Exploring the Role of Somatostatin in the Pathogenesis of Alzheimer’s Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2018

Abstract

The amyloid-β (Aβ) peptide is understood to be a critical factor in the pathogenesis of Alzheimer’s disease (AD), with soluble Aβ oligomers (oAβ) observed to be particularly toxic to neurons. For this reason, we undertook an in-depth search for oAβ binders in human brain, taking advantage of advanced mass spectrometry workflows. This analysis revealed the peptide somatostatin (SST) to be the most selective binder to oAβ in affinity capture experiments. Furthermore, SST was observed to interfere with Aβ aggregation kinetics and promote the formation of distinct 50-60 kDa Aβ assemblies. In a follow-up investigation of the SST interactome, it was revealed that SST predominantly binds to several members of the family of P-type ATPases, particularly the Na⁺/K⁺-ATPase. Subsequent validation experiments confirmed this interaction and identified a tryptophan residue within SST as critical for binding. These findings bring to light potentially new mechanisms for SST action in normal physiology and AD.
Acknowledgments

I would like to extend my sincerest gratitude to my supervisor, Dr. Gerold Schmitt-Ulms, whose passion for science and research is unrivaled by any professor I have encountered during my studies. His ability to extract the biological significance out of any piece of data and use it to develop a compelling narrative is truly inspiring. The two years spent under his supervision have certainly been a privilege.

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Lastly, I would like to thank my parents, for their continued support throughout my education, as well as my family and friends who always provided help when I needed it. I would like to give a special thanks to Callie O’Reilly for keeping me motivated on a daily basis and helping me stay sane during overwhelming times.

I would like to dedicate this thesis to my Dziadzia, Joseph Solarski, whose battle with dementia has served as my inspiration to pursue research in neurodegenerative diseases.
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<tbody>
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<td>4-VP</td>
<td>4-vinylpyridine</td>
</tr>
<tr>
<td>α-SN</td>
<td>Alpha-synuclein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid precursor protein intracellular domain</td>
</tr>
<tr>
<td>AP-MS</td>
<td>Affinity purification-mass spectrometry</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>BACE</td>
<td>β-site APP-cleaving enzyme</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CPEB1</td>
<td>Cytoplasmic polyadenylation element bind protein 1</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CST17</td>
<td>Cortistatin 17</td>
</tr>
<tr>
<td>CST29</td>
<td>Cortistatin 29</td>
</tr>
<tr>
<td>CTE</td>
<td>Chronic traumatic encephalopathy</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK2</td>
<td>Extracellular signal-related kinase 2</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>FTDP-17</td>
<td>Frontotemporal dementia and parkinsonism linked to chromosome 17</td>
</tr>
<tr>
<td>FTLD</td>
<td>Frontotemporal lobar degeneration</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
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<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
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<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HD6</td>
<td>Human defensin 6</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HTT</td>
<td>Huntingtin protein</td>
</tr>
<tr>
<td>IPI</td>
<td>International protein index</td>
</tr>
<tr>
<td>ISF</td>
<td>Interstitial fluid</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tag for relative and absolute quantitation</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumor</td>
</tr>
<tr>
<td>oAβ</td>
<td>Oligomeric amyloid β</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PHF</td>
<td>Paired helical filament</td>
</tr>
<tr>
<td>PiB</td>
<td>Pittsburgh compound B</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PPCST</td>
<td>Preprocortistatin</td>
</tr>
<tr>
<td>PPSST</td>
<td>Preprosomatostatin</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PSM</td>
<td>Peptide spectrum match</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RSP</td>
<td>Regulated secretory pathway</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong cation exchange</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SST14</td>
<td>Somatostatin 14</td>
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<tr>
<td>SST28</td>
<td>Somatostatin 28</td>
</tr>
<tr>
<td>SST25</td>
<td>Somatostatin 25</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and Tween 20</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phosphine</td>
</tr>
<tr>
<td>TE</td>
<td>Tetraethylammonium bromide</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoro-acetic acid</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem mass tag</td>
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</tbody>
</table>
UV    Ultraviolet
VIP    Vasoactive intestinal peptide
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Chapter 1: Introduction and Review of the Literature

Summary: This chapter provides a brief review of the literature pertinent to subsequent chapters in this thesis. A primary focus of this chapter is the pathobiology of Alzheimer’s disease with a similar emphasis on the biology of somatostatin. The final subchapter introduces specialized techniques employed in the current research.

1.1 Alzheimer’s disease

Regarded by many as one of the greatest threats to the global healthcare system in this century, Alzheimer’s disease (AD) is a relentless neurodegenerative disorder first characterized just over one hundred years ago. AD is the namesake of Dr. Alois Alzheimer, the physician to first associate a unique clinical phenotype of memory disturbance, paranoia, and confusion with a distinct neuropathological profile. Despite his untimely death at the age of 51, Dr. Alzheimer’s name would become infamous due to its association with the disease responsible for the majority of dementia cases worldwide. Today, it is estimated that nearly 50 million people are living with AD worldwide, a figure that is expected to double within the next thirty years (Alzheimer’s Disease International 2016). In the context of economic burden, it is estimated that healthcare costs for individuals with AD were $259 billion in the United States in 2017 alone (Alzheimer’s Association 2017).

The classical clinical presentation of AD includes a progressive worsening of memory and cognition that impacts speech (aphasia, apraxia) and the ability to process sensory information (agnosia), with changes in normal behavior patterns in the absence of other systemic or brain disorders that could account for the symptoms (McKhann et al. 1984). The gradual onset of these symptoms with a well-documented cognitive decline is suggestive of an evolving pathologic process which helps distinguish AD from other types of dementia (McKhann et al. 2011). However, the accurate diagnosis of AD remains challenging, particularly in the early preclinical and prodromal phases when cognitive impairments are subtle (Dubois et al. 2016). Further complicating the matter is the similarity in symptoms between AD and other neurodegenerative diseases such as Parkinson’s disease (PD) and frontotemporal lobar degeneration (FTLD). For this reason, close inspection of the disease neuropathology is critical to arrive at an accurate diagnosis.
1.2 Neuropathological hallmarks of Alzheimer’s disease

Insights into the neuropathological hallmarks of AD have been gleaned as early as Alois Alzheimer’s seminal case report of a “peculiar disease of the cerebral cortex” (Alzheimer 1907; translated by Strassnig and Ganguli 2005). Alzheimer’s description of an “atrophic brain” with “storage of peculiar matter” within the cortex and “peculiar changes of neurofibrils” would set the stage for a century of research dedicated to the characterization of these pathological changes. Today, it is well established that Alzheimer had observed extracellular plaques, consisting of insoluble aggregates of the amyloid-beta (Aβ) peptide (Glenner and Wong 1984), and intracellular aggregates of the microtubule-associated protein tau (Grundke-Iqbal et al. 1986) which are seen as neurofibrillary tangles within neurons (Masters et al. 2015). These two distinct pathologies are now regarded as the main neuropathological hallmarks of AD.

1.2.1 Amyloid-β

Despite the routine observation of neuritic plaques in the post-mortem brains of AD patients, the constituents of these deposits remained elusive for many years. It was apparent that these deposits consisted of amyloidogenic proteins, based on their green polarization color under polarization microscopy following Congo red staining (Glenner 1980b; Glenner 1980a). However, it was not until 1984, when a stringent purification protocol purification protocol using column chromatography and high performance liquid chromatography (HPLC) was applied to the brains of AD patients that the Aβ peptide was found to comprise the core of these extracellular plaques (Glenner and Wong 1984). At the time, no previously sequenced protein showed any homology to Aβ. This characterization of a novel AD-related amyloid protein would become the first to place Aβ, and the biology surrounding its biogenesis and clearance, at the core of the pathogenesis of AD.

1.2.1.1 Biogenesis and clearance of Aβ

Aβ is an endoproteolytic fragment of the amyloid precursor protein (APP), a transmembrane protein encoded on the long arm of chromosome 21 in humans (Kang et al. 1987). Although abundantly expressed in the brain, APP is also found in many peripheral tissues including the liver, skeletal muscle, and blood vessels, among others (Roher et al. 2009). The exact physiological function of APP remains unknown, despite countless in vitro and in vivo studies.
aimed at elucidating its function. Contrary to the pathological role of Aβ in AD, APP appears to have a positive impact on the development of the nervous system and neuronal homeostasis (van der Kant and Goldstein 2015). Much of this evidence is derived from studies of APP knockout mice, which display impaired long-term potentiation (Dawson et al. 1999), decreased dendritic spine density (Tyan et al. 2012), reduced brain weight (Magara et al. 1999), and axonal transport defects (Kamal et al. 2001). APP may also play a role in neuronal migration during embryogenesis, since rodent embryos injected with short hairpin RNA (shRNA) to reduce APP levels appear to have defects in the migration of neuronal precursors from the ventricular zone to the cortical plate (Young-Pearse et al. 2007). However, some adaptation to a complete loss of APP seems possible, because APP-knockout mice are typically viable and the phenotypic changes observed in these animals are often subtle (van der Kant and Goldstein 2015). While these observations all point towards a role of APP in neuronal development and maintenance, the function of APP in the adult brain remains disputed.

