumulation of Aβ, we examined the role of SST in *all-trans* retinoic acid (RA) induced progression of neurite outgrowth in SH-SY5Y cells. We also determined the morphological changes in prominent intracellular markers of neurite growth including microtubule-associated protein 2, Tuj1 and Tau. Here, we present evidence that SST is a molecular determinant in regulating the transition of SH-SY5Y cells from non-neuronal entity to neuronal phenotype in response to RA. Lastly, to elucidate the mechanism involved in SST mediated protection against Aβ-induced toxicity in neurons, phosphorylation level of

collapsing response mediator 2 (CRMP2), a well-established regulator of neurite homeostasis hyperphosphorylated in AD was monitored. We demonstrate that SST effectively inhibits the hyperphosphorylation of CRMP2 as Ser522, which plays a critical role in priming the phosphorylation of subsequent sites. Furthermore, we identified the underlying mechanism involved in the regulation of CRMP2 phosphorylation by monitoring the SST mediated regulation of calcium influx.

Taken together, results presented here suggest that SST might serve as a therapeutic intervention in AD via targeting multiple pathways responsible for neurotoxicity, impaired BBB function and disease progression.

Lay Summary

Alzheimer's disease (AD) is a neurodegenerative disease afflicting more than 60 million people worldwide. This debilitating disease harbors toxic environment to the brain causing neuronal cell death and causes general impairment of the cognitive function. Since the discovery of AD, significant efforts were made to identify the endogenous proteins that the body uses to fight against the onset and progression of the disease. Among them, somatostatin (SST) is one of the most potent neuroprotective protein identified yet. However, the exact mechanism of SST mediated neuroprotection against AD is not fully understood. In this thesis, we aim to find out how SST protects the brain from AD using various experimental techniques. The findings from this thesis may lead to the identification of potent therapeutic agent that may be used in treatment for the AD.

Preface

Chapter 2. A version of chapter 2 has been published. Seungil Paik, Rishi K. Somvanshi, Ujendra Kumar (2018) Somatostatin maintains permeability and integrity of blood-brain barrier in β-amyloid induced toxicity. *Molecular Neurobiology*. Epub ahead of print. I designed the study and experiments following discussion with Dr. Kumar. I have conducted the majority of the experiments, collected and analyzed the data. Dr. Somvanshi conducted Western blot analysis. I wrote the paper, with input and discussion from all of the co-authors. Dr. Kumar revised the manuscript.

Chapter 3. A version of chapter 3 has been submitted to the peer-review journal. Seungil Paik, Rishi K. Somvanshi, Ujendra Kumar (2018). Somatostatin mediated changes in microtubule-associated proteins and retinoic acid-induced neurite outgrowth in SH-SY5Y. I designed the study and experiments following discussion with my supervisor. I have conducted the majority of the experiments, collected and analyzed the data. Dr. Somvanshi conducted Western blot analysis. I wrote the paper, with input and discussion from all of the co-authors and revision from Dr. Kumar.

Chapter 4. I designed the study and experiments following discussion with my supervisor. I have conducted the majority of the experiments, collected and analyzed the data. Dr. Somvanshi and Dr. Zou conducted Western blot analysis. I wrote the paper, with input and discussion from all of the co-authors.

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List of Abbreviations

AD	Alzheimer's Disease
APP	Amyloid precursor protein
Aβ	β-amyloid peptide
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor;
CANP	Calcium-activated neutral proteinases
CDK5	Cyclin-dependent kinase
CNS	Central nervous system
CRMP2	Collapse response mediator protein 2
CSF	Cerebrospinal fluid
DMEM	Dulbecco's modified eagles medium
ECM	Extracellular matrix
ERK	Extracellular-signal-regulated kinase
FAM	Fluorescein amidite
FBS	Fetal bovine serum
GSK-3β	Glycogen synthase kinase-3β
hCMEC/D3	Human cerebral microvascular endothelial cell line
HD	Huntington's disease;
HRP	Horseradish peroxidase

i.c.v	Intracerebroventricular
IFN-γ	Interferon-γ
IL-1β	Interleukin 1β
JNK	c-Jun N-terminal kinases
ko	Knock out
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NGS	Normal goat serum
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nucleus antigen
PSEN	Presenilin
RA	Retinoic acid
RAGE	Receptor for advanced glycation end products
RT	Room temperature
SDS	Sodium dodecyl sulfate
Sema3A	Semaphorin3A

SH-SY5Y	Human neuroblastoma cell line
SST	Somatostatin
SSTR	Somatostatin receptor
TBST	Tris buffered saline
tg	Transgenic
TJP	Tight junction protein
TNF-α	Tumor necrosis factor-α
TUJ1	βIII tubulin
ZO	Zonula occluden

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Dedicated to my family and friends.

Special thanks to Hye Lim Ju and Seoyoon Olivia Paik.

Chapter 1: Introduction

1.1 Alzheimer's disease (AD)

Today, approximately 40 million people worldwide (mostly over the age of 65) suffer from dementia, with the number expected to quadruple by the year 2050 with an increase in aging population (Prince et al., 2013). High prevalence in AD patients is accentuated in developed countries such as Canada, Unites States and Western Europe due to the extended lifespan compared to the less developed countries. Accordingly, AD is one of the most common neurodegenerative diseases in the developed countries today (Citron, 2010).

In 1906, German neuropathologist Dr. Alois Alzheimer observed unique pathological features in patients with mental illness. In his short description, he reported the patient, a 51-year-old woman named Auguste D. displayed a 'peculiar disease of the cerebral cortex,' presented with multiple behavioral symptoms (delusion, paranoia, hallucination), language impairment and progressive memory loss (Maurer et al., 1997). Following her death, Alzheimer studied the patient's brain and noticed the presence of tangles (fibrils) and plaques (military foci). Although the presence of plaque-like formation in the brain has been described since the ancient Greeks, the observation of tangles was unique, and most importantly Dr. Alzheimer made the clear distinction of the disease apart from rest by stating "we must not be satisfied to force it into the existing group of well-known disease patterns." Following its initial discovery, however, clear criteria to define and distinguish AD from other neurodegenerative diseases were vaguely defined and were usually confined to cases with pre-senile (less than 65 years of age) onset. It was much later in 1968 when series of critical studies by Blessed et al., and Tomlinson et al., have reported that significant fraction of senile dementia also presented AD pathology, the modern understanding of AD was

established, drastically shifting AD from rarity to one of the major forms of mental disease. As the characteristic pathological changes could not be qualitatively assessed at the time, however, the AD could only be definitely diagnosed post-mortem. Today, with the advancement of diagnostic tools such as the development of magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) biomarkers and positron emission tomography (PET), AD-specific criteria for clinical diagnosis could be established. Currently, the diagnosis of AD follows a guideline published by National Institute of Aging and Alzheimer's Association (NIA-AA) workgroup (Table 1) (McKhann et al., 1984). To distinguish AD from other irreversible dementia and neurodegenerative diseases, such as Parkinson's disease, additional criteria such as family history, the age of the patient and symptoms are also outlined. Despite these clinical evaluations, however, diagnosis with certainty cannot be made until post-mortem pathological evaluation of the brain. As initially identified the presence and distribution of two hallmark indicators of AD, plaques and tangle formation, establish a definitive diagnosis of AD and the stage of the disease.

Table 1 NIA-AA Clinical criteria for Dementia [adapted from (McKhann et al., 2011)].

1. Cognitive deficits
a) Represent a decline from previous assessment
b) Interfere with daily activities
c) Not due to psychiatric disorder (ex. depression) or delirium
d) Observation corroborated by an objective informant (ex. friends or family member)
2. *Progressive* impairment in two or more areas
a) Changes in personality, behavior or mood
b) Memory (recollection and ability to learn)
c) Language (speaking, writing, reading)
d) Visuospatial (recognizing faces or objects)
e) Praxis (physical movement with purpose)
f) Executive function (planning and reasoning)

1.1.1 Genetic cause of AD

In the past 30 years, much of the focus in AD has been on the genetic investigation of AD pathogenesis. The underlying rationale for examining genetics of any disease, including AD, is multi-folded: 1) Identification of the genetic risk factors allows better understanding of the disease pathophysiology, 2) the identified genetic risk factors may be modified as a therapeutic target site, 3) possible environmental effectors on genetic risk factor may be elucidated, and 4) molecules identified to have derived from the genetic risk factor can be used as drug target and/or as a diagnostic biomarkers of the disease (Ertekin-Taner, 2007). The subsequent familial aggregation studies have reported that having a first-degree relative with AD significantly increased the one's risk of developing AD. Specifically, in one longitudinal study of AD, which surveyed 379 first-degree relatives of 79 AD subjects, striking 49% of the first-degree relatives developed AD by the age of 87 compared to less than 10% in the control population (Breitner et al., 1988). The more recent longitudinal study

also corroborates the importance of genetic risk factors in AD patients. The Multi-Institutional Research in Alzheimer Genetic Epidemiology (MIRAGE) project surveyed 12,971 first-degree relatives of 1,694 AD subjects (average age of AD onset 69.8) and have reported the incidence rate of AD onset among the first-degree relatives to be 39.0±2.1% by age 96, which was approximately double that of the control population (Lautenschlager et al., 1996). It is important to note that, however, that these incidence rates were significantly below the expected incidence rate among first-degree relatives of completely autosomal dominant disorders (50%), suggesting that AD is a much more complicated disease likely affected by environmental as well as genetic factors. Regardless, the importance of genetic risks in the onset and progression of AD has been established and significant efforts have been made to identify the common genetic risk factors found in AD patients. Through these studies, three genes that showed significant and consistent association with the onset of the familial AD has been identified: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Scheltens et al., 2016).

1.1.2 APP and Beta-amyloid (Aβ)

Beta-amyloid (Aβ) is a naturally occurring product of cellular metabolism derived from its membrane-embedded precursor APP (Thinakaran and Koo, 2008; Haass et al., 2012). Normally, APP is cleaved in the nonamyloidogenic pathway in which the APP is cleaved by α-secretase producing 83-amino acid C-terminal fragment followed by sequential cleavage by γ-secretase resulting in the short p3 peptide (Haass et al., 1993). In amyloidogenic pathway, however, APP is abnormally cleaved at 99-amino acid rather than 83-amino acid peptide from C-terminal of APP by β-secretase instead of α-secretase (Jarrett et al., 1993). The subsequent cleavage of the 99-amino acid peptide of the APP by γ-secretase results in Aβ

peptide containing an intact hydrophobic region called Aβ region, normally 40-amino acid in length (Aβ1-40) (Jarrett et al., 1993). Less frequently, subsequent cleavage by γ-secretase results in slightly longer 42-amino acid peptide (Aβ1-42), which is more hydrophobic than Aβ1-40 isoform and has significantly higher tendency to aggregate to form plaques and fibrils. The increased hydrophobicity of Aβ1-42 plays a critical role in the onset and progression of various neurodegenerative diseases as the extracellular Aβ aggregates target neurons directly by binding to the neuronal membrane leading to disruption in bilayer permeability (Sepulveda et al., 2010). Accordingly, it has been suggested that the Aβ1-42 is the prevalent isoform found in AD patients (Younkin, 1998). Following its initial discovery, studies have found that AD patients had significant intracellular deposition of Aβ prior to extracellular accumulation (Masters et al., 1985; Grundke-Iqbal et al., 1989). These clinical findings were further supported by *in vivo* studies using various transgenic mouse models of AD, where the researchers have found intraneuronal accumulation of Aβ prior to formation of extracellular Aβ plaques (Wirths et al., 2001; Wirths et al., 2002; Blanchard et al., 2003; Oddo et al., 2003; Casas et al., 2004; Lord et al., 2006; Oakley et al., 2006; Knobloch et al., 2007). Collectively, these findings corroborate the theory that Aβ is synthesized and accumulated intracellularly followed by extracellular accumulation.

1.1.3 Presenilin (PSEN1, PSEN2)

PSEN is one of the catalytic components of an intramembrane-cleaving protease γ-secretase (Wolfe, 2009). Currently, more than 90 type I transmembrane proteins are reported to be cleaved by a γ-secretase complex including APP (Zoltowska and Berezovska, 2018). In AD, specific mutations at PSEN1 and 2 alters the γ-secretase mediated cleavage of APP, leading to the preferential production of longer and highly hydrophobic Aβ1-42 over shorter

and aggregation-prone Aβ1-40 (Borchelt et al., 1996; Scheuner et al., 1996; Jankowsky et al., 2004; Chavez-Gutierrez et al., 2012). The preferential production of Aβ1-42 and the increasing ratio between Aβ1-42/1-40 is attributed to AD onset.

1.1.4 Tau

Despite not being identified as a genetic risk factor of the familial AD, Tau protein plays a critical role in both the pathogenesis and progression of the AD. Tau is a member of microtubule-associated protein (MAP) with six identified isoforms in the human brain (Goedert et al., 1989). All six isoforms are coded by a single gene in chromosome 17 following alternative splicing of mRNA (Himmler, 1989). Along with other members of neuronal MAPs, MAP1 and MAP2, Tau plays a critical role in the promotion of assembly of tubulin into microtubules and maintenance of its structural integrity (Weingarten et al., 1975). In AD and in other neurodegenerative diseases sharing similar characteristics broadly classified as tauopathies, the Tau protein is excessively phosphorylated causing its dissociation from microtubulin and aggregates into condensed filaments called neurofibrillary tangles (NFT) (Grundke-Iqbal et al., 1986). Particularly, the NFT formation is observed in dystrophic neurites in close proximity to Aβ plaques, suggesting a correlation between Aβ plaque and NFT formation (Braak et al., 1986).

1.1.5 Transgenic mice models of AD

With the understanding of APP, PSEN1 and 2 as the genetic risk factor of familial AD and Tau as a significant contributor, various transgenic mice models overexpressing one or combination of these genes have been developed. Some of the widely used transgenic lines include platelet-derived growth factor-β (PDGF) driving human APP transgene (PDAPP) and

hamster prion [prion protein (PrP)] driving human APP transgene containing the Swedish FAD mutation termed Tg2576 (Games et al., 1995; Hsiao et al., 1996). Utilizing the same promoter as the APP transgenic mice model, various PSEN1 and PSEN2 familial AD (FAD) mutant lines have been developed as well (Borchelt et al., 1996; Duff et al., 1996; Citron et al., 1997).

1.1.6 Neuropathology of AD

The AD is a progressive neurodegenerative disease causing significant disruptions in brain structure and function. Loss of cortical neurons, which mediates various cognitive functions has been documented (Mann, 1996; Norfray and Provenzale, 2004). In addition, reports have indicated disruption of synaptic plasticity in early stages of the AD, resulting in loss of memory and cognitive functions (Selkoe, 2002). It is now well established that the pathological features of AD start at the medial temporal lobe of the brain, with a specific focus on the hippocampus and entorhinal cortex which are critical in learning and memory (Jack et al., 1997). With the progression of the disease, structural damage can be found in areas such as temporal and parietal area, followed by degenerations in frontal cortex and ultimately on the remaining parts of the neocortex. Disease progression has also been reported to affect limbic system and combined with degeneration of neocortex, results in characteristic behavior changes found in AD patients (Holtzman et al., 2011).

Although the physiological role of Aβ in the normal state is not clearly understood, Aβ is produced in healthy individuals as well. One suggested a role of Aβ in the normal physiological state is the regulation of the activity of γ-secretase via negative-feedback loop (Kamenetz et al., 2003). In AD, the accumulation and oligomerization of Aβ is known as the primary reason for neurotoxicity (Walsh et al., 2002). Specifically, the increase of the

absolute level of Aβ1-42, increase of Aβ1-42 to Aβ1-40 ratio and increase of soluble oligomeric Aβ has all been reported to be directly associated with Aβ induced toxicity (Glabe, 2005; Walsh and Selkoe, 2007). Collectively, these processes lead to the formation of senile plaques. Excessive Aβ oligomers accumulation and senile plaques in turn directly bind to various cell surface receptors leading to apoptosis (Small et al., 2001; Zhu et al., 2015). In addition, Aβ promotes the production of reactive oxidative species (ROS) in the mitochondria by activating mitochondrial fission proteins Drp1 and Fis1 (Barsoum et al., 2006). The produced ROS causes oxidization of proteins and peroxided lipids, which is particularly damaging to the cell membrane integrity and the maintenance of critical enzymes of neuronal functions such as glutamine synthases (GS) and creatine kinases (CK) (Aksenov et al., 1995; Yatin et al., 1999). Furthermore, peroxidation of lipids results in synthesis of toxic products such as 2-propenal (acrolein) and 4-hydroxy-2-nonenal (HNE) which migrates to different regions of the neurons and elicit various damaging effects including inhibition of ATPase and Na^+-dependent glutamate, loss of calcium homeostasis and disruption of signaling pathways, which are all involved in neuronal cell death (Mark et al., 1995; Varadarajan et al., 2000; Ezeani and Omabe, 2016). In addition, Aβ is also known to damage DNA by causing DNA oxidation (Varadarajan et al., 2000).

In particular, the continuous accumulation and oligomerization of Aβ leads to the chronic activation of immunological receptors in microglia such as Toll-like Receptors 2 (TLR2), TLR4, TLR6 and their co-receptors such as CD14, CD36, and CD47 resulting in phagocytosis of functional neurons (Weggen et al., 2001; Neniskyte et al., 2011; Liu et al., 2012). Furthermore, over-activation of the immune system leads to hyperinflammation via the excess release of proinflammatory cytokines such as interleukin-1β and TNF-α, eicosanoids and chemokines. In turn, the Aβ induced hyperinflammation impairs the

microglial mediated clearance of intracellular Aβ and neuronal debris, resulting in neuronal death and ultimately AD pathogenesis (Weggen et al., 2001; Neniskyte et al., 2011; Liu et al., 2012). Interestingly, growing evidence suggests that Aβ hyperinflammation may be associated with tau pathology as well (Heneka et al., 2015).

In addition, the accumulation of APP at the mitochondrial membrane during the onset and progression of AD impairs normal mitochondrial activity by inhibiting the electron-transport chain [ETC;(Anandatheerthavarada et al., 2003; Devi et al., 2006)], which exacerbates the dysfunction by increasing the production of Aβ. Furthermore, localized Aβ in mitochondria binds to multiple pro-apoptotic factors such as cyclophin D (CypD) and Aβ-binding alcohol dehydrogenase (ABAD) leading to cell death (Lustbader et al., 2004; de Moura et al., 2010).

1.1.7 Origin of intracellular Aβ accumulation

Following the observation that the Aβ accumulates intracellularly, studies have aimed to identify the origin of Aβ. Specifically, it still remains elusive whether the primary source of intracellular Aβ accumulation is from the intracellular Aβ production or from the cellular uptake of extracellular Aβ. It has been reported that along with its prevalent presence in the plasma membrane, APP is also found on the endoplasmic reticulum (ER), autophagosomes, lysosomes, trans-Golgi network and mitochondria (Koo and Squazzo, 1994). More importantly, the enzyme complexes directly responsible for APP cleavage resulting in Aβ production, β- and γ-secretase has been detected in various subcellular compartments, indicating that intracellular production of Aβ is possible (Koo and Squazzo, 1994). Among other subcellular organelles, endosomes have been reported to be a significant site of Aβ production due to its acidic pH and co-expression of both β- and γ-secretase (Koo and

Squazzo, 1994). Despite the importance of intracellular accumulation of Aβ preceding extracellular accumulation, however, its implication in neurodegenerative disease is not ubiquitously accepted. Studies have reported that accumulation of intracellular Aβ occurs with age in healthy individuals (Tang, 2009). In another study, authors reported that the short time-frame fixation using cardiac perfusion, a common fixation procedure used in animal studies staining for Aβ, skews the observation toward more frequent intraneuronal Aβ observation (Bayer and Wirths, 2010). In parallel to the intracellular Aβ production, uptake of extracellular Aβ also presents itself as a significant source of intracellular Aβ accumulation. Tang BL et al have reported that the inhibition of Aβ endocytosis led to a significant reduction in intracellular Aβ level, suggesting that the uptake of extracellular Aβ may play a larger role in intracellular accumulation (Tang, 2009). Once deposited into the extracellular space, the Aβ can re-enter into the cells leading to further accumulation of intracellular Aβ. Although major portion of extracellular Aβ is cleared by glial phagocytic cells such as microglia and astrocytes through pinocytosis or endocytosis, significant fraction of Aβ are taken up by neurons as well (Matsunaga et al., 2003; LaFerla et al., 2007; Mandrekar et al., 2009; Amor et al., 2010; Bayer and Wirths, 2010). Due to its critical importance in various neurodegenerative diseases onset and progression, significant effort has been made to elucidate the functional mechanism of Aβ uptake by cells.

1.2 Mechanisms of Aβ uptake

1.2.1 Apolipoproteins

Apolipoproteins are a group of proteins involved in the uptake of Aβ in non-synaptic membranes. As previously described, Aβ is hydrophobic in nature which inhibits its ability to permeate lipid bilayer. Apolipoproteins are unique in its amphipathic properties, allowing it

to bind to the hydrophobic Aβ and enclosing it in the water-soluble milieu. In this way, some apolipoproteins can act as an Aβ chaperone, allowing the cellular uptake (Kim et al., 2009). Understandably, disruption in the regulation of apolipoprotein may enhance the cellular uptake of Aβ leading to neurotoxicity and onset of neurodegenerative disease. Kim J et al. has reported that in non-familial AD patients, there was a significant correlation between ε4 allele encoding apolipoprotein E (apoE) and genetic risk of the AD (Kim et al., 2009). In another study using apoE knockout transgenic mice, the authors reported significantly reduced intracellular Aβ accumulation, further corroborating the significant implication of apolipoprotein and Aβ uptake (Bales et al., 1999). Additionally, high colocalization of apoE and Aβ has been observed in intracellular immunoreactivity, suggesting that the apoE is internalized with Aβ (LaFerla et al., 1997). Based on these findings, efforts have been made to identify the membrane receptors that bind to apoE-Aβ complex as a potential therapeutic target of treatment in the AD. Although no particular receptor has been identified as a dedicated receptor for apoE, however, one particular receptor has been identified to play a critical role in Aβ uptake: low-density lipoprotein receptor protein (LRP).

1.2.2 LRP

LRP is a member of surface receptors with high endocytosis rate (Bu et al., 2006). Among the 12 isoforms of LRP, LRP1 in particular has been studied extensively for its role in Aβ uptake by acting as a binding site of apoE. Fuentealba RA et al. showed that in mouse neuroblastoma cell line, overexpression of LRP1 enhanced, while RNA interference of LRP1 inhibited, Aβ internalization (Fuentealba et al., 2010). In another study, mouse dorsal root ganglion (DRG) cells treated with a potent LRP1 antagonist, receptor-associated protein (RAP), showed significant inhibition of apoE-Aβ internalization compared to the control

(Gylys et al., 2003). Furthermore, these *in vitro* results were replicated in various *in vivo* studies where the LRP1 overexpressing transgenic mice displayed significantly higher accumulation of intraneuronal Aβ (Zerbinatti et al., 2006). It is now well established that the LRP1 mediated internalization of Aβ requires the presence of apoE, as in apoE free-environment the inhibition of LRP1 did not alter the internalization of Aβ (Saavedra et al., 2007). Collectively, these results suggest that apoE is required for Aβ uptake by LRP1.

1.2.3 Receptor for advanced glycation end products (RAGE)

Another receptor known to play a critical role in the neuronal uptake of Aβ is a receptor for advanced glycation end products (RAGE). RAGE is a 35-kDa transmembrane receptor protein of the immunoglobulin superfamily. Initially identified in 1992 by Neeper et al. it has since then been studied extensively for its involvement in activation of proinflammatory genes (Bierhaus et al., 2001). RAGE is known to interact with several classes of ligands, including advanced glycation end products (AGE), high mobility group box 1 protein (HMG-1 and amphoterin), and most importantly, with Aβ (Takuma et al., 2009). In a study using human brain endothelial cells, Giri R et al. have described in detail the formation of an intricate complex between RAGE and soluble Aβ (Giri et al., 2000). In *in vivo* studies, using RAGE overexpressing transgenic mice model, authors have observed early abnormalities in spatial learning/memory as well as exaggerated neuropathological symptoms compared to APP overexpressing littermates (Arancio et al., 2004). In parallel, cortical neurons derived from RAGE-deficient transgenic mice model showed significant reduction of intraneuronal Aβ, collectively suggesting that RAGE also plays a critical role mediating the Aβ uptake in neurons (Takuma et al., 2009).

1.2.4 Microglial Phagocytosis

The role of microglia in onset and progression of AD is a complicated one with stage-specific functions serving both neuroprotective and neurotoxic roles. In the early stages of AD, microglial clearance of Aβ through phagocytic degradation of both soluble and fibrillar Aβ has been well documented, serving as a critical agent of neuroprotection (Frautschy et al., 1998; Rogers et al., 2002; Wyss-Coray et al., 2003; Wyss-Coray, 2006). In particular, Yuyama et al. reported secretion of Aβ from neurons via exosome secretion resulted in activation of microglial cells, enhancing the phagocytic uptake of Aβ by microglial cells (Yuyama et al., 2012). In contrast, during the later stages of AD and with aging, microglial cells become hyper-responsive to stress such as Aβ and readily initiates pro-inflammatory reaction resulting in the neurotoxic environment. Another major glial cell responsible for Aβ phagocytosis is astrocyte. It has been reported that cultured astrocytes display high affinity to Aβ and actively internalize them for degradation (Nielsen et al., 2009). Accumulating evidence suggest that LRP1 receptor on astrocyte membrane is responsible for binding Aβ and activating astrocyte mediate phagocytosis of Aβ (Shibata et al., 2000).

1.2.5 Transportation across BBB via receptors

Since the initial discovery, the focal point of AD research has been laid on neurons and intrinsic neuronal components to identify the root cause of AD. However, as the paradigm slowly shifted to focus on clearance over synthesis, the importance of Aβ clearance via blood brain barrier has been gaining attention over regulation of Aβ synthesis. Among the various mechanisms in which BBB mediates clearance of Aβ from the brain, the LRP1 receptor has been studied most extensively for its critical role. Initially identified as a receptor for transportation and metabolism of cholesterol, LRP1 has since then been

identified to act as a scavenger receptor with more than 40 structurally different ligands. Most importantly, LRP1 has been found to actively transport the ligands through BBB. It is interesting to note that different isoforms of Aβ had different affinity to LRP1 resulting in a difference in change of clearance. For example, Aβ1-40 was rapidly transported through BBB via LRP1, while longer and more hydrophobic isoform Aβ1-42 was transported at a significantly slower rate (~50%) (Monro et al., 2002). Even among the Aβ1-40, mutated isoform Aβ1-40 (Dutch) was cleared at a significantly slower rate suggesting that LRP1 mediated clearance of Aβ largely depends on the structural composition (Monro et al., 2002). In support of these findings, Jaeger et al. reported that partial inhibition of LRP1 via LRP1-antisense treatment resulted in significant dysfunction in Aβ clearance resulting in accumulation of brain Aβ and cognitive impairment (Jaeger et al., 2009).

1.2.6 Aβ-degrading enzyme

Along with phagocytic glial cells that actively take up Aβ and degrade them, there exist multiple proteases of Aβ-degrading enzymes (ADE). Broadly, they can be classified into six categories including 1) zinc metalloendopeptidase [NEP-1 and -2, angiotensin-converting enzyme (ACE)], 2) thiol-dependent metalloendopeptidase [insulin-degrading enzyme (IDE)], 3) serine proteases [myelin basic protein (MBP), acylpeptide hydrolase], 4) cysteine protease 5) matrix metalloproteinase [MMP-9 and -2] and 6) others [mitochondrial peptidasome, GCPII, aminopeptidase A] (Yoon and Jo, 2012). Majority of ADEs contain endopeptidase activity targeting amino acid sequence within Aβ sequences, while some (such as MMP-9) cleaves Aβ from C-terminus (Nalivaeva et al., 2012). However, the effectiveness of ADE mediated clearance of beta-amyloid remains controversial due to the maintenance of toxicity in certain Aβ fragments from ADE mediated cleavages. For example, certain

truncated products of Aβ, such as Aβ25-35 or Aβ22-35 display similar toxicity and tendency to aggregate as the full-length counterparts Aβ1-40 or Aβ1-42 (Pike et al., 1995).

1.2.7 Current understanding of AD

Since the strong correlation between Aβ accumulation and AD progression has been established, significant efforts have been made to inhibit the production of Aβ as a potential therapeutic target. One strategy aimed to target β-secretase, responsible for cleaving APP to produce Aβ (Tomita, 2009; De Strooper et al., 2010). Despite the efforts, however, several Aβ synthesis blocking drug developments have recently failed phase three trials due to low clinical efficacy (Wischik et al., 2014). With the repeated failure of drugs targeting Aβ synthesis, the therapeutic development has recently shifted its aim to increasing Aβ clearance or degradation rather than blocking synthesis.

In normal physiology, the rate of Aβ synthesis and clearance measured in cerebrospinal fluid (CSF) is 7.6% and 8.3% of total volume, respectively, indicating that in healthy individuals Aβ accumulation will not occur (Bateman et al., 2006). However, even the slightest defect in Aβ clearance mechanism leads to rapid accumulation of Aβ resulting in cell toxicity. Accumulating evidence suggests that defective clearance of Aβ, rather than synthesis, is the main mechanism of Aβ accumulation (Weller et al., 2000). Specifically, the accumulation of Aβ is accentuated on the walls of capillaries and arteries leading to cerebral amyloid angiopathy (CAA) present in approximately 90% of AD patients (Love, 2004). Currently, the clearance mechanisms of Aβ from the brain can be broadly classified into four separate pathways 1) phagocytosis by glial cells such as microglia and astrocytes, 2) interstitial fluid (ISF) drainage, and 3) enzymatic degradation by proteins such as neprilysin (NEP), matrix metalloproteinase (MMP-9) and insulin-degrading enzyme (IDE), and 4)

active transport across blood brain barrier into blood vessel via various receptors such as LRP1, P-glycoprotein and very low-density lipoprotein receptor (VLDLR) (Shibata et al., 2000; Deane et al., 2004). In this project, we aimed to identify the role of somatostatin (SST) on active transport via receptors and enzymatic degradation via MMPs, as the cell line model we have utilized, human neuroblastoma cell line SH-SY5Y, were devoid of significant glial cell population and ISF drainage could not be replicated *in vitro*.

1.3 Somatostatin

SST, also known as somatotropin release-inhibiting hormone (SRIF), is a regulatory peptide initially identified in the hypothalamus as a growth hormone release inhibiting peptide (Brazeau et al., 1973). A phylogenetically ancient peptide, SST is found in both vertebrate and invertebrate as well as in the plant kingdom (Reichlin, 1983). Since the initial discovery, it has been reported that the production of SST is not restricted to hypothalamus but rather throughout the central and peripheral nervous system (Hokfelt et al., 1975; Patel and Reichlin, 1978; Reichlin, 1983). Outside of the CNS, SST expression has been observed in the gastrointestinal tract (GIT) and delta (δ) cells of pancreatic islets (Luft et al., 1974; Arimura et al., 1975; Dubois, 1975; Hokfelt et al., 1975; Orci et al., 1975; Pelletier et al., 1975; Polak et al., 1975; Patel and Reichlin, 1978). In alignment with its broad anatomical distribution in production, SST has also been found to act on a multitude of tissues by binding to its family of five different receptors identified as SSTR1-5, resulting in various biological effects (Reichlin, 1983). Today, two isoforms of SST, the original SST-14 discovered in the hypothalamus and a variant with longer N-terminus (SST-28) is recognized. The shorter form, SST-14 is commonly found in the neural tissues and pancreatic islets

whereas the longer variant SST-28 is found in gastrointestinal tracts (GIT) (Patel et al., 1981; Patel, 1999).

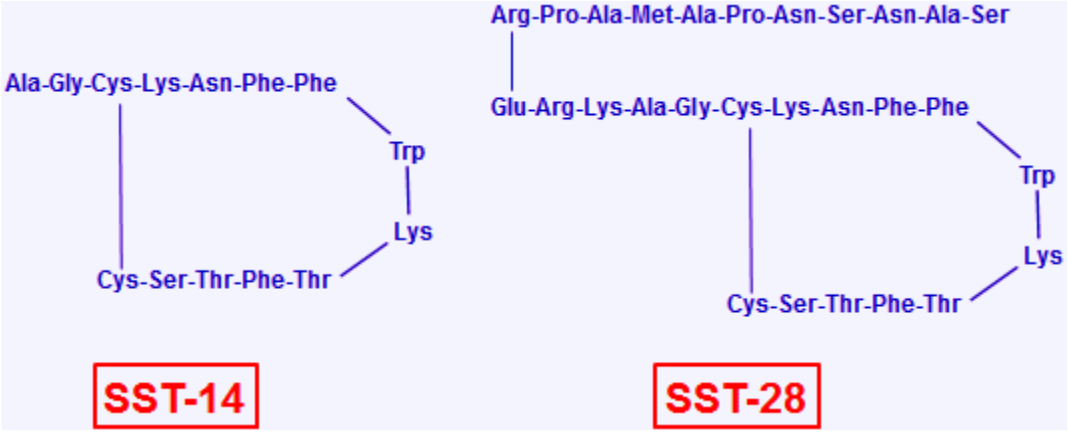

Figure 1 Schematic representation of SST-14 and SST-28 (reprinted with permission from War, S. A. (2013). Functional elucidation of human somatostatin receptor-3 : implications in breast tumor biology (T). University of British Columbia. Retrieved from https://open.library.ubc.ca/collections/ubctheses/24/items/1.0103354).

1.3.1 Processing and distribution of SST

Similar to other peptide hormones, SST is synthesized from a large precursor protein which undergoes series of enzymatic processes. In humans, 116-amino acid long preprosomatostatin (preproSST) undergoes a cascade of enzymatic cleavage to yield 92-amino acid long prosomatostatin (proSST) (Patel, 1999). The proSST is in turn enzymatically cleaved at C-terminal to yield two variants of bioactive isoforms, SST-14 and SST-28 (Patel, 1999). The shorter variant, SST-14, is predominantly expressed in neural tissues, stomach, pancreatic islets and in particular is the only isoform observed in the retina, enteric neurons, and in peripheral nerves (Patel et al., 1981). The longer variant, SST-28 constitutes approximately 20 – 30% of all SST positive neurons in the brain, however, whether the production of these two variants are derived from a single source or whether they are

predominantly produced in different regions of the brain remains elusive (Patel et al., 1981). The highest density of SST producing cells are found in CNS and in the endocrine pancreas along with smaller densities found in thyroid, kidneys, prostate and placenta (Reichlin, 1983). In CNS, the SST producing cells are localized in specific regions of hypothalamus and cortex along with all of limbic structures and striatum. A notable absence of SST producing cells can also be found in the cerebellum (Finley et al., 1981; Johansson et al., 1984). Site specific distribution of SST is shown in table 2.

Along with the production of SST, the mechanism of SST secretion has also been well documented. It has been reported that the secretion of SST is modulated by a broad range of stimulus including neuropeptides, neurotransmitters, cytokines, hormones, ions and nutrients (Patel et al.; Patel et al.; Reichlin, 1983). Some elicit universal effects on SST secretion regardless of the cell type or tissues, while others are more tissue-specific. For example, the majority of neuropeptides and neurotransmitters tested exerted a pro-secretory effect on SST release while opiates and gamma-aminobutyric acid (GABA) generally had an anti-secretory role on SST (Epelbaum et al., 1994). Insulin, on the other hand, stimulates SST release in hypothalamus while inhibiting the release in islet and gut (Berelowitz et al., 1981).

1.3.2 Biochemical, physiological and pharmaceutical actions of SST

As previously described, SST acts on a multitude of targets such as in pancreas, GIT, thyroid, immune cells and in the brain acting as a neurotransmitter, auto-, endo- and paracrine regulator, and as an inhibitor of cell proliferation (Reichlin, 1983; Patel, 1999; Barnett, 2003). Importantly, the role of SST in the brain includes modulation of cognitive functions as well as in locomotor, sensory and autonomic pathway (Reichlin, 1983; Epelbaum et al., 1994; Patel, 1999; Barnett, 2003). In the midbrain, SST mediates the

inhibition of dopamine release while in hypothalamus SST mediates the inhibition of various hormones including thyrotropin-releasing hormone, corticotrophin-releasing hormone, and norepinephrine as well as inhibiting its own release via a negative feedback loop (Barinaga et al., 1985; Moller et al., 2003). Given its wide range of effect in various tissues, dysfunction of SST has been implicated in numerous diseases such as in AD (Davies et al., 1980; Mouradian et al., 1991; Dournaud et al., 1994; Dournaud et al., 1995; Grouselle et al., 1998; Kumar, 2005; Geci et al., 2007), Huntington's disease (Aronin et al., 1983; Beal et al., 1988a; Patel et al., 1991; Kumar et al., 1997; Rajput et al., 2011), Parkinson's disease (Beal et al., 1988b; Soghomonian and Chesselet, 1991; Epelbaum et al., 1994; Strittmatter et al., 1996), AIDS encephalitis (Fox et al., 1997), excitotoxicity (Kumar, 2004; 2008; Rajput et al., 2012) and epilepsy (Strowbridge et al., 1992). For this study, the focus will be laid on the involvement of SST in Alzheimer's disease and in the neuronal differentiation.

1.3.3 Somatostatin receptors (SSTRs)

SST mediates its physiological effect by binding to the respective receptors. Currently, five different SSTR subtypes (SSTR1-5) have been identified that have nanomolar (nM) binding affinity to either SST-14 or SST-28 (Schonbrunn and Tashjian, 1978; Mandarino et al., 1981; Srikant and Patel, 1981; Kumar and Grant, 2010). Initially identified by Schonbrunn and Tasjian in 1978, all five different SST receptor subtypes have since then been cloned and sequenced (Bruno et al., 1992; Kluxen et al., 1992; Yamada et al., 1992a; Yamada et al., 1992b; Yasuda et al., 1992; Yamada et al., 1993a; Yamada et al., 1993b; Viollet et al., 2008). In addition to the classification into five receptors by structural differences, SSTRs can also be classified into two distinct class based on their affinity to octreotide (OCT), where SRIF-1 group (SSTR2, SSTR3, and SSTR5) are activated by OCT

while SRIF-2 group (SSTR1 and SSTR4) does not display such activation (Tran et al., 1985; Reisine and Bell, 1995; Kumar and Grant, 2010). With the development of SSTR specific antibodies, the tissue-specific distribution of SSTRs was assessed. In subsequent studies, SSTR-like immunoreactivity was observed in pancreas, GIT, spleen, lungs, thyroid, kidneys, immune cells and in the brain (Dournaud et al., 1996; Hukovic et al., 1996; Helboe et al., 1997; Kumar et al., 1997; Handel et al., 1999; Kumar et al., 1999; Schulz et al., 2000; Fischer et al., 2008; Watt et al., 2008; Lupp et al., 2011; Lupp et al., 2012).

Table 2 SST and SSTR distribution

Regions	Reference
Hypothalamus Deep layers of cortex	(Patel and Reichlin, 1978; Kumar, 2007)
Limbic system Subpopulation of C cells in thyroids	(Reichlin, 1983)
Stomach	(Arimura et al., 1975)
Pancreas	(Arimura et al., 1975; Dubois, 1975; Pelletier et al., 1975; Polak et al., 1975)
Gut	(Hokfelt et al., 1975)
Thyroid	(Fuller and Verity, 1989)
Adrenals	(Maurer and Reubi, 1986)
Submandibular glands	(Deville de Periere et al., 1988)
Kidney	(Bhandari et al., 2008)
Prostate	(Reubi et al., 1995)
Placenta	(Lee et al., 1982)
Blood Vessel Walls	(Reynaert et al., 2007)
Immune Cells	(Ferone et al., 2004)

1.3.4 SST in Alzheimer's disease

Among the various neuropeptides whose expression and/or activity is significantly changed in AD, SST is one of the most consistently reduced peptide in both the brain and CSF samples of AD patients (Davies et al., 1980; Beal et al., 1986; Davis et al., 1988; Bissette and Myers, 1992; Nemeroff et al., 1992; Molchan et al., 1993; Bissette et al., 1998;

Nilsson et al., 2001). In this regard, our laboratory has also reported significant reduction (~70%) of SST-positive neurons in AD patients brain sections compared to the healthy control (Kumar, 2005). In transgenic PS1xAPP AD mice model, a significant reduction in SST-positive neurons, as well as the loss of SST mRNA in the hippocampus, has been reported while other neuronal markers of glutamatergic or cholinergic system displayed limited to no detectable changes (Ramos et al., 2006). In addition, constitutive intracerebroventricular (i.c.v) infusion of Aβ in rat model resulted in significant reduction of SST-positive neurons in various regions of the brain including hippocampus, frontoparietal and temporal cortex (Nag et al., 1999; Aguado-Llera et al., 2005; Hervas-Aguilar et al., 2005; Burgos-Ramos et al., 2007). Similar results were also observed in postmortem human brains from AD patients (Davies et al., 1980). Currently, the correlation between reduction of SST during the onset and progression of the AD is well established and consequently, the SST expression level in the brain is gaining interest as a potential therapeutic marker of early stages of the AD (Ramos et al., 2006).

In addition to the temporal overlap between progression of AD and reduction of SST, there is also a significant spatial overlap between beta-amyloid and SST. Studies have reported significant overlap between SST14 and beta-amyloid in the hippocampus, frontal and parietal cortex, hypothalamus and amygdala of AD patients (Schettini, 1991; Gahete et al., 2010). In corroboration, histochemical analysis of brain slices of AD patients reveals colocalization of somatostatinergic neurons in close proximity with beta-amyloid plaques in the cortex, amygdala, and hippocampus (Armstrong et al., 1985; Morrison et al., 1985). Furthermore, more specific colocalization of somatostatinergic cells to beta amyloids in olfactory and piriform cortices has also been reported (Saiz-Sanchez et al., 2015).

1.3.5 SSTR and Alzheimer's disease

Along with correlation between SST and beta-amyloid, a significant interaction between various SSTRs and progression of the AD has been reported. In the postmortem brain sections of AD brains, up to 50% reduction in SSTR density has been reported in frontal and temporal cortices compared to the age-matched control (Beal et al., 1985). In another study, the continuous injection of Aβ via i.c.v infusion in rat resulted in a selective reduction of SSTR2 mRNA as well as protein expression in a cortex-specific manner with no effect in the hippocampus (Aguado-Llera et al., 2005; Hervas-Aguilar et al., 2005; Burgos-Ramos et al., 2007). Along with histochemical studies to monitor localization and changes in the expression of total SSTRs in postmortem AD brains, more specific analysis has also been performed using radio-ligand labeling. As previously reported by Krantic et al., a significant reduction in binding capacity of potent SST analog (SMS201-995) against SSTRs (specifically with SSTR2, 3 and 5) in cortices of AD patients was observed (Krantic et al., 1992). In this regard, our laboratory has also done extensive research in elucidating the changes in SSTRs during onset and progression of the AD, and have reported a marked reduction of SSTR2, 4, 5 with no significant changes in SSTR1 and a significant increase in SSTR3 (Kumar, 2005).

However, contradicting reports have been made with no significant changes of SSTRs in postmortem AD brains (Whitford et al., 1988). It is likely that the difference in different staining protocol, such antibodies used and fixation method utilized, as well as the difference in the severity of the disease, contribute to such discrepancies.

1.3.6 SST and neprilysin

Amyloid hypothesis emphasizes the neurotoxic effect of accumulating Aβ in the brain leading to onset and progression of the AD (Hardy and Selkoe 2002 Science). In a healthy brain, various components are involved in keeping Aβ level in equilibrium. APP cleaving enzymes, β- and γ-secretase have to be functional, naturally occurring Aβ degrading enzymes must be produced in sufficient level and the rate of subsequent oligomerization of Aβ must be kept in check (Turner and Nalivaeva, 2007). Specifically, the recent effort has been focused on identification of potent Aβ degrading enzyme for therapeutic applicability. Currently, a number of such enzymes have been identified including endothelin converting enzyme (Eckman et al., 2001), insulin-degrading enzyme (Farris et al., 2003), and family of zinc metallopeptidase enzyme (Iwata et al., 2000; Iwata et al., 2001). Among the known zinc metallopeptidase, growing evidence indicates neprilysin as the most potent enzyme for degradation of Aβ. Shirotani et al. have reported that neprilysin efficiently degraded both Aβ1-40 and Aβ1-42 *in vivo*, while Iwata et al. reported a significant increase of both Aβ1-40 and Aβ1-42 in neprilysin knockout mice (Iwata et al., 2001; Shirotani et al., 2001). Furthermore, enhanced neprilysin activity in APP transgenic mice resulted in a significant inhibition in the accumulation of both soluble and fibrillar variants of Aβ (Leissring et al., 2003; Iwata et al., 2004). Importantly, the level of neprilysin was observed to be decreased with age as well as in the early stages of the AD (Yasojima et al., 2001; Iwata et al., 2002).

With the growing evidence supporting the role of neprilysin as an efficient inhibitor of Aβ accumulation, regulation of neprilysin activity and expression has been gaining attention as a potential therapeutic agent. Interestingly, a number of studies have identified that SST is the most potent regulator of neprilysin. Saito et al. first reported that in cortical neurons, neprilysin activity was significantly elevated in presence of SST while the Aβ1-42

level in culture medium showed a significant reduction (Saito et al., 2005). The specificity of SST mediated effects has been confirmed as the use of SSTR specific antagonist (BIM23056) or G_i inhibitor (pertussis toxin) both negated the somatostatin-mediated increase in neprilysin activation, while G_s inhibitor (cholera toxin) had no effect. In the same study using SST knockout mice, Saito et al. report the significantly lower neprilysin activity compared to *wt* littermates with approximately 50% increase in Aβ1-42 levels (Saito et al., 2005). Collectively, these findings suggest that decrease of SST expression during aging results in a subsequent reduction of neprilysin activity, leading to increased Aβ accumulation and ultimately the onset of the senile AD (Hama and Saido, 2005) (Fig. 2).

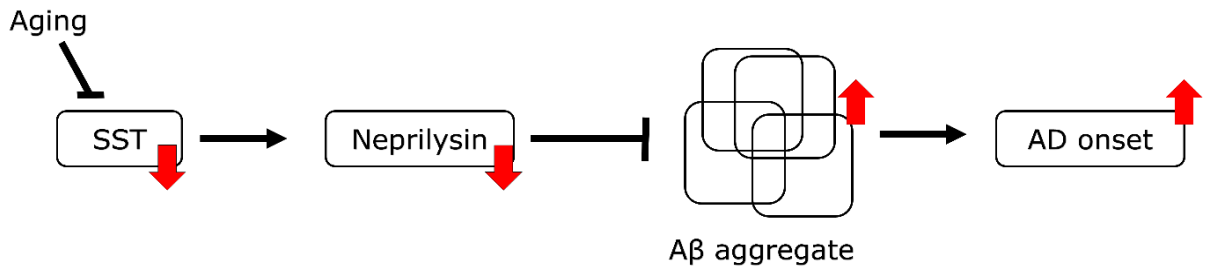

Figure 2 Schematic illustration of the effect of aging/Aβ accumulation in SST expression and AD onset. Decreased SST level with age leads to a reduction of Aβ degrading enzyme, neprilysin, attributing to the accumulation of Aβ and onset of the senile AD.

Previous studies have suggested a significant neuroprotective role of SST during onset and progression of the AD, however, the exact mechanism remains elusive. Accordingly, the present study aims to elucidate the role of SST in the AD and furthermore identify the role of SST in the maintenance of the normal nervous system by monitoring its effect on neuronal culture.

1.4 Blood Brain Barrier

In the mammalian system, circulating blood and interstitial fluid surrounding various tissues are separated by biological barriers. Among them, the barrier separating the blood vessels of the central nervous system (CNS), appropriately termed blood brain barrier (BBB), is perhaps the most extensively studied. As the brain requires maintenance of a precise environment for optimal transduction of electrical and chemical signaling between neurons, BBB is understandably extremely selective and tightly regulated. Specifically, BBB is responsible for the regulation of influx and efflux of various ions, transporting nutrients and oxygen from blood to brain, while also maintaining potentially harmful substrates out. In order to carry out this complex functionality, the CNS endothelial cells making up the BBB have distinct properties that other barriers do not have such as a relatively low level of transcytotic vesicles and absence of fenestrae, tight junction proteins (TJPs) with high electrical resistance to limit paracellular transport, BBB-specific transporters, and receptors regulating transcellular metabolite transportations (Abbott et al., 2010; Saunders et al., 2012).

1.4.1 Neurovascular unit (NVU)

Contrary to the previously accepted concept of 'brick wall,' studies indicate that BBB is a much more complex system (Lampron et al., 2013). To modulate the complex mechanism of selectively transporting various molecules at the highest efficiency, simple endothelial cell linings of CNS vasculature is not sufficient. Along with endothelial cells, functional BBB is composed of an intricate network of varying cell types including astrocytes, microglia, pericytes as well as numerous other components of brain parenchyma to collectively form 'neurovascular unit' (Fig. 3) (Obermeier et al., 2013).

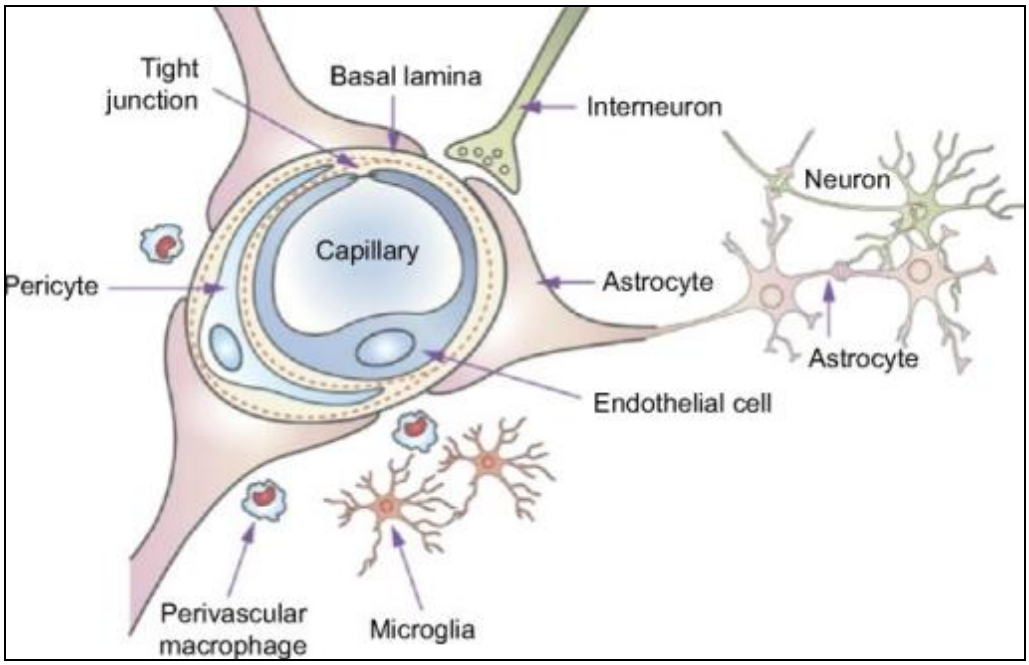

Figure 3 Schematic representation of the NVU [from (Feng et al., 2015)]. Capillary is encapsulated by intricate network NVU and the transportation of substances across blood-brain is tightly regulated.

1.4.1.1 Astrocytes

Astrocytes are 'star-shaped' glial cells that provide biochemical support and protection of neurons by modulating the release of neurotransmitters, regulating extracellular ion balance and immune reactions (Rodriguez-Arellano et al., 2016). In addition, astrocytes form end-feet projections that surround the abluminal side of cerebral capillaries made of endothelial cells (Abbott et al., 2006). In normal physiology, such physical interaction between astrocyte and endothelial cells are critical in the maintenance of metabolite levels (Zlokovic, 2008). Furthermore, multiple studies report that astrocytes release several effector molecules such as apoE and renin-angiotensin hormone which plays a critical role in maintaining BBB integrity (Wosik et al., 2007; Bell et al., 2012).

1.4.1.2 Pericytes

Pericytes are mesodermal derived perivascular cells that ensheath CNS vessel walls. Compared to peripheral vessels, the vasculature system of CNS has a significantly higher amount of pericytes which regulates cerebral blood flow by directly affecting capillary diameter while also affecting extracellular matrix (ECM) secretion (Winkler et al., 2011). The critical role of pericytes in normal BBB formation is well presented in series of knockout or knockdown studies of platelet-derived growth factor (*Pdgf*β) transgenic model. As the *Pdgf*β stimulates recruitment and proliferation of PDGFRβ-positive pericytes, the mice displayed significant deterioration such as dysfunctional TJs, increased microhaemorrhages and increased vascular permeability (Lindahl et al., 1997; Daneman et al., 2010). Collectively, these findings establish pericytes as a critical requirement in both the formation and maintenance of normal BBB.

1.4.1.3 Microglia

Unlike astrocytes or pericytes, the direct involvement of microglia during formation and maintenance of BBB is not well established. Rather, microglia acts as a main immune response in the CNS (Ginhoux et al., 2010). It has been established that microglia can be activated in two alternate pathways: M1 or M2. In M1 activation, microglia is mainly involved in the release of proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β). In M2 activation, microglia serves a neuroprotective role including phagocytosis of foreign materials and dysfunctional neurons, secretion of various growth factors and chemokines, and tissue repair (Aguzzi et al., 2013). Of particular interest in the AD, over-activation of microglia results in hyper-neuroinflammation associated with

disintegration of BBB (da Fonseca et al., 2014). Furthermore, brain endothelial cells from AD patients were observed to release microglia activating neurotoxic protease thrombin, where the resulting activation of microglia led to the loss of dopaminergic neurons (Huang et al., 2008; Yin et al., 2010).

1.4.2 Transportation across BBB

As previously indicated, transportation of molecule through BBB is tightly regulated. Passive diffusion across BBB is limited to substances that are 1) lipid-soluble, 2) contains less than 10 hydrogen bonds and 3) less than approximately 180 kDa in size (Pardridge, 2009). For essential nutrients, such as glucose, the substance is transported through BBB via carrier-mediated transportation (Abbott et al., 2010). For all Aβ, both brain-derived and peripheral, the transportation across BBB occurs via receptor-mediated transcytosis. Among the number of receptors identified, however, two receptors have been studied most extensively for their prominent role in Aβ transcytosis: low-density LRP1 and receptor for advanced glycation end products (RAGE).

1.4.2.1 Aβ transporting proteins

Along with mediating cellular uptake of Aβ in phagocytic cells such as astrocytes and microglia for subsequent degradation, LRP1 also plays a critical role as a brain to blood efflux transporter (Deane et al., 2004; Kanekiyo et al., 2012; Kanekiyo et al., 2013). The LRP1/Aβ binding on the abluminal side of the BBB results in clearance of Aβ, and reduction of the LRP1 level has been observed during normal aging as reported in various animal models including rodents and primates including humans (Deane et al., 2004). Most importantly, however, the LRP1 level was drastically decreased with progression of the AD,

in both transgenic mice models and human patients, suggesting that dysfunction of LRP1 activity and/or expression may result in inhibition of efficient Aβ clearance in AD patients (Bell et al., 2009). Although the exact mechanism of LRP1/Aβ binding remains elusive, studies have reported the involvement of phosphatidylinositol binding clathrin assembly protein (PICALM) as one of the potential protein necessary for successful transcytosis of the LRP1/Aβ complex across BBB (Harold et al., 2009; Zhao et al., 2015). Along with its prevalent presence on the surface of neurons and microglia, RAGE is also present on brain endothelial cells and plays a critical role in Aβ transcytosis from blood to brain and is reported to be drastically increased in AD patients (Deane et al., 2003).

1.4.2.2 Adherens and tight junctions

Along with transcellular transportation, BBB must maintain highly regulated and selective paracellular transportation to serve its neuroprotective role. Adherens junction proteins (AJPs) and tight junction proteins (TJPs) are a group of multiprotein complexes that interlocks adjacent endothelial cells and maintains its structural and functional integrity. AJPs are critical in initiating cell-cell contact between adjacent endothelial cells, interact with the actin cytoskeleton and promote endothelial cell survival (Hawkins and Davis, 2005; Tietz and Engelhardt, 2015). TJPs on other hand regulate the paracellular transportation of ions and molecules. Major TJPs at BBB include proteins such as occludin, claudin (specifically claudin-5 and -12) and junctional adhesion molecules (JAM) (Daneman et al., 2010; Haseloff et al., 2015). Importantly, the TJPs are interconnected to the cytoskeleton of BBB endothelial cells by class of interconnecting proteins such as zonula occluden-1 (ZO-1), which plays a critical role in maintaining the paracellular integrity of BBB (Fanning et al., 1998; Luissint et al., 2012). In addition to interconnecting TJPs to the cytoskeleton, ZO-1 also plays a crucial

role in regulating VE-cadherin activity involved in maintaining cell-cell tension, cytoskeletal organization and expression and localization of claudin-5 (Taddei et al., 2008; Tornavaca et al., 2015). Furthermore, ZO-1 mediated VE-cadherin activity is also involved in regulating barrier strength by modulating the level of occludin expression (Walsh et al., 2011). Understandably, dysfunction and disintegration of TJPs lead to increase in BBB permeability, implicated in various neurodegenerative diseases such as AD and multiple sclerosis (Zhong et al., 2008; Miyazaki et al., 2011; Hartz et al., 2012).

1.4.3 Systemic inflammation and AD

Although the exact role and mechanism of neuroinflammation in pathogenesis and progression of AD remains elusive, growing body of evidence suggests a significant contribution of inflammation in onset and progression of the AD (Akiyama et al., 2000; Lee et al., 2010; Holmes and Butchart, 2011). Along with other hallmarks of the AD, such as senile plaque and NFT formations, accumulation of activated immune cells in the brains of AD patients have been well documented (Dickson et al., 1993; Akiyama et al., 2000; Letiembre et al., 2009). Specifically, significant co-localization of astrocytes and microglia has been observed with clusters of Aβ plaque in parallel with increased release of inflammatory cytokines such as interleukin-1 and -6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) (Haga et al., 1989; Bauer et al., 1991; van der Wal et al., 1993; Griffin et al., 1995; Mrak et al., 1996; Tarkowski et al., 1999; Simard et al., 2006). In various transgenic mice models of the AD (ie. Tg2576, PS1xAPP and 3xTg), significant enhancement of inflammation is observed at immediate surrounding regions of Aβ plaques which are also observed in human AD patients (Frautschy et al., 1998; Matsuoka et al., 2001; Janelsins et al., 2005). Furthermore, although not yet conclusive, some studies suggest that

neuroinflammation precedes Aβ plaque formation and may contribute to disease pathogenesis. Corroborating this suggestion, certain markers of glial cell activation was observed prior to detectable accumulation of Aβ plaque while direct injection of IL-1β in mice resulted in significant up-regulation of APP (Sheng et al., 1996; Sheng et al., 2000).

1.4.3.1 Neuroinflammation and BBB

It has been previously reported that systematic inflammation induced by peripheral injection of LPS in APP mice showed significantly higher inflammatory cytokine in the brain compared to wildtype control, suggesting that leaky BBB in AD mice were more susceptible to inflammation (Takeda et al., 2013). In parallel, it has also been reported that the disintegration of BBB in transgenic APP mice precede the detectable formation of the pathological morphology of AD, such as accumulation of Aβ plaque (Ujiie et al., 2003). Specifically, the disintegration of BBB was detected as early as in 4-month-old APP transgenic mice, which significantly precedes the formation of Aβ plaque and measurable cognitive deficit. Collectively, these findings suggest that BBB may be particularly susceptible to inflammation-mediated damage, which may exacerbate neuroinflammation mediated neurotoxicity in AD patients. Accordingly, identification of agent capable of reducing neuroinflammation may be a viable therapeutic target in the treatment of various neurodegenerative diseases such as the AD.

1.4.3.2 Matrix metalloproteinase (MMP) and ECM remodeling

Matrix metalloproteinases (MMPs) are a family of 23 proteases in humans regulating various physiological activities including growth factor activation and extracellular matrix (ECM) remodeling. Among its various functions, the ECM remodeling functionality of MMPs has been gaining interest due to its implication in pathogenesis and progression of

neurodegenerative diseases. Particularly, MMP mediates breakdown of ECM and TJPs interlocking paracellular endothelial cells of BBB has been implicated in increased BBB permeability. Among them, MMP2 and MMP9 have been studied extensively for their role in ECM disruption. Specifically, elevated MMP2 levels were observed in immediate surroundings of Aβ affected vessels in human patients with grade 1 or 2 CAA as well as in APP transgenic mice models (Greenberg and Vonsattel, 1997; Yin et al., 2006). Recent findings have found a significant correlation between Aβ level and activity of MMPs. Hartz et al. reported the isolated rat brain microvessel treated with Aβ1-40 displayed a significant increase in MMP2 expression *in vitro* while transgenic APP mice showed a significant increase in MMP2 and MMP9 *in vivo* (Hartz et al., 2012).

1.4.3.3 JNK mediated regulation of MMP expression

During the reorganization of cytoskeleton system, various downstream signaling cascades are modulated including members of mitogen-activated protein kinase (MAPK), c-JUN NH2-terminal kinase (JNK), Rho-family GTPases (Rho, RAc, and Cdc42), focal adhesion kinase (FAK), p21-activated kinase (PAK) and phosphoinositide 3-kinase (PI3K) (Keely et al., 1997; Barberis et al., 2000; Birukov et al., 2002; Kiosses et al., 2002; Ailenberg and Silverman, 2003; Wojciak-Stothard and Ridley, 2003). Among these signaling molecules, ERK1/2 and JNK have been reported to have a significant regulatory role in the expression of MMP-2 (D'Angelo et al., 1995; Minden et al., 1995; Meadows et al., 2004; Boyd et al., 2005). In primary culture of rat skeletal muscle endothelial cells, Ispanovic et al. reported that the activation of JNK resulted in the subsequent elevation of MMP-2 mRNA (Ispanovic and Haas, 2006). In another study, inhibition of JNK, but not ERK1/2 signaling, resulted in MMP2 activity corroborating previous studies (Fromigue et al., 2008). Taken together, these

findings suggest that the regulation of MAPK activity may inhibit the hyperactivity of MMPs in response to Aβ.

To assess the role of SST in Aβ-induced toxicity of BBB, we have utilized hCMEC/D3 cell line. hCMEC/D3 is a cell line which has the capacity to closely resemble the physiological BBB structure with the formation of various TJPs and AJPs including ZO-1, claudin and occludin and have been widely used as an *in vitro* model of BBB. Along with the role of BBB in the maintenance of normal CNS physiology, maintenance of normal neuronal population during AD is also critical. In this regard, we studied the effect of somatostatin in promoting the neurite outgrowth using human neuroblastoma cell line SH-SY5Y.

1.5 Use of human neuroblastoma SH-SY5Y cells as an alternative model to study neuronal differentiation, maturation, and Aβ toxicity

Various immortalized cell lines have been developed to overcome the ethical and technical limitations in the use of animals for research. In particular, human neuroblastoma-derived SH-SY5Y cell is an established model to study neuronal differentiation in *in vitro* setting with well-characterized morphology and genotype (Rossino et al., 1991; Kaplan et al., 1993; Kito et al., 1997; Truckenmiller et al., 2001; Lopez-Carballo et al., 2002; Brill and Bennett, 2003; Conn et al., 2003; Ding et al., 2004; Miloso et al., 2004; Pan et al., 2005; Cuende et al., 2008; Cheung et al., 2009). Importantly, SH-SY5Y cells have the capacity to be differentiated into various neuronal subtypes including cholinergic, dopaminergic and adrenergic neurons based on different neuronal differentiation factors used (Pahlman et al., 1984; Xie et al., 2010). Among them, retinoic acid (RA; vitamin A metabolite) induced neuronal differentiation have been routinely used to yield a homogenous population of neuronal cells (Encinas M 2000 J Neurochemistry, Almeida A 2005 Neuroscience, Lasorella

A 2006 Nature). Specifically, the RA treatment results in neurite formation (Stio et al., 2001), increased acetylcholinesterase (AChE) activity (Sidell et al., 1984) and increased production of synaptic vesicle (Sarkanen et al., 2007). Another common differentiation method of SH-SY5Y cells is with the use of brain-derived neurotrophic factor (BDNF). Specifically, SH-SY5Y cells initially differentiated followed by treatment with BDNF displayed higher cell survival and further withdrawal from the cell cycle (Encinas M 2000 J Neurochem, Forster JI 2016 J Biomol Screen). In particular, translation of key synaptic components such as N-methyl-D-aspartate receptor [NMDA receptors (NR1 and 3)], PSD-95 and CamKII requires the presence of BDNF (Schratt et al., 2004). In one interesting study by Belinda J. Goldie et al. the authors surveyed all papers published between 2000 and 2014 which differentiated SH-SY5Y into neuronal phenotype and summarized the differentiation protocol used (Goldie et al., 2014). Specifically, Goldie et al. report that the vast majority of the studies (72% of all studies) used retinoic acid alone to induce neuronal differentiation while 11% of studies utilized sequential treatment of RA followed by BDNF. In 16% of studies, alternative differentiation agent such phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) was used, which promotes adrenergic receptor induction. In this regard, SH-SY5Y cells present itself as an excellent model to study various aspects of Aβ induced toxicity, as one can monitor the effect on differentiated neurons as well as in immature neurons undergoing differentiation.

1.5.1 MAP2, Tau and Tuj1 expression during neuronal differentiation

To assess the neuronal differentiation of neuroblastoma cell lines, various neuronal markers have been utilized. Among the neuron-specific markers, microtubule-associated protein 2 (MAP2) and Tau have been used extensively to establish neuronal commitment

(Sternberger et al., 1985). Structurally, MAP2 and Tau proteins both contain microtubule-binding domain near the carboxyl terminus (Lewis et al., 1988) and functions as microtubule stabilizing proteins. In addition, MAP2/Tau proteins are natively unfolded molecules, and only after binding to their targets such as microtubules, F-actin and possibly more unidentified cytoskeletal subunits are expected to display folded conformation (Uversky, 2002).

In mammals, the MAP2 expression is mainly in neurons, with limited expression detected in non-neuronal cells such as oligodendrocytes. In undifferentiated precursor neurons, the expression of MAP2 is very weak but gradually becomes strong with the progression of neuronal differentiation. Specifically, the expression of MAP2 becomes significant approximately 24 hours following the expression of neuron-specific tubulin isoform βIII (TUJ1) (Menezes and Luskin, 1994). MAP2 has 4 isoforms (MAP2a-d), with temporal-specific expression during differentiation. Among them, the shorter (~70kDa) MAP2c is the juvenile isoform which is only expressed during early stages of neuronal differentiation followed by a gradual loss, while longer isoforms (~220kDa) MAP2a and b are considered mature isoforms with expression in both differentiating and in mature neurons (Chung et al., 1996). MAP2d has limited expression throughout neuronal differentiation.

Similar to MAP2 expression during neuronal differentiation, Tau protein also shows the temporal specification of specific isoforms during differentiation. Specifically, shorter isoform (~65kDa) of Tau with three microtubule-binding protein domains are the major form of Tau expressed during early stages of differentiation. Later, in more mature neurons, longer Tau isoform (~120kDa) with four microtubule-binding protein domain become the major isoform of Tau (Goedert et al., 1989; Kosik et al., 1989). Along with temporal specification, both MAP2 and Tau displays spatial localization during neuronal differentiation as well.

Following the commitment of single neurite into axon in a process called axonogenesis, MAP2 gradually localizes into nascent dendrites (also called 'minor neurites' at this stage) while Tau becomes segregated specifically into axons (Matus, 1990). This spatial specification of MAP2 and Tau is thought to be mediated through a combination of multiple regulatory mechanisms including differential protein sorting, protein stability, and specific transportation of MAP2 mRNA (Garner et al., 1988; Kanai and Hirokawa, 1995; Hirokawa et al., 1996). Collectively, the expression of MAP2 is restricted to dendrites while Tau is found in axons in the mature neuron.

Moreover, microtubules play a critical role in various cellular functions including intracellular transport, regulating cell morphology, mitosis, and differentiation (Desai and Mitchison, 1997; Howard and Hyman, 2003). Tubulin, a subunit protein component of microtubulin, exist as an α/β-heterodimer with various isotypes of both α- and β-tubulin with unique amino acid sequences and preferential tissue distribution. In mammals, seven β-tubulin isoforms have been identified and are referred to as βI to βVI (Luduena, 1998). The functional difference between β-tubulin isoforms has revealed that any of the isoforms had the capacity to carry out the basic functions such as forming an interphase network and mitotic spindle (Joshi et al., 1987; Lewis and Cowan, 1988). However, certain isotype preference was also identified in a cell type-specific manner as βI- and βIV-tubulin were observed to be specifically enriched in axonemal microtubules (Renthal et al., 1993; Jensen-Smith et al., 2003). In particular, βIII-tubulin (Tuj1) shows a significant expression and distributional specification in the nervous system to an extent where Tuj1 specific antibodies are one of the most common markers of neurons in both *in vitro* and *in situ* studies (Lee et al., 1990; Ferreira and Caceres, 1992; Easter et al., 1993; Luduena, 1998). In addition,

fluorescent proteins driven by Tuj1-promoter has been developed to study neuronal development in mice (Liu et al., 2007).

1.5.2 Functional role of MAP2 and Tau during differentiation and maintenance of normal neuronal morphology

As previously described, both MAP2 and Tau contains multiple microtubule binding domain. Ultrastructural analyses of MAP2 and Tau showed significant colocalization with microtubules (Hirokawa et al., 1988a; Hirokawa et al., 1988b; Al-Bassam et al., 2002). Along with spatial localization found in structural analyses, the functional analyses identified that the binding of MAPs with microtubules resulted in significant increase in stability and rigidity of microtubules (Felgner et al., 1997). In support of *in vitro* results, various studies from knockout mice model have corroborated the supportive role of MAPs on microtubule assembly. In mice model, homozygous knockout of MAP2 did not result in significant changes in overall brain morphology but displayed reduced microtubule density in dendrites (Harada et al., 2002). Furthermore, the cultured neurons derived from homozygous knockout mice displayed significantly reduced dendrite length suggesting a positive correlation between MAP2, microtubule and neurite elongation. In double-knockout of MAP1B, a MAP2 isoform, in combination with Tau or MAP2 resulted in much more severe morphological changes compared to double-knockout model (DiTella et al., 1996; Takei et al., 2000; Teng et al., 2001). These results indicate that different MAPs may share redundant roles and act in a cooperative manner in the normal physiological state. Furthermore, in homozygous Tau knockout, mice did not show significant changes in brain morphology but displayed a significant loss of microtubule density in axons indicative of the essential role of Tau in maintaining normal microtubule formation (Harada et al., 1994).

1.5.3 MAP2/Tau in microtubule regulation during neuronal differentiation

Microtubules are highly regulated structure constantly undergoing phases of rapid growth, pausing and retraction. Specifically, the transition from shortening to growth phase is called ‘rescue,’ while the transition from growth to shortening is called ‘catastrophe.’ This highly dynamic transition is in part regulated by binding of MAP2 and Tau proteins with microtubules (Gamblin et al., 1996; Al-Bassam et al., 2002; Panda et al., 2003). In particular, the shorter variant of MAP2, MAP2c promotes rescue while inhibiting catastrophe by reducing the duration and frequency of each catastrophe cycle (Gamblin et al., 1996). Furthermore, MAP2 may inhibit catastrophe by forming a cluster and binding to microtubule at which regions catastrophe event is blockaded (Ichihara et al., 2001). Along with MAP2, Tau also induces rescue phase of microtubule assembly by binding to microtubules and inhibiting depolymerization in these regions of contact (Panda et al., 2003). A comprehensive summary of the identified binding partner of Tau and MAP2 is listed in Table 2. However, the effect of MAP2 and Tau is not sufficient enough to induce complete activation of rescue phase or complete inhibition of catastrophe, as microtubules still undergo constant changes in phases even in presence of high concentrations of MAP2 and/or Tau (Kaech et al., 1996). Collectively, these findings suggest that MAP2/Tau activity is further regulated by various other factors including level of phosphorylation.

Table 3 Selected interaction partners of MAP2/Tau family proteins and proposed functions of the interaction. (Table modified from Dehmelt and Halpain., (2004) Genome Biology 6: 204).

Family	Interacting protein	Observed interaction	Reference
Tau	Microtubule (MT)	MT stabilization; catastrophe (depolymerization) inhibition; increase MT rigidity/stability	(Butner and Kirschner, 1991)
	Calmodulin	MT assembly	(Baudier et al., 1987)
	Calmodulin-related protein S100b	Tau phosphorylation by protein kinase C	(Baudier et al., 1987)
	Fyn	MT organization; involved in the pathogenesis of Alzheimer's disease	(Lee et al., 1998)
	Presenilin I	Involved in Tau interaction with glycogen synthase kinase 3β; pathogenesis of Alzheimer's disease	(Takashima et al., 1998)
MAP2	Microtubule (MT)	MT stabilization; catastrophe (depolymerization) inhibition; increase MT rigidity/stability, neurite outgrowth	(Kim et al., 1979)
	F-actin	Modulation of neurite outgrowth	(Roger et al., 2004)
	Regulatory subunit RII of PKA	Modulation of neurite outgrowth; cAMP-responsive element binding protein (CREB) phosphorylation	(Obar et al., 1989)
	MAP2-RNA trans-acting proteins MARTA1 and MARTA2	Interaction with MAP2 mRNA; induces localization of MAP2 mRNA to dendrites	(Rehbein et al., 2000)
	Neurofilaments	Crosslink between MT and neurofilaments	(Leterrier et al., 1982)
	Tyrosine kinase Src	Signal transduction and integration	(Lim and Halpain, 2000)

1.5.4 ERK1/2 activity and neuronal differentiation

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinase with a wide range of functions including modulation of cell cycles such as mitosis and apoptosis, inflammation, cell survival and differentiation (Lei et al., 2014). In eukaryotic cells, the activation of MAPK leads to ligand and cell type-specific response (Robinson and Cobb, 1997; Lewis et al., 1998). Particularly, the activation of specific MAPK, extracellular signal-regulated kinase (ERK) has been well established to be involved in regulation of neurite outgrowth (Watanabe et al., 2004). The initial neurite outgrowth process during induced neuronal differentiation of neurons and/or neuronal cell lines by stimulation of neural cell adhesion molecule such as nerve growth factor (NGF) requires activation of ERK (Klesse et al., 1999; Doherty et al., 2000). Another reported that the activation of ERK regulates neuronal differentiation in both *in vivo* mice model and *in vitro* culture of PC12 cells (Riese et al., 2004; Chen et al., 2009c). In particular, ERK-activation was absolutely required in the NGF-induced neuronal differentiation of PC12 cells (Meng et al., 2007; Obara et al., 2009; Washio et al., 2009). Furthermore, Robinson MJ et al., have reported that constitutively active ERK2 was sufficient to induce neurite outgrowth and neuronal differentiation of PC12 cells (Robinson et al., 1998). In turn, activation of ERK during neuronal differentiation induces the expression of p35, a subunit for cyclin-dependent kinase (CDK5) activation. The activated p35-CDK5 complex subsequently initiates neurite outgrowth in *in vitro* culture of neuronal cell lines and in *in vivo* differentiation (Nikolic et al., 1996; Harada et al., 2001).