The mechanisms involved in the processing of APP at the cell surface have been extensively studied and directly linked to the generation of the pathologic Aβ peptide (Figure 1.1). APP has three predominant cleavage sites at which endoproteinases, referred to as secretases, can act: one within the transmembrane region (cleaved by the γ-secretase) and two in the plasma membrane-adjacent segment of its extracellular domain (cleaved by the α- or β-secretase) (Thinakaran and Koo 2008). In the amyloidogenic pathway, sequential cleavage by β- and γ-secretases releases the APP intracellular domain (AICD) and yields two extracellular peptides, APPsβ and Aβ. In contrast, in the non-amyloidogenic pathway, APP is cleaved by the α- and γ-secretases. Since the cleavage site of the α-secretase is located within the Aβ domain, the non-amyloidogenic pathway yields AICD, APPsα, and an N-terminally truncated Aβ peptide, termed p3. While Aβ has been thoroughly investigated because of its association with AD, the fate and function of the alternative cleavage products are not fully understood (Chow et al. 2010).

Although secretase is the general term used to describe the proteases that cleave APP, there has been much work done to identify and characterize the individual proteins involved in APP processing. α-secretases encompass members of the ADAM family of zinc metalloproteinases, with ADAM9, ADAM10, ADAM17, and ADAM19 being able to cleave APP at the α-secretase site (Allinson et al. 2003). The aspartyl protease, BACE2, also has the ability to cleave at the α-secretase site, although its low level of expression in the brain suggests it is only a minor
contributor (Farzan et al. 2000). The primary β-secretase is β-site APP-cleaving enzyme 1 (BACE1), which has been confirmed by observations that Aβ secretion is abolished in BACE1-deficient mice (Cai et al. 2001; Vassar 2004). Finally, the γ-secretase, which cleaves within the transmembrane domain of APP, is a multi-protein complex consisting of at least four proteins: presenilin-1 or -2, nicastrin, APH-1, and PEN-2 (De Strooper 2003). Unlike the α- and β-secretases, the γ-secretase can cleave APP at multiple sites, resulting in proteolytic fragments of varying length at the C-terminus. This non-specificity accounts for the existence of Aβ peptides with lengths ranging from 37 to 43 amino acids (Beher et al. 2002).

Following the sequential cleavage by the β- and γ-secretases, Aβ is ultimately released into the extracellular space where it must be cleared. Since an imbalance between the production and clearance of Aβ is thought to be one of the inciting factors in AD, understanding the mechanisms by which Aβ is removed from the brain is critical. In general, there are three potential mechanisms by which Aβ is cleared from the brain: phagocytosis, proteolytic degradation, and release into the peripheral circulation (Tarasoff-Conway et al. 2015). Extracellular Aβ can be taken up by a number of different cell types within the brain, including microglia and astrocytes, where it is phagocytosed and degraded (Nielsen et al. 2009; Rogers et al. 2002). Furthermore, a number of proteases, including neprilysin, matrix metalloproteinases, and insulin-degrading enzyme, are capable of degrading extracellular Aβ (Iwata et al. 2001; Miners et al. 2008; Qiu et al. 1998). Possibly the most important route of Aβ clearance, however, involves efflux into the periphery, which may account for the clearance of 40-60% of brain Aβ (Wang et al. 2017). Aβ can enter the peripheral circulation via enzyme-mediated transport across the blood-brain barrier (BBB) and/or blood-CSF barrier, in addition to bulk flow clearance from the interstitial fluid (ISF) to the cerebrospinal fluid (CSF) or perivascular space (Zlokovic 2011; Iliff et al. 2012). Once in the systemic circulation, Aβ can be catabolized by peripheral organs such as the liver, kidney, gastrointestinal tract, and skin (Xiang et al. 2015).
Figure 1.1 **APP processing and Aβ biogenesis.** Proteolytic processing of APP can occur following one of two routes: the non-amyloidogenic or the amyloidogenic pathway. In the non-amyloidogenic pathway (left), APP is first cleaved by the α-secretase, to produce an extracellular fragment (APPsα). Subsequent cleavage by the γ-secretase yields another extracellular peptide (p3) and the APP intracellular domain (AICD). In contrast, in the amyloidogenic pathway (right), APP is initially cleaved by the β-secretase to produce APPsβ, which is released into the extracellular space. Additional cleavage by the γ-secretase yields the Aβ peptide and AICD. Since the γ-secretase can cleave APP at multiple sites, Aβ peptides with lengths varying from 37-43 residues are produced. The predominant form of Aβ, Aβ1-40, accounts for approximately 90% of the secreted peptide. While the more pathogenic version, Aβ1-42, normally accounts for less than 10% of the total peptide, mutations in APP or presenilin-1/2 can increase its relative production.
1.2.1.2 Aβ aggregation in Alzheimer's disease

When Aβ clearance mechanisms are compromised, or the metabolic balance is shifted towards the production of Aβ, the brain becomes inundated with Aβ peptides, which can form pathological aggregates in AD. The propensity for Aβ to convert from a normally soluble peptide into an insoluble state, which makes it prone to self-aggregation, is a characteristic shared by a number of amyloidogenic proteins in various neurodegenerative diseases. Indeed, a similar phenomenon is observed in PD, Huntington’s disease (HD), and prion disease, with alpha-synuclein (α-SN), Huntingtin (HTT), and the prion protein (PrP), respectively (Knowles, Vendruscolo, and Dobson 2014). Although these proteins are coded by different genes and possess different amino acid sequences, they are all capable of assuming an amyloid state, in which individual polypeptide chains, enriched in β-sheet structure, form highly ordered, fibrillar aggregates. Typically, under electron microscopy (EM) or atomic force microscopy (AFM), amyloid fibrils are seen as unbranched filamentous structures, with diameters of only a few nanometers but lengths on the micrometer scale (Fitzpatrick et al. 2013). Mature fibrils consist of several protofilaments twisted around each other, with individual protofibrils displaying a characteristic cross-β pattern seen under X-ray fiber diffraction analysis (Eisenberg and Jucker 2012). The cross-β pattern of amyloid fibrils allows the formation of a continuous network of hydrogen bonds along the fibril axis, which imparts great stability to the mature fibril. In fact, it has been proposed that the amyloid state is more thermodynamically stable than the native state of many proteins at critically high concentrations, which may suggest why these structures form under pathological conditions (Baldwin et al. 2011).

The assembly of Aβ into higher ordered, multimeric structures is one of the characteristics of AD pathogenesis (Figure 1.2A). In general, the process begins with soluble, monomeric Aβ, which exists in an unstructured state under native conditions (Nelson and Eisenberg 2006). While multiple Aβ variants exist, those with longer C-terminal extensions (particularly the 42-amino acid version, Aβ1-42) appear to be more hydrophobic and display a greater tendency to self-aggregate (Jarrett, Berger, and Lansbury 1993). After undergoing a conformational change to a partially folded monomer, the peptides self-aggregate to initially form low molecular weight oligomers, which further assemble into protofibrils and fibrils rich in β-sheet structure (Roychaudhuri et al. 2009). This so-called ‘on-pathway’ of fibrillogenesis follows a nucleation-dependent polymerization process, in which the nucleation of Aβ monomers to form paranuclei
proceeds at a very slow rate (Jarrett and Lansbury 1993). This period is known as the ‘lag-phase’ of fibrillogenesis. The self-association of paranuclei to form protofibrils and the maturation of protofibrils into amyloid fibrils are both kinetically favored and occur rapidly. While this relatively simple model accounts for the kinetics of amyloid fibril formation, it is complicated by the identification of several distinct Aβ assemblies that don’t develop into fibrils. These ‘off-pathway’ structures include Aβ globulomers, which are formed in vitro in the presence of 2% SDS; Aβ*56, a dodecameric Aβ assembly found in the brains of transgenic APP mouse models and human AD brains; and amylospheroids (ASPDs), which are large, spherical Aβ aggregates formed in vitro using synthetic Aβ peptides and have been isolated from the brains of AD patients (Barghorn et al. 2005; Lesné et al. 2006; Lesné et al. 2013; Noguchi et al. 2009).

The identification of numerous Aβ assemblies with unique sizes and morphologies has prompted researchers to question which species are the most neurotoxic in AD. While early observations of AD brains led investigators to believe that the highly abundant amyloid plaques were toxic structures in the disease, current evidence suggests that soluble aggregates of Aβ are, in fact, the toxic species (Haass and Selkoe 2007). Early evidence in support of this theory came from the analysis of the number and size of amyloid plaques (referred to as the amyloid burden) in patients with AD with respect to age and disease duration (Hyman, Marzloff, and Arriagada 1993). The conclusion that the amyloid burden does not worsen as the duration of dementia increases was surprising, and led the authors to hypothesize that the deposition of Aβ in AD is a dynamic process involving resorption and/or resolution. Furthermore, it was observed around the same time that amyloid burden also correlated poorly with cognitive impairment in patients with AD (Terry et al. 1991). These findings led to a shift in the field, with investigators beginning to search for new correlates of disease progression. Just before the turn of century, a number of studies emerged, reporting strong correlations between the levels of soluble, non-fibrillar Aβ in the brain parenchyma with disease severity and synaptic loss (McLean et al. 1999; Lue et al. 1999). Although these studies established that soluble Aβ oligomers are the likely neurotoxic species in AD, the mechanisms by which Aβ causes synaptic loss and neuronal death remain controversial.
1.2.1.3 The amyloid cascade hypothesis