1.5.5 Somatostatin and neurite outgrowth

Numerous studies have reported the involvement of neuropeptides in the morphogenesis of the neuronal circuitry during normal neuronal differentiation and support the maintenance of healthy brain (Schecterson and Bothwell, 1992; Buchman and Davies, 1993; Maubert et al., 1994). Among the various neuropeptides studied, SST has been gaining attention due to its distinctive role in influencing the neuronal development and nerve cell growth (Epelbaum et al., 1994; Maubert et al., 1994; Taniwaki and Schwartz, 1995). SST has also been reported to stimulate the neuronal differentiation and neurite outgrowth in cerebellar granule cells (Gonzalez et al., 1992; Taniwaki and Schwartz, 1995). Furthermore, as in the normal process of neurogenesis, cells treated with SST displayed significant inhibition of cell proliferation indicative of commitment of cells into terminally differentiated morphology (Bodenant et al., 1997). In addition, SST has been reported to induce neurite outgrowth in regenerating neurons of various invertebrates (Bulloch, 1987; Grimm-Jorgensen, 1987). Interestingly, the SST-like molecule has also been identified in both hemocytes and nervous systems of invertebrates acting as a growth hormone (Grimm-Jorgensen, 1987). Most notably, SST mediated promotion of neurite outgrowth has also been observed in neuroblastoma cells as well as rat adrenal medulla derived PC12 cells (Kentroti and Vernadakis, 1991; Ferriero et al., 1994). Despite these observations, however, the exact mechanism involved in SST mediate promotion of neurite outgrowth remains elusive.

Collectively, to assess the effect of SST in promoting RA induced neurite outgrowth, we have utilized human neuroblastoma cell line derived SH-SY5Y cell line. Next, to assess the effect of Aβ-induced toxicity in the differentiated SH-SY5Y cells and to study the neuroprotective role of SST, Aβ-mediated toxicity was assessed in SH-SY5Y cells.

1.6 Beta-amyloid toxicity of Alzheimer's disease: a breakdown in neuronal cytoskeleton

In AD patients, cumulative defects in dendritic spines are reported to be the main cause of cognitive impairment (Zhao et al., 2006). With the progression of the AD, significant distortion in dendritic postsynaptic proteins is observed with the loss of postsynaptic proteins such as drebrin reaching as high as 95% compared to age-matched control (Gylys et al., 2004; Ma et al., 2008). In this regard, the growth cone collapse mediated by the phosphorylation of microtubule-associated protein collapsing response mediator 2 (CRMP2) has been attributed to the loss of neurites in AD patients (Arimura et al., 2000). Previous reports have indicated hyperphosphorylation of CRMP2 in the cortex of postmortem AD brains while another study reported a direct correlation between beta-amyloid and increased phosphorylation of CRMP2 in SH-SY5Y cell line (Cole et al., 2007; Petratos et al., 2008). In the same study using APP (Swe) Tg2576 AD mouse model, authors have indicated an increased level of CRMP2 in close proximity to aggregates of beta-amyloid suggesting a direct effect of beta-amyloid and CRMP2 phosphorylation (Petratos et al., 2008).

1.6.1 CRMP2

The cytoskeletal reorganization is a highly regulated process involved in various cellular processes including neuronal development. In particular, significant cytoskeletal reorganization occurs during neuronal morphogenesis of immature neurons to allow changes in morphology, adhesion and directional movement (Bielas and Gleeson, 2004; Barnes and Polleux, 2009). Over the years, significant efforts have been made to identify the key cytoskeletal regulators, and among the numerous proteins reported, collapsing response mediator protein (CRMP) family has

been gaining attention due to its multifunctional role in neurodevelopment (Ip et al., 2014). Initially identified as a subcomponent involved in Semaphorin 3A (Sema3A)-induced growth cone collapse in chick dorsal root ganglion, CRMPs are comprised of five homologous cytosolic tubulin-binding proteins (CRMP1-5), with conserved expression in the mammalian nervous system (Goshima et al., 1995; Charrier et al., 2003). Since its initial discovery, CRMP is reported to play a critical role in various aspect of neuronal development including neurotransmission (Brittain et al., 2009), kinesin and dynein-mediated axonal transport (Kimura et al., 2005; Arimura et al., 2009), neuronal polarity (Inagaki et al., 2001) and transmembrane protein endocytosis (Nishimura et al., 2003). Specifically, CRMP1 and 3 plays a critical role in the maintenance of proper dendritic patterning and spine development, while CRMP4 is involved in a dendritic bifurcation in the hippocampus (Yamashita et al., 2007; Quach et al., 2008; Niisato et al., 2013). CRMP5 is also reported to be involved in dendritic development in the cerebellum (Yamashita et al., 2011). Among the five homologous cytosolic proteins, CRMP2 was the first identified and therefore the most extensively studied and is recognized as a key modulator in axonal guidance and neuronal development. In the mammalian CNS, CRMP2 expression was localized in post-mitotic neurons with particular localization in hippocampus, cerebellum and olfactory bulb (Wang and Strittmatter, 1996; 1997). In neurons, CRMP2 is involved in differentiation via assembly and trafficking of microtubules (Morita and Sobue, 2009). Various functional studies have reported the preferential binding of non-phosphorylated CRMP2 with tubulin heterodimers of neuronal microtubules leading to tubulin polymerization and GTPase activity (Fukata et al., 2002; Chae et al., 2009). Importantly, CRMP2 induces increased stability of actin filaments (Kawano et al., 2005).

1.6.2 CRMP2 and Alzheimer's disease

Taking into consideration the significant interaction CRMP2 has with neuronal tubulin and filaments stability, it is easily conceivable how various neurodegenerative diseases might occur with malfunction of CRMP2. Similar to Tau, another microtubule-associated protein, CRMP2 is subjected to phosphorylation by various kinases. Cyclin-dependent kinase 5 (CDK5) preferentially phosphorylates S522 site of CRMP2, which 'primes' downstream phosphorylation by glycogen synthase kinase-3β (GSK-3β) at sites T509 and T514 (Cole et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005; Li et al., 2006). In addition to GSK-3β and CDK5, Rho kinase is also involved in phosphorylation of CRMP2 at site T555 (Arimura et al., 2000). Once phosphorylated at these sites, CRMP2 loses significant affinity for tubulin heterodimers and dissociates, leading to reduced neuronal microtubule stability as well as an axonal retraction (Arimura et al., 2000; Arimura et al., 2005; Khanna et al., 2012). In addition to the dissociation between CRMP2 and tubulin, hyperphosphorylation of CRMP2 blocks the tubulin transport to the plus end of microtubule for neurite assembly leading to hindered neurite outgrowth and elongation (Petratos et al., 2008). In another study, overexpression of full-length CRMP2 led to increased axonal elongation in primary neurons and neuroblastoma cell line, while microtubule binding region devoid of overexpression of CRMP2 resulted in inhibited axonal growth (Inagaki et al., 2001). Collectively, these results suggest a critical role of CRMP2 in mediating proper axon growth. Notably, increased CDK5 and GSK-3β mediated phosphorylation has been observed in the frontal cortex in postmortem brain of AD patients compared to the age-matched controls (Cole et al., 2007). In both double (PS1/APP mutant) and triple (PS1/APP/Tau mutant) transgenic mouse that develops AD-like phenotype including the formation of plaques and NFT, a drastic increase in both CDK5 and GSK-3β has been reported (Cole et al., 2007). Interestingly,

in the same study, mutant tau (P301L) transgenic mice shows NFT, however, failed to show CDK5 mediated phosphorylation of CRMP2 along with no plaque formation, These observations suggest a possible correlation between beta-amyloid tangle formation and CRMP2 phosphorylation (Cole et al., 2007). Interestingly, CRMP2 hyperphosphorylation was observed well before the formation of beta-amyloid plaques or NFTs, suggesting that CRMP2 may be an early indicator of AD pathogenesis. Furthermore, Williamson R et al. reported that the CRMP2 hyperphosphorylation was not observed in other neurodegenerative disease and was specific to AD, suggesting a significant implication of CRMP2 and AD (Williamson et al., 2011).

1.6.3 CDK5 activity and CRMP2 hyperphosphorylation

CDK5 is a Ser-Thr kinase playing a pivotal role in both developing and maturing neurons, including neuronal migration (Xie et al., 2003), neurogenesis and neurite outgrowth (Jessberger et al., 2008; Lagace et al., 2008), and in synapse formation and plasticity (Fischer et al., 2005; Johansson et al., 2005; Samuels et al., 2007). In the normal state, physiological activity of CDK5 is required for neurogenesis (Jessberger et al., 2008; Lagace et al., 2008). In the nervous system, CDK5 is mainly activated by forming a complex with either p35 or p39 subunit (Dhavan and Tsai, 2001; Smith et al., 2001). Among the two subunits, p35 is reported to be a key modulator of CDK5 activity in synaptic plasticity (Fischer et al., 2003; Fischer et al., 2005) and neuronal development (Cicero and Herrup, 2005). However, the dysfunction in CDK5 activity and its regulatory subunit p35 and p25 leads to hyperphosphorylation of numerous downstream proteins such as CRMP2 resulting in senescence of mature neurons in the AD (Patrick et al., 1999; Liu et al., 2003). In addition, hyperactivation of CDK5 has been observed in brains of AD patients (Lee et al., 1999). In support, Crews et al. have previously reported that dysfunctional activation of

CDK5 significantly hindered normal neurogenesis and neurite outgrowth in both *in vitro* and in mice model of study (Crews et al., 2011). The hyperactivation of CDK5 is largely mediated by conversion of CDK5 substrate p35 into more stable p25 by calpain (Patrick et al., 1999; Liu et al., 2003; Wang et al., 2010; Patrick et al., 2011).

1.6.4 Calcium influx and Somatostatin

Understandably, stringent regulation of calpain is required for maintenance of normal physiological state. Among the various stress identified to induce abnormal activation of calpain, high intracellular Ca^{2+} concentration is reported to be the main inducer of calpain-mediated truncation of p35 into the more stable form of p25 (Lee et al., 2000; Dhavan and Tsai, 2001; Amin et al., 2002). In this regard, it is plausible that a potent inhibitor of Ca^{2+} influx may reduce the over-expression/activation of calpain, resulting in maintenance of normal activity of all downstream pathway including and up to CRMP2 phosphorylation. Among the various modulators of calcium influx, it is well established that somatostatin inhibits Ca^{2+} influx by binding to SSTR2 (Reisine, 1990; Johnson et al., 2001; Petrucci et al., 2001).

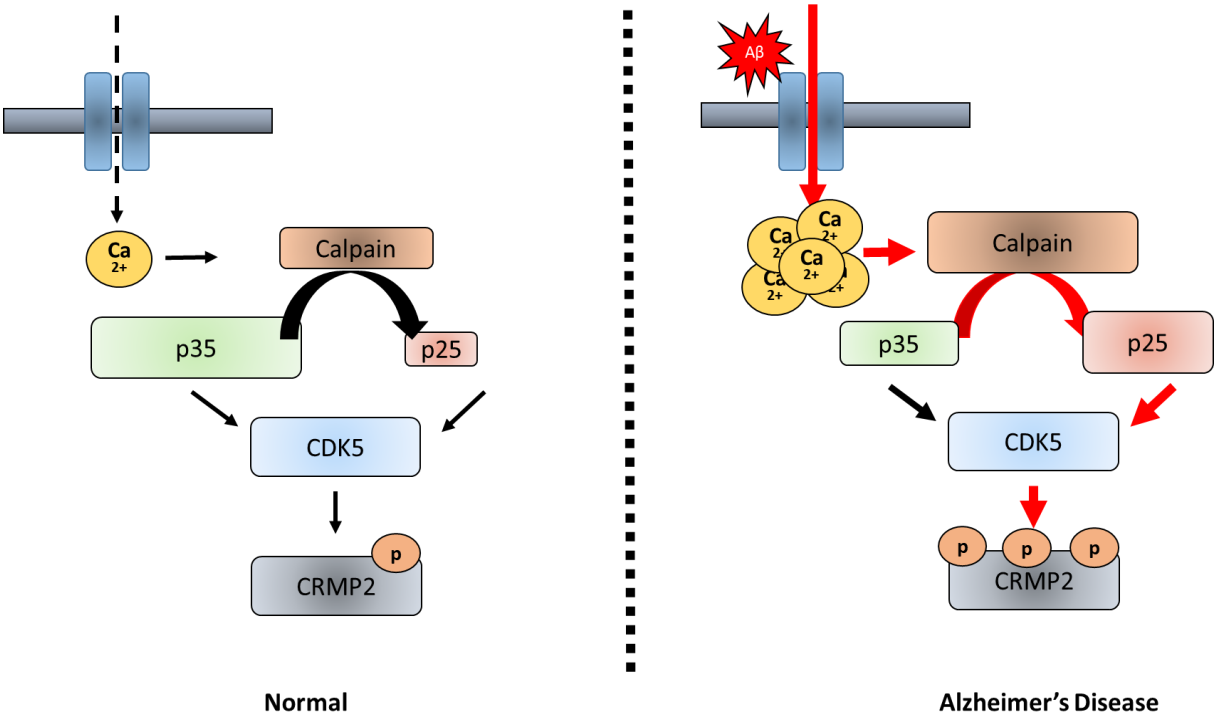

Figure 4 Aβ induced hyperactivation of calpain leads to hyperphosphorylation of CRMP2 leading to tubulin instability and Alzheimer's disease. Regulated Ca^{2+} influx is significantly increased in presence of Aβ, which in turn activated Ca^{2+} dependent calpain activity. The increased calpain activity promotes excess truncation of p35 to a more stable form of p25. Both p35 and p25 acts as a subunit of CDK5 activity and increased p25 in AD patients lead to constitutively active CDK5. The hyperactivity of CDK5 results in hyperphosphorylation of downstream targets including CRMP2.

1.7 Background overview and summary

Previous studies corroborate the significant involvement of SST in serving a protective role against beta amyloid induced toxicity. Specifically, beta-amyloid accumulation in AD brain and a decrease in production of SST showed significant temporal as well as spatial overlap suggesting the likely possibility of a correlation between the two.

In BBB, accumulation of beta-amyloid results in hyperactivation of JNK which in turn induces expression of ECM degrading MMP2. Furthermore, accumulation of beta-amyloid in extracellular fluid leads to eventual accumulation of beta-amyloid in intracellular fluid resulting in neurotoxicity. In this regard, somatostatin mediated inhibition of JNK as well as maintenance of beta-amyloid transporter proteins may lead to protection of BBB integrity against beta amyloid induced toxicity.

During the neuronal differentiation of various neuroblastoma cells, somatostatin has been reported to promote neurite outgrowth. Activation of ERK1/2 has been reported as a key component in maintaining proper neurite outgrowth. In this study, the role of somatostatin mediated activation of ERK1/2 in promoting neurite outgrowth will be assessed.

In neuronal culture, beta-amyloid is known to elicit neurotoxicity resulting in retraction of neurites and cell death. Specifically, microtubule-associated protein CRMP2 is hyperphosphorylated via beta-amyloid hyperactivation of kinases involved in the CDK5 pathway. In this, the role of somatostatin in inhibiting CDK5 pathway by inhibiting Ca^{2+} influx will be assessed.

1.8 Research hypothesis

Growing evidence suggest the neuroprotective role of SST against beta amyloid induced toxicity/damage in various tissues of the CNS. Furthermore, SST mediated promotion of neurite outgrowth has been established.

- To characterize the neuroprotective role of SST in blood brain barrier using hCMEC/D3 cell line as an *in vitro* model and elucidate the mechanisms involved (Chapter 2)

- To characterize the SST mediated promotion of neuronal differentiation/neurite outgrowth using the SH-SY5Y cell as an *in vitro* model and elucidate the mechanisms involved (Chapter 3)
- To elucidate the role of SST in mediating neuroprotective role against beta-amyloid-induced toxicity in neurons with a specific focus on inhibiting beta-amyloid-induced hyperphosphorylation of CRMP2 (Chapter 4).

Chapter 2: Somatostatin maintains permeability and integrity of Blood Brain Barrier in β-amyloid induced toxicity

2.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a common form of dementia characterized by the formation of neurofibrillary tangle, senile plaques and the accumulation of β-amyloid peptide (Aβ) (Yankner, 1996; Selkoe, 2001). The prominent cause of neuronal cell death in AD is associated with proteolytic cleavage of amyloid precursor protein along with excessive accumulation and aggregation of Aβ in the central nervous system (CNS). The activation of microglia and astrocytes have also been associated with neuronal loss in AD (Mhatre et al., 2004; Yin et al., 2010; Grammas, 2011). The release of pro-inflammation cytokines such as prostaglandins and matrix metallopeptidase (MMP) from brain microvessels in AD patients demonstrates the role of inflammation in the progression of AD. We have observed increased expression of astrocytes in close proximity of blood capillaries in AD patients (Kumar, 2005). In addition, a pro-inflammatory cytokine interleukin-1β (IL-1β), is increased in neuroinflammatory diseases and is associated with the impaired permeability of the blood brain barrier (BBB). Previous studies have shown that defective clearance of Aβ from the brain in AD pathology is due to impaired BBB homeostasis. Normal brain homeostasis is prominently maintained by proper development and function of BBB. The main constituent of BBB is endothelial cells connected by tight- and adherens-junctions which exert a critical role in BBB permeability. The BBB maintains homeostasis of the CNS by regulating transportation of some molecules while restricting the permeability of others (Begley and Brightman, 2003; Hawkins and Davis, 2005; Wolburg et al., 2009; Abbott et al., 2010). Moreover, such selective

permeability of BBB is regulated by proper organization and expression of tight junction proteins (TJP), constituted with proteins such as ZO-1, ZO-2, occludin and claudin (Furuse et al., 1993; Furuse et al., 1998; Itoh et al., 1999; Tsukita et al., 1999). By binding directly to other TJPs such as occludin and claudin, ZO-1 orchestrates the formation of tight junction complexes in BBB (Eisenhauer et al., 2000). Previous studies have shown diminished levels of TJPs in the pathogenesis of AD (Zlokovic, 2008; Kalaria, 2010). In addition, it has been noted that functional integrity of the BBB is crucial in the regulation of Aβ efflux and influx. In addition to the changes in the organization of TJPs, the Aβ efflux and influx through BBB is also regulated by low-density lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE). LRP exhibit high binding affinity to Aβ, whereas RAGE is crucial for neurite outgrowth, and also serves as a prominent receptor at BBB involved in regulation in the influx of peripheral and/or plasma Aβ (Huttunen et al., 2000). The loss of LRP expression and increased RAGE activity has been reported in an age-dependent manner and believed to be a critical determinant of Aβ clearance and accumulation. The role of LRP in Aβ clearance is further supported by studies in LRP1 *ko* mice displaying over-accumulation of Aβ (Kanekiyo et al., 2013). Furthermore, the functional relation between RAGE and TJPs is also reported in AD model (Kook et al., 2013).

MMPs are a family of proteases involved in various physiological functions including cleavage of zymogens, activation of growth hormones and remodeling of extracellular matrix (ECM). Specifically, the remodeling of ECM by MMPs has been studied extensively for its potential implication in cognitive impairment as evident in various neurodegenerative diseases. Furthermore, MMPs have been reported to break down TJPs forming BBB, leading to hemorrhage. Among the family of MMPs, MMP2 and 9 has been studied extensively for their

involvement in ECM disruptions. MMPs via many proteolytic reactions have been associated with several cell surface entities including receptors and junction proteins (Chen et al., 2009a). Moreover, previous studies have emphasized the role of MMPs in TJPs and impaired BBB permeability (Gu et al., 2005). The degradation of occludin and claudin has also been reported in response to MMPs (Dhanda and Sandhir, 2017).

Somatostatin (SST), a growth hormone inhibitory peptide widely expressed in central and peripheral tissues, exert a variety of endocrine and non-endocrine functions. In addition to a neuroprotective role in excitotoxicity, decreased level of SST has been reported in several neurological diseases (Davies et al., 1980; Kowall and Beal, 1988; Beal, 1990; Epelbaum et al., 1994; Fox et al., 1997; Binaschi et al., 2003). Furthermore, the cortical and cerebrospinal fluid (CSF) levels of immuno-reactive SST is markedly decreased in AD and have become a reproducible biochemical marker of the disease (Beal et al., 1985). We have shown changes in SST secretion and accumulation in cultured cortical neurons in response to Aβ-induced neurotoxicity and expression of SST receptors (SSTRs) in AD brain tissue (Kumar, 2005; Geci et al., 2007). SST increases neprilysin, an enzyme involved in degradation of Aβ, and SST *ko* mice exhibit decreased neprilysin and increased Aβ deposit (Saito et al., 2005).

We previously demonstrated changes in the expression of SSTR subtypes as well as modulation of cytokine and SST release in the human cerebral microvascular endothelial cell line (hCMEC/D3) in response to lipopolysaccharide (LPS) treatment. Moreover, the same study also supports the role of SST in the organization of TJPs surface expression perturbed in the presence of LPS and cytokines (Basivireddy et al., 2013). Furthermore, another study reported *Sstr2*$^{-/-}$ mice showed selective degeneration of their central noradrenergic projections, suggesting the role of specific SST receptors in the progression of AD (Adori et al., 2015). It is unknown

whether SST is involved in the regulation of Aβ influx or efflux and permeability of BBB. In the present study, using a combination of morphological, biochemical and molecular approaches, we aimed to investigate the role of SST in protection against Aβ induced damage in hCMEC/D3 cells. Here, we demonstrate that the presence of SST protects BBB integrity by maintaining the organization of TJPs against Aβ induced damage.

2.2 Material and Methods

2.2.1 Materials

The human brain microvascular endothelial cell line (hCMEC/D3) was a kind gift from Dr. P.O. Couraud (Institut Cochin, INSERM, CNRS, Université Paris Descartes, Paris, France). Endothelial Basal Medium-2 (EBM-2) was purchased from Lonza (Basel, Switzerland). The culture medium was purchased from Thermo Fisher Scientific (Massachusetts, USA) and was supplemented with ascorbic acid and lipid concentrate. HEPES, basic fibroblast growth factor (bFGF) and hydrocortisone were obtained from Sigma-Aldrich Inc (St. Louis, MO, USA). SST-14 was purchased from Bachem (Torrance, CA, USA). Aβ-peptides were purchased from EZ Biolab (Westfield, IN, USA) and the scrambled Aβ-peptide was purchased from Anaspec (Fremont, CA, USA). All other reagents were of analytical grade and purchased from various sources.

2.2.2 Cell Culture

hCMEC/D3 cells were cultured and maintained as previously described (Basivireddy et al., 2013). Briefly, the cells were cultured and maintained in flasks or culture dishes precoated with rat tail collagen-I and grown until complete confluence in all experimental setup. The EBM-

2 media was supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 5µg/mL ascorbic acid (AC), 1.4µM hydrocortisone (HC), 10mM HEPES, 1ng/mL bFGF and chemically defined lipid concentrate at 1:100 dilution. All experiments were carried out using cells within passages 27 - 33.

2.2.3 MTT Assay

Cells were treated with increasing concentration of Aβ for 24 hours and were processed for MTT assay. Briefly, 300µg/mL of methylthiazolyldiphenyl-tetrazolium bromide solution (Sigma) was prepared in DMEM. Following dose-dependent treatment with Aβ1-42 (0, 1, 5, 25µM) for 24 hrs, hCMEC/D3 cells were incubated with prepared MTT solution for 3 hours at 37°C. Following subsequent washes with PBS, 100µL of isopropanol was added to each well and the resulting change in color from dissolving formazan salt was immediately quantified using spectrophotometer at a wavelength of 550nm with background absorbance measured at 695nm. Results are presented as a percentage of control versus treated group.

2.2.4 Caspase/Apoptosis Activity Assay

The apoptosis in hCMEC/D3 cells treated with Aβ1-42 in presence or absence of SST was assessed using Caspase-3/7 Green Apoptosis Assay (Essen Bioscience, Michigan, USA) following manufacturer's instructions. Briefly, cells were treated with Aβ1-42 (5µM) alone or in combination with increasing concentrations of SST (1nM, 100nM, 1µM) in presence of a DNA intercalating dye NucView^{TM}488 (Essen Bioscience). Immediately after treatments, the plate was placed in live-cell imaging system (Essen Bioscience) and caspase-3/7 activity was monitored up

to 48 hours as an index of cells undergoing apoptosis and calculated using IncuCyte basic analyzer (Essen Bioscience).

2.2.5 Live/Dead Cell Assay

The cell viability upon treatments with Aβ1-42 in the presence or absence of SST was analyzed using LIVE/DEAD Cell Vitality Assay (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, the cells were treated with Aβ1-42 (5μM) alone or in combination with SST (1, 30, 100, 300, 1000nM) for 24 hours. Following treatment, cells were washed with PBS and collected using 0.05% trypsin-EDTA (Thermo Fisher Scientific). Trypsinized cells were then re-suspended in 100μL of PBS and incubated with C_{12}-resazurin (20ng/μL) and SYTOX dye (1μM) for 15 minutes at 37˚C. 400μL of PBS was then added to make total suspension of 500μL and the cells were immediately analyzed on LSR II (BD Bioscience, CA, USA) with fluorescence excitation at 488nm and emission at 530 and 570nm. All resulting data was analyzed using FlowJo workstation (BD bioscience).

2.2.6 Western blotting

To determine time and dose dependent effect of Aβ on protein expression, hCMEC/D3 cells were treated with two concentrations of Aβ (1 and 5μM) for 2 and 6 hours. In addition, the dose dependent effect of Aβ (200nM, 1μM, 5μM) with or without increasing concentration of SST (400nM, 2μM, 10μM) was further assessed for 24 hours at 37˚C. Post-treatment, cells were lysed using RIPA (radio-immune precipitation assay) buffer supplemented with phosphatase and protease-inhibitor (1:100). Total protein content was measured by Bradford Protein Assay (Biorad, Hercules, California, USA). 15μg of protein per sample were fractionated on an SDS-

PAGE gel and transferred to nitrocellulose membrane. The membranes were incubated with mouse monoclonal-ZO-1 (1:500, Abcam, Cambridge, UK), rabbit polyclonal-Occludin (1:1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA), rabbit monoclonal-LRP1 (1:1000, Abcam), rabbit monoclonal-RAGE (1:1000, Abcam), mouse monoclonal-pJNK and -total JNK (1:1,000, Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal-MMP2 (1:1000, Abcam) primary antibodies overnight at 4°C. The membranes were washed and incubated with species-specific, HRP-conjugated secondary antibodies (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) and developed using enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) on Alpha Innotech FluorChem 8800. β-actin was used as internal control. Densitometric analysis of immunoblot was performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.2.7 Enzyme linked immunosorbent assay (ELISA)

For the detection of SST and IL-1β release, ELISA analysis was performed following manufacturers' instruction. Briefly, culture media from control and treated cells were collected and added to 96-well plate pre-coated with SST or IL-1β antibody and incubated for 1 to 2 hours, respectively (SST, Cloud-Clone Corp, TX, USA and IL-1β, BD Bioscience). The plate was then incubated with provided detection reagent for 1 hour at 37˚C for SST and 30 minutes for IL-1β at room temperature (RT). Subsequently, the plates were incubated for 30 minutes at 37˚C in 100µL of Detection Reagent B or Enzyme Working Reagent for SST and IL-1β, respectively. The substrate was added to respective kits and the incubation was terminated by adding 50µL of Stop Solution to each well. The absorbance was read at 450nm using spectrophotometer (BMG Labtech, Germany). Data were quantified and analyzed as per manufacturer's instructions.

2.2.8 Immunofluorescence Staining

hCMEC/D3 cells were grown to confluence on glass coverslip pre-coated with rat tail collagen I. Cells were treated with Aβ (5μM), SST (2μM) alone or in combination for 24 hours. Following treatment, control and treated cells were fixed with 4% paraformaldehyde at 4˚C for 15 minutes. Cells were permeabilized with 0.1% Triton-X solution for 15 minutes at RT, washed with PBS and incubated in 5% normal goat serum (NGS) for 1 hour at RT. Cells were then incubated with primary antibodies, ZO-1 (1:200), Occludin (1:150), LRP1 (1:150), RAGE (1:150) in TBS containing 5% NGS overnight at 4˚C. Following the wash, cells were incubated for 1 hour at RT with Alexa-594 tagged secondary antibody (1:200). To visualize the nucleus, cells were exposed to Hoechst 33258 dye (0.5μg/mL, Calbiochem, CA, USA) for 10 minutes at RT. Coverslips were then mounted on slides and photographed using Zeiss LSM700 confocal microscope (Carl Zeiss, Germany).

2.2.9 Permeability Assay

hCMEC/D3 cells were grown to confluence on 0.4μM pore-transwell inserts (Greiner bio-one, Austria) pre-coated with rat tail collagen-I. The cells were treated with either increasing concentration of Aβ (1, 5, 25μM), and SST (80nM, 400nM, 2μM, 10μM) alone or in combination (Aβ: 5μM, SST: 80nM, 400nM, 2μM, 10μM) for 24 hours. The paracellular permeability of hCMEC/D3 cells to FITC-dextran (40 kD, Sigma Aldrich, St. Louis, MO, USA) was then quantified as previously described (Weksler et al., 2005). Briefly, 2mg/mL of 40kDa FITC-dextran was loaded onto apical chamber of transwell insert for 30 minutes at 37˚C. To determine the amount of FITC-dextran that passed through, 100μl of media from the basolateral

chamber was collected and the fluorescence intensity (FITC-dextran excitation wavelength 495 nm, emission 519 nm) was measured using spectrophotometer (BMG Labtech, Germany).

2.2.10 Cellular uptake and efflux quantification

The quantification of Aβ influx and efflux in hCMEC/D3 cells was determined as previously described (Alemi et al., 2016). Briefly, for the quantification of Aβ uptake (influx), hCMEC/D3 cells were cultured until 100% confluency. Cells were washed twice with PBS and incubated with either fluorescein (FAM)-Aβ1-42 (100nM) alone, in combination with SST (2μM), or 15-minute pre-treatment of cells with SST (2μM) followed by FAM-Aβ1-42 (100nM) for 30 minutes at 37°C. Following treatment, cells were washed with PBS and enzymatically detached using 0.05% Trypsin (Thermo Fisher Scientific). The cells were resuspended in PBS, washed and fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were then washed in PBS, centrifuged and re-suspended in FACS solution (PBS containing 2% FBS and 2mM EDTA) for analysis. The cells were analyzed in LSR II (BD Bioscience) using Blue laser excitation at 488nm.

For efflux study, hCMEC/D3 cells were plated and treated as described above for 10 minutes at 37°C. The media was removed and the cells were washed three times with PBS to remove residual FAM-Aβ1-42. Media was then replaced with FAM-Aβ1-42 free medium, and cells were incubated for an additional 20 minutes. The cells were then processed and analyzed as described above. All resulting data was analyzed using FlowJo workstation.

2.2.11 Statistical Analysis

All experimental data are presented as mean ± SEM of minimum three independent experiments. All statistical analysis was performed using GraphPad Prism 5. For analysis of data with one independent variable in more than two groups, one-way analysis of variance (ANOVA) was used. For data with one independent variable in two groups, two-tailed unpaired Student's *t*-test was performed as indicated. In all cases, *p* value less than 0.05 were considered significant.

2.3 Results

2.3.1 Concentration-dependent effect of Aβ1-42 induced toxicity in hCMEC/D3 cells

To establish Aβ-induced toxicity models, we first determined the concentration-dependent effect of Aβ on hCMEC/D3 cell survival. At lower concentrations, Aβ1-42 (5, 10μM) had no significant effect on cell viability, however, at relatively higher concentrations (25μM), Aβ exhibited 50% cell death (Fig. 5a). In support of MTT assay, Hoechst nuclear staining was performed to visualize changes in cells treated with Aβ1-42 (Fig. 5b).

Previous reports have indicated Aβ1-42 induced toxicity is mediated via apoptotic pathway (Loo et al., 1993). To further elucidate and establish the correlation between Aβ1-42 and apoptosis, hCMEC/D3 cells treated with Aβ1-42 (5μM) alone or in combination with SST in a dose-dependent manner were incubated with caspase-specific NucView^{TM}488. Cells were monitored up to 48 hours to assess caspase-3/7 activation following treatment. In the presence of Aβ1-42 alone, caspase-3/7 activity showed a gradual, time-dependent increase (Fig. 5c). In contrast, the untreated control and cells treated with SST (1μM) alone displayed minimal changes in caspase-3/7 activity. Cells treated with Aβ1-42 in the presence of increasing concentrations of SST showed significantly lower caspase-3/7 activity when compared to the

cells treated with Aβ1-42 alone, indicating an inhibitory effect of SST on caspase-activity in hCMEC/D3 cells.

To further elucidate and support our results from caspase-3/7 activity, we next investigated the cell viability using LIVE/DEAD Cell Vitality Assay. By assessing the reduction of C_{12}-resazurin in metabolic cells as well as the inclusion of impermeable SYTOX dye, the LIVE/DEAD Cell Vitality Assay allowed accurate quantification of cell viability. In alignment with caspase-3/7 activity results, cells treated with Aβ1-42 alone showed an increase in FITC positive cells, indicating increased cell permeability of cells and apoptosis/toxicity compared to untreated control (Fig.5 d, e). In contrast, the cells treated with SST alone showed a comparable number of FITC positive cells with untreated control. In parallel with caspase activity, cells treated with Aβ1-42 in combination with increasing concentrations of SST showed a dose-dependent decrease in FITC positive cells, suggesting a protective role of SST against Aβ1-42 induced toxicity. Taken together, results obtained from caspase activity and Live/Dead cell viability assay suggesting a protective role of SST in Aβ induced toxicity.