The amyloid cascade hypothesis, which has been revised over the years, is one of the most widely accepted, yet scrutinized, models for the pathogenesis of AD (Hardy and Higgins 1992; Hardy and Selkoe 2002). It posits that the accumulation of Aβ in the brain is the primary influence promoting downstream pathological changes. Early support for this theory came from genetic studies, which established that mutations in the APP gene, in addition to the genes coding for the presenilin-1 or -2 proteins, are causative for early-onset, familial forms of AD (Goate et al. 1991; Rogaev et al. 1995; Sherrington et al. 1995). These mutations shift the balance of Aβ production to favor the more pathologically relevant Aβ1-42. Further observations that pathologic alterations to the tau protein, seen in humans with FTLD and mice with mutations in the MAPT gene (which encodes the tau protein), are insufficient to induce significant Aβ deposition add support for the amyloid cascade hypothesis (Hardy et al. 1998; Lewis et al. 2001). Perhaps the most compelling pieces of evidence for this hypothesis are the repeated reports of endogenous Aβ oligomers causing impairments in long-term potentiation (LTP), loss of dendritic spines, and tau hyperphosphorylation at disease-relevant epitopes (Larson and Lesne 2012). On a behavioral level, these pathological changes manifest as impairments in cognition in various experimental paradigms. The overall framework of the amyloid cascade hypothesis, in its most recent version, is as follows: changes in Aβ production/clearance mechanisms cause elevated levels of the Aβ peptide (particularly Aβ1-42), promoting the formation of soluble oligomers which cause progressive impairments in synaptic function. In parallel to the continued impairment of neuronal function by oligomeric Aβ, through the interference of ion homeostasis and promotion of tau hyperphosphorylation, Aβ is deposited in the brain parenchyma as diffuse plaques. The cascade ultimately ends in widespread neuronal dysfunction and synaptic loss, resulting in progressive dementia (Figure 1.2B).
Figure 1.2 The amyloid cascade hypothesis. (A) Following its cleavage from APP, Aβ, which exists in a natively unstructured state, undergoes a conformational change to adopt a partially folded structure. As Aβ levels in the brain increase, the monomers begin to self-associate into low-n oligomers (e.g. dimers, trimers). These small oligomers can enter the “on-pathway” and develop into protofibrils, fibrils, and ultimately amyloid plaques. Alternatively, Aβ can enter the “off-pathway” and form non-fibrillar aggregates (e.g. globulomers, Aβ*56, ASPDs), which cause LTP impairment, dendritic spine loss, and tau hyperphosphorylation in neurons. (B) According to the amyloid cascade hypothesis, AD is the result of elevated levels of Aβ, which forms assemblies of varying sizes, ultimately leading to widespread neuronal dysfunction/death and dementia.
1.2.1.4 Mechanisms of Aβ toxicity

While the outcomes of prolonged Aβ exposure, which include impairments in synaptic plasticity (Shankar et al. 2008), dendritic spine loss (Wei et al. 2010; Shankar et al. 2007), and tau hyperphosphorylation (Jin et al. 2011), have been well established in several paradigms, the molecular mechanisms involved have yet to be fully elucidated. Indeed, a number of signaling pathways initiated by Aβ have been proposed. Since Aβ assemblies have been observed to impair synaptic plasticity through the inhibition of LTP, the NMDA receptor (NMDAR) has been scrutinized as a potential mediator of Aβ’s toxic effects. Indeed, NMDAR surface expression is reduced following exposure to soluble Aβ (Snyder et al. 2005). While Aβ has been observed to colocalize with NMDARs at neuronal synapses, it is unclear whether the two proteins interact directly. Perhaps, an indirect scenario is at play that involves the activation of α7-nicotinic acetylcholine receptors (α7nAChR), which recruits the Ca\(^{2+}\)-dependent protein phosphatase, calcineurin, and the striatal-enriched tyrosine phosphatase (STEP), ultimately leading to NMDAR internalization (Snyder et al. 2005). Aβ has also been observed to bind to the receptor tyrosine kinase EphB2 and trigger its degradation in the proteasome, an event which also reduces NMDAR surface expression (Cisse et al. 2011). In 2009, a controversial interaction between oligomeric Aβ and the cellular prion protein (PrP\(^C\)) was reported that may represent an additional mechanism by which Aβ perturbs NMDAR signaling (Lauren et al. 2009; Um et al. 2012). The latter is thought to involve binding of oligomeric Aβ to PrP\(^C\), with subsequent activation of the protein kinase, Fyn. Once activated, Fyn can phosphorylate the NR2B subunit of the NMDAR leading to a progressive loss of receptor expression at the synapse.

Countless other proteins have also been proposed to act as receptors for Aβ. Among these are the receptor for advanced glycation end products (RAGE); insulin receptor-sensitive Aβ binding protein; P/Q-type calcium channels; and the Na\(^+\)/K\(^+\)-ATPase (Origlia et al. 2008; De Felice et al. 2009; Nimmrich et al. 2008; Ohnishi et al. 2015). The failure to reach a consensus regarding the identity of the primary Aβ receptor may have several explanations. Poorly standardized protocols for the preparation of synthetic Aβ assemblies are one possibility. Inconsistencies in the concentration of exogenously applied Aβ assemblies are another impediment to the identification of biologically relevant receptors. Indeed, while nanomolar (nM) concentrations of Aβ are sufficient to trigger dendritic spine loss and induce tau hyperphosphorylation, micromolar (µM) concentrations are routinely used in the literature (Larson and Lesne 2012). It is to be anticipated
that many discrepancies in the proposed Aβ signaling pathways will be resolved once the experimental paradigms and methods in the field become more standardized and representative of the authentic physiological milieu.

1.2.1.5 Challenges to the amyloid cascade hypothesis

Although the amyloid cascade hypothesis remains the leading theory of AD pathogenesis at this time, several reports have challenged the notion of a linear pathway beginning with Aβ production and ending with dementia. One of these data points emerged following the development of Aβ-directed positron emission tomography (PET) ligands, such as Pittsburgh compound B (PiB), that allow the visualization of Aβ plaques in the intact human brain (Mathis et al. 2002). Longitudinal assessments of the amyloid burden by PET scanning in individuals with AD or mild cognitive impairment (MCI) revealed that the amount of Aβ in the brain correlates poorly with cognitive decline (Villemagne et al. 2011). Furthermore, up to a third of cognitively normal individuals were found to have substantial amyloid burdens (Klunk et al. 2009). Minimally, these data indicated that if Aβ is critical also for sporadic AD, it’s not the highly aggregated forms of the peptide that correlate with severity and cognitive decline. Another line of research that cast doubt on the significance of the amyloid cascade hypothesis for sporadic forms of the disease, has been the development of therapeutic Aβ-directed monoclonal antibodies (Herrup 2015). Although these reagents showed promise in preclinical studies in mice, they have so far failed to improve measures of cognition in AD patients in multiple clinical trials (Doody et al. 2014; Salloway et al. 2014). This failure occurred despite a significant reduction in plaque number and reduced levels of phosphorylated tau in the CSF. However, the last word has not been spoken on this issue, and it is possible that these antibody treatments were administered too late in the clinical course of the disease to have a significant impact, since amyloid accumulation precedes the onset of AD symptoms by many years.

Regardless of emerging data that conflict with this theory, there can be little doubt that Aβ is of critical importance in AD. The fact that mutations in APP and the genes coding for the presenilin proteins lead to familial AD remains irrefutable evidence that Aβ is a key player in the disease pathogenesis. However, it is conceivable that comorbidities, such as inflammation, play a larger role than initially appreciated. Indeed, in addition to the proteins involved in Aβ processing, several proteins with roles in pro-inflammatory signaling pathways have been found to be
associated with AD in several genome-wide association studies (Lambert et al. 2009; Hollingworth et al. 2011; Jonsson et al. 2013; Lambert et al. 2013). Specifically, variants in the genes encoding the proteins TREM2, CD33, CR1, and ABCA7, among several others involved in microglial inflammation, appear to influence the risk of developing late-onset AD. Furthermore, recent evidence has begun to emerge implicating human herpesvirus 6A and 7, which are elevated in the brains of patients with AD, as novel regulators of APP metabolism and potential contributors to the development of AD neuropathology (Readhead et al. 2018). The possibility that specific viral species may interact with AD risk genes (e.g. PSEN1 and BACE1) opens up an exciting new area of AD research and highlights the importance of understanding all aspects of AD etiology in addition to Aβ.

1.2.2 Tau

As previously alluded to, another key player in the pathogenesis of AD is the tau protein, which is the primary constituent of the neurofibrillary tangles seen in the disease. In the mature neuron, tau is the predominant microtubule associated protein (MAP), along with MAP1 and MAP2. Under normal physiological conditions, tau interacts with tubulin and aids in the formation and stabilization of microtubules (Weingarten et al. 1975). In neurons, microtubules are one of the major cytoskeletal components, aiding in the processes of neuronal migration, axon and dendrite formation, and the establishment of synaptic connections (Kapitein and Hoogenraad 2015). Like the other MAPs, the ability of tau to promote the formation of microtubules is regulated by phosphorylation. The degree of phosphorylation that optimizes the interaction between tau and tubulin appears to be 2-3 moles of phosphate per mole of tau (Kopke et al. 1993). However, in AD tau becomes hyperphosphorylated, making it less soluble and therefore more likely to self-aggregate (Grundke-Iqbal, Iqbal, Tung, et al. 1986). Much like Aβ, hyperphosphorylated tau can adopt a number of aggregation states, predominantly soluble oligomers and fibrillar structures called paired helical filaments (PHF). Not only does tau lose its ability to bind tubulin and promote microtubule formation in its self-aggregated state, it also disrupts microtubule structure by sequestering normal tau, MAP1, and MAP2 (Alonso et al. 1994; Alonso et al. 1997). Destabilization of the microtubule network within neurons is thought to be the mechanism by which tau causes neurodegeneration. It is currently believed that soluble tau oligomers are the pathogenic form of the protein, rather than PHFs, based on observations that (1) microtubule disruption does not coincide with the presence of PHF in neurons from AD brain specimens; (2)
behavioral deficits in mice are reduced when mutant tau expression is turned off but neurofibrillary tangle formation persists; and (3) self-association of tau into PHF abolishes its toxic ability to sequester MAPs (Cash et al. 2003; Santacruz et al. 2005; Alonso et al. 2006). The fact that fibrillar tau aggregates don’t appear to be neurotoxic suggests that the polymerization of monomeric tau into neurofibrillary tangles might be a defense mechanism by the cell to abrogate the toxic effects of soluble tau (Spires-Jones et al. 2009). This neuroprotective mechanism, however, is ultimately futile since the loss of functional tau compromises axonal transport along the microtubule network.

The cause of tau hyperphosphorylation in AD and related neurodegenerative diseases has been the subject of intensive investigation in recent years. It is well established that the phosphorylation of tau is a dynamic process regulated by various kinases and phosphatases. The most relevant tau kinases are glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (CDK5), extracellular signal-related kinase 2 (ERK2), and microtubule affinity-regulating kinases (MARK1, 2, 3 and 4) which are potential therapeutic targets for AD (Mazanetz and Fischer 2007; Drewes et al. 1997). Protein phosphatase 2A (PP2A), which is the primary tau phosphatase, appears to have reduced expression levels and activity in AD (Gong et al. 1995). It is therefore possible that reduced expression of PP2A is the underlying mechanism leading to abnormal tau hyperphosphorylation. Another potential mechanism proposed to induce tau hyperphosphorylation is a reduction in the amount of tau modified with O-linked N-acetylglucosamine (GlcNAc) (Arnold et al. 1996). This process, called O-GlcNAcylation, inhibits tau phosphorylation and is decreased by impaired glucose metabolism, suggesting a role for glucose metabolism in neurofibrillary degeneration (Gong et al. 2006). Lastly, as previously described, it is possible that tau hyperphosphorylation is triggered by the action of soluble Aβ assemblies at cell surface receptors (Jin et al. 2011). While there remains much to be learned about the mechanisms leading to tau pathology, new insights not only advance the understanding of AD pathogenesis but also of other tauopathies, including FTLD, frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), and chronic traumatic encephalopathy (CTE).
1.2.3 Neuroanatomical progression of Alzheimer's disease

Understanding the progression of pathological changes occurring in the brain in AD is critical in order to accurately assess a patient’s clinical course. The most widely accepted staging method for AD was established by Braak & Braak nearly thirty years ago (Braak and Braak 1991; Braak and Braak 1997). In this system, stages A-C correspond to the progression of amyloid pathology, with A indicating an early disease stage and C indicating a late stage. Typically, amyloid deposits first appear in the basal portions of the cerebral cortex, specifically the frontal and temporal lobes (Stage A). As the disease progresses, amyloid deposition increases and spreads to the adjacent neocortical association areas and the hippocampus (Stage B). In the later stages of AD the entire cerebral cortex, including primary sensory and motor areas, displays marked amyloid deposition (Stage C). At this point subcortical structures, including the striatum, thalamus, and hypothalamus are also affected. The development of neurofibrillary changes, on the other hand, encompasses six stages (I-VI) (Braak and Braak 1991; Braak and Braak 1997). The transentorhinal region within the medial temporal lobe is preferentially affected in early stages of AD and is the first to display neurofibrillary tangles (Stage I & II). As neurofibrillary pathology begins to extend beyond the transentorhinal region, regions of the temporal cortex and hippocampus become affected (Stage III), followed by the cortical association areas (Stage IV). Ultimately, the entire hippocampus becomes affected and the entire neocortex, including primary areas, develops increasingly severe pathology (Stage V & VI). Subcortical structures, including the claustrum and thalamus, also display severe neurofibrillary pathology in these later stages. Braak and Braak note that the progression of neurofibrillary pathology generally follows a better-defined pattern than amyloid, making the differentiation of stages more reliable (Braak and Braak 1991). They suggest that the ‘transentorhinal’ stage (I & II) represents the pre-clinical phase of the disease, with patients showing few, if any, symptoms, while the ‘limbic’ stage (III & IV) represents the initial stages of clinically apparent AD and the ‘neocortical’ stage (V & VI) corresponds to fully developed AD. Although these staging methods were established based on post-mortem analyses of AD brains, it may soon become possible to assess these pathological changes in the living human brain with the development of novel imaging techniques, such as PiB-PET (Mathis et al. 2002).
1.3 Somatostatin in Alzheimer’s disease

Although there is no doubt that Aβ and tau are the primary pathological mediators in AD, a number of other proteins and molecules have been shown to have secondary roles in the disease process. For example, apolipoprotein E (APOE), a glycoprotein predominantly expressed by astrocytes, has been extensively studied for its influence on Aβ aggregation and clearance (Kim, Basak, and Holtzman 2009). In fact, possession of the ε4 allele of APOE is one of the strongest genetic risk factors for AD (Corder et al. 1993). Furthermore, variants in the gene coding for triggering receptor expressed on myeloid cells 2 (TREM2), a transmembrane receptor expressed by microglia, have recently been identified as significant risk factors for non-familial AD (Guerreiro et al. 2013). This protein has been proposed to play a role in the regulation of microglial responses to Aβ (Colonna and Wang 2016). A particularly intriguing body of literature exists, dating back over 30 years, that connects the etiology of AD with the small neuropeptide, somatostatin (SST). In fact, one of the earliest biochemical differences observed in the brains of AD patients was a reduction in SST immunoreactivity (Davies, Katzman, and Terry 1980). While the role of SST in the pathogenesis of AD remains unclear, a wealth of data places this small peptide at the epicenter of AD pathology. An understanding of SST biology is therefore critical in order to understand how this small peptide might influence the course of the disease.

1.3.1 Biogenesis and physiological function of somatostatin

Somatostatin (SST) is a regulatory neuropeptide and hormone produced by a wide range of tissues including the brain, pancreas, and parts of the gastrointestinal (GI) tract (Reichlin 1983a; Reichlin 1983b). The 14 amino acid SST peptide (SST14) was first discovered in the hypothalamus as a negative regulator of growth hormone (GH) release from the anterior pituitary (Brazeau et al. 1973). A predominant N-terminally extended version of the peptide consisting of 28 amino acids (SST28) was later found to also possess biological activity (Pradayrol et al. 1980). SST14 and SST28, as well as other SST peptides of varying lengths (e.g. SST25), are derived from the proteolytic cleavage of an inactive precursor protein, preprosomatostatin (PPSST), which is coded by the SST gene on chromosome 3 in humans (Figure 1.3A) (Shen, Pictet, and Rutter 1982). Recently, an additional cleavage product of PPSST, named neuronostatin, was discovered that encompasses residues 31-43 of the preprohormone, although
the function of this peptide appears to be independent of SST (Samson et al. 2008). A similar phenomenon is observed for the closely related SST paralog, cortistatin (CST), which is generated primarily in the cerebral cortex by the cleavage of preprocortistatin (PPCST) (Spier and de Lecea 2000). Following its translation by ribosomes of the rough endoplasmic reticulum (ER), PPCST is further processed in the trans-Golgi network (TGN) where it undergoes dibasic or monobasic cleavage to generate SST14 or SST28, respectively (Goodman, Aron, and Roos 1983; Lepage-Lezin et al. 1991). In its final biologically active form, SST is a circular peptide that cyclizes through the formation of a disulfide linkage between Cys3 and Cys14 in SST14 or Cys17 and Cys28 in SST28 (Figure 1.3B,C). After traversing the regulated secretory pathway (RSP), SST is ultimately stored within electron-dense secretory granules where it awaits an appropriate stimulus to trigger its release (Xu and Shields 1993).
**Figure 1.3 Sequence alignment of PPCST & PPSST and structure of SST14.** (A) Sequence alignment of preprocortistatin (PPCST) and preprosomatostatin (PPSST). Horizontal bars indicate the signal sequence and the boundaries of the bioactive cortistatin and somatostatin. Identical residues are highlighted by black background shading. (B) and (C) Stick and surface models of SST14 with colors emphasizing the relative hydrophobicity of the constituent amino acid residues. In its functional state, SST14 cyclizes through the formation of a disulfide linkage between Cys3 and Cys14. A binding epitope spanning residues 7-10 (FWKT) within cyclic SST confers it the ability to bind with high affinity to its cognate receptors, the SSTRs.
1.3.1.1 Somatostatin as a functional amyloid

It has become apparent in recent years that in order for SST, and related peptide hormones, to be stored in secretory granules at exceedingly high concentrations, they must adopt an amyloid-like aggregation state (Maji et al. 2009). Indeed, in vitro studies using optical and electron microscopy, X-ray scattering, and vibrational spectroscopy to examine the self-assembly of SST14 under native conditions (pH ~5) revealed that SST spontaneously forms laterally associated nanofibrils with characteristics similar to amyloid fibrils (van Grondelle et al. 2007). Such amyloid-like characteristics include the propensity to bind Congo red, a fixed β-hairpin backbone of the constituent peptides, and an intramolecular hydrogen bonding network arranged with cross-β symmetry relative to the fibril axis (van Grondelle et al. 2007). Not only does this structure offer an elegant solution to a storage challenge within the cell, it also confers protection of the peptide against degradation and unwanted interactions with other molecules through the exclusion of water molecules (Sawaya et al. 2007). An important observation is that, under reducing conditions that disrupt the disulfide linkage of the endogenous peptide, SST adopts a conformation with much higher flexibility and a greater propensity to form amyloid-like aggregates (Anoop et al. 2014). Whereas it is understood that subtle changes to the native structure of SST have profound effects on its aggregation kinetics, the steps and kinetics of the disassembly of inactive, amyloid-like SST aggregates to functional, non-aggregated SST are less well defined. In addition to providing an efficient storage method, the amyloid-like conformation may also act as a sorting mechanism to direct SST, and similar peptides, through the RSP (Maji et al. 2009). It is possible that amyloid aggregation is initiated in the Golgi when prohormone concentrations are critically high. As the precursor is cleaved into the mature peptide, the rate of fibrillation may increase, thereby encouraging the formation of granule cores that exclude other proteins with lesser propensity to undergo the necessary conformational transitions. These dense granule cores can then interact with membrane lipids, which surround the SST amyloid aggregates to form the mature granule. The peptide can be stored within the secretory granules for long durations, protected from undesirable interactions and degradation until it is required. For the reasons described, SST has been added to a growing list of endogenous proteins that adopt an amyloid-like state in order to perform their physiological role. These so called ‘functional amyloids’ include cytoplasmic polyadenylation element bind protein 1 (CPEB1), a
protein that adopts an amyloid-like state to aid in long-term potentiation, and human defensin 6 (HD6), an important antimicrobial protein (Si et al. 2010; Szyk et al. 2006).