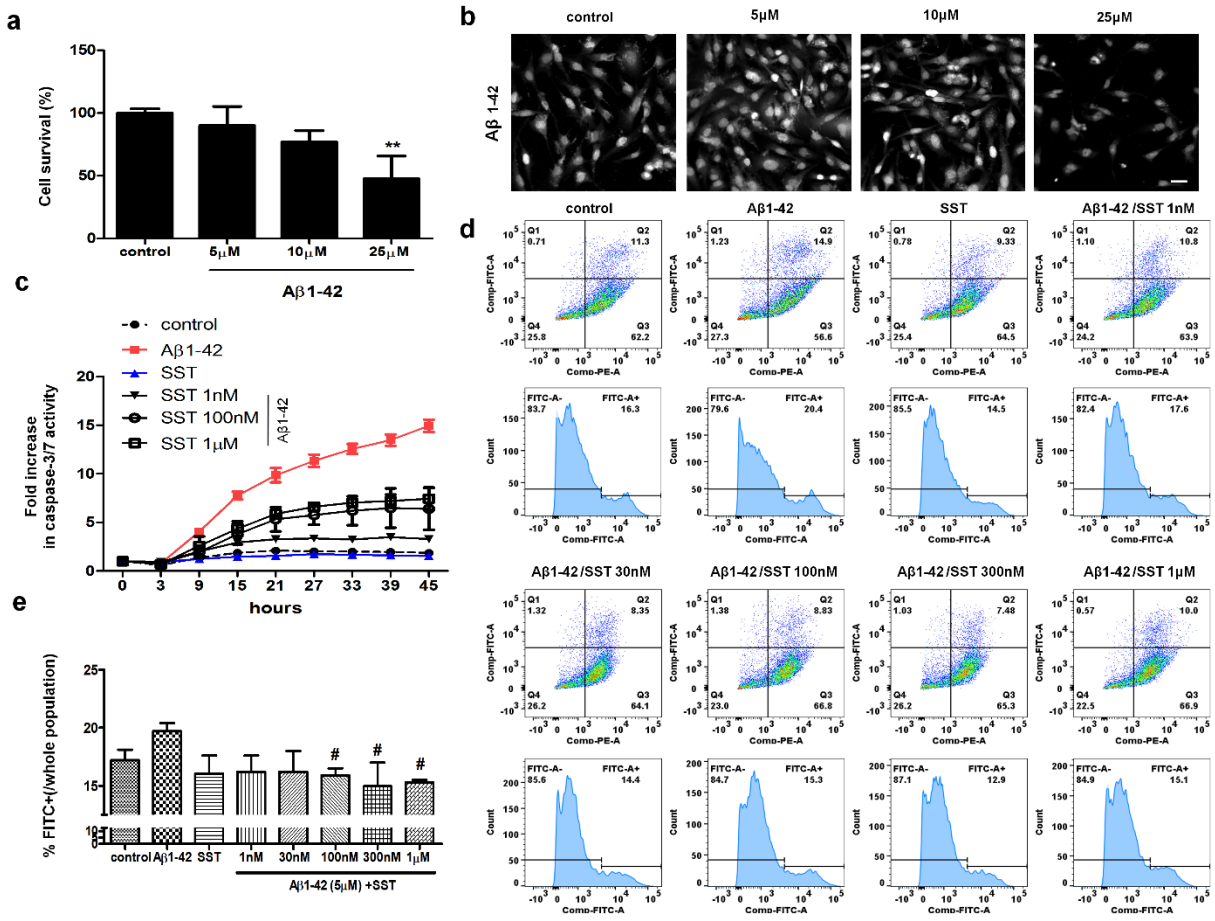

Figure 5 SST inhibits Aβ induced toxicity in hCMEC/D3 cells. **a** hCMEC/D3 cells were treated with increasing concentration of Aβ1-42 (5-25μM) for 24 hr at 37˚C and processed for MTT assay. Aβ induced cell death in dose-dependent manner. **b** Hoechst staining of hCMEC/D3 cells treated with increasing concentration of Aβ1-42 shows dose-dependent toxicity. **c** SST inhibits Aβ1-42 induced caspase-3/7 activation. Cells co-treated with Aβ1-42 and SST shows significant inhibition of caspase-3/7 activity. **d** Dot plot data of C_{12}-resazurin and SYTOX fluorescence intensity (dot plot) and FITC intensity distribution (histogram) displays distribution of cells based on viability. FITC positive cells on histogram represents dead cells with higher SYTOX inclusions. **e** SYTOX cell permeability assay indicates significant increase in permeability in cells treated with Aβ1-42 (5μM) alone, whereas dose-dependent inhibition in

presence of increasing concentrations of SST. **p <0.01 against control, $^{\#}p$<0.05 against Aβ1-42 treated alone. *Scale bar* = 20 μm

2.3.2 Aβ1-42 induced release of IL-1β in hCMEC/D3 cells is blocked in presence of SST

To investigate the cytokine release, hCMEC/D3 cells grown to confluency were treated with Aβ1-42 (5μM) in absence or presence of SST (2μM) for 24 hours at 37˚C. Post-treatment culture medium was collected and processed for IL-1β quantification using ELISA according to manufacturer's instructions. The IL-1β release was approximately fourfold higher upon treatment with Aβ1-42 in comparison to control (Fig. 6a). Augmented IL-1β release in response to Aβ1-42 was suppressed significantly in the presence of SST and was comparable to SST treatment alone.

2.3.3 Release of SST is unaffected in response to Aβ1-42

In addition to the decreased levels of SST in CSF of AD patients, previous studies using neuronal culture and hCMEC/D3 cells have shown changes in SST release in response to Aβ1-42 and LPS treatment (Geci et al., 2007). To address whether Aβ is involved in regulation of SST release, hCMEC/D3 cells were treated with increasing concentration of Aβ (400nM, 2μM, 10μM) for 24 hours. Post-treatment, culture medium was collected and processed to determine SST content by ELISA according to manufacturer's instruction. SST release in response to Aβ remained comparable to control, although a decreasing trend in SST release was observed with increasing concentrations of Aβ1-42 (Fig. 6b).

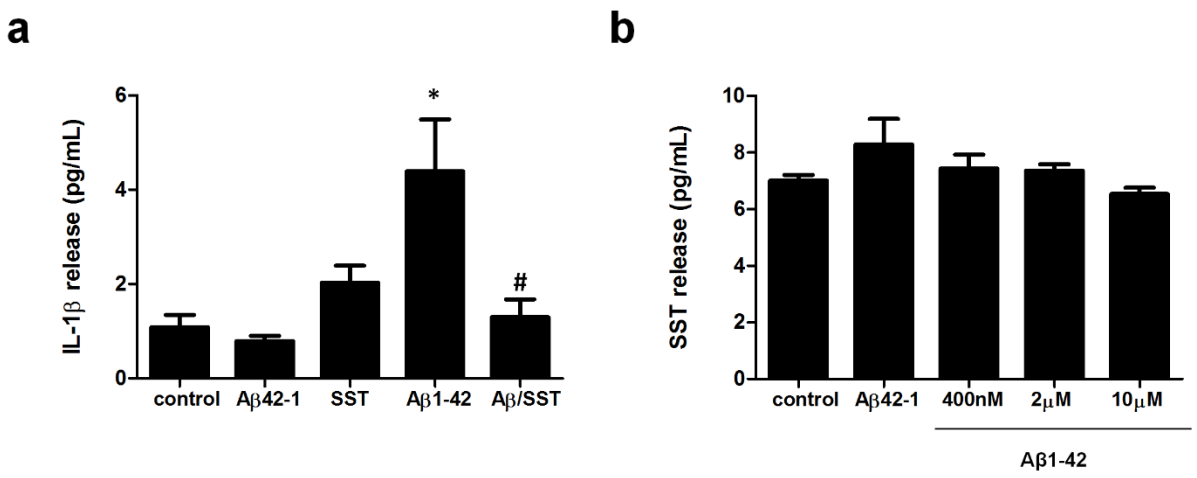

Figure 6 Aβ1-42 induced cytokine release in hCMEC/D3 cells. a Pro-inflammatory cytokine IL-1β release was significantly increased in presence of Aβ (5μM), which was inhibited in presence of SST. **b** SST release was not changed in presence of Aβ as indicated (n=3). *p<0.05 against control.

2.3.4 Concentration and time dependent effect of Aβ1-42 on TJPs expression in hCMEC/D3 cells

The release of cytokines from hCMEC/D3 cells in response to Aβ1-42 is an indication of inflammation and impaired cell permeability. Consistent with previous studies demonstrating that TJPs are crucial for BBB integrity, we next explored whether Aβ1-42 induced toxicity and/or IL-1β release is associated with the disruption of TJPs expression in cells. To address this, we first determined the expression of ZO-1 and occludin in control and cells subjected to Aβ treatment in a concentration and time dependent manner. hCMEC/D3 cells grown to confluency were exposed to Aβ1-42 (1 and 5μM) for 2 and 6 hours at 37˚C (Fig. 7). Cell lysates prepared

from control and treated cells were immunoblotted for the expression of ZO-1 and occludin. The expression level of both ZO-1 and occludin was decreased significantly in cells treated with Aβ in concentration and time dependent manner (Fig. 7a). Unlike ZO-1, occludin showed rapid recovery of expression at a lower concentration of Aβ1-42 (1μM) treatment, while under the higher concentration of Aβ1-42 (5μM), the expression remained inhibited.

2.3.5 Concentration and time-dependent effect of Aβ1-42 on LRP1 and RAGE in hCMEC/D3 cells

Along with para-cellular integrity maintained by ZO-1 and occludin, various transporters play a pivotal role in the transcellular transportation of Aβ across BBB (Deane et al., 2003). To delineate whether the loss in TJPs expression is also linked to abnormal efflux and/or influx in hCMEC/D3 cells, we first attempted to determine the expression level of LRP1 and RAGE in cell lysate prepared from hCMEC/D3 cells following treatment with Aβ1-42 in concentration and time dependent manner. LRP1 and RAGE expression levels were significantly decreased upon treatment with Aβ in time and dose dependent manner (Fig. 7b). Similar to occludin, RAGE showed significant recovery following 6 hours post-treatment with a lower concentration of Aβ1-42 (1μM). These results suggest transient changes in transporter proteins, which tend to return to the normal depending on intensity and duration of insult.

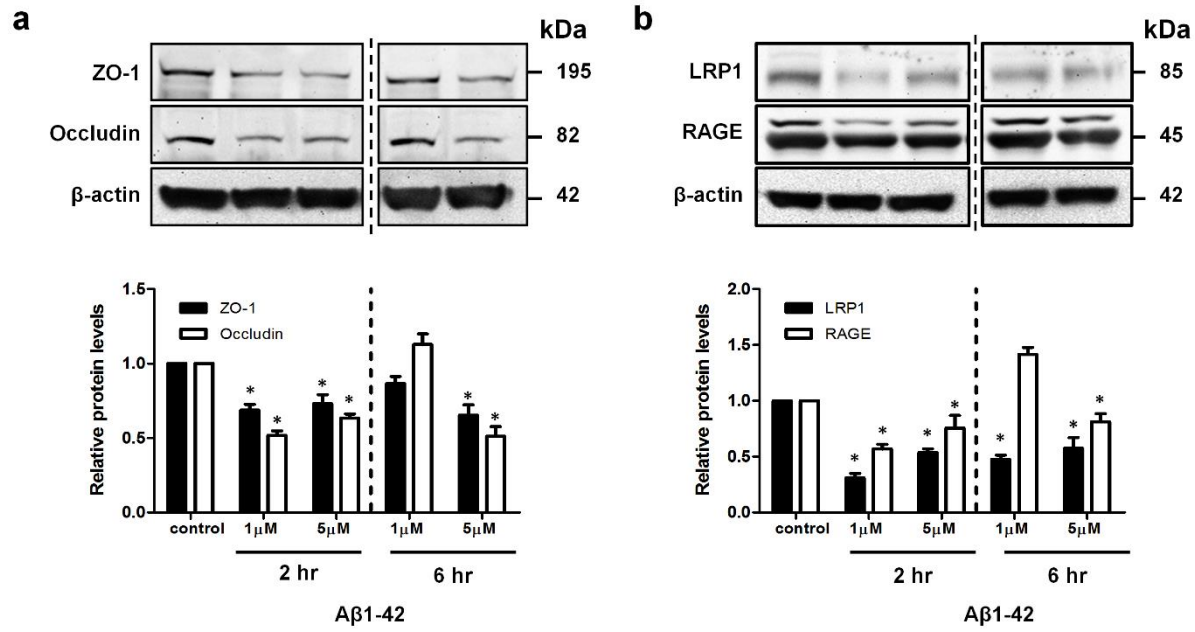

Figure 7 Aβ modulates TJPs and Aβ transporters expression in time and dose dependent manner. a Representative Western blot showing decreased level of ZO-1 and occludin (upper panel). Histograms represent densitometric analysis of Western blot (bottom panel). **b** Representative immunoblot analysis (upper panel) and densitometric analysis of Western blot (bottom panel) show decreased level of Aβ transporters LRP1 and RAGE. Note significant recovery of RAGE at a lower concentration with prolonged treatment (6 hr). β-actin was used as loading control. Data represents mean ± SEM of three independent experiments. *$p < 0.05$ against control.

2.3.6 Exogenous SST improved Aβ induced disruption of TJPs in BBB

In order to assess whether SST restores Aβ induced disruption of ZO-1 and occludin expression levels at the cell surface, cells were treated with Aβ in the presence of increasing concentration of SST (400nM - 10μM) for 24 hours. Scrambled Aβ was used as negative control.

Post-treatment, cells were processed for subcellular distribution and expression of ZO-1 and occludin using immunofluorescence immunocytochemistry and Western blot analysis. The distributional pattern of ZO-1 immunostaining in the presence of SST alone was comparable to control group (Fig. 8a). However, ZO-1 immunostaining at the cell surface displayed discontinuation in the expression in cells treated with Aβ. Interestingly, the treatment of cells with SST in combination with Aβ resulted in an intact and continuous expression pattern of ZO-1 immunoreactivity at the cell-cell interface that was comparable to the control or cells treated with SST (Fig. 8a). Western blot was performed to determine whether SST restores ZO-1 immunoreactivity at the cell surface as well as the expression of ZO-1 in presence of Aβ. Immunoblot analysis revealed dose dependent decrease in ZO-1 expression upon treatment with Aβ (Fig. 8c). Such decreased ZO-1 expression was recovered to the control level in presence of SST at lower concentrations (400nM and 2μM). However, at higher concentration, SST had no effect on ZO-1 expression.

We next determined the distributional pattern and expression of occludin in similar experimental conditions as described for ZO-1. No significant changes were seen in the distribution of occludin immunoreactivity at cell surface upon treatments as indicated (Fig. 8b). The expression level of occludin was increased in all treatment conditions excluding 400nM SST in presence of 5μM of Aβ (Fig. 8c). However, SST treatment alone (2μM) also resulted in increased expression of occludin when compared with control. Taken into consideration, the results described here delineate that lower concentration of SST can normalize Aβ1-42 induced disruption in TJPs organization and its expression.

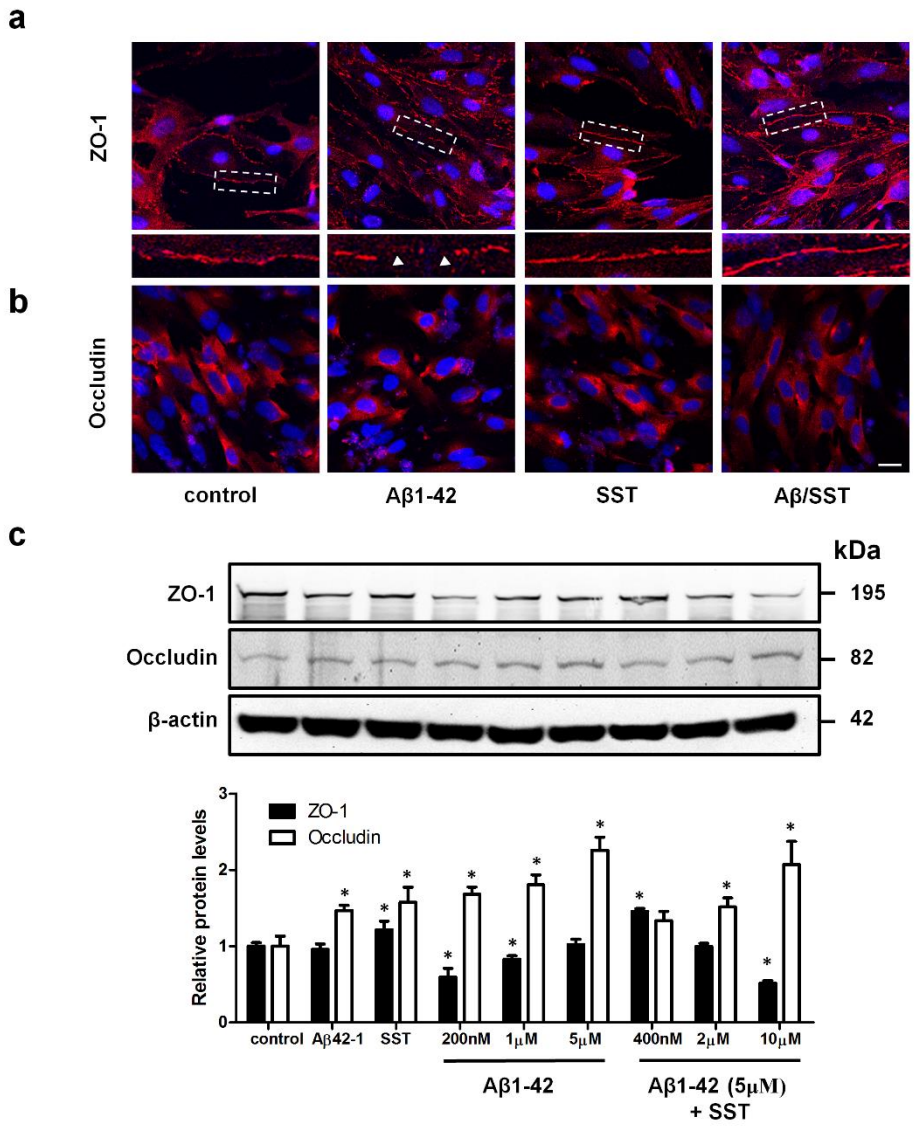

Figure 8 SST inhibits Aβ induced damage on TJP integrity and expression. a Immunofluorescence staining of ZO-1 and occludin. Control hCMEC/D3 cells exhibited continuous staining of ZO-1 on cell periphery, whereas Aβ induced discontinuation in ZO-1 staining as indicated by fragmented staining. Note the normal staining of ZO-1 in presence of SST alone and restoration of ZO-1 like immunoreactivity at cell surface when used in combination with Aβ. High magnification of selected area at cell membrane displayed pattern of ZO-1 disturbance (arrowhead). **b** Occludin immunofluorescence was not changed upon treatment

as indicated. **c** Representative Western blot depicts changes in ZO-1 and occludin expression following dose dependent treatment with either Aβ alone or in presence of increasing concentration of SST as indicated. Densitometry analysis revealed the opposing effect of Aβ on ZO-1 and occludin expression, which was significantly inhibited in presence of SST. β-actin was used as a loading control. *$p < 0.05$ against control. *Scale bar*=20 μm

2.3.7 SST mediated protection against Aβ induced toxicity modulate LRP1 and RAGE expression and activity

Aβ clearance from the brain to CSF and its influx to the neurons from peripheral tissues via circulation is mediated by two prominent receptors protein, LRP1 and RAGE, respectively (Deane et al., 2003; Deane et al., 2008). Previous studies have also demonstrated decreased expression of LRP1 in contrast to increased expression of RAGE in AD brain, however, the role SST might play in regulation of LRP1 and RAGE is not well understood. Both LRP1 and RAGE was not significantly changed in subcellular distribution of immunofluorescence staining (Fig. 9a). However, immunoblot revealed the basal expression of LRP1 in cell lysate was down-regulated in comparison to control when cells were treated with SST (2μM) alone (Fig. 9b). In addition, LRP1 expression was significantly increased when Aβ was used in combination with SST. Western blot analysis also revealed upregulation of RAGE expression in cell lysate prepared from Aβ treated cells. Such enhanced expression of RAGE was down-regulated to the basal level in presence of SST when used in combination with Aβ (Fig. 9b). The suppression of RAGE expression in the presence of SST alone and in combination with Aβ was significantly lower than control.

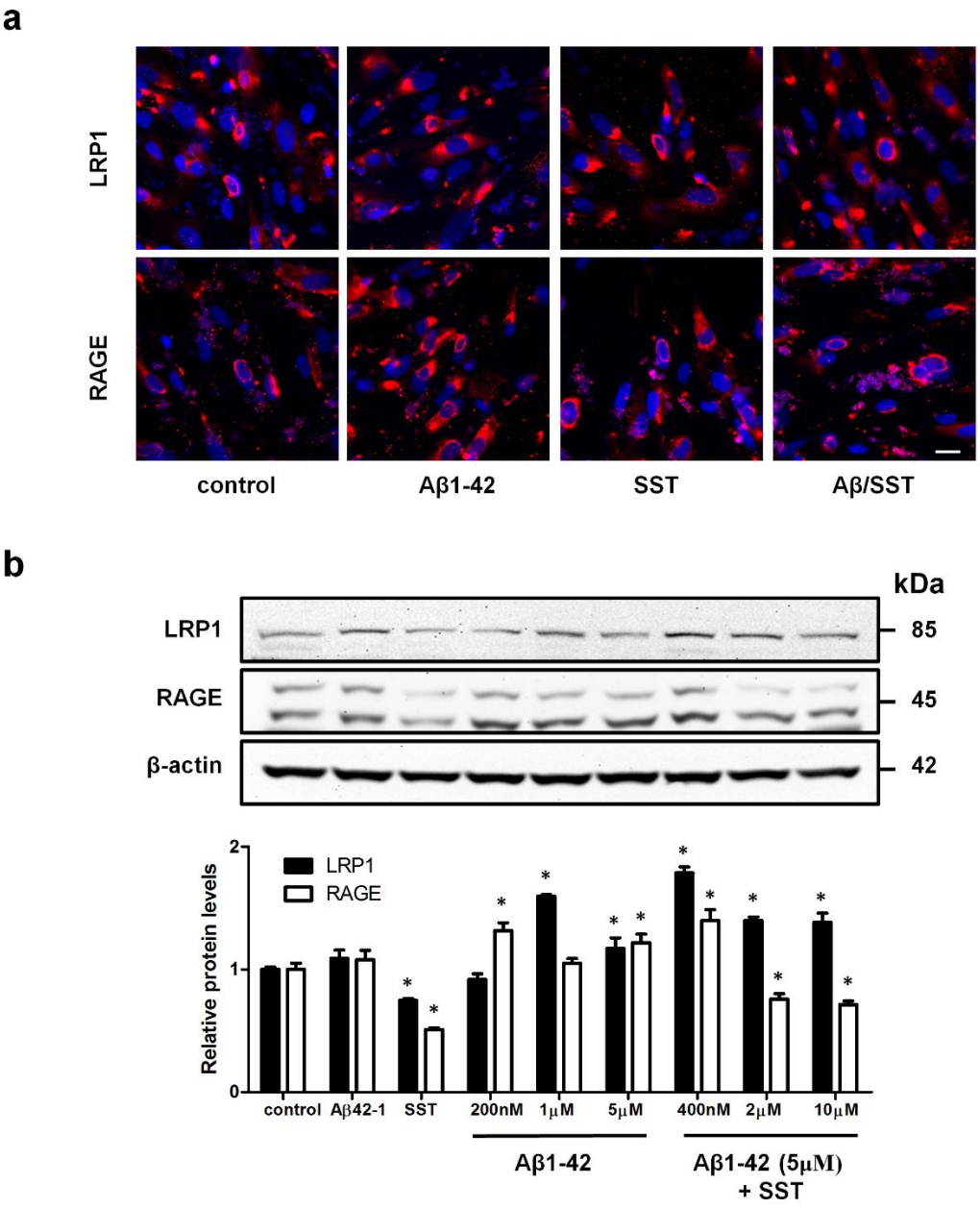

Figure 9 SST restores Aβ induced changes on Aβ1-42 transporter protein expression. a Subcellular distributional pattern of both LRP (upper panel) and RAGE (lower panel) was not visibly affected by the presence of Aβ or SST. **b** Western blot analysis revealed concentration dependent changes in expression of LRP1 and RAGE in presence of Aβ alone. Note the significant inhibition of Aβ mediated changes in presence of SST. Histograms indicate that the

presence of SST increased the expression of LRP1 while reducing the expression of RAGE. β-actin was used as a loading control. *$p < 0.05$ against control. *Scale bar*=20 μm

2.3.8 Impaired cell permeability with Aβ is improved with the use of SST

Transwell permeability assay was performed to determine whether the perturbed organization of ZO-1 and occludin along with changes in expression of LRP1 and RAGE are directly associated with cell permeability (Fig. 10a). Increased level of FITC-dextran was observed in the basolateral chamber in response to Aβ in a concentration-dependent manner due to enhanced cell permeability (Fig. 10b). To assess the effect of SST on BBB permeability, cells were then treated with increasing concentration of SST (80nM-10μM). Increasing concentration of SST exhibit decreased FITC-dextran levels in the basolateral chamber when compared with control, scrambled and Aβ treatment (Fig. 10c). In order to assess whether the presence of SST block Aβ mediated increase in permeability, cells were treated with Aβ alone and in combination with different concentration of SST (80nM-10μM) for 24 hours. SST displayed inhibition of FITC-dextran permeability in the basolateral chamber in comparison to control and/or Aβ treated cells (Fig. 10d). Scrambled Aβ was used as a negative control. Collectively, these results suggest that SST provide protection against Aβ1-42 induced damage to BBB permeability either directly or indirectly through improving the expression of TJP and/or limiting the active influx of Aβ by inhibiting Aβ-binding to transporters such as LRP1 and RAGE.

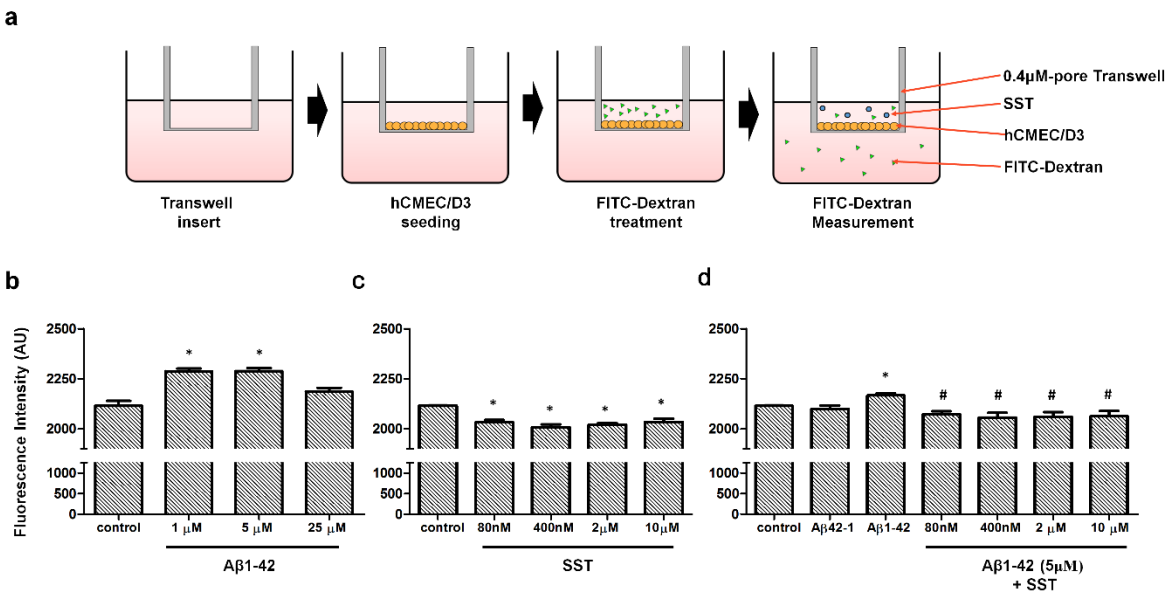

Figure 10 Effects of Aβ and SST on hCMEC/D3 cell permeability. a Schematic representation of FITC-dextran permeability assay using transwell insert. **b** Following 1 and 5μM treatment of Aβ, the FITC-dextran fluorescence in the basolateral chamber was significantly higher in comparison to control. In presence of higher concentration of Aβ (25μM), the FITC-dextran intensity was enhanced however insignificantly. **c** SST exhibited inhibition of FITC-dextran fluorescence intensity in basolateral chamber compared to untreated group in all treatment conditions. **d** Aβ (5μM) treatment resulted in significantly increased FITC-dextran fluorescence intensity. Note the significant inhibition of FITC-dextran in presence of SST and effective restoration of cell permeability. Aβ42-1 scrambled peptide was used as internal **control. ****p* **<0.05 against control; #*p* <0.05 against Aβ1-42 treated alone.**

2.3.9 Pretreatment with SST affects Aβ1-42 influx and efflux in hCMEC/D3 cells.

To investigate whether changes in cell permeability are linked to impaired efflux and influx of Aβ in cells, FACS analysis was used to determine the influx/efflux of FAM-dye

conjugated Aβ in hCMEC/D3 cells with or without SST. Fluorescently labeled Aβ (FAM- Aβ1-42) was used to treat confluent hCMEC/D3 cells, and the uptake was monitored through the bright field and fluorescence microscopy (Fig. 11a) and immunostaining with phalloidin (Fig. 11b). In both preliminary assessments, we observed significant uptake of FAM- Aβ1-42 by cells as early as 10 minutes post-treatment. FACS analysis was performed to quantify the amount of FAM- Aβ1-42 inside the cells following each treatment as indicated. Briefly, the cells were treated with either FAM- Aβ1-42 alone and in combination with SST, or incubated with SST for 15 minutes prior to the addition of FAM- Aβ1-42 and processed for the influx of Aβ (Fig. 11c, upper panel) and efflux (Fig. 11c, bottom panel). To determine the Aβ influx, the geometric fluorescence mean intensity (GMFI) was measured following 30 minutes of treatment. Aβ influx was not significantly changed in cells treated with Aβ alone or in combination with SST (Fig. 11d). However, cells pre-treated with SST (SST+Aβ) for 15 min before the addition of Aβ resulted in significant inhibition of influx (Fig. 11d). We next determined Aβ efflux under similar treatment conditions as described for the influx. As seen in influx, in cells co-treated with FAM- Aβ1-42 and SST, the GMFI was similar to FAM- Aβ1-42 alone. However, when the hCMEC/D3 was pre-incubated with SST for 15 minutes prior to the addition of FAM-Aβ1-42, there was a significant decrease in GMFI (Fig. 11e). These data suggest that the presence of SST effectively inhibit the Aβ uptake by hCMEC/D3 cells.

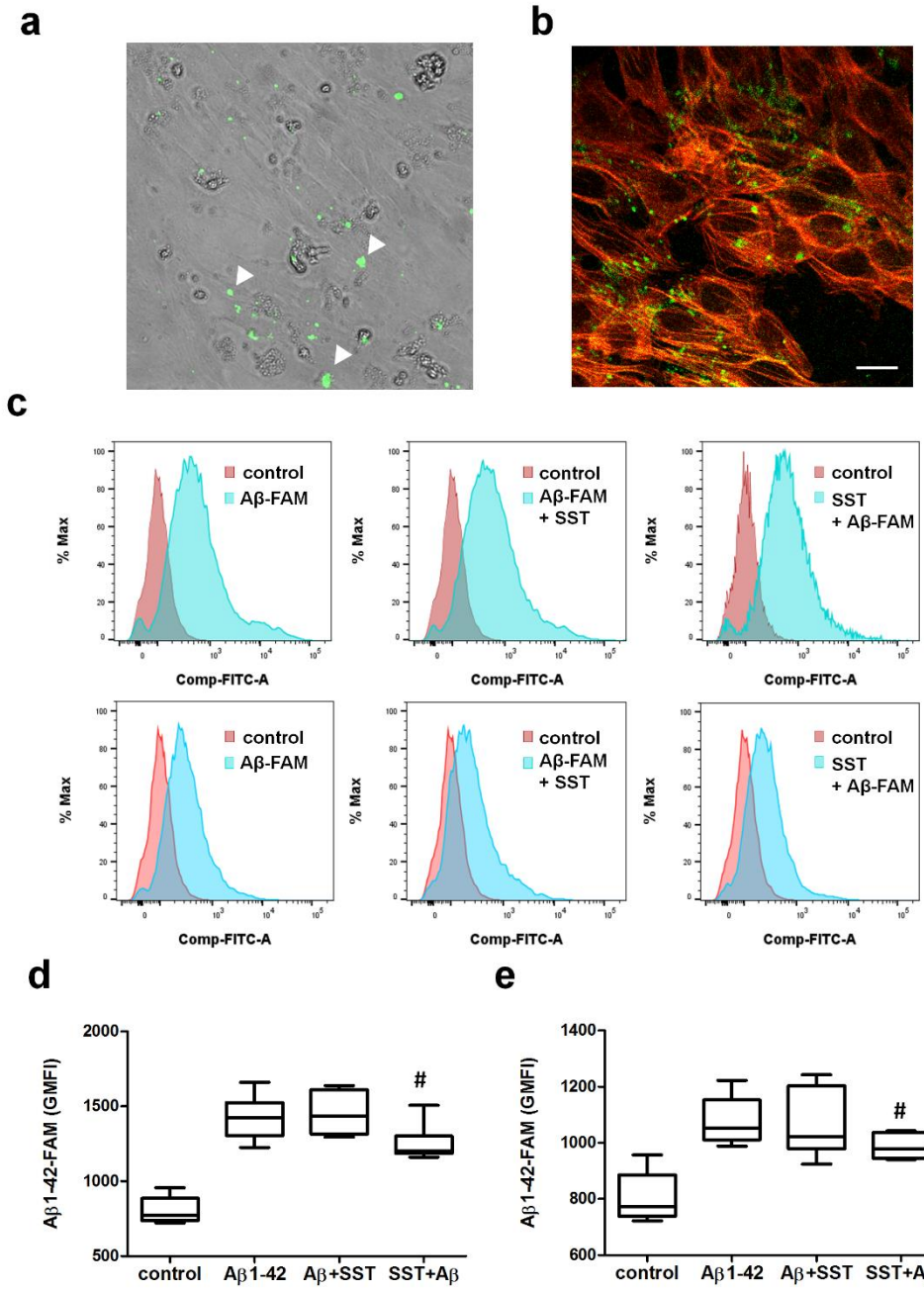

Figure 11 Aβ uptake by BBB is regulated by SST. a Brightfield photograph of confluent hCMEC/D3 cells showing FAM-Aβ as bright green dots (arrowheads) following 10 minute treatment. **b** Immunofluorescence staining of actin marker, phalloidin (red) with the intracellular uptake of FAM-Aβ (green). **c** Representative histogram of FAM-Aβ fluorescence intensity (blue)

compared to negative control (red). Influx (upper panel) and efflux (bottom panel) is shown in three different conditions: FAM-Aβ treated alone (Aβ-FAM; left panel), FAM-Aβ co-treated with SST (Aβ-FAM + SST; middle panel), and cells treated with FAM-Aβ following 15-minute treatment of SST (SST+Aβ-FAM; right panel). **d, e** Representative box blot displaying a summary of GMFI for each treatment group in influx (d) and efflux (e). #$p < 0.05$ against FAM-Aβ alone. These results are representative of six independent experiments and presented as mean ± SEM. *Scale bar*=20 μm.

2.3.10 Role of SST in regulating Aβ-mediated changes in JNK phosphorylation and MMP2 expression in hCMEC/D3 cells

To dissect out the possible mechanism of Aβ-mediated disruption of BBB, the status of JNK expression and phosphorylation and expression of MMP2 was determined in hCMEC/D3 cells following treatment with Aβ in presence or absence of SST. SST and Aβ-scramble displayed no significant effect on JNK activation. Phosphorylation of JNK was significantly enhanced in the presence of Aβ (200nM), whereas decreasing trend of JNK activation was observed with Aβ treatment in a dose-dependent manner. In contrast, SST treatment in presence of Aβ displayed inhibition of JNK phosphorylation and was comparable to control (Fig. 12a).

To determine whether SST interfere in Aβ mediated changes in MMP activity, cell lysate prepared from control and treated cells were immunoblotted for MMP2 expression. MMP2 expression was increased in presence of Aβ in a dose-dependent manner and the increased level of MMP2 was ameliorated in presence of SST when used in combination with Aβ (Fig. 12b). Taken together, our results convincingly support the role of SST in the regulation of Aβ-induced changes in signal transduction pathways that might be associated with perturbed BBB function.

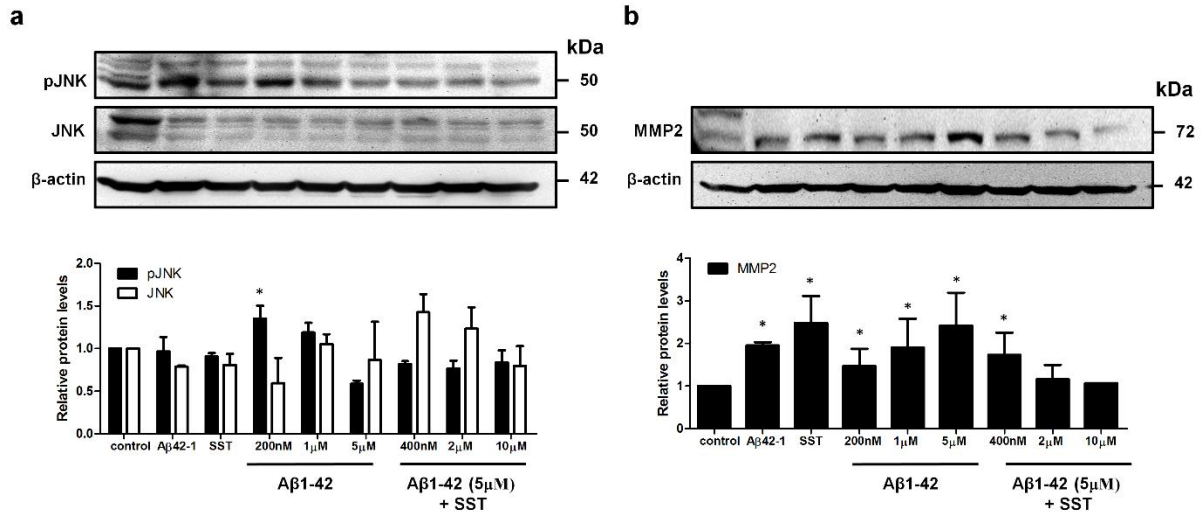

Figure 12 SST inhibits Aβ-induced phosphorylation of JNK and expression of MMP2 in hCMEC/D3 cells. hCMEC/D3 cells were treated with increasing concentration of Aβ (200nM-5μM) alone or in combination with increasing concentration of SST (400nM - 10μM) with Aβ (5μM) for 24hr. Cell lysate prepared from control and treated cells was processed for immunoblot. **a** Aβ mediated up-regulation of pJNK was inhibited in presence of SST. **b** Immunoblot showing increased expression of MMP2 in cells treated with Aβ. Note, the concentration dependent inhibition of Aβ induced expression of MMP2. Histograms represent the densitometric analysis of expression when adjusted with β-actin as a loading control. Mean ± SEM from three independent experiments. *$p < 0.05$ against control.