1.3.1.2 Somatostatin release and mechanisms of action

The release of SST from secretory granules is triggered by a number of different factors depending on the tissue. For example, SST release from the hypothalamus is stimulated by dopamine, GH, neurotensin, glucagon, acetylcholine, α₂-adrenergic agonists, and vasoactive intestinal peptide (VIP), among others (Barnett 2003). It is evident that this process involves secretory granule fusion with the plasma membrane in a Ca²⁺-dependent manner. Indeed, the influx of Ca²⁺ through voltage-gated Ca²⁺-channels following depolarization has been shown to be the primary trigger for SST release (Drouva et al. 1981). Other factors that modulate SST release include glucose and γ-aminobutyric acid (GABA), which appear to be negative regulators of this process (Berelowitz, Dudlak, and Frohman 1982; Harty and Franklin 1983). Following secretory granule fusion with the plasma membrane, SST is ultimately liberated from the cell and can exert its biological effect on a multitude of target tissues.

In general, SST exerts inhibitory actions on a broad range of cell types, including neurons, neuroendocrine cells, and various cells within the GI tract. Table 1.1 summarizes some of the key physiologic actions of SST on various target organs. The main method by which SST exerts its widespread biological effects is through binding to five specific cell-surface somatostatin receptors (SSTRs). The genes coding for the five SSTRs (SSTR1-5) are located on chromosomes 14, 17, 22, 20, and 16, respectively (Reisine and Bell 1995). Since SSTR2 is the only SSTR that harbors an intron, two forms of SSTR2, SSTR2A and SSTR2B, exist due to alternative splicing (Vanetti et al. 1992). A four amino acid sequence (Phe-Trp-Lys-Thr), encompassing residues 7-10 of SST14, has been found to be critical for SST binding to the SSTRs (Figure 1.3C) (Veber et al. 1979). Since the SST paralog, CST, contains this sequence as well, it can also interact with the SSTRs and activate SSTR-mediated signaling pathways (Spier and de Lecea 2000). The expression of SSTRs varies depending on the tissue and cell type. For example, SSTR2 is the primary subtype in the brain, while SSTR1 is the major subtype in the GI tract (Reisine and Bell 1995).
<table>
<thead>
<tr>
<th>Target Organ</th>
<th>Biological Effect</th>
<th>References</th>
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<tbody>
<tr>
<td>Brain</td>
<td>• Modulation of neuronal synaptic inputs through presynaptic inhibition</td>
<td>(Epelbaum 1986; Tallent 2007; Barnett 2003; Boehm and Betz 1997)</td>
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<tr>
<td></td>
<td>• Inhibition of neurotransmitter release (e.g. dopamine, glutamate, norepinephrine)</td>
<td></td>
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<td></td>
<td>• Dampening of central and peripheral nervous system functions (e.g. behavior, cognition, sensory, motor)</td>
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<tr>
<td>Anterior pituitary</td>
<td>• Inhibition of growth hormone, thyroid stimulating hormone, and prolactin release</td>
<td>(Brazeau et al. 1973; Epelbaum 1986)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>• Inhibition of insulin and glucagon release from the endocrine pancreas</td>
<td>(Boden et al. 1975; Heintges, Luthen, and Niederau 1994; Rutter 2009)</td>
</tr>
<tr>
<td></td>
<td>• Inhibition of bicarbonate and digestive enzyme release from the exocrine pancreas</td>
<td></td>
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<tr>
<td>Stomach</td>
<td>• Inhibition of gastrin release from G-cells; pepsin release from chief cells; gastric acid release from parietal cells</td>
<td>(Krejs 1986; Creutzfeldt and Arnold 1978)</td>
</tr>
<tr>
<td></td>
<td>• Reduction of gastric motility</td>
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<tr>
<td>Small intestine</td>
<td>• Inhibition of nutrient absorption and secretin release</td>
<td>(Boden et al. 1975; Krejs 1986)</td>
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<tr>
<td></td>
<td>• Modulation of intestinal motility</td>
<td></td>
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<tr>
<td>Gallbladder</td>
<td>• Inhibition of contraction and bile release</td>
<td>(Fisher et al. 1987)</td>
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<tr>
<td>Kidney/Adrenal gland</td>
<td>• Increased urinary volume, renal plasma flow, and glomerular filtration rate</td>
<td>(Vora et al. 1986; Gomez-Pan et al. 1976; Boscaro et al. 1982)</td>
</tr>
<tr>
<td></td>
<td>• Inhibition of renin and aldosterone secretion</td>
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Table 1.1 Biological effect of SST on various target organs.
SSTR1-5 are members of the family of G protein-coupled receptors (GPCRs), which are membrane receptors characterized by seven transmembrane α-helices (Patel 1997). A key feature of this family of receptors is the interaction with several intracellular GTP binding proteins (G proteins), which mediate the downstream signaling pathways following ligand binding. SSTR activation initiates four predominant signaling pathways, each involving a specific G protein: (1) recruitment and inhibition of adenylyl cyclase via Ga_{i1} and Ga_{i2}; (2) coupling to K^+ channels via Ga_{i3}; (3) coupling to Ca^{2+} channels via Ga_{o2}, β1, β3, and γ3; and (4) coupling to protein phosphatases via an unclear process (Reisine and Bell 1995; Patel 1997; Ben-Shlomo and Melmed 2010). The inhibition of adenylyl cyclase is a feature shared by all SSTRs and results in a reduction in the intracellular concentration of cyclic adenosine monophosphate (cAMP), an important second messenger that typically promotes hormone release (Koch and Schonbrunn 1984; M. Tallent and Reisine 1992; Liu et al. 1994). Coupling of SSTRs to K^+ channels results in channel activation and subsequent K^+ efflux, resulting in reversible hyperpolarization of the cell (Yatani et al. 1987; Sims, Lussier, and Kraicer 1991). A consequence of this hyperpolarization is a decrease in intracellular levels of Ca^{2+} due to the inhibition of voltage-gated Ca^{2+} influx channels (Koch, Blalock, and Schonbrunn 1988). A further blockade of inward Ca^{2+} currents is induced by a direct inhibition of voltage-gated Ca^{2+} channels by Ga_{o2} (Ikeda and Schofield 1989). This pathway, which culminates in reduced intracellular Ca^{2+} levels, is thought to be a major mechanism involved in the acute inhibition of hormone release by the SSTRs. Lastly, SSTR activation has been shown to stimulate a number of intracellular phosphatases, including calcineurin, which further inhibits hormone exocytosis, serine/threonine phosphatases, which dephosphorylate and activate additional K^+ and Ca^{2+} channels, and phosphotyrosine phosphatases, that inhibit a number of factors involved in cell proliferation (Renstrom et al. 1996; White, Schonbrunn, and Armstrong 1991; Liebow et al. 1989). While the SSTRs collectively activate these signaling pathways as a receptor family, it appears that some of these signaling mechanisms are distinct between individual SSTRs, with other mechanisms overlapping between SSTR subtypes. For example, the functional coupling to adenylyl cyclase appears to be a common feature of SSTR1-5, while only SSTR3 is capable of promoting the dephosphorylation and induction of the tumor suppressor protein, p53, leading to apoptosis (Sharma, Patel, and Srikant 1996; Patel 1997). Furthermore, SSTRs are capable of forming homo- and heterodimers, which has consequences for ligand binding and receptor functional
activity (Rocheville et al. 2000). These characteristics of the SSTRs allow for an intricate level of modulation of receptor function, leading to cell-type specific effects of SST binding.

1.3.2 The role of somatostatin in human disease

Given the breadth of SST’s physiological functions and anatomical distribution, it is no surprise that SST biology is involved in several human diseases. Owing to SST’s role in the inhibition of hormone release from various endocrine organs, long-lasting SST analogs are the primary pharmacological treatment for various neuroendocrine tumors (NETs) that over secrete a particular hormone. The drug octreotide, a cyclic octapeptide intended to mimic endogenous SST, was the first SST analog to be developed and displays increased pharmacological efficacy at SSTR2 and SSTR5, increased resistance to degradation, and longer half-life than SST14 (Bauer et al. 1982). These properties of octreotide allow it to inhibit GH release from the anterior pituitary with 45 times more potency than SST14, making octreotide particularly well suited to treat acromegaly, a disorder caused by the excess release of GH by a pituitary adenoma (Freda 2002). Other SST analogs have since been developed, including lanreotide, which also activates SSTR2 and SSTR5, albeit with less potency, and pasireotide, which has high affinity for SSTR1-3 and SSTR5 (Murphy et al. 1987; Bruns et al. 2002; Freda 2002). The indications for these drugs have since expanded to include diarrhea related to AIDS and chemotherapy, postoperative complications following pancreatic surgery, acute variceal bleeding, and portal hypertension (de Herder and Lamberts 2003).