2.4 Discussion

The disintegration of BBB functionality with the progression of AD has been well documented, however, the molecular mechanisms involved in Aβ induced damage to BBB remains elusive. In the present study, using hCMEC/D3 cells as an *in vitro* model of BBB, we

demonstrate the expression and localization of TJPs, Aβ transporter LRP1 and RAGE and cell permeability in presence of Aβ. In addition, we also determined the status of JNK phosphorylation and expression of MMP2 might play a crucial role in impairing BBB function in Aβ induced damage. Furthermore, the role of SST in abrogating the deterious effect of Aβ in cell permeability and TJPs organization at the cell-cell interaction has been studied. Our results uncovered an important role of SST in restoring the TJPs distribution and expression of LRP1 and RAGE to regulate Aβ influx and efflux in hCMEC/D3 cells. Furthermore, the presence of SST suppressed the activation of JNK and expression of MMP2. Several studies have shown that SST improves cognitive function and memory recovery, work as a neurotrophic factor, improve the formation of synapses, and most importantly blocks the aggregation of Aβ (Matsuoka et al., 1994; Saito et al., 2005; Liguz-Lecznar et al., 2016). Our results establish a functional association between the disruption of TJPs, changes in LRP1 and RAGE as well as signal transduction pathways in Aβ induced toxicity model. To our knowledge, this is the first comprehensive description providing evidence in support of SST mediated protection of BBB in Aβ induced toxicity.

The release of pro-inflammatory cytokines such as IL-1β and TNF-α in AD patients lead to the loss of BBB functionality and increase barrier permeability. Aβ activates RAGE and inflammatory toll-like receptors, resulting in increased cytokines release (Abbott, 2000; Ramasamy et al., 2009; Reed-Geaghan et al., 2009). In turn, the released cytokines form a positive feedback loop and promote further production of Aβ (Smith et al., 2007). We recently reported that SST mediates suppression of LPS-induced release of TNF-α and IFN-γ, and recovered ZO-1 expression at the cell surface in hCMEC/D3 cells. Also, SST effectively inhibits inflammation-induced damage to TJPs *in vitro* upon co-treatment with pro-inflammatory

cytokines (Basivireddy et al., 2013). The anti-inflammatory effect of SST via SSTRs has been well-established using *ko* mice (Green et al., 1992; Szolcsanyi et al., 1998a; Szolcsanyi et al., 1998b). Consistent with these observations, we have demonstrated that Aβ induces the release of IL-1β, which was significantly blunted in the presence of SST whereas SST release in response to Aβ tends to decrease albeit insignificantly. These results are consistent with previous studies suggesting that Aβ impaired SST processing from its precursor (Epelbaum et al., 2009).

ZO-1, occludin and claudin have been studied extensively for their prominent role in maintaining the paracellular integrity of BBB. Specifically, ZO-1 is a membrane protein at brain parenchyma localized to blood vessels forming an integral part of BBB (Tornavaca et al., 2015). Previous studies have reported that cytokines activate pro-inflammatory pathways in mouse brain endothelial cells leading to hyperphosphorylation of occludin, ZO-1 and claudin. This results in dissociation of these proteins from tight junction complexes, ultimately leading to the loss of barrier integrity and increased permeability (Stamatovic et al., 2003; Stamatovic et al., 2006). Most importantly, suppression of cytokines attenuates BBB permeability and restore inflammation induced expression and organization of TJPs. Consistent with these findings, we observed a systematic increase of IL-1β in the presence of Aβ1-42, which led to a dose-dependent decrease in ZO-1 and occludin expression as well as disrupted organization. Importantly, such loss was effectively ameliorated in presence of SST.

It is now well established that Aβ elicits significant changes in transporter proteins involved in clearance of Aβ and pathogenesis of AD involving LRP1 and RAGE as key modulators of such activity (Cirrito et al., 2005; Deane et al., 2008; Qosa et al., 2012). Previous *in vitro* study using hCMEC/D3 monolayer have shown decreased LRP1 while increased expression of RAGE upon treatment with Aβ1-40 or Aβ1-42 (Qosa et al., 2014b). Recent studies

have reported that LRP1 regulates matrix-degrading proteases such as MMPs and plasminogen activators (PAs) (Etique et al., 2013). The clearance of MMP is primarily mediated via LRP-dependent endocytosis, where the decreased level of LRP1 in AD leads to accumulation of MMPs (Barmina et al., 1999; Yang et al., 2001; Van den Steen et al., 2006).

The role of pro-inflammatory cytokines IL-1β and TNFα in the regulation of MMP2 and its association with impaired BBB function in AD is well established (Iwata et al., 2000; Gao et al., 2004; Lynch et al., 2006; Chen et al., 2009b). In addition, MMPs levels were found to be significantly higher in the hemorrhagic areas of the cerebral amyloid angiopathy brains, while MMP2 levels were also seen to be highly localized to astrocytes surrounding Aβ affected vessels (Hernandez-Guillamon et al., 2012). In *tg* mice model tg-SwDI, MMP2 levels were especially high in reactive astrocytes immediately surrounding Aβ plaques and cerebral microvascular fibrillar amyloid deposits (Yin et al., 2006). Since hCMEC/D3 cells were cultured in the absence of astrocytes in the present study, changes seen in MMP2 expression might be associated with some other unknown mechanism. Subsequent studies using isolated rat brain microvessels have also shown decreased expression of claudin with increased expression of MMP2 and 9 in response to increasing concentration of Aβ1-40 (Hartz et al., 2012). Previous studies have also described that activation of JNK increases MMPs expression (Fromigue et al., 2008). Consistent with these observations, our results describe increased phosphorylation of JNK and MMP2 expression in presence of Aβ, which was inhibited in presence of SST. In support, the inhibition of JNK resulted in blockade of MMP2 expression implicating the direct role of JNK in regulation of MMP2 (Fromigue et al., 2008). Taken together, the disturbed organization of TJPs and transporter proteins were effectively ameliorated in presence of SST, resulting in inhibition of Aβ uptake.

Our results revealed that at least in acute exposure to Aβ, the suppression of TJPs and changes in transporter proteins and signaling molecules are interconnected. With these observations, we speculate that anti-inflammatory effect of SST may alleviate the Aβ induced pro-inflammatory cytokines, which in turn would prevent or inhibit the loss of integrity of BBB. We have convincingly demonstrated that Aβ induced disruption in cell surface organization of TJPs and imbalance in LRP and RAGE expression in brain endothelial may be abrogated by SST. Most importantly, neprilysin (NEP) and insulin degrading enzyme (IDE) are the two most well-established enzymes identified to mediate proteolytic cleavage of Aβ (Iwata et al., 2000; Gao et al., 2004; Lynch et al., 2006; Chen et al., 2009b; Qosa et al., 2014a). Interestingly, SST has also been identified as a potent activator of neprilysin, leading to increased catabolism of Aβ (Saito et al., 2005), further supporting the notion that SST plays a pivotal role in the homeostatic maintenance of BBB integrity.

With these observations, we hypothesize that gradual loss of SST in AD patients not only associated with a loss of memory and cognitive function but is also responsible for the disrupted and impaired BBB functionality and integrity. Whether this effect of SST on LRP1 and RAGE is mediated directly via activation of five different SST receptor subtypes or indirectly through some other possible mechanism is not known and future studies are in progress in this direction. Taken together, these finding represents a novel insight into the role of SST in improving BBB permeability in Aβ induced toxicity and strengthens SST as a potential target for treatment and prevention of AD. Collectively, our data indicate that SST may serve as a crucial signaling mechanism involved in the protection of BBB by blocking the JNK-MMP pathways.

Chapter 3. Somatostatin mediated changes in microtubule-associated proteins and retinoic acid-induced neurite outgrowth in SH-SY5Y

3.1 Introduction

The optimization of efficient *in-vitro* neuronal culture has been a challenge due to the senescence of mature neurons. To compensate, studies have utilized the immortalized neuronal cell lines derived from neuroblastoma. The prominent caveat, however, is that despite neuronal phenotype acquired through culture manipulation, there exist certain differences between differentiated cells versus mature neurons. Human neuroblastoma derived SH-SY5Y cell has been extensively used as an experimental model in either undifferentiated or differentiated state due to its well-characterized genotype and relative ease in culture compared to primary neurons (Rossino et al., 1991; Kaplan et al., 1993; Kito et al., 1997; Truckenmiller et al., 2001; Lopez-Carballo et al., 2002; Brill and Bennett, 2003; Conn et al., 2003; Ding et al., 2004; Miloso et al., 2004; Pan et al., 2005; Cuende et al., 2008; Cheung et al., 2009). SH-SY5Y cells are capable of undergoing various differentiation processes with the use of different inducers of neuronal differentiation including retinoic acid (RA), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Pahlman et al., 1995; Encinas et al., 2000). RA, a vitamin A metabolite, is known to play a critical role in proper development of the mammalian nervous system (Maden, 2002; Clagett-Dame et al., 2006). RA induced differentiation of SH-SY5Y cells have been widely used to yield genomic and morphological changes during differentiation (Encinas et al., 2000; Almeida et al., 2005; Lasorella et al., 2006). Furthermore, studies have revealed that SH-SY5Y cells have the ability to differentiate into specific neuronal subtypes such as cholinergic, dopaminergic and adrenergic neurons depending on the differentiation-promoting factors used

(Pahlman et al., 1984; Xie et al., 2010). With these properties, SH-SY5Y cell lines have served as an ideal model to study various physiological, biochemical and pharmacological aspects of the neuronal cells including neurogenesis (Abemayor and Sidell, 1989).

Previous studies have used neuron-specific markers such as MAP2 and Tau to determine the commitment of SH-SY5Y cells into neuronal lineage which showed progressive increase upon treatment with RA (Cuende et al., 2008). Growing evidence suggests a critical role of MAPs in the neuronal development. The initial characterization revealed that MAP2/Tau family proteins preferentially bind to microtubules (Hirokawa et al., 1988a; Hirokawa et al., 1988b; Al-Bassam et al., 2002). In addition to the direct binding of MAPs to microtubules, functional analysis has revealed that the presence of MAP2 and Tau also increased the microtubule rigidity (Felgner et al., 1997). Today, MAP2 is generally accepted as a key member of the family of proteins responsible for stabilizing microtubules (Caceres et al., 1986). In addition to the role of microtubules associated proteins in neurite outgrowth, MAP2 also mediated downstream signal transduction pathways.

Numerous studies have also reported the critical role of mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK1/2) activation in cellular differentiation (Qui and Green, 1992; Nguyen et al., 1993; Cowley et al., 1994; Kharbanda et al., 1994). In particular, studies have reported that the activation of MAPK/ERK1/2 is required for neuronal differentiation and in protection against drug-induced injury (Riese et al., 2004; Abdul and Butterfield, 2007; Chen et al., 2009c; Chen et al., 2010). Furthermore, ERK1/2 activation has also been observed in the NGF-induced differentiation of PC12 cells (Morooka and Nishida, 1998). Among the numerous activators of ERK1/2, we recently demonstrated that SST activated

ERK1/2 pathway in *in-vitro* model of multiple sclerosis using human brain microvascular endothelial cells (hCMEC/D3) (Basivireddy et al., 2013).

SST has been extensively studied for its role in neuronal cell growth, maturation and migration as well as for the anti-proliferative effect on adult nerve cells. SST plays a critical role in the maintenance of normal cell physiology and has been associated with protective roles in numerous pathological conditions including neurodegenerative diseases (Holst et al., 1990; War et al., 2015). In the central nervous system (CNS), SST serves as neuromodulator and neurotransmitter. SST also promotes neuronal differentiation and neurite outgrowth in cerebellar granule cells (Epelbaum et al., 1994; Maubert et al., 1994; Taniwaki and Schwartz, 1995). The SST promotes neurite outgrowth in re-generating neurons of invertebrates as well as in cultured neuroblastoma cells and PC12 cell line (Bulloch, 1987; Grimm-Jorgensen, 1987; Kentroti and Vernadakis, 1991; Ferriero et al., 1994), however the molecular mechanism involved remains elusive.

In Alzheimer's Disease (AD), the gradual loss of SST has been associated with impaired cognitive function and memory loss, increased beta-amyloid (Aβ) degrading peptidase neprilysin activity with blockade of Aβ aggregation and toxicity (Saito et al., 2005). In addition, studies have also shown the preferential survival of SST positive medium sized aspiny interneurons in Huntington’s disease (HD) and decreased level of SST in cerebrospinal fluid (CSF) in multiple sclerosis (MS) patients (Sorensen, 1987; Roca et al., 1999). Furthermore, dysfunction of somatostatin-positive interneurons have been correlated with memory loss in AD model (Schmid et al., 2016). The anti-inflammatory role of SST has been associated with a neuroprotective role in excitotoxicity (Patel, 1999).

Accordingly, in the present study, we examined the role of SST in response to RA mediated differentiation and elucidated the progressive development of neurite outgrowth and molecular mechanisms that might be associated with this transition using SH-SY5Y cells. We uncovered the novel function of SST in promoting and potentiating of RA induced neuronal differentiation with a significant increase in both MAP2 and Tau expression and neurite outgrowth. Furthermore, we have identified that SST activates ERK1/2 in presence of RA leading to improved neurite outgrowth. Taken together, results described here suggest that in addition to the neuroprotective role in excitotoxicity, SST in combination with growth factors may promote and maintain proper neurite outgrowth in CNS.

3.2 Material and Methods

3.2.1 SH-SY5Y cell culture and differentiation

Human SH-SY5Y neuroblastoma cell line (kindly provided by Dr. Neil Cashman, University of British Columbia, Vancouver, Canada) were grown and maintained in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100µg/ml) in a 5% CO_2 humidified incubator at 37°C. Cells were differentiated as previously described (Encinas et al., 2000). Briefly, cells were seeded on culture dishes pre-coated with Matrigel (10mg/mL, BD Bioscience) and grown for 7 days in medium containing 10µM all trans-retinoic acid (RA, Sigma, St.Louis, USA). To examine the effect of SST, cells were treated with RA in combination with increasing concentrations of SST (80nM, 400nM, 2µM, 10µM) for 3 to 7 days. All experiments were carried out using cells within passage 9 – 15.

3.2.2 Cell morphology and Quantitative analysis of neurite outgrowth

Brightfield photographs of SH-SY5Y cells were obtained using Leica DM IRBE inverted microscope (Leica, Wetzlar, Germany) to monitor morphological changes during RA induced differentiation in presence of SST, receptor-specific agonists and/or SSTR specific sense or anti-sense oligonucleotides treatments. Multiple confocal images of the SH-SY5Y cells collected post-treatment with RA or SST alone and in combination were analyzed for total neurite length using Neurite Tracer as described earlier (Pool et al., 2008). Briefly, the control and treated SH-SY5Y cells were stained with neurite marker MAP2 and Hoechst for the nucleus. Neurite Tracer plug-in on ImageJ software was used to detect the neurites strongly stained for MAP2. Following optimization of parameters to separate neurites from the cell body, and tracing the neurite through skeletonization, positively-labeled neurites and its respective lengths were quantified.

3.2.3 Immunofluorescence immunocytochemistry

Control and treated cells were fixed with 4% paraformaldehyde for 20 minutes on ice followed by permeabilization with 0.1% Triton-X100 prepared in phosphate-buffered saline (PBS) for 15 minutes at room temperature (RT). Following 3 washes in PBS, cells were blocked with PBS containing 5% Normal Goat Serum (NGS) for 1 hour at RT. Cells were then incubated with primary antibodies, beta-III tubulin (TUJ1, 1:5000; BioLegend, Cat# MMS-435P), MAP2 (1:500, Sigma, Cat# M1406), Tau (1:250; Invitrogen, Cat# AHB0042), anti-rabbit SSTR2 and 4 (1:1000, Santa Cruz Biotechnology, Cat# sc25676 and sc25678) and SST (1:250; Santa Cruz Biotechnology, Cat# L-74556) in PBS (containing 5% NGS) overnight at 4˚C. Following incubation, cells were washed with PBS and incubated with species-specific, Alexa-conjugated secondary antibody (Alexa-488, -594) for 1 hour at RT (1:200; Invitrogen). For nucleus

visualization, cells were incubated with Hoechst dye 33258 (0.5μg/mL, Calbiochem, CA, USA) for 10 minutes at RT. The coverslips were then mounted onto the slides and photographed using Zeiss LSM700 confocal microscope (Carl Zeiss, Germany). Image panels were constructed using Carl Zeiss Zen software.

3.2.4 Agonist, Sense and antisense oligonucleotide treatment

The phosphorothioate modified antisense oligonucleotide against the first six codons of preproSST, hSSTR2 and 4 were obtained from University Core DNA services, University of Calgary (Alberta, Canada). The exact sequence of used are preproSST Sense 5' ATG CTG TCC TGC CGC CTC 3', Antisense 5' GAG GCG GCA GGA CAG CAT 3', SSTR2 Sense 5'ATG GAC ATG GCG GAT GAG 3', Antisense 5' CTC ATC CGC CAT GTC CAT 3', SSTR4 Sense 5' ATG AGC GCC CCC TCG ACG 3', Antisense 5' CGT CGA GGG GGC GCT CAT 3'. Cells were treated with sense- or antisense oligonucleotides (10μg/mL) for 48 hours prior to RA induced differentiation and were maintained for 3 to 7 days. The culture medium was supplemented with sense and antisense-oligonucleotides every 48 hours.

hSSTR2 and 4 specific non-peptide agonist (L-779976 and L-803087) were kindly provided by Dr. S.P. Rohrer from Merck. Briefly, SSTR specific agonists (3, 10, 30nM) were added to culture medium every 48 hours during RA induced differentiation.

3.2.5 Western blot analysis

For Western blot analysis, control and the treated cells were harvested and lysed in cold lysis buffer (150mM NaCl, 50mM Tris-HCl, 1% Triton X-100, 0.1% SDS, 0.5% Sodium deoxycholate and 1% protease-phosphotase inhibitor mixture). The supernatants were collected

and protein concentrations were determined using Bio-Rad protein assay (Bio-Rad Laboratories). Whole cell lysates (15μg) were subjected to 10% SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hr at RT and blotted overnight in presence of monoclonal anti-mouse MAP2 (1:1000, Sigma, Cat# M1406), Tau (1:1000, Invitrogen, Cat# AHB0042), PCNA (1:1000, Sigma, Cat# P8825) and TUJ1(1:5000, BioLegend, Cat# MMS-435P) and rabbit polyclonal total- and phophorylated ERK1/2 (1:1000, Cell Signaling, Cat# 9101 and 9102), anti-rabbit SSTR2 and 4 (1:1000, Santa Cruz Biotechnology, Cat# sc25676 and sc25678) antibodies. The membranes were washed and incubated for 1 hr in RT with either horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:2000) or goat anti-rabbit antibodies (1:2000) (Jackson Lab). The membranes were washed and developed using chemiluminescence detection kit (Millipore, Billerica, MA, USA) on Alpha Innotech FluorChem 8800. β-actin was used as a loading control. Densitometric analysis of protein expression levels was performed with ImageJ Software.

3.2.6 Statistical Analysis

Results are presented as mean ± SD of minimum three independent sets of experiments. All statistical analysis has been performed in Graph Prism5.0. One-way analysis of variance (ANOVA) or Student's *t*-test was used as indicated. *p<0.05, **<0.01 against control or #p < 0.05 against RA treated alone was taken into consideration as significant.

3.3 Results

3.3.1 RA-induces neurite outgrowth and morphological changes in SH-SY5Y cells

Several previous studies have shown successful use of RA in the differentiation of SH-SY5Y cells into highly homogeneous populations of neuron-like cells (Encinas et al., 2000; Wang et al., 2006). Following RA-induced neuronal differentiation, SH-SY5Y cells displayed a gradual extension of neurite outgrowth from day 1 to 7 (Fig. 13A). In addition to neurite formation and elongation, cells also displayed morphological changes in shape and size (Fig. 13A). During differentiation, cells became elongated in comparison to round shape of the undifferentiated cells. Next, we quantified neurite outgrowth at day 1, 3 and 7 in comparison to day 0 control cells. As shown in Fig.13B, neurite length was consistently increased during differentiation with an average length of 52.1±18.1μm on day 1, 87.88±15.45μm on day 3 and 104.35±29.96μm on day 7.

3.3.2 RA induced differentiation of SH-SY5Y cells to neuronal phenotype is associated with changes in MAP2 and TUJ1 expression

To determine whether microtubule-associated proteins are involved in RA induced neurite outgrowth and neuronal differentiation of SH-SY5Y cells, the expression level of MAP2 and neuronal marker TUJ1 was analyzed using Western blot analysis. Total protein extract prepared from SH-SY5Y cells at day 0, 1, 3 and 7 of differentiation upon treatment with RA were immunoblotted for the expression of PCNA, MAP2, and TUJ1. As shown in Fig. 13C and D, TUJ1 expression level was comparable to control without any significant changes at days 1 to 7 of differentiation. In comparison to the control, microtubule-associated MAP2 was significantly upregulated at days 3 and 7 of differentiation in response to RA (Fig. 13, C and D).

Furthermore, gradual loss of the cell-division marker PCNA during differentiation indicates the commitment of SH-SY5Y cells into the terminally differentiated neurons. The gradual loss of PCNA in combination with an increased MAP2 further supports the neuronal differentiation capacity of SH-SY5Y cells in presence of RA.

3.3.3 SH-SY5Y cells differentiation and colocalization between MAP2 and TUJ1

To determine whether differentiated cells co-expressed MAP2 and TUJ1, double immunofluorescence staining was performed. As shown in Fig. 13E, in comparison to control, differentiated cells expressed strong MAP2 and TUJ1 like immunoreactivity and colocalization by day 3. Colocalization was confined to the apical endings of the cell body and neurites (Fig. 13E, arrows). In addition, we also observed sparsely distributed cells which were devoid of MAP2 and TUJ1 expression and failed to bear neurites (Fig. 13E, arrowheads). TUJ1 like immunoreactivity in cells at day 7 following differentiation with RA was reduced at the apical ending of the cell body and enhanced in neurite formation with no significant changes in MAP2 distribution (Fig. 13E). This significant site-specific variation in the distributional pattern of MAP2 and TUJ1 increased colocalization in neurites (Fig. 13E). Together, these results indicate increased expression of MAP2 in differentiated SH-SY5Y cells with site-specific cellular colocalization with TUJ1 that might exert a crucial role in neurite elongation.

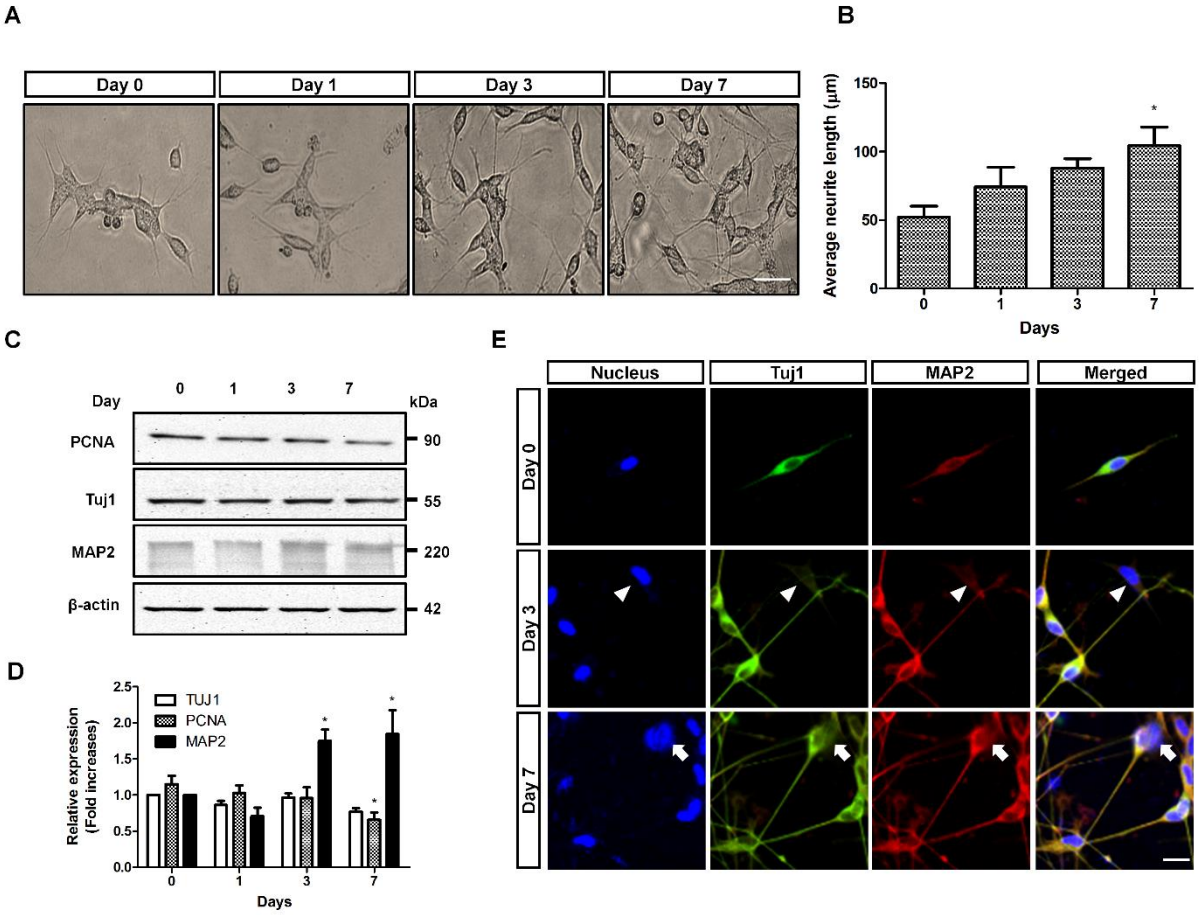

Figure 13 Morphological and biochemical characterization of RA-induced differentiation of SH-SY5Y cells. A. Representative bright field photographs of SH-SY5Y cells at day 0-7 of differentiation in presence of RA displayed morphological changes in shape and size of cells along with neurite growth in time dependent manner. **B.** Representative histogram showing gradual increase in neurite length in time dependent manner. **C.** SH-SY5Y cells differentiated with RA (10μM) for 0-7 days as indicated. Post-treatment cell lysate prepared was immunoblotted to detect neuronal differentiation markers PCNA, MAP2 and TUJ1. PCNA level is decreased in a time-dependent manner. MAP2 showed time-dependent increase indicative of neurite formation. Note no detectable changes in TUJ1 expression. **D.** Histogram showing densitometric analysis of relative expression of PCNA, MAP2 and TUJ1. β-actin was used as a

loading control. **E.** Representative confocal photomicrographs showing cellular distribution and colocalization of MAP2 and TUJ1 in RA induced differentiated SH-SY5Y cells at day 0, 3 and 7. In comparison to day 0, MAP2 and TUJ1 displayed strong colocalization at day 3 and 7. At day 7, cells exhibited neurite growth and colocalization at the apical ending of cells. Note the cells which are devoid of colocalization and neurite formation at day 3 (arrowhead), and mild colocalization in cell body due to translocation of TUJ1 to neurites (arrow). Results are mean ± SD of three separate experiments. *$p< 0.05$ against control. Scale bar = 20μm.

3.3.4 SST enhances MAP2-like immunoreactivity and neurite length during RA induced differentiation

Next, we determined the distributional pattern of MAP2-like immunoreactivity in cells treated with RA alone and in combination with increasing concentration of SST (80nM, 400nM, 2μM, 10μM). The extent of neurite formation and elongation was quantified using image analysis software to trace MAP2 expression in neurites. In comparison to the cells treated with RA alone, cells treated with RA with increasing concentration of SST resulted in an increase of MAP2 expression and total neurite length at day 3 and 7 in a dose-dependent manner (Fig. 14, A and B). Importantly, the SST mediated increase in MAP2 expression was pronounced at day 3 but was indistinguishable from RA alone by day 7.

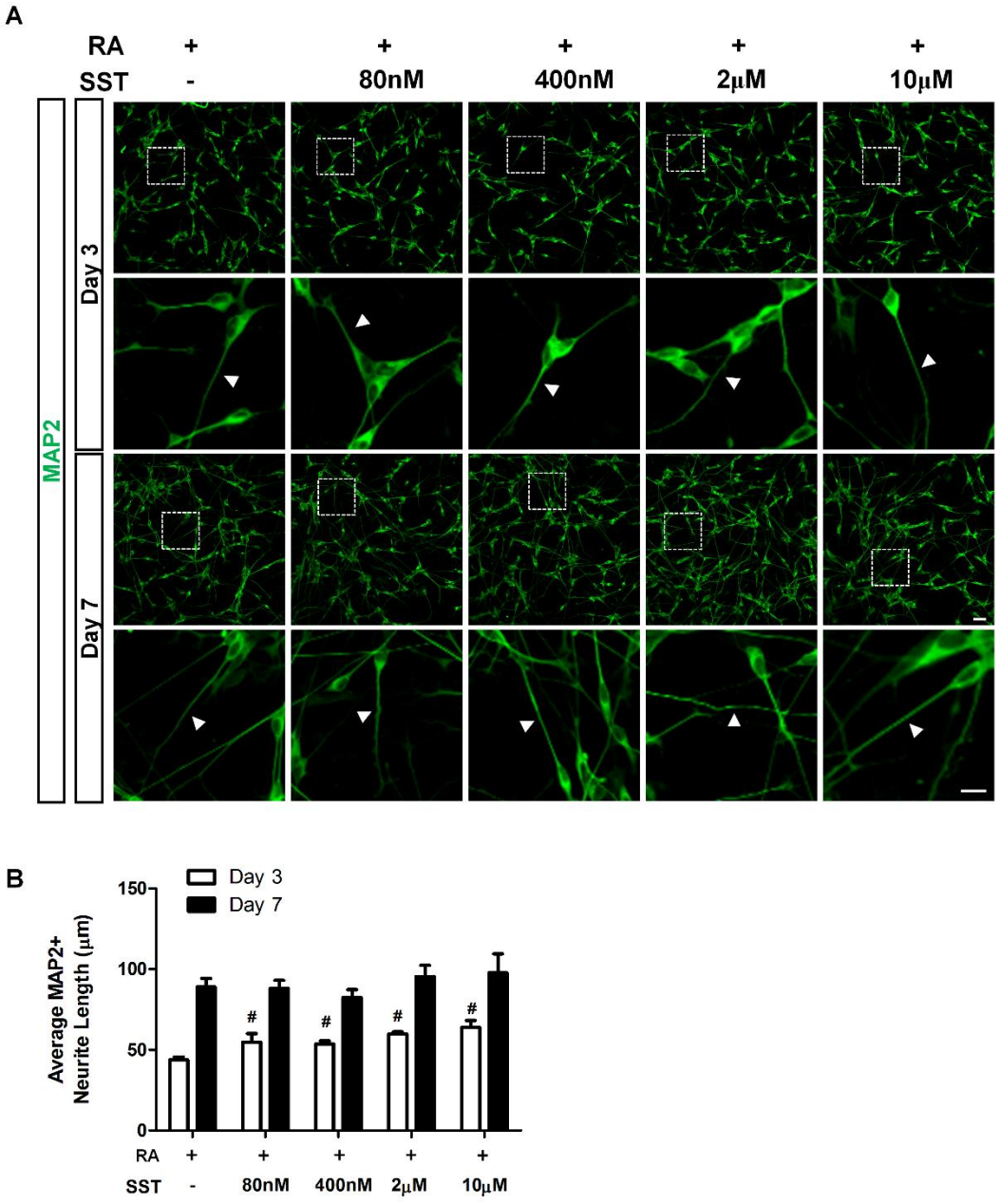

Figure 14 Concentration and time dependent effect of SST on cellular distribution of MAP2 in SH-SY5Y cells. **A.** Representative photomicrographs of low- and high-magnification illustrating immunofluorescence staining of MAP2 in differentiated cells. In response to increasing concentration of SST, MAP2 positive neurites display increased length at day 3 and 7. **B.** Histogram depicts the quantitative analysis of neurite length. Arrowheads in representative

panels indicate MAP2 positive neurites. Results are mean ± SD of three separate experiments. *#p* < 0.05 against RA treated alone. Scale bar = 20μm.

3.3.5 SST enhances microtubule stabilizing Tau expression in SH-SY5Y cells

Similar to MAP2, the role of Tau in axonal and neurite outgrowth is well established. However, SST mediated elongation of neurite outgrowth in SH-SY5Y cells associated with the changes in Tau expression remains elusive. Accordingly, we next determined the cellular distribution of Tau in SH-SY5Y cells upon treatment with RA alone and in combination with increasing concentrations of SST. Similar to the distributional pattern of MAP2 expression, Tau like immunoreactivity was increased in a dose-dependent manner in response to SST (Fig. 15A). Tau immunostaining was significantly increased at day 7 in presence of the higher concentration of SST (2, 10μM) when compared to RA alone. As shown in Fig. 15B, quantitative analysis of the intensity of Tau immunoreactivity revealed no significant variation between day 3 and 7, but remained augmented in comparison to the cells treated with RA alone. Collectively, these data suggest that SST increases tubulin stabilizing proteins MAP2 and Tau, which in turn promotes neurite formation and elongation.

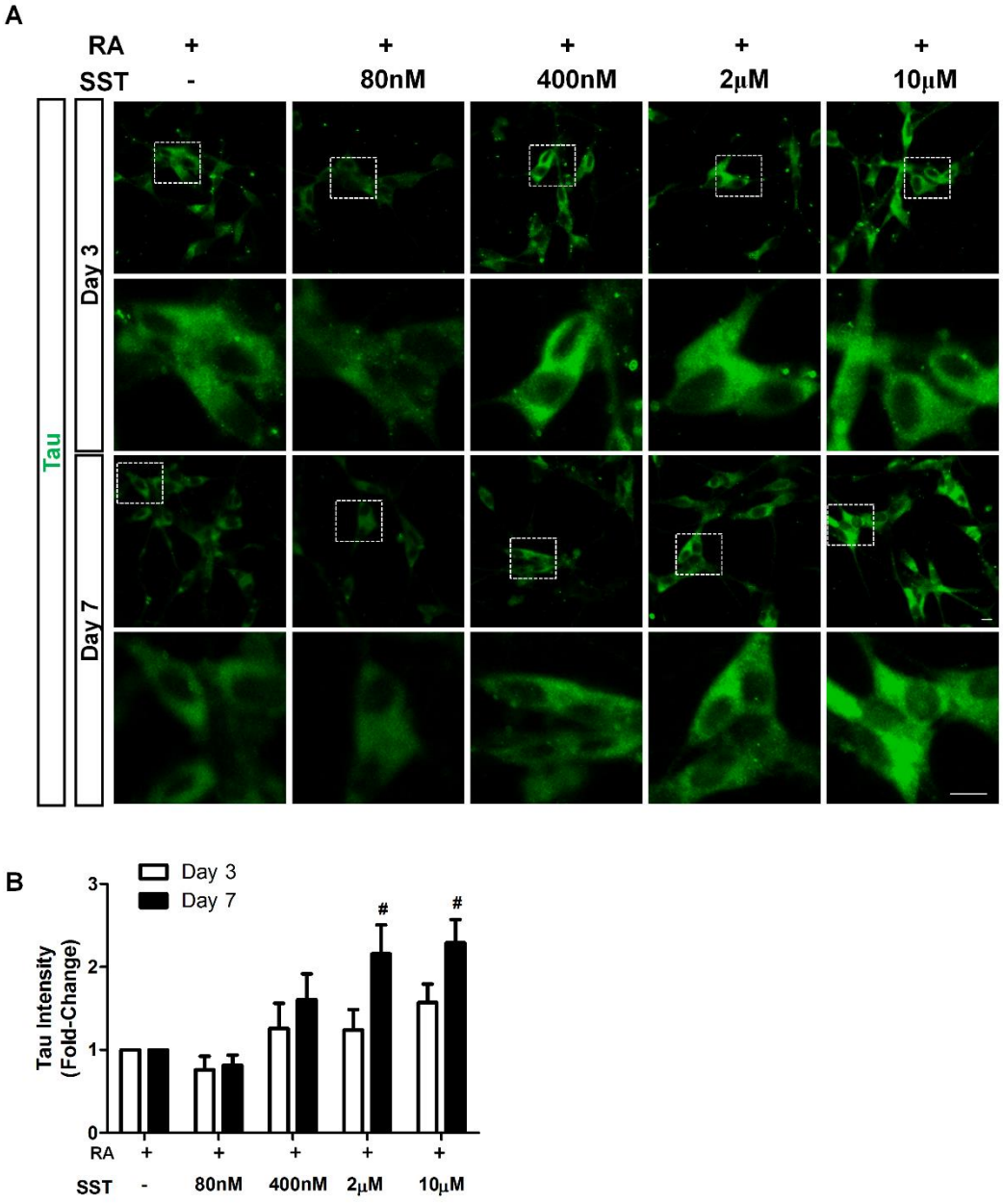

Figure 15 Concentration and time dependent effect of SST on cellular distribution of Tau in SH-SY5Y cells. A. Representative confocal photomicrographs illustrating immunofluorescence staining of Tau in differentiated cells. In response to increasing concentration of SST, enhanced Tau staining was seen at day 3 and 7 of differentiation. Representative panels show high magnification view of positive cells. **B.** Histogram depicts the

quantitative analysis of Tau immunoreactivity. Results are mean ± SD of three separate experiments. *#p* < 0.05 against RA treated alone. Scale bar = 10μm.

3.3.6 SST-mediated promotion of RA-induced neurite outgrowth in SH-SY5Y involve up-regulation of MAP2 expression

Previous reports have suggested the role of SST in promoting neurite outgrowth (Ferriero et al., 1994). To assess whether SST exerts a positive role in RA induced differentiation of SH-SY5Y cells, we next determined the effect of SST on RA induced differentiation and neurites outgrowth. SH-SY5Y cells were treated with RA (10μM) and SST (2μM) alone or in combination for 3 and 7 days. Post-treatment, cells were processed to visualize neurite growth using MAP2 and TUJ1 immunofluorescence staining. Control cells maintained round morphology with minimal neurite formation. In comparison, differentiated SH-SY5Y cells displayed significant neurite formation at day 3 and 7 (Fig. 16A; arrow). As shown in Figure 16A, cells treated with SST alone displayed sporadic neurite developments suggesting spontaneous neurite formation to a certain extent. Importantly, cells treated with RA and SST in combination exhibit extended neurite outgrowth and strong staining of MAP2 and TUJ1 as well as colocalization (Fig. 16A). These results indicate that SST enhanced RA induced neurite formation possibly by increasing MAP2 expression.

To further assess the concentration-dependent effect of SST in promoting neuritogenesis, SH-SY5Y cells were treated with increasing concentration of SST (80nM, 400nM, 2μM, 10μM) in combination with RA (10μM) and cell lysate was processed to determine the expression of MAP2 and TUJ1 by Western blotting. Surprisingly, the presence of SST alone or low concentration of SST (80nM) in presence of RA inhibited the MAP2 expression at the earlier

time point of day 3 (Fig. 16B). However, in the presence of increasing concentration of SST (400nM-10μM), MAP2 was significantly increased in comparison to cells differentiated with RA alone. At Day 7, SST treatment alone resulted in significant increase in MAP2 expression, which was also maintained in co-treatment with RA. No significant changes were observed for TUJ1 expression at any time point.

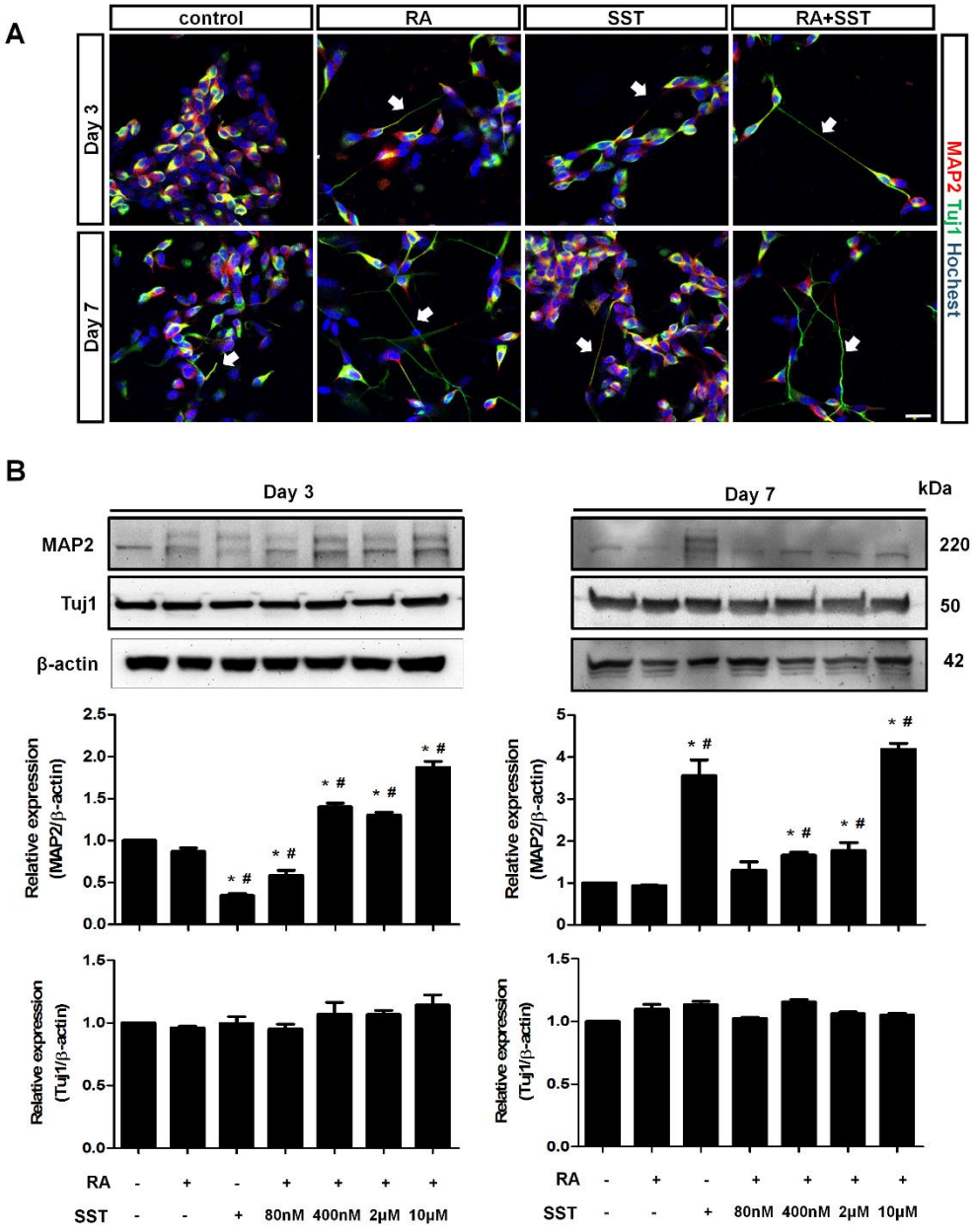

Figure 16 SST promotes neurite outgrowth by enhancing tubulin stabilizing MAP2. **A.** Confocal photographs showing immunofluorescence colocalization of MAP2 and TUJ1 in RA differentiated SH-SY5Y cells in presence or absence of SST. Combined treatment of cells with SST and RA induced longer neurite formation indicated by MAP2 staining. Presence of SST alone did not induce significant neurite outgrowth, however, spontaneous neurite formation was

observed at both day 3 and 7 in presence of SST. Neurites formation in representative panels is indicated by arrows. **B.** Western blot analysis of MAP2 and TUJ1 following differentiation at day 3 and 7. MAP2 shows a significant dose-dependent increase with increasing concentration of SST whereas no significant changes were seen in TUJ1 expression. Histograms represent a densitometric analysis of relative expression of MAP2 and TUJ1. β-actin was used as a loading control. Results are mean ± SD of three separate experiments. *$p < 0.05$ against control. #$p < 0.05$ against RA treated alone. Scale bar = 20μm.

3.3.7 Enhanced neurite formation in SH-SY5Y cells prominently confined to the cells exhibiting SST and TUJ1 colocalization.

Having seen the promotion of neurite formation and increased expression of MAP2 and Tau in response to SST, we next determined whether neurite formation is restricted to SST positive cells. SH-SY5Y cells were differentiated with RA for 7 days and processed for immunofluorescence colocalization of TUJ1 and SST. As can be seen in Fig. 17, cells with strong colocalization between SST and TUJ1 displayed extensive neurite outgrowth in comparison to the cells devoid of co-expression (Fig. 17, arrow). In addition, extensive neurite branching was seen in presence of SST (Fig. 17, arrowhead). Together, these results indicate that neurite formation and elongation in differentiated cells require association between SST and TUJ1.

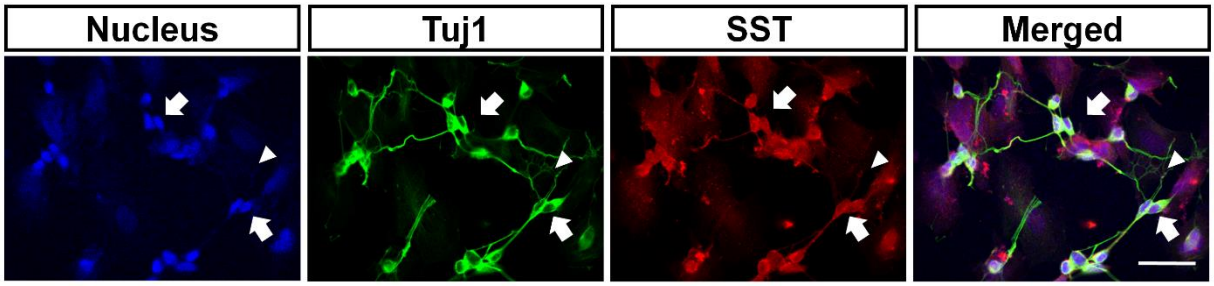

Figure 17 Neurite formation is more pronounced in cells with colocalization of SST and TUJ1 in RA-induced differentiated SH-SY5Y cells. In RA differentiated SH-SY5Y cells, neurite formation and extension was mainly confined to the cells displaying strong colocalization between SST and TUJ1 (arrows). Note the fine communicating network of TUJ1 positive neurite branching (arrowheads). Scale bar = 20μm.

3.3.8 Concentration-dependent effect of SSTR 2 and 4 specific agonists in the modulation of MAP2 and Tau expression

SST exerts its effect via binding to five different receptor subtypes (SSTR1-5) which are distributed at different levels in several tissues or cells of different origin including SH-SY5Y cells. Among them, multiple studies have reported significant contribution of SSTR2 and SSTR4 in the regulation of calcium uptake and increasing inward potassium current in neurons which are critical in the maintenance of maturity and functionality of healthy neurons (Fujii et al., 1994; Van den Steen et al., 2006). Accordingly, we first determined the expression and distributional pattern of endogenous expression of SSTR2 and 4 like immunoreactivity in undifferentiated and differentiated SH-SY5Y cells using immunofluorescence immunocytochemistry and Western blot analysis (Fig. 18A, B). In both undifferentiated and differentiated state, SH-SY5Y cells displayed strong SSTR2 and 4 expressions. Receptor-like immunoreactivity was observed in

both cell soma and neurites (Fig. 18A; arrow and arrowhead). In cell tissue lysate, SSTR2 and 4 are expressed at the expected molecular size of 57 and 45 kDa, respectively (Fig. 18B).

Accordingly, to elucidate the functional role of these SSTR subtypes in the regulation of RA induced neurite outgrowth and expression of MAP2 and Tau, SH-SY5Y cells were treated with increasing concentrations (3nM-30nM) of SSTR2 (L-779976) and SSTR4 (L-803087) specific agonists in combination with RA for 7 days. Following treatment, cell lysate was immunoblotted for the expression of MAP2 and Tau. In alignment with cells treated with SST alone or in combination with RA, cells treated with either L-779976 and L-803087 resulted in significant up-regulation of MAP2 (Fig. 18C, D). In particular, L-779976 treatment showed a dose-dependent increase of MAP2. L-803087 treatment resulted in a maximal increase at lower concentration (3nM) whereas displayed decreasing effect in response to higher concentrations (10, 30nM). In case of Tau, L-779976 had little to no effect on expression, with limited inhibition of Tau at lower concentrations (3, 10nM) and no significant changes at higher concentration (30nM). In contrast, L-803087 had a significant effect on Tau expression at lower concentration (3nM) with an approximately 1.5-fold increase in Tau expression. Moreover, increasing concentration of L-803087 resulted in dose-dependent inhibition of Tau expression (Fig. 18D).

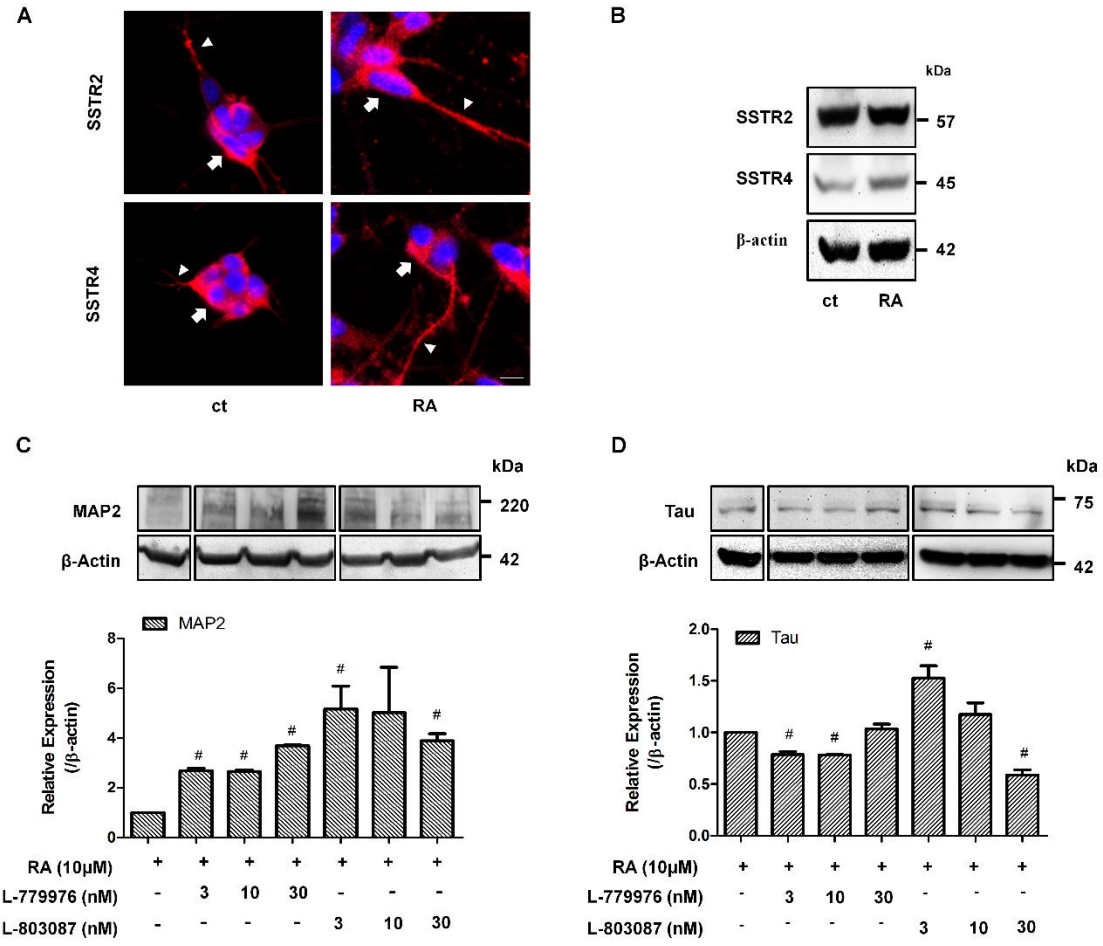

Figure 18 SSTR 2 and 4 specific agonist and receptor-dependent changes in MAP2 and Tau expression in differentiated SH-SY5Y cells. A. Immunofluorescence photomicrographs showing SSTR2 and 4 staining in undifferentiated and differentiated SH-SY5Y cells. Receptors positive cell body and neurites are indicated by arrows and arrowheads. **B.** Cell lysate from undifferentiated and differentiated SH-SY5Y cells are analyzed by immunoblotting for endogenous expression of SSTR2 and 4. β-actin was used as a loading control. Scale bar = 20μm **C.** RA differentiated SH-SY5Y cells were treated with increasing concentration (3-30nM) of SSTR2 (L-779976) and 4 (L-803087) specific agonists. The cell lysate was immunoblotted to detect MAP2 and Tau expression. Note, receptor subtype agonists induced a significant increase

of MAP2 expression in the presence of RA. **D.** Cells treated with L-779976 induced inhibition of Tau expression at lower concentrations (3, 10nM) with no significant changes at 30nM. In presence of L-803087 (3nM), Tau expression was increased significantly and decreased dose-dependently at higher concentrations (10, 30nM). β-actin was used as a loading control. Results are mean ± SD of three separate experiments. #$p < 0.05$ against RA treated alone.

3.3.9 SST, SSTR2 and 4 knockdown changes MAP2 and Tau expression and contribute to the suppression of neurite outgrowth

SST and receptor-specific agonist modulate MAP2 and Tau expression as well as the regulation of neurite formation. Further in support of these results, we next determined the effect of SST/SSTR suppression using SST/SSTR subtype specific antisense oligonucleotides. To verify the specificity of SST and SSTR antisense oligonucleotides, the cells were treated with respective sense and antisense oligonucleotides for 4 days and the expression level of target proteins were assessed using IFC. As expected, all antisense oligonucleotide used induced significant reduction in target protein expression in comparison to the sense oligonucleotide treated groups (Fig. 19A, B). Next, SH-SY5Y were differentiated with RA in presence of respective antisense oligonucleotide and the cellular distribution of MAP2, Tau and TUJ1 was used as an index of neuronal differentiation in SH-SY5Y cells. As presented in Fig. 19C, following treatment with antisense oligonucleotides, expression levels of MAP2 and Tau was decreased in comparison to cells treated with sense oligonucleotide or untreated control. TUJ1 expression was relatively comparable in all conditions as indicated. As shown in Fig. 20, SH-SY5Y cells treated with RA showed significant neurite formation with intense staining of MAP2 and TUJ1 when compared to control cells. In comparison to control, cells with SST blockade

displayed significant loss of neurite formation and MAP2/TUJ1 immunoreactivity even in the presence of RA (Fig. 20). SSTR2 and 4 specific blockade using antisense resulted in inhibition of neurite formation, but to the lesser extent in comparison to SST. Important observation emerged from this experiment were the suppression of neurite growth and elongation in cells exposed to antisense oligonucleotides for SST or SSTR subtypes 2 and 4 prior to RA treatment. Taken together, these results support the role of SST via SSTR2 and 4 in potentiation of RA induced differentiation of SH-SY5Y cells.

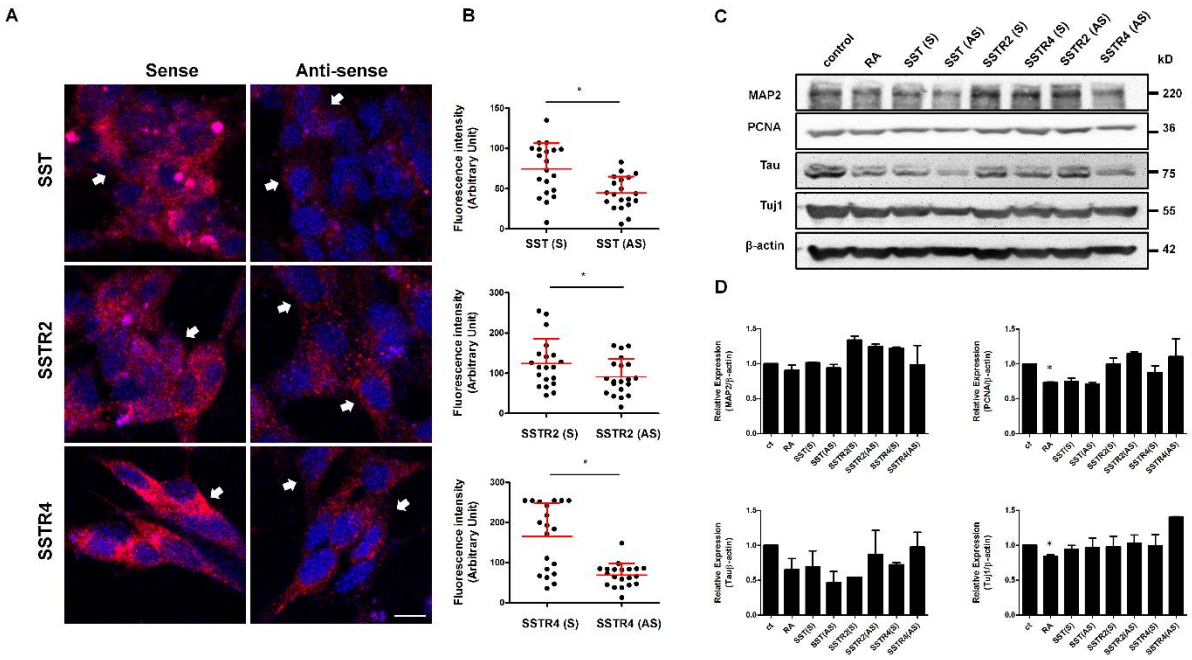

Figure 19 Antisense blockade of SST and SSTR subtypes modulate the expression of neuronal markers and neurite formation. Briefly, cells were treated with antisense oligonucleotide (10μg/mL) for 48 hr and then exposed to RA. Both antisense oligonucleotide and RA were re-supplemented every 48 hours. **A.** Immunofluorescence staining image of SST, SSTR2 and SSTR4 expression following treatment with respective sense and antisense

oligonucleotide for 4 days (arrow; respective protein staining). **B.** The dot plot summary of protein expression level from immunofluorescence staining quantified using fluorescent intensity as an index for protein level (n=20). **C.** The cell lysate was processed for Western blot to detect proliferation marker PCNA and neurite tracer MAP2, Tau and TUJ1 expression. Expression levels of all the markers as indicated was decreased in antisense-treated cells when compared to sense treatment but remains comparable to the control. **D.** Histograms represent a densitometric analysis of relative protein expression. β-actin was used as a loading control. *$p < 0.05$ against control.

3.3.10 Time and concentration-dependent effect of SST in RA-mediated activation of ERK1/2 in SH-SY5Y cells

Previous studies reported that ERK1/2 activation is required in RA induced neuronal differentiation of SH-SY5Y cells (Wang et al., 2006). Whether ERK1/2 activation is involved in SST mediated neurite elongation remains elusive. Accordingly, the status of ERK1/2 phosphorylation upon treatment with RA and SST alone or in combination was determined in SH-SY5Y cells. In comparison to the control, cells treated with RA exhibited significant activation of ERK1/2 at day 3 and 7 of differentiation (Fig. 21A). In alignment, cells treated with SST alone also exhibited increased pERK1/2 at day 3 and 7 (Fig. 21A). Interestingly, cells treated with RA with increasing concentration of SST (80nM-10μM) exhibited significantly higher ERK1/2 phosphorylation in comparison to the untreated control but failed to show any concentration dependency at day 3 and 7 of differentiation (Fig. 21A). Lack of concentration dependency suggests the saturation of ERK1/2 activation and association with neurite outgrowth.

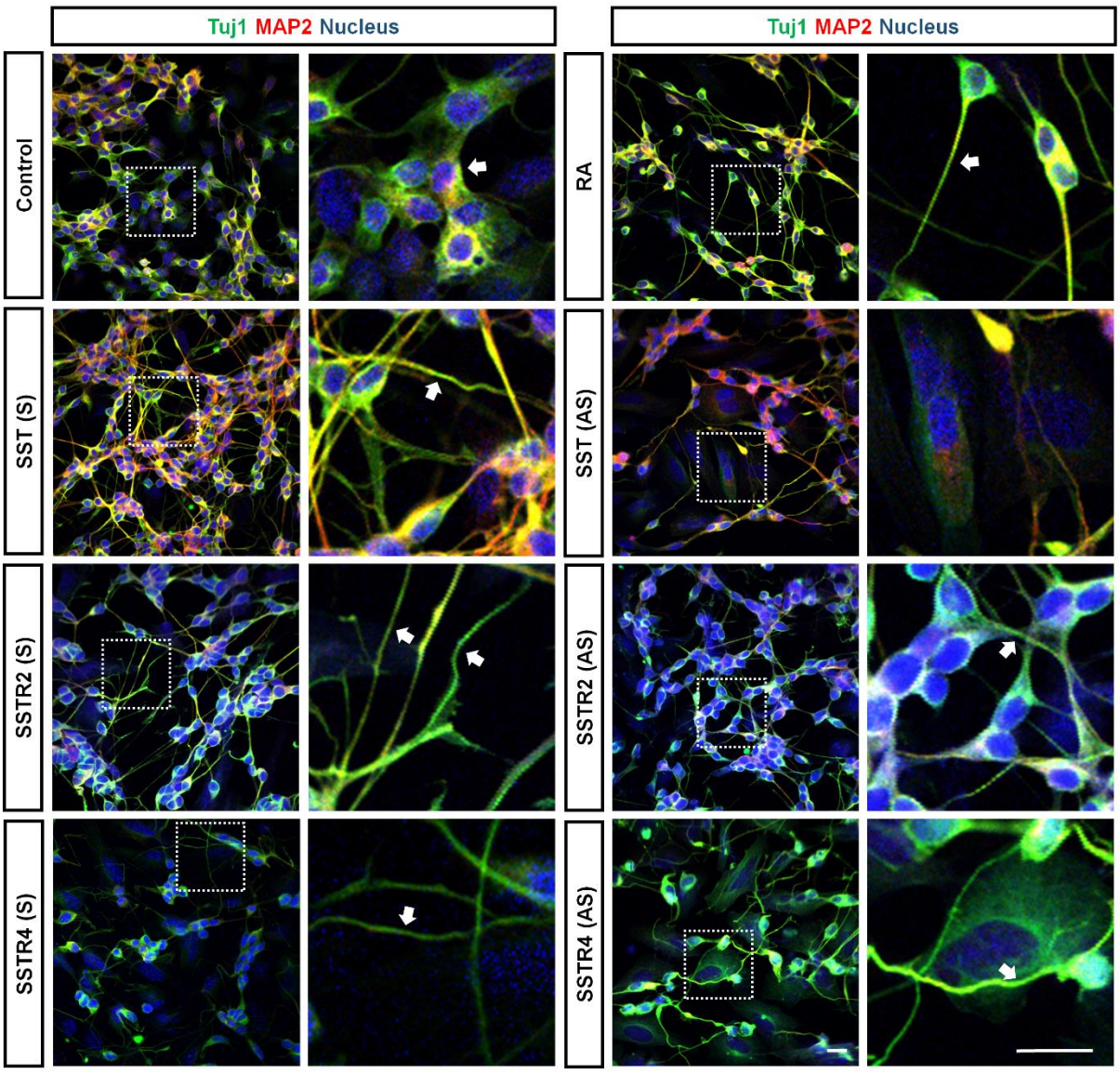

Figure 20 The effect of SST, SSTR2 and SSTR4 antisense on RA-induced neurite outgrowth. Photomicrograph showing immunofluorescence colocalization between MAP2 and TUJ1 at day 7 of differentiation in cells exposed to SST and SSTR specific sense and antisense oligonucleotides. RA differentiated cells exhibited pronounced differentiation with elongated neurite formation. Cells subjected to antisense oligonucleotides either SST or SSTR subtypes resulted in diminished neurite outgrowth as well as the distribution of MAP2 and TUJ1 and

colocalization. In high magnification images, changes in neurite formation following antisense oligonucleotide when compared to RA alone or sense oligonucleotide are indicated by the arrow. Results are mean ± SD of three separate experiments. *$p < 0.05$ against control. Scale bar = 20μm.

3.3.11 SSTR2 and 4 mediated activation of ERK1/2 in differentiation of SH-SY5Y cells

To determine whether the trophic effect of SST in RA induced upregulation of ERK1/2 is directed via activation of specific SSTR subtypes, cells were treated with increasing concentration (3-30nM) of receptor-specific agonist and RA for 24hr at 37˚C and cell lysate was processed for ERK1/2 expression and phosphorylation. As shown in Fig. 21B, we found significant ERK1/2 activation in cells treated with SSTR2 specific agonist in comparison to RA alone. Importantly, no noticeable changes were observed between different concentrations used. In comparison to SSTR2, cells treated with SSTR4 agonist displayed augmented ERK1/2 phosphorylation in presence of higher concentration (10-30nM) when compared to control. Together, these results indicate a prominent role of SSTR2 and 4 in the regulation of neurite growth via activation of ERK1/2.

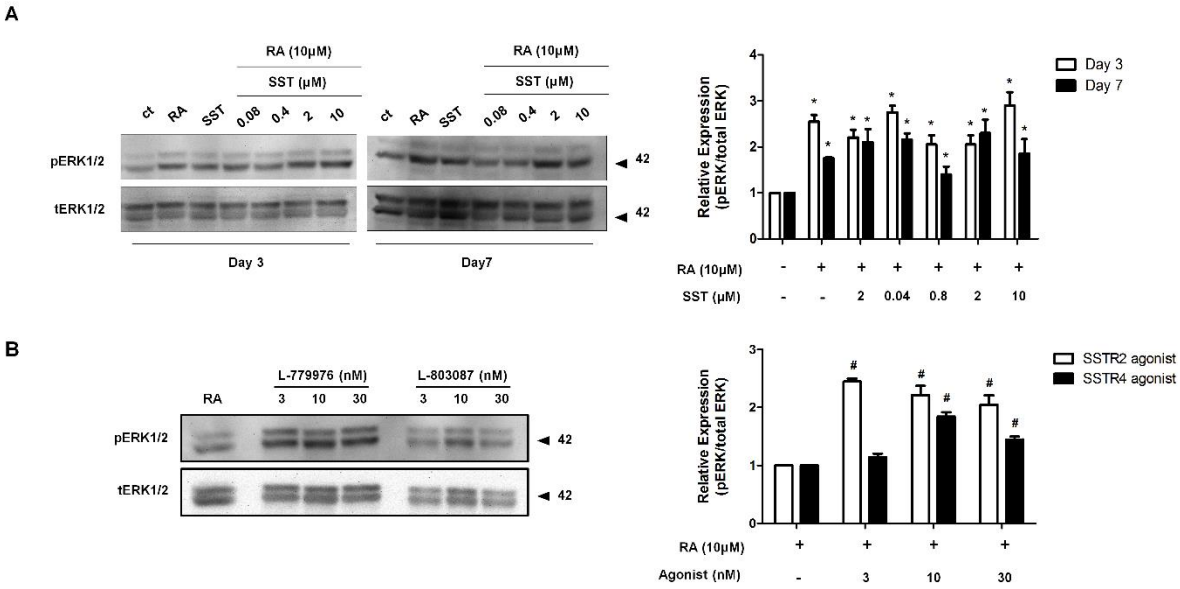

Figure 21 Time and concentration-dependent effect of SST and SSTR specific agonist on ERK1/2 activation in differentiated SH-SY5Y cells. A. SH-SY5Y cells were differentiated with RA in the presence of increasing concentrations of SST for 3 to 7 days and processed for Western blot to detect the status of total and phosphorylated ERK1/2. In comparison to the control, cells treated with RA with or without increasing concentration of SST exhibited increased phosphorylation of ERK1/2. **B.** ERK1/2 phosphorylation was also increased in cells treated with SSTR2 and SSTR4 specific agonist in comparison to control. Histograms represent a quantitative analysis of ERK1/2 activation against total ERK1/2. Results are mean ± SD of three separate experiments. *$p < 0.05$ against control. #$p < 0.05$ against RA treated alone.

3.4 Discussion

SH-SY5Y cells have been widely used for its ability to differentiate into neuronal lineage in presence of RA (Rossino et al., 1991; Kaplan et al., 1993; Kito et al., 1997; Truckenmiller et al., 2001; Lopez-Carballo et al., 2002; Brill and Bennett, 2003; Conn et al., 2003; Ding et al.,

2004; Miloso et al., 2004; Pan et al., 2005; Cuende et al., 2008; Cheung et al., 2009). Considering the positive neurotrophic effect of SST in neurodegenerative diseases and neurite-promoting effect in PC12 cells, we found enhanced neurite outgrowth with SST when used in combination with RA. Differentiated SH-SY5Y cells in presence of RA exhibit morphological changes along with positive regulation of neuronal specific markers Tau, TUJ1 and MAP2 at day 3 and 7. Furthermore, cell proliferation is reduced with diminished expression of proliferation marker PCNA likely due to the transition in the post-mitotic stage, a well-defined property of differentiated neuronal cells. Two prominent neurite stabilizing proteins, MAP2 and Tau were selected to assess the effect of SST on RA-induced differentiation. SST mediated promotion and regulation of neurite growth were further confirmed in the presence of SSTR-specific agonist and knocking down of SST and SSTRs. Here, we demonstrate that cells differentiated with RA in presence of either SSTR2 or 4 specific agonists resulted in a significantly higher level of MAP2 and Tau expression. Furthermore, in our knockdown experiments, we observed a significant reduction in neurite growth in cells treated with SST antisense oligonucleotide. In contrast, receptor-specific ablation elicited only partial suppression of neurite growth. These results suggest the role of other SSTR subtypes probably due to the protein-protein interaction. Our results demonstrate increased ERK1/2 phosphorylation in presence of SST and SSTR specific agonist. SST upregulate MAP2 expression and importantly modulate localization of MAP2 and TUJ1 in differentiated SH-SY5Y cells. We also uncovered that presence of TUJ1 and SST seems to be the prerequisite for promotion and elongation of neurite growth in presence of RA. To our knowledge, this is the first comprehensive demonstration of the role of SST in the promotion of RA induced differentiation of SH-SY5Y cells to a neuronal phenotype.

MAP2 is essential in the RA-induced differentiation of embryonal carcinoma cells (Dinsmore and Solomon, 1991; Dehmelt et al., 2003). In addition to *in-vitro* analyses, knock out studies have elucidated the role of MAPs in microtubule assembly (Liu et al., 2015). Homozygous MAP2 knockout mice did not show a defect in brain morphology, however, displayed significantly lower microtubule density and shorter dendrites in cultured neurons, supporting the critical role of MAP2 in dendrite elongation (Harada et al., 2002). Consistent with previous studies, we found that SST increases the expression of MAP2 and Tau during RA induced neuronal differentiation of SH-SY5Y cells and suggest that SST might be involved in microtubule organization. Our speculation is further supported by the loss of MAP2 and neurite formation following knockdown of SST as well as SSTR subtypes.

Previous studies have shown the crucial role of Tau not only in the maintenance of neuronal polarity but also in the survival of mature neurons (Chen et al., 2014). In parallel to previous observations, we found increased expression of Tau in response to SST and SSTR specific agonist supporting the crucial role of SST in upregulation of Tau and neurites outgrowth in differentiated SH-SY5Y cells. Although it remains elusive whether SST is directly associated with Tau phosphorylation in our experiments, the increased level of total Tau is an indication of suppressed phosphorylation in differentiated cells. Previous studies have also shown the role of Tau in the expression of cell cycle protein and modulation of signaling pathways independent of its binding to microtubule as seen with the use of NGF and epidermal growth factor (Leugers and Lee, 2010). In addition, overexpression of Tau promotes neurite growth in neuroblastoma 2a and cortical neurons. Results presented here suggest that Tau expression is stabilized in response to SST at an early stage and sustained at prolonged differentiation. Taken into consideration, these previous observations and data presented here, we speculate that SST induced activation of

ERK1/2 leads to upregulation of total Tau expression and promote neurite outgrowth in SH-SY5Y cells. RA has been shown to increase total Tau but suppress phosphorylation correlate with neurite formation (Chen et al., 2014). Furthermore, Tau like immunoreactivity in undifferentiated cells were confined to cell body but showed gradual translocation to neurites upon differentiation with weaker expression in the soma (Chen et al., 2014). Consistent with these reports, we also observed Tau expression in neurite with expression in the cell body.

The neuronal entity of SH-SY5Y cells following differentiation was also confirmed by TUJ1 expression and subcellular distribution in cells. Like MAP2, the presence of TUJ1 is required in neuronal morphogenesis and maintenance of axons and dendrites. Consistent with these results, we found significant colocalization of SST with TUJ1 in RA-differentiated SH-SY5Y cells. Our results uncovered that it is not the level but the distribution site of TUJ1 that is a critical determinant in neurite formation and growth. In addition, an association of MAP2 and TUJ1 is required for progressive changes in neurite growth. In the present study, we also revealed that neurite outgrowth in differentiated SH-SY5Y cells are prominently confined to the cells displaying colocalization between SST and TUJ1. Previously, increased SST immunoreactivity in the nucleus has been reported in presence of RA (Hashemi et al., 2003). In contrast to these results, in the present study, we did not see any nuclear translocation of SST in differentiated cells. Taken together results indicate that SST is essential for neurite growth and may serve in the organization of cytoskeleton proteins and association with neurites formation.