Since SSTRs are expressed by various cancer cell types, they have become an appealing cancer target and biomarker. The expression of SSTRs by cancer cells can be exploited for the purposes of in vivo tumor imaging by SST scintigraphy, in which a radiolabelled SST analog is administered to a patient with a suspected SSTR-positive tumor and is subsequently internalized by the cancer cells after receptor binding (Lamberts et al. 1990). The tissues that uptake the SST analog can be visualized by planar and emission computed tomography images taken by a γ-camera. This method has allowed for the imaging of various types of cancer and their metastases, including small cell lung cancer, breast cancer, Merkel cell tumors, and meningiomas, among others (Kwekkeboom, Krenning, and de Jong 2000). The SSTRs have also been studied as a potential target for cancer therapies, since they can initiate downstream signaling pathways that induce cell cycle arrest, inhibit cell invasion, and induce apoptosis (Pyronnet et al. 2008). Other
strategies for the targeting of cancer cells via the SSTRs include the conjugation of cytotoxic agents to SST, allowing for the efficient internalization of these drugs following receptor binding (Huang, Wu, and Chen 2000; Hofland and Lamberts 2003). SST has also been observed to indirectly inhibit tumor growth by suppressing the release of growth factors and inhibiting angiogenesis, which is a critical process for tumor growth, invasion, and metastasis (Pyronnet et al. 2008).

As alluded to earlier, a number of observations in the literature connect SST to the pathobiology of AD. The first link between SST and AD came from a systematic investigation of neuropeptides in autopsied brain tissue from AD patients and cognitively normal individuals (Davies, Katzman, and Terry 1980). By using a radioimmunoassay (RIA) for SST in human brain extracts, the immunoreactivity of SST in the hippocampus, frontal cortex, parietal cortex, and superior temporal gyrus was measured and observed to be significantly lower in AD brains compared to controls. This finding has since been corroborated by a number of investigators using similar RIA techniques as well as quantitative real time polymerase chain reaction (PCR) (Beal et al. 1985; Gahete et al. 2010; Gabriel et al. 1993). It has since been discovered, through the transcriptional profiling of human frontal cortex from individuals aged 26 to 106 years of age, that SST is amongst a subset of gene transcripts that steadily decline in abundance throughout adult life (Lu et al. 2004). Furthermore, SST has been visually observed to be involved in AD pathology, with reports of degenerating SST-immunoreactive neurons being colocalized with Thioflavin-S stained amyloid plaques in AD brains (Morrison et al. 1985; Armstrong et al. 1985). Strikingly, in these studies, 20-50% of amyloid plaques were found to contain some SST immunoreactivity with the highest incidence of co-localization occurring in layer III of the cingulate, temporal, and frontal cortices in addition to the amygdala and hippocampus. Moreover, SST has been found to regulate the transcription of two enzymes involved in the degradation and clearance of Aβ, nephrilysin and insulin-degrading enzyme (Saito et al. 2005; Tundo et al. 2012). With regard to tau pathology in AD, early data suggested that SST-expressing neurons were particularly susceptible to forming neurofibrillary tangles and undergoing neurodegeneration (Roberts, Crow, and Polak 1985). However, more recent studies examining the vulnerability of various neuron types in AD have found that the association between somatostatinergic neurons and neurofibrillary tangles is relatively weak (Daniel Saiz-Sanchez et al. 2015). The last notable connection between SST and AD was the identification of
the SST gene as a genomic region with the potential to modulate the risk of acquiring AD (Vepsalainen et al. 2007; Xue, Jia, and Jia 2009). This discovery was reported by two independent genome-wide association studies using Finnish and Chinese patient cohorts. Despite these numerous observations of SST overlapping AD pathobiology, it is unclear how or to what extent SST contributes to the pathogenic processes in AD.

1.4 Proteomic approaches to the study of neurodegenerative diseases

In recent years, the study of proteins has continued to shift from reductionist approaches in which highly purified proteins are examined in isolation to more systematic approaches where complex biological systems can be interrogated. This transition makes sense as it reflects a reality of proteins not functioning independently but within intricate regulatory networks. The systematic analysis of proteins at the level of the tissue, cell or biofluid is commonly referred to as proteomics. For decades, proteomics studies were hampered by a lack of sensitive protein sequencing technologies capable of identifying multiple proteins within a complex sample. Prior to the mid-1990s, the most common sequencing technology was Edman degradation, a technique that relies on the sequential degradation of a peptide from the N- to the C-terminus, releasing a single amino acid during each cycle that can be identified by ultraviolet (UV) absorbance spectroscopy or a similar detection method (Edman 1949). Over the past thirty years, advances in mass spectrometry (MS) instrumentation have allowed these devices to be applied to the study of larger biomolecules, including nucleic acids and proteins, rendering Edman sequencing a niche technique in use today only for specialized applications (Andersen and Mann 2000). Compared to other protein sequencing techniques, MS has the advantage of higher sensitivity, specificity, and throughput, with new experimental strategies and optimizations continuing to emerge. It is important to recognize that these advances in MS technology and the growth of the proteomics field were a result of the breakthroughs made in the related field of genomics, which highlighted the utility of exploring biological systems in a systematic, high-throughput manner (Domon and Aebersold 2006). Moreover, the databases generated by the ongoing efforts in genomics research provide the genomic DNA sequence data necessary to match mass spectra to protein sequences following MS analysis (Pandey and Lewitter 1999). In the field of neurodegenerative disease research, proteomics applications have fueled the discovery of candidate biomarkers for AD, PD,
and HD, among others, and have provided countless insights into the molecular mechanisms involved in these diseases (Shi, Caudle, and Zhang 2009; Zhang et al. 2008).

1.4.1 Mass spectrometer components & function

Despite the growing number of mass spectrometer types, which vary in their functional applications, performance characteristics, and physical principles, there are three main components that are shared across instruments: an ionization source, a mass analyzer, and a detector. The first step in any MS analysis is the conversion of analytes into gas-phase ions. For many years, the use of mass spectrometry was restricted to the study of thermostable and low-molecular-weight compounds that are more readily volatilized and ionized. The study of biomolecules, which are typically large and non-volatile, has been made possible through the development of revolutionary ionization techniques, including electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Yamashita and Fenn 1984; Karas et al. 1987). In ESI, analytes in solution are passed through a capillary with a small internal diameter in the presence of a strong electric field to generate an aerosol of charged droplets. Once freed of solvent molecules, the charged ions are directed through electric fields to the orifice of the mass analyzer. All mass analyzers measure the mass-to-charge (m/z) ratio of ions, albeit through fundamentally different mechanisms. Recently, a new state-of-the-art mass analyzer, the Orbitrap, was developed that displays high mass resolving power and mass accuracy (Zubarev and Makarov 2013). The Orbitrap analyzer is particularly well suited for the study of large biomolecules, given its ability to distinguish ions with similar m/z ratios. Three electrodes comprise this device: two are outer electrodes facing each other in a barrel-like configuration and a third spindle-like electrode located at the center of the barrel. When voltage is applied between the outer and central electrodes, ions that enter the analyzer are influenced to adopt a circular spiral-like flight path around the central electrode. The ions are then pushed to the center of the trap by an axial electric field, which initiates harmonic axial oscillations. These oscillations are detected by the outer electrodes as an image current that is Fourier-transformed to obtain the oscillation frequencies of various ions, from which their mass spectrum can be inferred (Makarov 2000).

For the study of complex mixtures of compounds, such as biological samples, the technique of tandem mass spectrometry (MS/MS) can be used, in which a second stage of MS analysis is
performed. This method is commonly used for the purposes of protein sequencing, which initially involves the chemical/enzymatic degradation of proteins in a mixture, followed by a first stage of MS to separate these ‘parent ions’ according to their $m/z$ ratio (Hunt et al. 1986). These isolated ions are then further fragmented by various dissociation methods, such as collisions with an inert gas, into ‘product ions’ and separated in a second round of MS. The amino acid sequences of these fragments can then be predicted and matched to the peptide sequences of proteins found in publicly available databases.

### 1.4.2 Discovery based applications of mass spectrometry

A powerful application of mass spectrometry is the analysis of complex mixtures of peptides, a process colloquially referred to as ‘shotgun’ proteomics. This type of discovery-based approach can be used to study the global proteome of a biological system, for example to identify as many proteins as possible in an organism, a tissue, or a specific cell type (Brewis and Brennan 2010). Such global proteome analyses are particularly useful when comparing protein expression across multiple experimental conditions, such as healthy versus disease, wild-type versus mutant, or wild-type versus knockout. The information generated in such experiments can provide valuable insights into protein signaling pathways or generate new candidate pharmacological targets and biomarkers for a given disease. In a more targeted approach, the molecular interactions that a given protein undergoes can be studied in order to generate a comprehensive list of its binding partners, a network referred to as an interactome (Charbonnier, Gallego, and Gavin 2008). A very common and efficient method to identify interacting proteins is based on affinity purification-mass spectrometry (AP-MS) (Gingras et al. 2007). As the name would suggest, this technique consists of two steps: (1) an affinity purification step to enrich proteins that bind specifically to a bait protein, followed by (2) the identification of these proteins by mass spectrometry (Dunham, Mullin, and Gingras 2012). In the first step, a ligand (bait) is immobilized on a solid support (e.g. agarose beads) in order to capture interacting proteins (prey) present in a soluble phase. Although several different approaches can be used to capture the bait, including bait-specific antibodies or ligands or cross-linking of the bait protein to a solid phase, the goal of this step always is to generate a stationary phase that has the bait protein enriched at its surface (Roque and Lowe 2008). Following the capture of the bait protein’s interacting partners, the solid phase must be stringently washed in order to remove non-specific binders and/or contaminants. A thorough post-purification wash step using salt and detergent containing
buffers will ensure a reduction in the number of false positives in the final dataset. After washing, the bait-bound proteins must be eluted from the solid phase, which can be done by pH drop, competition with a soluble antigen, or boiling in detergent-containing buffers. In the second step of AP-MS, the eluted proteins are prepared for MS analysis, which involves protease digestion (commonly by trypsin) resulting in a mixture of peptides. Analysis of the peptide mixture by MS/MS allows for the identification of all proteins present in the affinity capture eluates, which is used to generate an interactome dataset for the bait protein studied.