ERK has been reported in the induction of neurite outgrowth in primary neurons as well as in functional recovery of nerve injury in mice (Desbarats et al., 2003). In extension to these observations, we found SST mediated activation of ERK1/2 in the promotion of neurite outgrowth in SH-SY5Y cells. Whether this effect of SST is due to upregulation of microtubules

stabilizing protein MAP2/Tau or directly by activation of ERK1/2 is not known. However, the prolonged activation of ERK1/2 in presence of SST and SSTR specific agonist support the positive role on neurites growth. Growing evidence support the role of signal transduction pathways in the regulation of neurite outgrowth and also in modulation of microtubules associated proteins. Taken together, we summarized that SST mediated activation of ERK1/2 possibly enhances MAP2 and Tau activity in a receptor-specific manner and promote neurite formation and elongation.

There is growing evidence that SSTR subtypes play a critical role in neuronal maturation and migration during development as well as in inflammation. The role of SSTR2 in the regulation of Ca^{2+} and SSTR4 in via modulation of K^{+} inward current is well established. Furthermore, with the concept of receptor homo-and heterodimerization, it is conceivable that co-activation of SSTR2 and 4 might provide better neurite elongation in SH-SY5Y cells and act as a potential therapeutic intervention in neurological diseases. Tau is involved in the stabilization of microtubule in Ca^{2+} dependent manner and in this process of dissociation of Tau from microtubule is require for Tau phosphorylation. The role of SST via regulation of Ca^{2+} and K^{+} activated channel cannot be avoided from the discussion and warrant future studies.

Our results provide direct evidence in support of SST in promoting RA induced neurite outgrowth through the stabilization of microtubules proteins MAP2 and Tau in association with ERK1/2. However, the possibility of an indirect effect of SST via regulation of growth factors cannot be avoided from the discussion. In this direction, the role of BDNF is critical, as BDNF is required in neuronal differentiation and exert a neuroprotective role in many neurological diseases (Chen et al., 2013; Shipley et al., 2016). Furthermore, BDNF is also associated with the modulation of microtubules proteins (Hu et al., 2011). Importantly, BDNF induces SST release

in excitotoxicity and possibly affords neuroprotection. Whether SST elicits similar functions as other growth factors such as BDNF in neurite outgrowth and differentiation is not known and need to be determined. The role of SSTR subtypes in neurite formation and elongation is largely elusive.

In conclusion, the present study describes a significant role of SST in the promotion of neurite outgrowth during differentiation of SH-SY5Y cells via regulation of microtubule-associated proteins. These observations not only attest the role of SST in the pathogenesis of neurological diseases but also recognize SST as a critical mediator of neurite outgrowth. Our systematic analysis of SST mediated progression of neurite formation and outgrowth support previously reported a neurotrophic effect. SST seems to facilitate the development of therapeutic strategies to improve functional recovery of cognitive function and memory loss in neurological diseases. Moreover, these findings identify SSTR2 and 4 as key receptor subtypes that might play a prominent role in neurite outgrowth. In support of the notion that SST improves cognitive function and memory loss, we further speculate the significance of SST mediated promotion of neurite outgrowth in improving cognitive function in neurodegeneration associated with aggregated and misfolded proteins. Therefore, the role SST plays in the maintenance of neurite formation predicts SST as a potential therapeutic target. Hence, further studies are required to elaborate molecular mechanism that might contribute to SST mediated neurite outgrowth in the pathogenesis of neurological disease.

Chapter 4. Somatostatin ameliorates neuronal toxicity by inhibiting β-amyloid induced hyperphosphorylation of CRMP2

4.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia. A common clinical feature of the disease includes memory loss, abnormal social behavior, and deterioration of cognitive function (Jorm and Jolley, 1998; Chung et al., 2005; Cole et al., 2007). AD is characterized by the formation of amyloid plaques, composed of abnormally truncated fragments of the amyloid precursor protein (APP) called β-amyloid (Aβ), and intracellular neurofibrillary tangles (NFT), composed of hyperphosphorylated Tau protein (Sternberger et al., 1985; Cork et al., 1986). The accumulation of plaques and the formation of NFTs, along with other pathological changes, resulted in synaptic dysfunction, excitotoxicity, dendritic spine loss and overall destabilization of the neural network (Haass and Selkoe, 2007; Palop and Mucke, 2010). Although the precise mechanism interconnecting the AD onset and progression is not fully elucidated, however signaling pathways that exert determinant role have been identified. One such important signaling molecule that may represent a common denominator is collapse response mediator 2 (CRMP2).

Initially identified as a signaling molecule of a repulsive axon guidance molecule Semaphorin3A (Sema3A), CRMP2 has since then identified as a marker in synapse formation, the establishment of neuronal cell polarity, dendritic patterning, learning and memory (Arimura and Kaibuchi, 2007; Yamashita and Goshima, 2012). In particular, CRMP2 regulates neuronal microtubule dynamics by binding to the tubulin heterodimers leading to polymerization while also co-localizing with the actin cytoskeleton (Fukata et al., 2002; Arimura et al., 2005; Varrin-

Doyer et al., 2012; Tan et al., 2015). Furthermore, CRMP2 also plays a critical role in the transportation of soluble tubulin and vesicles by acting as a cargo adaptor (Kimura et al., 2005; Namba et al., 2011). Like many other microtubule binding proteins, such as Tau or microtubule-associated proteins (MAP), CRMP2 is phosphorylated by cyclin-dependent kinase (CDK5) and glycogen synthase kinase-3β (GSK-3β) near its C-terminus. Specifically, CDK5-mediated phosphorylation of CRMP2 at Ser522 primes the subsequent phosphorylation by GSK-3β at sites Ser518, Thr514 and Thr509 (Brown et al., 2004; Cole et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005). In addition to Cdk5 and GSK-3β, Rho/ROCK-kinase has also been identified to phosphorylate CRMP2 at Thr555 (Yamashita and Goshima, 2012). Taken together, the phosphorylation of CRMP2 at these sites has been associated with regulation of neurite outgrowth, likely due to the modifications of microtubule dynamics (Cole et al., 2004; Yoshimura et al., 2005). Several previous studies have reported hyper-phosphorylation of CRMP2 in AD patients when compared to the age-matched control (Cole et al., 2004; Uchida et al., 2005; Cole et al., 2007). However, the exact mechanism of CRMP2 phosphorylation during the progression of AD remains elusive. Although controversial, it is reported that the hyperphosphorylation of CRMP2 occurs before the onset of pathology in AD mouse model, implicating CRMP2 hyperphosphorylation as an early indicator of AD (Cole et al., 2007).

As previously reported, CDK5 is the main kinase responsible for the CRMP2 phosphorylation at Ser522. CDK5 has been reported to play a critical role in CNS, including neuronal migration, synapse formation, plasticity and neurogenesis (Xie et al., 2003; Fischer et al., 2005; Johansson et al., 2005; Samuels et al., 2007; Jessberger et al., 2008; Lagace et al., 2008). In contrast to other members of CDK family, which are regulated by p21 and p27, CDK5 activity is mainly regulated by metabolism of p35 (Sherr and Roberts, 1999; Hisanaga and Saito,

2003). In addition, while activation of CDK5 by p35 in physiological condition is essential for normal neuronal development, synaptic activity and axonal transport, abnormal activation of CDK5 leads to cell death and neurodegeneration (Patrick et al., 1999; Lee et al., 2000; Copani et al., 2001; Fischer et al., 2003; Cicero and Herrup, 2005; Fischer et al., 2005; Johansson et al., 2005; Neve and McPhie, 2006). In AD, the abnormal increase in CDK5 activation leading to hyperphosphorylation of various tubulin-associated proteins including Tau and CRMP2 is reported to be associated with accumulation of truncated fragment of p35 called p25, which induces constitutive activation of CDK5 and mislocalization *in vivo* (Patrick et al., 1999). In this regard, the same study also has identified that calpain I mediate cleavage of p35 into p25 (Patrick et al., 1999). Calpain (also known as calcium-activated neutral proteinases or CANP) is a key enzyme involved in calcium-mediated neurodegeneration (Saito et al., 1993). In AD, accumulation of Aβ leads to increase in intracellular Ca^{2+} levels, leading to the mitochondrial Ca^{2+} overload, production of pro-apoptotic proteins such as cytochrome *c*, and generation of superoxide radicals leading to cell death and neurodegeneration (Stutzmann, 2007). Furthermore, we have previously demonstrated the effect of SST in promoting RA-induced differentiation of SH-SY5Y cells (Chapter 3). We hypothesize that the identification of a molecule capable of inhibiting Aβ-induced hyperphosphorylation of CRMP2 via blockade of Ca^{2+} accumulation may serve as a novel therapeutic agent. We observed a SST-mediated increase in overall neurite length in differentiated SH-SY5Y cells, with specific effects on microtubule-associated proteins such as MAP2 and Tau. In the present study, we we aim to elucidate the mechanism involved in phosphorylation of Ser522 using Aβ1-42 in SH-SY5Y cell as a model. Our results revealed SST as a novel molecule capable of inhibiting Aβ induced hyper-influx of Ca^{2+} leading to the inhibition of calpain activity.

4.2 Material and Method

4.2.1 SH-SY5Y cell culture

Human SH-SY5Y neuroblastoma cells were provided by Dr. Neil Cashman, University of British Columbia, BC, Canada. Briefly, the cells were grown on Matrigel-coated (10mg/mL, BD Bioscience, CA, USA) 75cm^2 culture flask. The culture medium comprised of Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/mL), streptomycin (100µg/ml) in a 5% CO_2 humidified incubator at 37°C. For neuronal differentiation, cells were treated with all trans-retinoic acid (RA, 10µM, Sigma, St. Louis, USA) for 7 days following previously established protocols (Encinas et al., 2000). All experiments were performed on cells differentiated for 7 days unless otherwise stated.

4.2.2 MTT assay

The cell viability in response to Aβ was monitored using MTT assay. Briefly, the differentiated SH-SY5Y cells were treated with increasing concentrations of Aβ1-42 (0, 1, 5, 25µM) for up to 48 hours. Following Aβ1-42 treatment, the cells were washed with PBS and incubated with 300µg/mL of methylthiazolyldiphenyl-tetrazolium bromide solution (Sigma) prepared in serum-free DMEM for 3 hours at 37˚C. The cells were subsequently washed in PBS and the resulting formazan salt in cells was dissolved using 100µL of isopropanol for 15 minutes on rotating shaker. The changes in color were analyzed using spectrophotometer at a wavelength of 550nm with background absorbance measured at 695nm. The results are presented as a fold difference between the treated versus the control group.

4.2.3 Caspase/Apoptosis Activity Assay

The Aβ1-42 induced apoptosis in differentiated SH-SY5Y cells was analyzed using Caspase-3/7 Green Apoptosis Assay Kit (Essen Bioscience, Michigan, USA) following manufacturer's instructions. Briefly, SH-SY5Y cells were treated with Aβ1-42 (5μM) alone or in combination with increasing concentration of SST (0.4, 2, 10μM) in presence of a DNA intercalating dye NucView^{TM} 488 (Essen Bioscience). The resulting fluorescence was analyzed in the live-cell imaging system (Essen Bioscience) and the caspase-3/7 activity was assessed as an index of cells undergoing apoptosis using IncuCyte basic analyzer (Essen Bioscience).

4.2.4 Live/Dead Cell Assay

The Aβ1-42 mediated toxicity in presence or absence of SST was analyzed using LIVE/DEAD Cell Vitality Assay (Thermo Fisher Scientific, Massachusetts, USA) following manufacturer's instructions. The differentiated SH-SY5Y cells were treated with Aβ1-42 (5μM) or SST (10μM) alone or in combination for 48 hours. Post treatment, cells were washed with PBS and were collected using 0.05% trypsin-EDTA (Thermo Fisher Scientific). The collected cells were re-suspended in 100μL of PBS in presence of C_{12}-resazurin (20ng/μL) and SYTOX dye (1μM) for 15 minutes at 37˚C. Following incubation, cells were immediately assessed on LSR II (BD Bioscience, CA, USA) with excitation at 488nm and emission at 530 and 570nm and analyzed using FlowJo workstation (BD Bioscience).

4.2.5 Western blot analysis

For Western blot analysis, the differentiated control and treated SH-SY5Y cells were harvested. The whole cell lysates (15μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hr at RT and blotted overnight in the presence of respective rabbit polyclonal primary antibodies: C-terminal CRMP2 (1:1000; Cat # CP2161; ECM Bioscience, KY, USA), Thr514-CRMP2 (1:1000, Cat# ab62478; Abcam, Cambridge, UK), Ser522-CRMP2 (1:1000, Cat# CP2191; ECM Bioscience), Thr555-CRMP2 (1:1000, Cat# CP2251; ECM Bioscience), SSTR2 (1:500, Cat# sc-25676; Santa Cruz Biotechnologies), SSTR4 (1:500, Cat# sc-25678; Santa Cruz Biotechnologies), Calpain I (1:500; Cat# 2556; Cell Signaling). Other antibodies used are mouse monoclonal CDK5 (1:2000; Cat# 05-364; Millipore) and rabbit monoclonal p35/25 (1:250; Cat# 64310; Cell Signaling). Following the overnight incubation with respective primary antibody, the membranes were washed in TBST for three times and were incubated for 1 hr in RT with either horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:2000) or goat anti-rabbit antibodies (1:2000) (Jackson Lab). The membranes were washed and developed using chemiluminescence detection kit (Millipore, Billerica, MA, USA) on Alpha Innotech FluorChem 8800. β-actin was used as a loading control. Densitometric analysis of protein expression levels was performed with ImageJ Software.

4.2.6 Immunofluorescence immunocytochemistry

The control and treated cells were fixed with 4% paraformaldehyde for 20 minutes and were permeabilized with 0.1% Triton-X100 in PBS for 15 minutes at RT. Following 3 washes in

PBS, cells were blocked with 5% Normal Goat Serum (NGS) for 1 hour at RT. Cells were then incubated with rabbit polyclonal primary antibody Ser522-CRMP2 (Cat# CP2191; ECM Bioscience) in 5% NGS overnight at 4˚C. Following the overnight incubation with primary antibody, cells were washed with PBS and incubated with Alexa-conjugated goat anti-rabbit secondary antibody (1:200; Invitrogen) for 1 hour at RT. For nucleus visualization, cells were incubated with Hoechst dye 33258 (0.5μg/mL, Calbiochem, CA, USA) for 10 minutes at RT. The coverslips were then mounted onto the slides and photographed using Zeiss LSM700 confocal microscope (Carl Zeiss, Germany). Image panels were constructed using Carl Zeiss Zen software.

4.2.7 Agonist treatment

SSTR2 and 4 specific non-peptide agonists (L-779976 and L-803087) were kindly provided by Dr. S.P. Rohrer from Merck. Briefly, the differentiated SH-SY5Y cells were treated with SSTR specific agonists (3, 10, 30nM) with or without Aβ for 24 hours. Following treatment, the whole cell lysate was collected and changes in protein expressions were analyzed using Western blot analysis.

4.2.8 Fluo-4 calcium assay

The intracellular calcium levels were assessed using Fluo-4 Direct^{TM} calcium assay kit (Invitrogen) following manufacturer's instructions with modification as previously reported (Yaron et al., 2015). Briefly, the SH-SY5Y cells were plated onto a 96-well plate coated with Matrigel and differentiated with RA for up to 7 days. Following differentiation, cells were treated with Aβ1-42 alone (5μM), SST alone (10μM) or in a combination of both. Untreated cells

were used as internal control. Post-treatment, an equal volume of 2X Fluo-4 Direct^{TM} calcium reagent loading solution was added directly to each wells and incubated at 37˚C for 30 minutes. The resulting fluorescence intensity was measured with excitation at 494nm and emission at 516nm in a spectrophotometer. Results are presented as a fold-difference between the treatment versus control (n=6).

4.2.9 Statistical Analysis

Results are presented as mean ± SD of minimum three independent sets of experiments. All statistical analysis has been performed in Graph Prism5.0. One-way analysis of variance (ANOVA) or Student's *t*-test was used as indicated. *p<0.05 or **<0.01 against control was taken into consideration as significant.

4.3 Results

4.3.1 SST inhibits Aβ-induced toxicity in differentiated SH-SY5Y cells

To assess the Aβ1-42 effect on differentiated SH-SY5Y cells, the changes in cell metabolism was assessed using MTT assay. We recently described Aβ1-42 induced toxicity in hCMEC/D3 cell line (Paik et al., 2018). In alignment with these findings, the differentiated SH-SY5Y cells also exhibited dose-dependent toxicity with increasing concentration of Aβ1-42 (Fig. 22A). Next, we assessed the Aβ1-42 induced induction of cell death by assessing the activity level of caspase-3/7 as an index of apoptosis (Fig. 22B). As expected, the SH-SY5Y cells treated with Aβ1-42 displayed a significant increase in caspase-3/7 activity compared to the control. Importantly, the cells treated with SST alone showed a significant inhibition of caspase-3/7 activity. As shown in Figure 22B, upon treatment with Aβ1-42 in combination with SST, the

cells displayed significant inhibition of caspase-3/7 activity compared to the cells treated with Aβ1-42 alone, suggesting SST-mediated inhibition of Aβ-induced apoptosis. To determine the changes in metabolism as well as cell membrane integrity in response to the Aβ1-42 induced toxicity in SH-SY5Y cells Live/Dead cell assay was performed. Interestingly, Live/Dead assay did not show significant changes in metabolic activity, which may be due to the metabolic demand of cells undergoing apoptosis (Fig. 22C, D). However, when assessed strictly for the cell membrane integrity, the Live/Dead assay showed an increasing trend in cell permeability in cells treated with Aβ1-42 alone, albeit insignificantly, indicative of the toxic effect of Aβ (Fig. 22C).

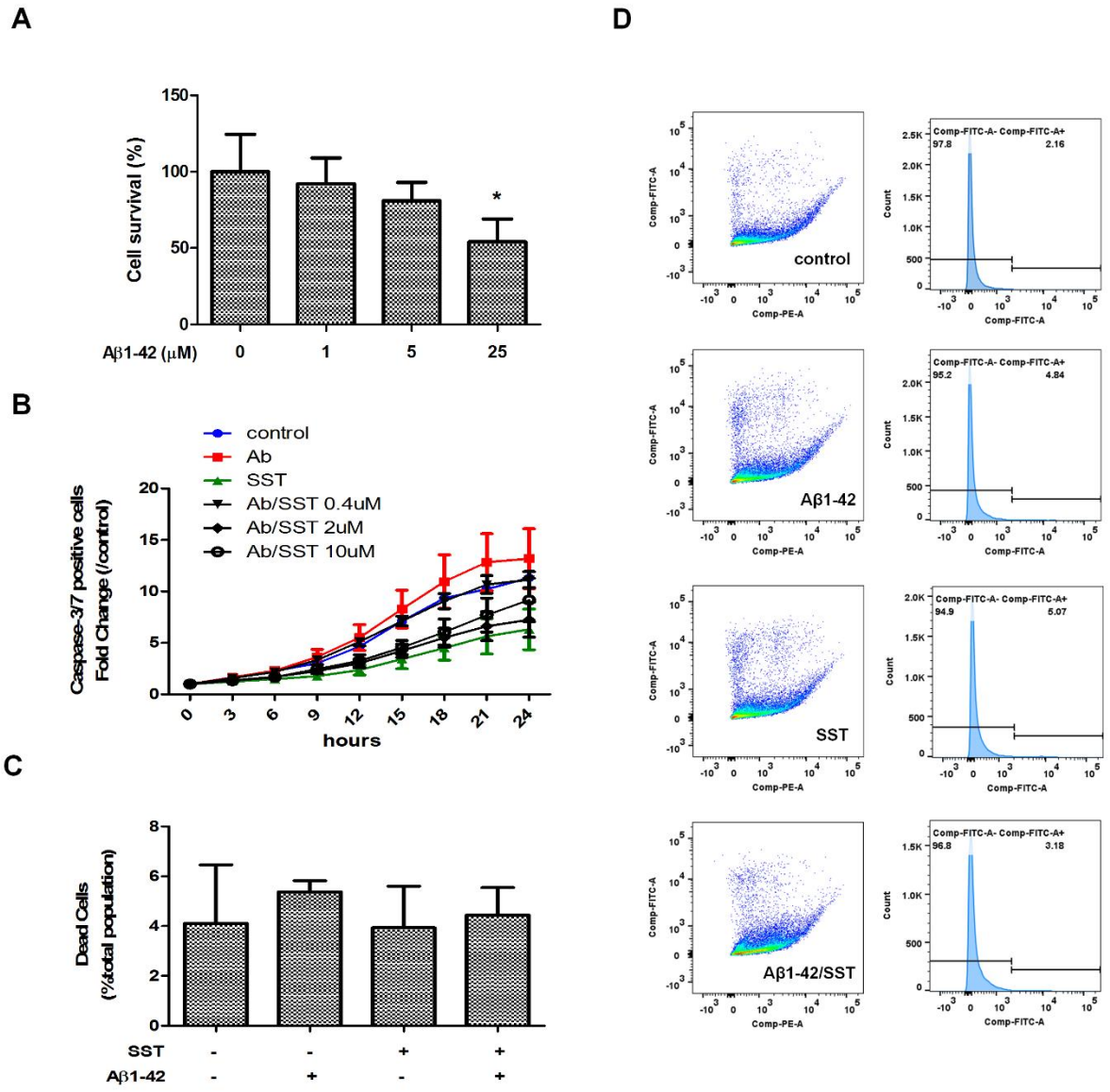

Figure 22 SST inhibits Aβ-induced activation of apoptosis. A. Changes in cell survival following treatment with increasing concentration of Aβ assessed by MTT assay. Aβ1-42 induced dose-dependent toxicity on differentiated SH-SY5Y cells with maximal toxicity observed at 25μM of Aβ1-42. **B.** Apoptosis induction assessed by measuring caspase-3/7 activity. Cells treated with Aβ alone displayed a significant elevation of caspase-3/7 activity while cells treated with SST alone displayed lowest caspase-3/7 activity. Co-treatment of Aβ1-42 and SST

resulted in reduced caspase-3/7 activity compared to the cells treated with Aβ1-42 alone. **C.** Cell viability assessed by live/dead assay using metabolic activity and cell permeability as an index. **D.** Representative FACS data of C_{12}-resazurin and SYTOX fluorescence intensity (dot plot) and FITC intensity distribution (histogram) displaying the distribution of cells based on viability. Data represent mean ± SD of three independent experiments.

4.3.2 Somatostatin inhibits $A\beta_{1-42}$ induced hyperphosphorylation of CRMP2 at Ser522 site

Previous studies have demonstrated that SST enhances neurite outgrowth in various neuronal cell lines when co-treated with growth factors such as neuronal growth factor (NGF), brain development growth factor (BDNF) or RA (Ferriero et al., 1994). Furthermore, we have also identified that SST increases the neurite outgrowth in differentiated SH-SY5Y cells when co-treated with RA (Chapter 3;). Whether CRMP2 is directly associated with SST induced neurite outgrowth is not well understood. CRMP2 plays a critical role in mediating tubulin stability and neuronal outgrowth, we examined whether SST may be involved in maintaining neurite integrity against Aβ1-42 induced damage by modulating CRMP2 phosphorylation. To test whether the presence of SST attenuates Aβ1-42 induced hyperphosphorylation of CRMP2, differentiated SH-SY5Y cells were treated with increasing concentrations of SST (0.4 - 10μM) in the presence of Aβ1-42 (5μM). Untreated cells or the cells treated with Aβ42-1 were used as a control. To determine site specific phosphorylation, three phosphorylation sites of CRMP2 which has been previously reported to be highly phosphorylated in AD patients, Thr514-, Ser522- and Thr555 were selected (Mokhtar et al., 2013). As shown in Fig.23, SST did not alter the phosphorylation levels of Thr514- or Thr555-CRMP2 at any of the concentrations used. However, SST significantly lowered the Ser522-CRMP2 phosphorylation level in a dose-

dependent manner (Fig. 23). The reduction of phosphorylation level at Ser522-CRMP2 was observed in cells treated with 2μM of SST, and the maximal reduction was observed at 10μM. Therefore, all subsequent experiments were performed using 10μM of SST with focus on Ser522-CRMP2 phosphorylation.

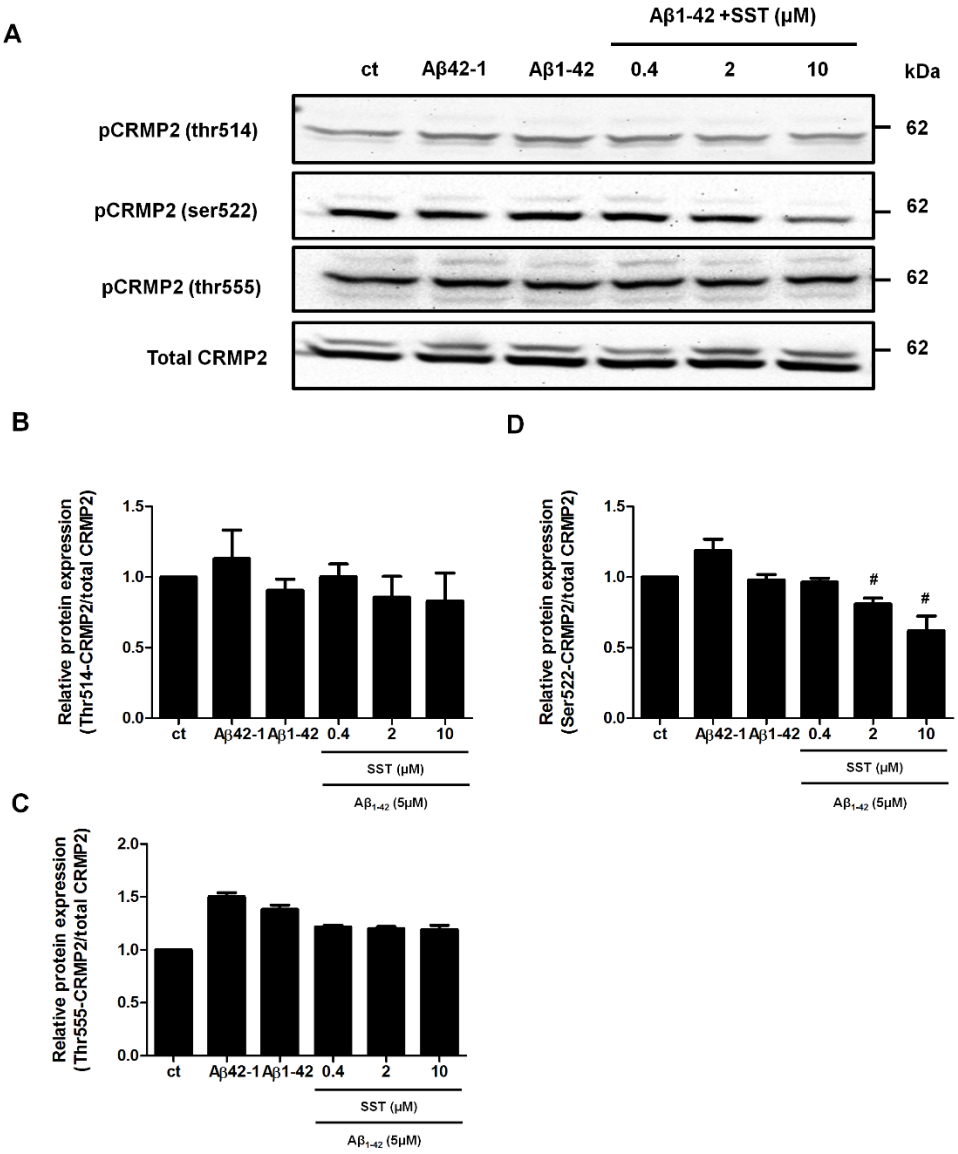

Figure 23 SST inhibits phosphorylation of Ser522-CRMP2. A. Representative Western blot showing decreasing phosphorylation level of Ser522-CRMP2 with increasing concentration of

SST. Total CRMP2 used as loading control. **B-D.** The densitometric analysis of Western blot data. **(B)** Thr514-CRMP2, **(C)** Thr555-CRMP2, **(D)** Ser522-CRMP2. Ser522-phosphorylation level is inhibited in dose-dependent manner with increasing concentration of SST used. Data represents mean ± SD of three independent experiments. #$p < 0.05$ against Aβ1-42 treated alone.

To further test the hypothesis that the phosphorylation of CRMP2 at Ser522 is regulated by SST, differentiated SH-SY5Y cells were treated with SST alone or in combination with Aβ1-42, and the phosphorylation level of CRMP2 at Ser522 was monitored using Western blot and immunofluorescence staining. As shown in Figure 24A, cells treated with SST displayed a significant inhibition of phosphorylation at Ser522. Taken together, these results suggest that SST displayed significant inhibition of phosphorylation. The quantitative analysis of inhibition in phosphorylation at Ser522 is also determined by densitometric analysis (Fig. 24A, right panel). To further verify the effect of SST on Ser522-phosphorylation, SH-SY5Y cells were stained with Ser522-CRMP2 specific antibody along with neuronal tubulin marker βIII-tubulin. Consistent with the Western blot results, the introduction of SST resulted in significant inhibition of Ser522 phosphorylation, which was maintained in the presence of Aβ1-42 (Fig. 24B).

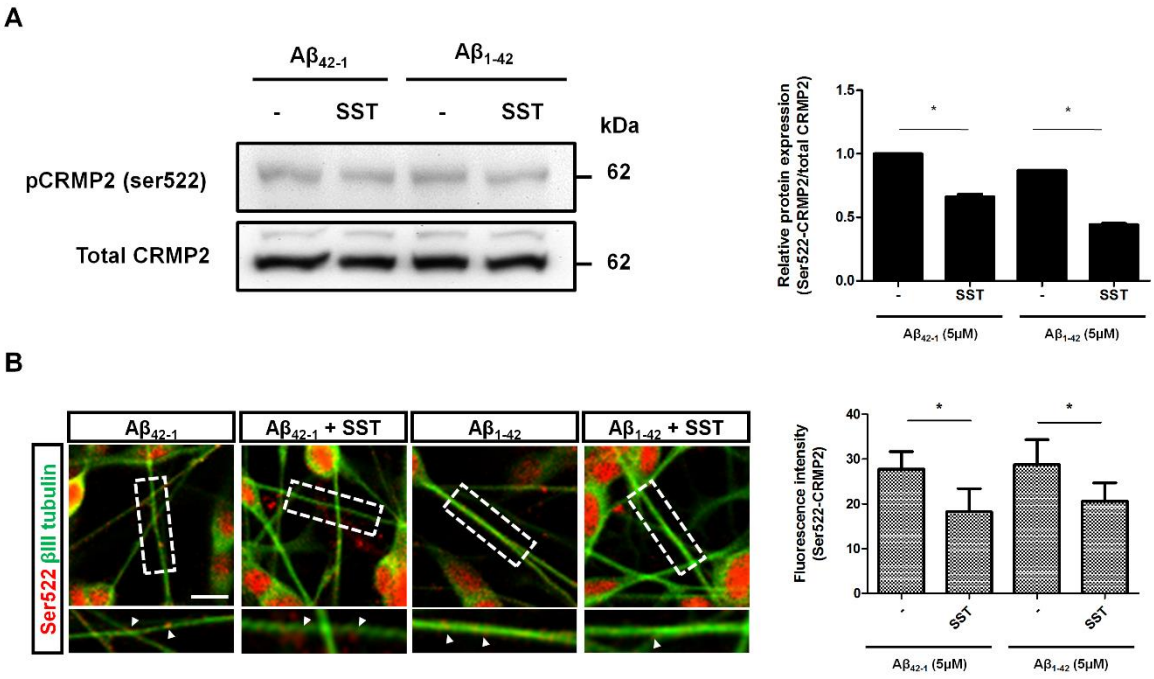

Figure 24 SST inhibits S522-CRMP2 phosphorylation. A. Representative Western blot showing inhibited phosphorylation at Ser522-CRMP2 in presence of SST (left panel). Densitometric analysis of the Western blot corroborates significant reduction in phosphorylation level in the presence of SST in both Aβ42-1 and Aβ1-42 treated cells (right panel). **B.** Immunofluorescence staining image of Ser522-CRMP2 (red) co-stained with neuronal tubulin marker βIII-tubulin (green). The colocalization of Ser522-CRMP2 on neurites with βIII-tubulin is indicated (arrowhead; inset). Data represent mean ± SD of three independent experiments. *$p < 0.05$ against control.

4.3.3 SST inhibits Aβ1-42 induced over-expression of SSTR4

As the biological effects of SST are mediated by binding to its receptors (SSTR1-5), we next monitored the changes in SSTR expression following the treatment of cells with either Aβ1-

42 alone or in combination with SST. Previously, we have reported the critical role of SSTR2 and 4 in promoting RA induced neuronal differentiation in SH-SY5Y cells (Chapter 3). SST resulted in significant up-regulation of SSTR2 (Fig. 25). The cells treated with Aβ1-42 displayed an increased expression of both SSTR2 and 4 compared to the untreated control. In the co-treatment group, SSTR2 expression remained higher than untreated control but remained comparable to cells treated with SST or Aβ1-42 alone. Interestingly, the cells treated with Aβ1-42 in combination with SST showed a significant inhibition SSTR4 expression compared to cells treated with Aβ1-42 alone, suggesting that SSTR4 may be the receptor responsible for SST mediated effect on Aβ1-42.

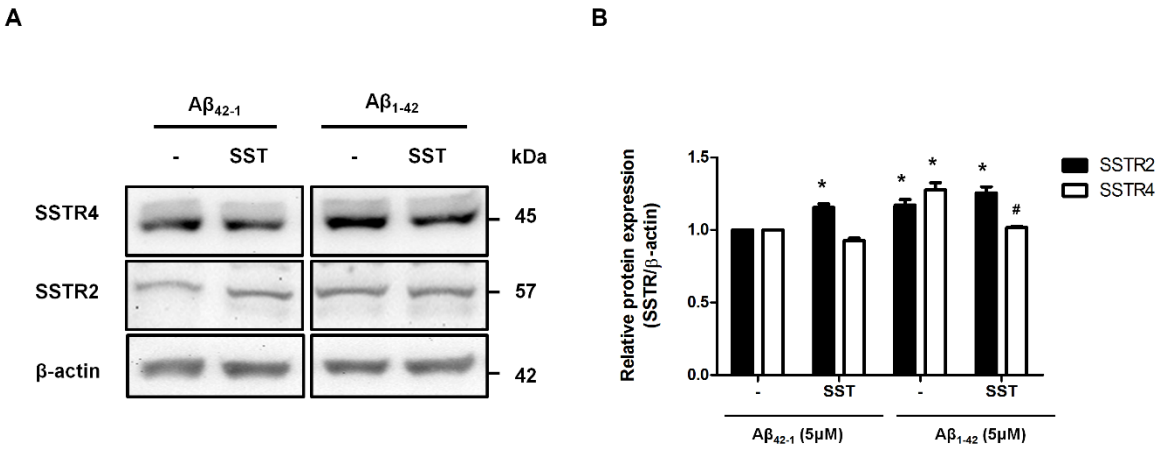

Figure 25 SST induced changes in SSTR2 and 4 expressions. A. Representative Western blot showing the effect of SST in the expression of SSTR2 and 4. **B.** The densitometry analysis of the Western blot shows that the treatment of cells with SST resulted in significant increase of SSTR2 expression in presence of either Aβ42-1 or Aβ1-42. SSTR4 expression increased following the treatment with Aβ1-42. In contrast, the co-treatment of cells with Aβ1-42 and SST resulted in

inhibition of Aβ1-42-induced increase of SSTR4. Data represent mean ± SD of three independent experiments. *$p < 0.05$ against control.

4.3.4 SSTR specific agonist elicit significant changes in CRMP2 phosphorylation

To determine the receptor subtype involved in SST mediated inhibition of CRMP2 phosphorylation, we next assessed the status of CRMP phosphorylation in differentiated SH-SY5Y cells exposed to SSTR2 and 4 specific agonists. As shown in Figure 26, 3nM of SSTR2 agonist L-779976 inhibited phosphorylation at Ser522 in absence of Aβ1-42. At higher concentrations (10, 30nM), L-779976 moderately increased Ser522 phosphorylation. In contrast, SSTR4 agonist induced CRMP2 phosphorylation at lower concentration (3nM) which was inhibited at higher concentrations. We next extended our study and determined whether Aβ1-42 mediated increase in CRMP2 phosphorylation is suppressed in presence of SSTR2 or 4 agonist. As shown in Figure 26, SH-SY5Y cells treated with Aβ1-42 in presence of SSTR2 agonist at 3nM displayed no significant changes in phosphorylation compared to the control. CRMP2 phosphorylation remained elevated in presence of higher concentration of SSTR2 agonist with no noticeable difference with or without Aβ1-42.