Since the goal of shotgun proteomics often is to determine differences in protein abundance across multiple samples, it is desirable to differentially label samples and analyze them simultaneously to avoid run-to-run variances (Rauniyar and Yates 2014). The development of isobaric mass tags has facilitated this relative quantitation of proteins by MS. These tags consist of three components: a reporter group, a mass-normalization spacer, and a reactive group (Thompson et al. 2003). Although with isobaric tagging strategies individual tags have the same mass and chemical structure, they are designed such that they vary according to the distribution of heavy isotopes ($^{13}$C and/or $^{15}$N) in the reporter group and the mass-normalization spacer. Therefore, identical peptides labeled with different isobaric tags appear as a single peak in MS scans, but upon MS/MS analysis, they can be distinguished based on characteristic reporter ions that emerge upon fragmentation of the isobaric tag (Wiese et al. 2007). The isobaric labeling follows disulfide reduction, cysteine alkylation and trypsin digestion (Dayon and Sanchez 2012). Once labeled, peptides derived from multiplex analyses can be pooled equistoichiometrically and analyzed simultaneously by MS/MS (Figure 1.4). The most common isobaric tags are tandem mass tags (TMT) and isobaric tags for relative and absolute quantitation (iTRAQ) reagents, which are most often used to simultaneously analyze up to 6 or 8 samples, respectively (Thompson et al. 2003; Ross et al. 2004; Choe et al. 2007; Dayon et al. 2008). Isobaric labeling-based quantification strategies are therefore appealing for a number of reasons, including the ability to enhance the throughput of MS analyses by reducing analytical time, eliminate run-to-run variances, and promote the consistent selection of parent ions due to the multiplexing of samples (Dayon and Sanchez 2012).
1.5 Project rationale

The goal of this research project is to systematically explore the molecular interactions of the Aβ peptide, in order to shed light on the mechanisms by which it contributes to neurodegeneration in AD. Furthermore, this project aims to investigate the long-standing, but poorly understood, relationship between AD and the biology surrounding the SST peptide. Our experimental design involves the generation of interactome datasets, using advanced mass spectrometry-based workflows, and the validation of key interactors using various orthogonal biochemical assays.
**Figure 1.4 Overview of the procedure for isobaric tagging for the relative quantitation of peptides.** In a quantitative mass spectrometry experiment, samples are first denatured then reduced and alkylated to maintain the peptide in an unfolded state. Following enzymatic (e.g., tryptic) or chemical cleavage the peptides are labeled with unique isobaric tags (e.g., TMT or iTRAQ reagents) through covalent attachment to primary amines present at their N-terminus and/or within lysine side-chains. Once labeled, the samples are pooled and analyzed in a single tandem mass spectrometry run. The first mass spectrometry stage produces a mass spectrum of all the peptides (i.e., parent ions) present in the pooled sample. Following fragmentation of these peptides, a second stage of mass spectrometry reveals the spectra of the resulting ‘product ions.’ In these spectra, the presence of unique reporter ions, derived from the fragmentation of the isobaric tags, can be observed and used to quantify the relative amount of the corresponding peptide in each sample.
Chapter 2: Identification of Somatostatin as a Novel Interactor of Aβ

Please note that the majority of this chapter, with minor revisions, was published in the following articles:


Candidate’s role: Participated in the conceptualization and execution of SST epitope mapping experiments in addition to manuscript writing, reviewing, and editing.

Summary: The amyloid beta (Aβ) peptide is central to the pathogenesis of Alzheimer’s disease (AD). Insights into Aβ-interacting proteins are critical for understanding the molecular mechanisms underlying Aβ-mediated toxicity, yet a systematic investigation of Aβ binding partners has not been reported. In order to address this shortcoming, we undertook an in-depth in vitro interrogation of the Aβ1–42 interactome using human frontal lobes as the biological source material and taking advantage of advances in mass spectrometry performance characteristics. These analyses uncovered the small cyclic neuropeptide somatostatin (SST) to be the most selectively enriched binder to oligomeric Aβ1–42. Subsequent validation experiments revealed that SST interferes with Aβ fibrillization and promotes the formation of Aβ assemblies characterized by a 50–60 kDa SDS-resistant core. Further to this discovery, the distributions of SST and Aβ overlap in the brain and SST has been linked to AD by several additional observations. The fact that SST is one of several neuropeptide hormones that acquire amyloid properties before their synaptic release places the interaction between SST and Aβ among an increasing number of observations that attest to the ability of amyloidogenic proteins to influence each other. A model is presented which attempts to reconcile existing data on the involvement of SST in the AD etiology.
2.1 Materials and methods

2.1.1 Peptides
Synthetic human Aβ1-42 (catalog number AS-24224), Aβ1-40 (catalog number AS-24236), [Arg8]-Vasopressin (catalog number AS-24289) and SST14 (catalog number AS-24277) were purchased from Anaspec, Inc. (Fremont, CA, USA). CST17 (catalog number H-5536) and CST29 (catalog number H-6458) were obtained from Bachem Americas, Inc. (Torrance, CA, USA). The biotinylated Aβ peptides, including biotin-Aβ1-42, Aβ1-42-biotin, and truncated or mutant SST peptides were synthesized by LifeTein LLC (Hillsborough, NJ, USA).

2.1.2 Antibodies
Primary antibodies used in this study were the anti-Aβ1-16 antibody (6E10) (catalog number 803015), the anti-Aβ17-24 antibody (4G8) (catalog number 800701), and the anti-Aβ1-42 antibody (12F4) (catalog number 805501), which were all sourced from BioLegend (San Diego, CA, USA).

2.1.3 Western blot
All Western blot reagents were purchased from Thermo Fisher Scientific (Burlington, ON, Canada). For denaturing gels, peptide samples were mixed with Bolt LDS Sample Buffer (catalog number B0007) in the presence of 2.5% 2-mercaptoethanol and boiled at 70°C for 10 minutes before loading. The samples were separated on Bolt 12% Bis-Tris Plus gels (catalog number NW00125BOX) in MES SDS Running Buffer (catalog number NP0002) at 100 to 120 V for 1.5 to 2 hours. For native gels, the peptide samples were mixed with Novex Tris-Glycine Native Sample Buffer (catalog number LC2673) without boiling before loading. The samples were separated on SDS-free 4-20% Tris-Glycine Mini Gels (catalog number XP04202BOX) in Novex Tris-Glycine Native Running Buffer (catalog number LC2672) at 150 V for 1.5 hours. 0.3 to 0.5 mg of Aβ peptide were loaded to each lane for both denaturing and native gels. For all immunoblot analyses, peptides were transferred to polyvinylidene difluoride (PVDF) membranes at 50 V in Tris-Glycine buffer containing 10-20% methanol for 1.5 to 2 hours. Membranes were blocked for 2 hours in conventional Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% fat-free milk and probed overnight with the respective primary antibodies. After at least three washes with TBST, membranes were incubated for 2 hours with 1:2000 to 1:5000 diluted anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad
Laboratories, Inc., Hercules, CA, USA). The band signals were visualized using enhanced chemiluminescence reagents (catalog number 4500875; GE Health Care Canada, Inc., Mississauga, ON, Canada) and X-ray films.

2.1.4 Preparation of monomeric versus pre-aggregated Aβ, SST and other neuropeptides
Aβ peptides were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg peptide per mL for one hour at room temperature, then dried in a centrifugal evaporator and stored at 80°C until use. Monomeric Aβ peptides were prepared by dissolving peptides in dimethyl sulfoxide (DMSO) at a concentration of 2 mM and further diluting them in phosphate buffered saline (PBS, pH 7.4) to 100 mM, followed by centrifugation (14,000 g, 20 minutes) to remove traces of insoluble aggregates. Oligomeric Aβ was created by incubating the monomeric preparation at 4°C for 24 hours. The Aβ oligomers were then purified by centrifugation (14,000 g, 20 minutes). The supernatant containing Aβ oligomers was further diluted as required and indicated in figure legends describing specific experiments. When high-purity monomeric Aβ peptides at concentrations below 10 mM were required for interactome and ThT assay analyses, the solubilized Aβ preparations were passed through a size-exclusion column (see below for details). Pre-aggregated SST was generated by incubating 1 mM SST in PBS at 37°C for 1 hour with shaking at the speed of 700 rpm.