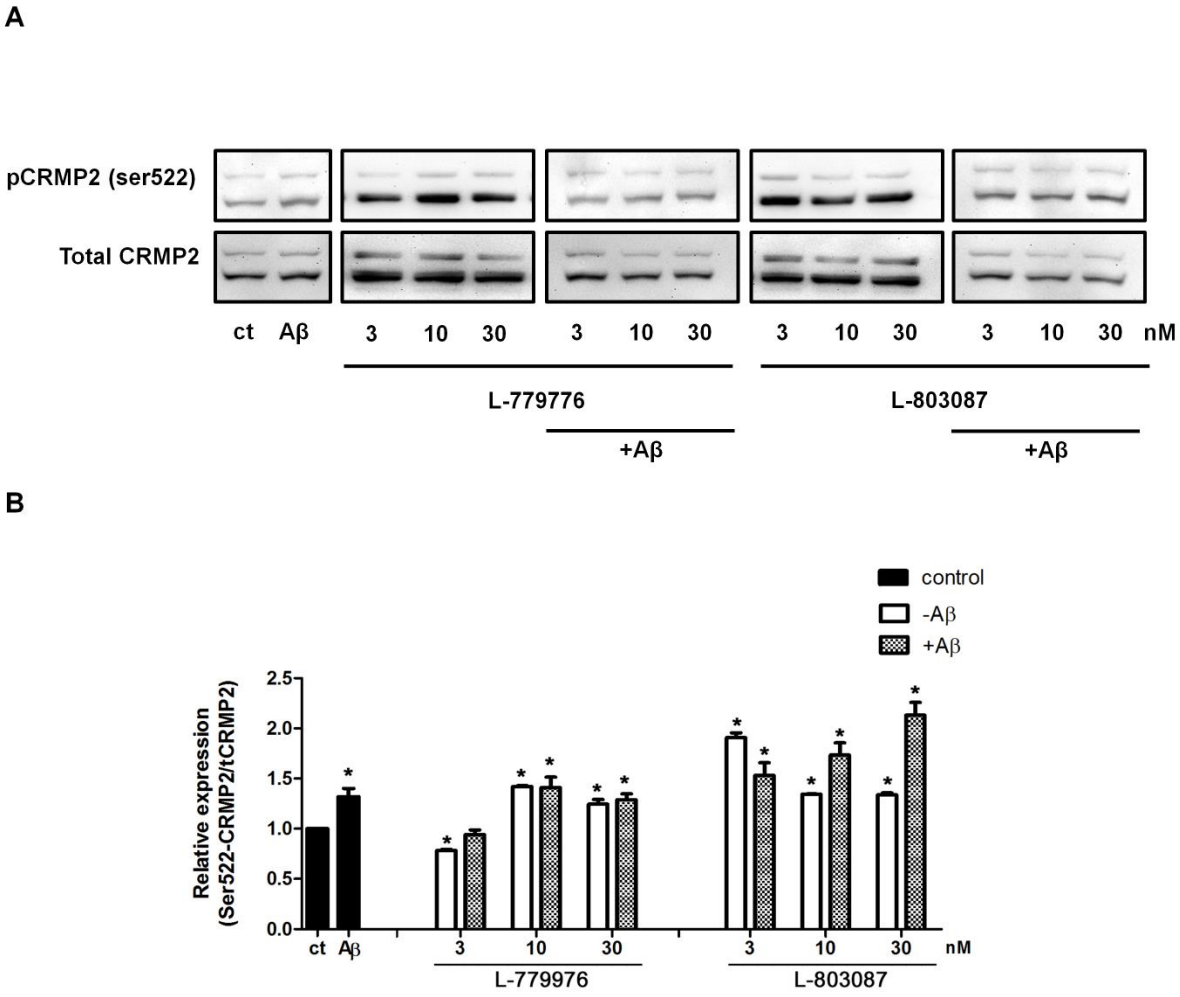

Figure 26 SSTR specific agonist effect on Ser522-CRMP2 phosphorylation. A. Representative Western blot showing changes in the level of S522-CRMP2 phosphorylation in cells following treatment with increasing concentration of SSTR2 and SSTR4-specific agonist (3, 10, 30nM) in presence or absence of Aβ1-42 (5μM). **B.** The densitometric analysis of Western blot shows a significant inhibition of Ser522 phosphorylation in presence of 3nM of L-779976 in absence of Aβ1-42. 10 and 30nM of L-779976 resulted in the moderate elevation of phosphorylation at Ser522 site compared to the untreated control. 3nM of L-803087 in absence of Aβ1-42 caused a significant increase in Ser522 phosphorylation, whereas 10 and 30 nM

treatment resulted in moderate increase compared to the untreated control. In presence of Aβ1-42, L-803087 induced a dose-dependent increase of Ser522 phosphorylation, resulting in a highest level of elevation at the 30nM treatment of L-803087. Data represent mean ± SD of three independent experiments. *$p < 0.05$ against control.

4.3.5 Somatostatin mediated inhibition of Ser522-CRMP2 is mediated through calcium pathway

Increased intracellular Ca^{2+} accumulation is a well-documented mechanism of Aβ toxicity. Furthermore, increased Ca^{2+} influx is an established inducer of calpain activity leading to the over-activation of CDK5 by catalyzing the conversion of CDK5 substrate p35 into a more stable variant p25. In turn, the hyperactivity of CDK5 leads to the hyperphosphorylation of CRMP2 at Ser522, leading to disassembly of the CRMP2 complex. Previous reports have suggested that SST inhibits Ca^{2+} by binding to SSTR2 (Reisine, 1990; Johnson et al., 2001; Petrucci et al., 2001). To assess whether SST inhibits the Aβ induced increase in Ca^{2+} influx, intracellular Ca^{2+} content was monitored using Fluo-4 calcium indicator. As shown in Figure 27A, cells treated with Aβ (1μM) exhibited no significant changes in Ca^{2+} influx in comparison to control but displayed time dependent increase in Ca^{2+} in presence of higher concentration of Aβ (10μM). We next compared time and concentration dependency on Ca^{2+} influx in response to SST. Ca^{2+} influx in cells treated with SST (1μM) remained comparable to the control, however, decreased in the presence of higher concentration of SST (Fig. 27B). As shown in Figure 27C, intracellular Ca^{2+} was relatively higher than control in comparison to cells exposed to SST (1μM) in combination with Aβ (10μM). Conversely, cells displayed decreased intracellular Ca^{2+} upon treatment with Aβ (10μM) in combination with SST (10μM) (Fig. 27C). Taken together, these

results displayed SST-concentration dependency on the inhibition of Aβ -mediated increase in Ca^{2+} influx in SH-SY5Y cells.

Next, we observed whether the changes in intracellular Ca^{2+} level had an effect on calpain expression and CDK5 activity. In addition, as the p35 is the key modulator of CDK5 activity, wherein AD patients the truncation of p35 to more stable p25 form induces hyperactivation of CDK5 leading to hyperphosphorylation of CRMP2, we also monitored the changes in p35/25 expression in presence of SST (Fig. 27D-G). The CDK5 expression was also significantly increased in the presence of SST alone or in presence of Aβ1-42 compared to the untreated control (Fig. 27D, E). In particular, the cells treated with Aβ1-42 and SST together also resulted in significant increase in CDK5 expression compared to cells treated with Aβ1-42 alone. Interestingly, cells treated with SST with Aβ42-1 showed a significant increase in calpain expression while Aβ1-42 treatment alone did not result in significant changes. However, the co-treatment of cells with Aβ1-42 and SST displayed significant inhibition of calpain expression compared to the cells treated with Aβ1-42 alone (Fig. 27D, F). Interestingly, such changes in calpain expression did not translate into changes in p35 expression, and the SST treatment in both control and Aβ1-42 treated group resulted in significant increase in p35 expression (Fig. 27D, G).

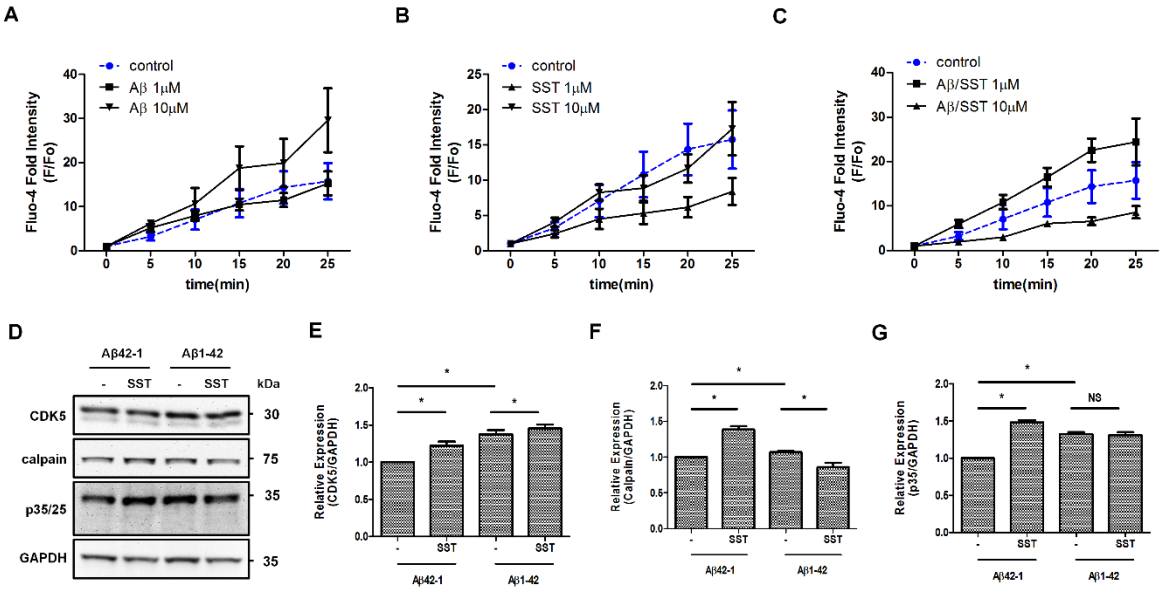

Figure 27 SST mediated effect on calcium signaling and downstream mediators. A. Intracellular level of Ca^{2+} was assessed using Fluo-4 calcium indicator. Cells treated with high concentration of Aβ1-42 (10μM) resulted in significant increase of Ca^{2+} influx compared to the untreated control. The lower concentration of Aβ1-42 (1μM) had no significant changes in Ca^{2+} influx. **B.** Cells treated with high concentration of SST (10μM) resulted in noticeable inhibition of Ca^{2+} influx compared to the cells treated with 1μM of SST or control. **C.** In co-treatment, cells treated with Aβ1-42 (10μM) with low concentration of SST (1μM) had no significant effect in the inhibition of Ca^{2+} influx. In presence of high concentration of SST (10μM) with Aβ1-42 (10μM), however, the Ca^{2+} influx displayed significant inhibition. **D.** Representative Western blot displaying the changes in the expression of CDK5, calpain and p35/25 expression in cells treated with Aβ1-42 in presence or absence of SST. **E-G.** Histograms represent the densitometric analysis of Western blot. Data represents mean ± SD of three independent experiments. *p < 0.05 against control.

4.4 Discussion

The neuroprotective role of SST in AD has been well documented. Specifically, among the various neuropeptides whose expression is changed during the onset and/or progression of AD, SST is one of the most consistently reduced peptide in both cerebrospinal fluid and brain tissues of AD patients (Davies et al., 1980; Beal et al., 1986; Davis et al., 1988; Bissette and Myers, 1992; Nemeroff et al., 1992; Molchan et al., 1993; Bissette et al., 1998; Nilsson et al., 2001). Importantly, the accumulation of Aβ and the reduction of SST showed a significant correlation, suggesting a crucial role in loss of SST and AD progression. We previously reported the neuroprotective role of SST against various neurotoxic molecules such as pro-inflammatory lipopolysaccharide (LPS) and Aβ1-42 in human cerebral microvessel cell line (hCMEC/D3) (Basivireddy et al., 2013; Paik et al., 2018). Furthermore, previous studies from this lab have also reported approximately 70% reduction of SST-positive neurons from brain sections of AD patients compared to the age-matched control (Kumar, 2005). In another study, constitutive intracerebroventricular (i.c.v) infusion of Aβ in rat led to significant reduction of SST-positive neurons in various brain regions including hippocampus, temporal and frontoparietal cortex (Nag et al., 1999; Aguado-Llera et al., 2005; Hervas-Aguilar et al., 2005; Burgos-Ramos et al., 2007). A histochemical analysis of AD patients brain slices also displayed a significant colocalization between the somatostatinergic-neurons and Aβ plaques in various regions including amygdala, cortex and hippocampus (Armstrong et al., 1985; Morrison et al., 1985). Most importantly, Saito et al. reported that the activity of a potent inhibitor of Aβ accumulation neprilysin was significantly elevated following the introduction of SST, resulting in a subsequent reduction of Aβ aggregation in the culture medium (Saito et al., 2005). Collectively, these findings suggest a

significant neuroprotective role of SST against AD and more specifically against Aβ induced toxicity. In the present study, using SH-SY5Y cell line and Aβ1-42 as a model of AD, we have demonstrated that SST effectively inhibits hyperphosphorylation of CRMP2 by modulating intracellular calcium level, which plays a pivotal role in the CDK5 activity. To our knowledge, this is the first comprehensive study correlating the effect of SST on calcium regulation and modulation of CRMP2 phosphorylation. Further, the changes in downstream mediators of calcium signaling pathway such as calpain and CDK5 has been associated with the changes in CRMP2 activity.

The close association of CRMPs, particularly CRMP2, with various characteristics of neurite homeostasis such as the formation, outgrowth and guidance has been well established (Inagaki et al., 2001; Nishimura et al., 2003; Quinn et al., 2003; Deo et al., 2004; Arimura et al., 2005; Yoshimura et al., 2005). CRMP plays a critical role in maintaining the proper microtubule assembly by binding to the microtubule heterodimers and inducing polymerization while also directly regulating tubulin GTPase activity (Fukata et al., 2002; Chae et al., 2009). Understandably, the dysfunction of CRMP2 expression or activity may lead to a significant disruption in overall neurite structure and cognitive impairments. It was reported that the phosphorylation of CRMP2 by CDK5 and GSK3β was significantly increased in the post-mortem brains of AD patients compared to the age-matched controls (Cole et al., 2007). Furthermore, the hyperphosphorylation of CRMP2 has been observed in NFTs as well as in the soluble-fragments of the brain tissues derived from AD patients (Yoshida et al., 1998; Cole et al., 2007). The implication of CRMP2 in the onset and progression of the AD is also observed in various transgenic mice models of AD. In both double (PSEN1 (M146V) KI; Thy1.2-AβPP (swe)) and triple (PSEN1 (M146V) KI; Thy1.2- AβPP (swe); Thy1.2-tau (P301L) transgenic

mice models, a significant increase in CRMP2 phosphorylation was observed in hippocampus and cortices (Cole et al., 2007). On the other hand, in other transgenic mice models of AD such as Tg2576, P301L or P301s tau, mice failed to show a significant increase in CRMP2 phosphorylation, suggesting that the combination of AβPP and PSEN1 mutation may be a prerequisite for dysfunctional CRMP2 phosphorylation. Despite the established association of AβPP and PSEN1 with CRMP2 phosphorylation, however, the exact mechanism involved in regulation of CRMP2 activation remains elusive.

CDK5 is a serine/threonine kinase which is activated by association with its substrate p35 or p39. Reported as a neural-specific kinase due to its spatial restriction in expression to the nervous system, CDK5 is closely associated with neuronal differentiation, migration, synaptic development and functionality (Cheung et al., 2006). The abnormal CDK5 expression or activity has been closely associated with neurotoxicity in various neurodegenerative diseases including AD, HIV neurotoxicity and prion-related encephalopathies. Specifically, the overstimulation of CDK5 activity resulted in hyperphosphorylation of various downstream targets leading to subsequent effects on overall cellular functionality. Until recently, significant focus has been laid on tau hyperphosphorylation by CDK5 during the progression of neurodegenerative diseases (Patrick et al., 1999; Cruz and Tsai, 2004; Han et al., 2005). However, growing evidence suggested the critical role of CRMP2 in homeostasis of neuronal organization and the effect of CDK5 mediated hyperphosphorylation on CRMP2 has been gaining attention.

In the present study, the toxic effect of Aβ was corroborated via various toxicity assay including MTT, caspase-3/7 activity assay and LIVE/DEAD toxicity assay which were in alignment with the previous findings. In addition, we also demonstrated that the presence of SST effectively inhibited the phosphorylation of CRMP2 at Ser522 site, which is primarily

phosphorylated by CDK5. Furthermore, the effect of SST on the regulation of intracellular calcium level was analyzed, which directly affect the activity of calpain. Calpain, in turn, catalyzes unstable CDK5 substrate p35 into much more stable p25, resulting in a significant increase in CDK5 activity and hyperphosphorylation of CRMP2 at Ser522 site.

Previous studies have suggested that an elevated intracellular calcium level leads to cell death via apoptosis and the hyperphosphorylation of key proteins resulting in various pathogenesis or toxicity (Mattson and Rydel, 1992; Li et al., 1996; Berridge et al., 1998; Tucker et al., 1998; LaFerla, 2002; Pierrot et al., 2004). Furthermore, the disruptions in intracellular calcium homeostasis have been associated with the onset and progression of AD and other amyloidogenic diseases such as Parkinson's disease (Kawahara et al., 2000; Blanchard et al., 2004; Mattson, 2004). The accumulation of prefibrillar amyloid aggregates has been reported to increase cytosolic calcium level in neurons (Kawahara et al., 2000; Bucciantini et al., 2004). Various mechanisms have been suggested for the Aβ mediated increase in calcium influx including disruption of lipid integrity (Kayed et al., 2004), the formation of cation-selective channels by Aβ (Kawahara et al., 2000; Kagan et al., 2002), or from activation of selective cell surface receptors to calcium (Guo et al., 1996; Mattson and Chan, 2003; Blanchard et al., 2004). It is likely that the Aβ induced increase of calcium influx is not solely dependent on one particular pathway, but mediated through a complex network as discussed above. In particular, the excess influx of calcium in presence of Aβ leads to calpain-mediated truncation of CDK5 substrate p35 into the much more stable form of p25 (Lee et al., 2000; Dhavan and Tsai, 2001; Amin et al., 2002). In turn, the accumulation of p25 leads to the prolonged activation of CDK5, leading to hyperphosphorylation of downstream mediators such as CRMP2. In this regard, the

regulation of calcium influx plays a critical role in the maintenance of proper CRMP2 phosphorylation.

Among the various modulators of calcium influx, SST is one of the most established regulators of intracellular calcium homeostasis. By binding to SSTR 2, SST is known to inhibit the calcium influx (Reisine, 1990; Johnson et al., 2001; Petrucci et al., 2001). In the present study, we have observed the significant increase of SSTR2 expression following the treatment with SST in presence or absence of Aβ, further supporting SSTR2 as the key receptor involved in SST mediated inhibition of Ca^{2+} influx. Furthermore, we have also observed significant inhibition of calpain expression in cells co-treated with Aβ and SST compared to the cells treated with Aβ alone. Although this inhibition of calpain expression by SST did not result in inhibition of CDK5 expression, it did cause a significant decrease of CRMP2 phosphorylation at Ser522, suggesting that SST might inhibit hyperphosphorylation of CRMP2 by modulating calcium influx. Furthermore, as the CDK5 mediated phosphorylation of a downstream target such as CRMP2 depends on the activity rather than the expression level of CDK5, significant changes in CDK5 activity by SST is conceivable and future study is warranted in this direction. It is interesting to note that although we have observed SST mediated inhibition of CRMP2 phosphorylation at Ser522, SSTR specific agonists induced noticeable increase. This contradicting effect may be due to the fact that the effect of SST is mediated by corroboration of all five SST receptors, whereas the specific activation of one particular receptor may elicit opposing effect. Furthermore, it is possible that unlike SST, which is highly associated with calcium uptake, specific SSTRs may work independently of calcium pathway via modulation of downstream signaling pathways.

In conclusion, the findings from current study describe a significant regulatory role of SST in both intracellular calcium homeostasis and CRMP2 phosphorylation. These observations

corroborate with the neuroprotective role of SST in neurotoxicity and neurodegenerative diseases by suggesting a novel mode of action. Furthermore, as calcium homeostasis is not restricted to the neurodegenerative disease, the effective regulation of calcium level by SST may have a great therapeutic applicability.

Chapter 5: Overall discussion and significance

The pathogenesis of various neurodegenerative diseases such as Alzheimer's and Huntington's disease cannot be solely contributed to a single factor as it often involves systematic physiological dysfunction leading up to the onset of disease. Understandably, the commonly utilized approach in identifying the potential therapeutic targets of such diseases is the elucidation of specific cells that are selectively spared during the progression of the disease. The identification and the subsequent characterization of such cells may lead to the confirmation of genetic factors involved in its selective resistance to the onset and progression of the disease, opening the door for its potential therapeutic applicability. In the striatum, it has been reported that more than 80% of neuronal cells are composed of medium-sized projection neurons that are particularly vulnerable to excitotoxicity (Torres-Peraza et al., 2007). Among them, a subclass of medium-sized aspiny neurons co-expressing SST along with neuropeptide Y and bNOS were observed to be selectively spared in both excitotoxicity model (Beal, 1990; Patel et al., 1991; Kumar et al., 1997; Kumar, 2004). Such selective sparing of SST positive cells in various models of disease along with the widespread distribution of SST in the brain led to the studies examining the correlation and effects of SST in various neurodegenerative diseases (Beal, 1990; Patel, 1999). In parallel, corroborative studies suggested the role of SST in promoting the expression and activity of a potent Aβ degrading enzyme, neprilysin (Shirotani et al., 2001; Saito et al., 2005; El-Amouri et al., 2008). Specifically, Saito et al, reported that SST knockout mice displayed approximately 50% increase in Aβ1-42 level compared to age-matched wild-type littermates (Saito et al., 2005). Here, we explored the mechanistic details of SST mediated neuroprotection in various models of AD to provide a substantial proof of concept.

In chapter 2, the neuroprotective role of SST against Aβ induced damage in BBB was monitored using hCMEC/D3 cell line. hCMEC/D3 is an ideal *in vitro* model of BBB as it forms various junctional proteins (Weksler et al., 2005; Afonso et al., 2008), display significant restriction of small molecules (Cucullo et al., 2008), exhibit high transendothelial electrical resistance (TEER) (Butt et al., 1990; Poller et al., 2008; Hatherell et al., 2011) and have functional transport barriers (Dauchy et al., 2009; Ohtsuki et al., 2013). By monitoring these parameters of physiological BBB in presence of Aβ in absence or presence of SST, we aimed to elucidate the role of SST in protecting BBB integrity against Aβ induced damage. The Aβ induced activation of inflammatory toll-like receptors have been implicated in the pathological increase of proinflammatory cytokines (Abbott, 2000; Ramasamy et al., 2009; Reed-Geaghan et al., 2009). In turn, the released proinflammatory cytokines are reported to induce further production of Aβ (Smith et al., 2007). In this regard, we have previously reported the anti-inflammatory effect of SST against LPS-induced inflammation and hypothesized that this anti-inflammatory effect could also work against Aβ-induced inflammation (Basivireddy et al., 2013). As expected, in the present study using pro-inflammatory cytokine IL-1β as an index of inflammation, we have observed significant inhibition of Aβ-induced inflammation by SST. Interestingly, the release of other pro-inflammatory cytokine such as TNFα did not show a significant change in presence of SST, suggesting multiple pathways might be involved in Aβ-induced inflammation. Next, the changes in various TJP and AJP integrity were examined with specific focus on ZO-1, claudin and occludin as these proteins were identified to be hyperphosphorylated under hyperinflammatory conditions leading to the dissociation and loss of barrier integrity (Stamatovic et al., 2003; Stamatovic et al., 2006). Among the TJPs assessed, SST ameliorated Aβ-induced damage on ZO-1, which showed significant improvement in both

the overall expression level and cellular distribution in presence of SST as assessed by Western blot analysis and immunofluorescence staining. Furthermore, we have assessed the SST mediated effect on Aβ transporters of BBB, namely LRP1 and RAGE. Previous studies have reported that Aβ induces significant changes in LRP1 and RAGE expression and activity, leading to dysfunctional Aβ clearance and accumulation (Cirrito et al., 2005; Deane et al., 2008; Qosa et al., 2012). In the present study, we have established that the presence of SST ameliorated LRP1 expression during Aβ-mediated damage, actively promoting the transportation of Aβ to outside. Further, LRP1 activity has been reported to regulate MMP activity (Etique et al., 2013). Specifically, the clearance of excess MMP is largely mediated by LRP-dependent endocytosis where the Aβ-mediated decrease of LRP1 expression during progression of AD led to the pathological accumulation of MMPs (Barmina et al., 1999; Yang et al., 2001; Van den Steen et al., 2006). In this regard, the hyperinflammation by the accumulation of IL-1β and TNFα has also been reported to cause dysfunction of MMP activity (Iwata et al., 2000; Gao et al., 2004; Lynch et al., 2006; Chen et al., 2009b). In addition to inflammation, hyperactivation of JNK is also associated with an increase in MMP expression (Fromigue et al., 2008). Taken together, we hypothesized that the inhibition of Aβ-mediated hyperphosphorylation and maintenance of Aβ-transporters LRP1 and RAGE by SST may, in turn, regulate the expression and activity of MMP2 and JNK. As expected, we have observed significantly increased phosphorylation of JNK and expression of MMP2 following the treatment with Aβ, which were normalized in presence of SST. Collectively, we inferred that gradual loss of SST expression during the progression of AD may be involved in exacerbating BBB impairment (Fig. 28). Whether this neuroprotective role of SST mediated directly through specific SST receptor or indirectly remains unidentified and future studies are in progress in this direction.

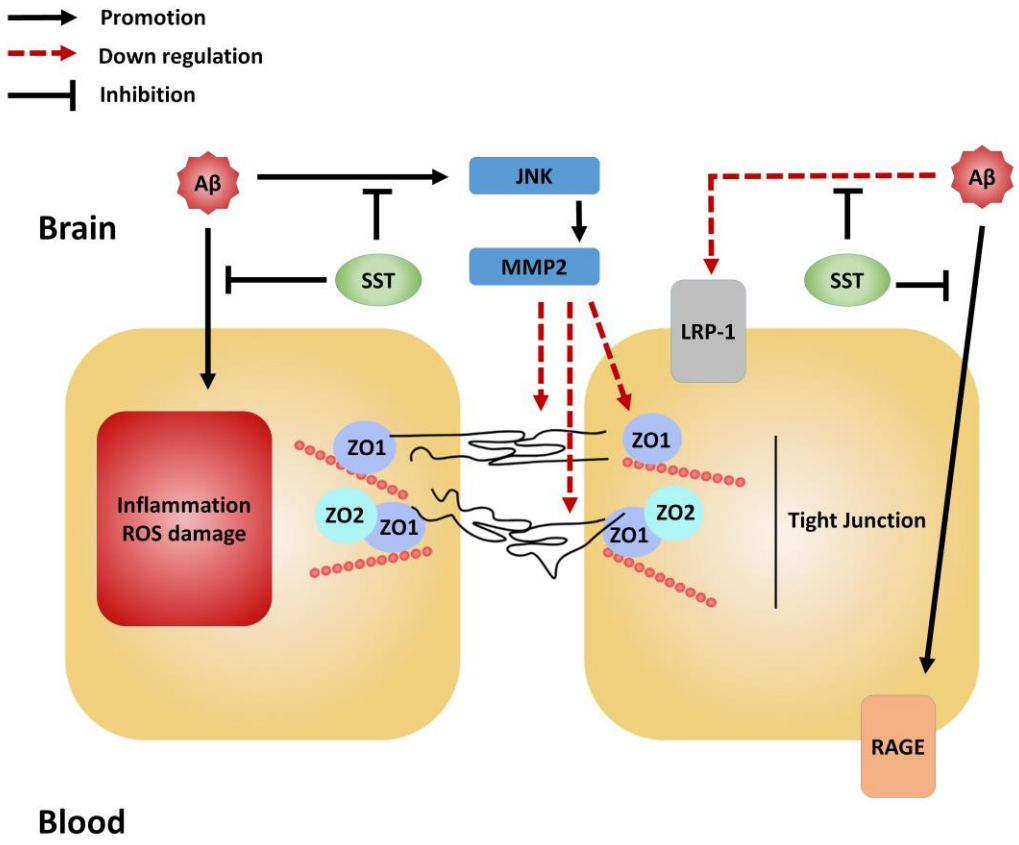

Figure 28 SST-mediated protection of BBB integrity against Aβ-induced damage. SST inhibits the Aβ-induced hyper-activation of JNK and release of pro-inflammatory cytokine IL-1β. Furthermore, expression level of Aβ-transporters LRP-1 and RAGE is maintained in presence of SST, along with maintenance of ZO-1 expression.

In chapter 3, the role of SST in promoting the RA-induced neuronal differentiation of human neuroblastoma-derived SH-SY5Y cell was assessed. SH-SY5Y cell is a well-established *in vitro* model of neuronal differentiation (Rossino et al., 1991; Kaplan et al., 1993; Kito et al., 1997; Truckenmiller et al., 2001; Lopez-Carballo et al., 2002; Brill and Bennett, 2003; Conn et

al., 2003; Ding et al., 2004; Miloso et al., 2004; Pan et al., 2005; Cuende et al., 2008; Cheung et al., 2009). Previously, SST has been reported to enhance neurite outgrowth in PC12 cells, however, the exact mechanism involved remained unidentified (Traina et al., 1998). In the present study, by focusing on neuronal specific βIII tubulin (TUJ1), and two pivotal microtubule-associated proteins known to stabilize tubulin assembly, MAP2 and Tau, we have identified that SST enhances the expression of MAP2 and Tau to promote neurite outgrowth. The essential role of MAP2 in maintaining proper neurite integrity is well established (Dinsmore and Solomon, 1991; Dehmelt et al., 2003). The homozygous knockout mice of MAP2 does not develop defects in overall brain morphology, however displayed significantly reduced microtubule density and truncated dendrite formations in culture (Harada et al., 2002). Along with MAP2, Tau also plays a critical role in neurite integrity. The overexpression of Tau resulted in enhanced neurite outgrowth in neuroblastoma 2a cell line and in cultured cortical neurons (Leugers and Lee, 2010). Further, we have identified that SST mediated its effect in promoting MAP2 and Tau by specifically binding to SST receptors 2 and 4. In our SST-specific knockdown experiment, we have observed significantly reduced neurite outgrowth. Interestingly, the SSTR2 and 4 specific knockdowns displayed only limited effect on neurite outgrowth, suggesting that the SST mediated effect on neurite outgrowth may require multiple receptors and their subsequent interactions to be fully functional. Consistent with this assumption, we have observed significant colocalization between SST and TUJ1 in RA-differentiated SH-SY5Y cells suggesting that SST is required for neuronal differentiation. Among the various potential intermediary mechanism between SST and neurite outgrowth, we suspected ERK pathway as a likely candidate ERK has been reported to play a role in the induction of neurite outgrowth in primary neurons as well as in functional recovery of nerve injury in mice (Desbarats et al., 2003). In corroboration, we

observed that SST indeed mediated activation of ERK1/2. Furthermore, this increase in ERK1/2 activity was observed following SSTR2 and 4 specific agonists as well. Collectively, our findings in the present study suggest that SST promotes RA induced neurite outgrowth by enhancing MAP2 and Tau. Moreover, these findings not only suggest a critical role of SST in neurite outgrowth but also its implication in various neurodegenerative diseases given the importance of neurite homeostasis in the maintenance of normal neuronal functions. In addition, the identification of SSTR2 and 4 as a key receptor subtypes involved in SST induced promotion of neurite outgrowth presents these receptors as a potential therapeutic target. Taken together, SST mediated promotion of neurite outgrowth may provide novel insight into improving cognitive function in various neurodegenerative disease affecting neurite integrity.

In chapter 4, the role of SST in maintaining neurite integrity against Aβ induced damage has been assessed. CRMP is a member of microtubule-associated protein initially identified as a signaling molecule of Semaphorin3A (Sema3A) reported to be involved in establishing neuronal polarity, synapse formation, learning and memory (Arimura and Kaibuchi, 2007; Yamashita and Goshima, 2012). CRMP is also reported to maintain microtubule integrity by binding to the microtubule heterodimers and inducing polymerization (Fukata et al., 2002; Chae et al., 2009). Among the various isoforms of CRMPs, CRMP2 in particular has been studied extensively for its involvement in neurite formation, outgrowth and guidance (Inagaki et al., 2001; Nishimura et al., 2003; Quinn et al., 2003; Deo et al., 2004; Arimura et al., 2005; Yoshimura et al., 2005). In AD, it has been reported that CRMP2 was hyperphosphorylated (Yoshida et al., 1998; Cole et al., 2007). In both double (PSEN1 (M146V) KI; Thy1.2-AβPP (swe)) and triple (PSEN1 (M146V) KI; Thy1.2- AβPP (swe); Thy1.2-tau (P301L) transgenic mice models of AD, hyperphosphorylation of CRMP2 was observed (Cole et al., 2007). Similar to MAP2 or Tau,

CRMP2 is also phosphorylated at the C-terminal by CDK5 and GSK3β. Specifically, CDK5 phosphorylates CRMP2 at Ser522, which also serves as the prime site for subsequent phosphorylation at Thr509, Thr514 and Ser518 by GSK3β (Brown et al., 2004; Cole et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005). As Ser522 site served a dual role as a primary phosphorylation site of CDK5 as well the prime site for GSK3β, we have focused on monitoring the phosphorylation level at the Ser522 site in this study. The increased activity of CDK5 occurs due to the increased conversion of CDK5 substrate p35 into more stable isoform of p25. This cleavage of p35 into p25 is mediated by calpain, which is sensitive to the intracellular level of calcium (Saito et al., 1993; Patrick et al., 1999). In AD, the accumulation of Aβ leads to increase in intracellular calcium level resulting in mitochondrial calcium overload, generation of superoxide radicals and excess production of pro-apoptotic proteins such as cytochrome *c* (Stutzmann, 2007). Accordingly, it became plausible that effective inhibition of Aβ-induced calcium influx may inhibit this pathway leading to CRMP2 phosphorylation. In this regard, previous studies have reported the role of SST in inhibition of calcium influx through SSTR2 (Reisine, 1990; Johnson et al., 2001; Petrucci et al., 2001). In the present study, using Aβ1-42 induced toxicity in SH-SY5Y cell line and as a model of AD, we demonstrate that SST effectively inhibits the Aβ1-42 induced increase in intracellular calcium level, resulting in subsequent inhibition of hyperactive calpain and CDK5, and in normalization of CRMP2 phosphorylation. Our results described here establish a neuroprotective role of SST by regulating calcium level in neuronal cells, thereby regulating the downstream kinase activity and modulating the phosphorylation of microtubule-associated proteins such as CRMP2. To our knowledge, this is the first comprehensive study correlating the effect of SST on calcium regulation and modulation of CRMP2 phosphorylation

5.1 Limitations

In the present study, all experiments were carried out in a homogeneous population of single cell line hCMEC/D3 (Chapter 2) or SH-SY5Y cells (Chapter 3 and 4) in an isolated system. In normal physiological condition, the system is hardly a closed system with single cell population but rather composed of the heterogeneous cell population with an intricate network of activities. Specifically, the role of astrocytes and microglia cannot be ignored and their absence in current system presents a significant limitation in interpolating the results to direct therapeutic application. Furthermore, a number of studies have reported that Aβ induced toxicity requires the presence of multiple isoforms of Aβ (ex. Aβ25-35, Aβ1-40, Aβ1-42, etc) and that a single isoform often lacks the full toxic effect regardless of the concentration used. However, both hCMEC/D3 and SH-SY5Y cells are well-established and most widely used *in vitro* models to study blood-brain barrier and neuronal differentiation, respectively, and have provided excellent *in vitro* model to study AD despite the limitations. To overcome some of these limitations, future studies should incorporate following changes.

5.2 Future Studies

1. To better represent the physiological conditions in *in vitro*, a number of studies recently attempted to culture blood-brain barrier or neuronal cells in presence of astrocyte/microglia (Appelt-Menzel et al., 2017). Having heterogeneous cell population does introduce significant variability and increased room for error, but does offer more physiologically relevant system. Accordingly, the results would also have significantly higher therapeutic applicability. Further, it could provide a realistic alternative to *in vivo* studies if sufficiently optimized.
2. During the onset and progression of AD, multiple isoforms of Aβ with varying length are produced (Haass et al., 1993; Jarrett et al., 1993). Despite the initial hypothesis that only the highly hydrophobic Aβ1-42 isoform causes a toxic effect, later studies have suggested that the presence of other isoforms are required to cause pathologically relevant toxicity in *in vitro* models (Snyder et al., 1994; Yoshiike et al., 2003; Yan and Wang, 2006; Kuperstein et al., 2010; Pauwels et al., 2012). Recently, studies incorporating the mixture of various isoforms of Aβ are becoming more common. In this context, future studies utilizing Aβ should incorporate these changes and use a cocktail of Aβ isoforms rather than using single Aβ population.
3. The kinase activity involved in Chapter 4 could be analyzed in more detail. Specifically, in the current study, GSK3β was omitted as we did not initially observe significant changes in the target site of GSK3β, namely Thr514. However, as the GSK3β is a well-established kinase involved in CDK5 mediated phosphorylation, the changes in GSK3β activity and/or expression should be elucidated.

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