2.1.5 Affinity capture of Aβ1-42 and its binding proteins in human frontal lobe extracts
The biotinylated Aβ oligomers or monomers were captured on Streptavidin UltraLink Resin beads (Thermo Fisher Scientific, Burlington, ON, Canada) by overnight incubation in PBS at 4°C and continuous agitation on a slow-moving turning wheel. Additional negative control samples were generated by saturation of Streptavidin UltraLink Resin (catalog number 53113, Thermo Fisher Scientific, Inc.) with biotin. Subsequently, the bait peptide- or biotin-saturated beads were washed with lysis buffer (0.15% digitonin, 150 mM NaCl, 100 mM Tris, pH 8.0). Human frontal lobe tissue samples from individuals (two males and two females) who had died in their early 70s of non-dementia causes served as the biological source material. These samples were adopted from a former Canadian Brain Tissue Bank at the Toronto Western Hospital and are held in -80°C freezers in the bio-bank of the Tanz Centre for Research in Neurodegenerative...
Diseases. 1 g pieces each of these brain tissue samples were combined and homogenized in Lysis Buffer supplemented with cOmplete Protease Inhibitor Cocktail (Roche, Mississauga, ON, Canada). Following the removal of insoluble debris by centrifugation for 30 minutes at 14,000 g, the protein concentration was adjusted to 2 mg/mL before the brain homogenates were added to the pre-saturated affinity capture beads for overnight incubation at 4°C. Following the affinity capture step, the affinity capture beads (100 mL per biological replicate) were extensively washed in three consecutive wash steps with a total of 150 mL of lysis buffer. Subsequently, the beads were additionally washed with 50 mL of 20 mM HEPES, pH 7.0, and transferred to Pierce Spin columns (catalog number 69705, Thermo Fisher Scientific, Inc.) to remove primary amines stemming from the Tris buffer and to prepare the samples for elution. Captured proteins were finally eluted by rapid acidification mediated by a solution comprising 0.2% trifluoro-acetic acid and 20% acetonitrile in deionized water (pH 1.9).

2.1.6 Sample preparation for interactome analyses
Affinity-capture eluates were essentially processed as described before (Gunawardana et al. 2015; Mehrabian et al. 2014; Jeon and Schmitt-Ulms 2012). Briefly, sample tubes were moved to a centrifugal evaporator to remove the organic solvent. Additional acidity of the sample was removed following the addition of water and continuous evaporation. Subsequently, protein solutions were denatured by the addition of 9 M urea (to achieve a final concentration of 6 M urea) and 10 minutes incubation at room temperature. Next, the pH was raised by the addition of 100 mM HEPES, pH 8.0, and proteins were reduced for 30 minutes at 60°C in the presence of 5 mM Tris (2-carboxyethyl) phosphine (TCEP), and alkylated for 1 hour at room temperature in the presence of 10 mM 4-vinylpyridine (4-VP). To ensure that the residual urea concentration did not exceed 1.5 M, protein mixtures were diluted with 50 mM tetraethylammonium bromide (TE), pH 8.0, to a total volume of 100 mL. Samples were then digested with side-chain-modified porcine trypsin (Thermo Fisher Scientific, Burlington, ON, Canada) overnight at 37°C. The covalent modifications of primary amines with isobaric labels provided in the form of tandem mass tag (TMT) reagents (Thermo Fisher Scientific, Inc.) or isobaric tagging for relative and solute quantitation (iTRAQ) reagents (Applied Biosystems, Foster City, CA, USA) followed instructions provided by the manufacturers. Equal amounts of the labeled digests were pooled into a master mixture and purified with C18 (catalog number A5700310) or SCX (catalog number A5700410) Bond Elut OMIX tips (Agilent Technologies, Inc., Mississauga, ON,
Canada) using manufacturer instructions. Peptide mixtures were finally reconstituted in 0.1% formic acid and analyzed by tandem mass spectrometry analysis on a Tribrid Orbitrap Fusion instrument. Instrument parameters during the data acquisition were as described in detail before (Gunawardana et al. 2015).

2.1.7 Post-acquisition data analyses

The post-acquisition data analyses of interactome data sets was conducted against the human international protein index (IPI) database (Version 3.87) which was queried with Mascot (Version 2.4; Matrix Science Ltd, London, UK) and Sequest HT search engines within Proteome Discoverer software (Version 1.4; Thermo Fisher Scientific, Burlington, ON, Canada). Spectra exceeding a stringent false discovery rate (FDR) target of DCn of 0.05 for input data and a FDR of 0.01 for the decoy database search were detected and removed by the Percolator algorithm (Käll et al. 2007) as described before (Gunawardana et al. 2015). PEAKS Studio software (Version 6.0; Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used to assess the reproducibility of nano-HPLC separations. A maximum of two missed tryptic cleavages and naturally occurring variable phosphorylations of serines, threonines and tyrosines were considered. Other posttranslational modifications considered were carbamylations, oxidation of methionines and deamidation of glutamines or asparagines. Mass spectrometry data sets have been deposited to the ProteomeXchange Consortium (Vizcaíno et al. 2014) via the PRIDE partner repository (Vizcaíno et al. 2013) with the dataset identifier PXD004867 and have been made fully accessible.

2.1.8 Kinetic aggregation assay

Assay procedures were largely based on a protocol described by Sarah Linse’s group (Hellstrand et al. 2010). Briefly, Aβ1-42 and/or SST14 were prepared in assay buffer (20 mM sodium phosphate, pH 8.0, 200 mM EDTA and 0.02% NaN₃) or in PBS, pH 7.4, at concentrations specified in the individual figures. 100 mL of peptide solutions were supplemented with 25 mM Thioflavin T (ThT) (catalog number T3516, Sigma-Aldrich Canada, Oakville, ON, Canada) and loaded into 96-Well Half-Area Microplates (catalog number 675096, Greiner Bio One International, Kremsmünster, Austria). The subsequent plate incubation proceeded at 37°C with shaking at 700 rpm for 4 of every 5 minutes in a microplate reader (CLARIOstar, BMG Labtech, Guelph, ON, Canada) for overall durations specified in individual figures. ThT fluorescence was
measured every 5 minutes at excitation and emission wavelengths of 444 nm and 485 nm, respectively.

2.2 Results

2.2.1 The Aβ interactome
The primary objective of this study was to generate an in-depth inventory of human brain proteins that oligomeric preparations of Aβ1-42 can bind to using an unbiased in vitro discovery approach. Synthetic Aβ1-42 peptides and brain extracts generated from adult human frontal lobe tissue served in these studies as baits and biological source materials, respectively. oAβ1-42 was prepared by aggregating the peptide at 4°C for 24 hours, using previously described procedures known to generate amyloid-β-derived diffusible ligands (ADDLs) (De Felice et al. 2009; Krafft and Klein 2010). Because the interaction with a given binding partner may involve a binding epitope that comprises N- or C-terminal residues of Aβ1-42, initially two separate experiments (I and II) were conducted, which differed in the orientation designated for tethering the oAβ1-42 bait to the affinity matrix. To facilitate meaningful comparisons across experiments, the method of Aβ1-42 capture was not based on immunoaffinity reagents. Instead, alternative Aβ1-42 baits were equipped with biotin moieties attached to the N- or C-terminus by a 6-carbon linker chain, enabling their consistent affinity-capture on streptavidin agarose matrices. Large aggregates were removed prior to the bait capture step by centrifugal sedimentation. Biotin-saturated streptavidin agarose matrices served as negative controls and three biological replicates of samples and controls were generated for each interactome dataset by reproducing the affinity-capture step side-by-side on three separate streptavidin agarose affinity matrices that had been saturated with the biotinylated baits. To identify differences in protein-protein interactions of monomeric versus oligomeric Aβ1-42, a third interactome experiment (III) was conducted in which oAβ1-42-biotin or mAβ1-42-biotin served as baits. Digitonin-solubilized brain extracts, which are known to primarily comprise extracellular and cellular proteins (except for nuclear proteins) served as biological starting materials, consistent with the main subcellular areas previously reported to harbor Aβ. Following extensive washes of affinity matrices in their protein-bound state, binders to the bait peptides were eluted by rapid acidification, fully denatured in 9 M urea, and trypsinized. To avoid notorious confounders related to variances in the subsequent handling and analysis of samples, individual peptide mixtures were labeled with distinct isobaric tandem mass
tags (TMT) in a six-plex format, then combined and concomitantly subjected to ZipTip-based pre-analysis cleanup by strong cation exchange (SCX) and reversed phase (RP) separation. Four-hour split-free reversed phase nanospray separations were online coupled to an Orbitrap Fusion Tribrid mass spectrometer, which was configured to run an MS³ analysis method. Tandem MS spectra were matched to peptide sequences by interrogating the human international protein index (IPI) using Sequest and Mascot algorithms. The relative levels of individual peptides in the six samples could be determined by comparing the intensity ratios of the corresponding TMT signature ions in the low mass range of MS³ fragment spectra.

The three comparative Aβ1-42 interactome analyses (Experiments I-III) conducted in this study led to mass spectrometry datasets, which were characterized by similar benchmarks of data quality and enabled confident assignments of several thousand mass spectra to human peptides. For example, Experiment I generated 9661 spectra, which passed confidence criteria applied. These filtered spectra could be matched to 4352 unique peptides, which in turn formed the basis for the identification of 1074 protein groups. The designation ‘protein groups’, as opposed to ‘proteins’, reflects a reality of a subset of tryptic peptide sequences not being uniquely associated with a specific protein. Whenever encountered, only ambiguous assignments are possible. For this specific dataset, the 1074 protein groups were annotated to comprise 1619 unique proteins. No attempt was made to resolve this residual source of ambiguity at the individual peptide level. Instead, a majority of uncertain identifications were removed by requiring protein identifications to be based on the confident assignment of at least three unique peptides with a minimum length of six amino acids.

A more detailed analysis of the list of biotin-oAβ1-42 (B-oAβ1-42) candidate interactors revealed that all top-listed 50 candidates were identified on the basis of more than ten peptide-to-spectrum matches (PSMs) and with sequence coverages exceeding 50% of their primary structures. Moreover, indicative of high selectivity of the affinity capture procedures applied, the abundance levels of these 50 candidate interactors in the B-oAβ1-42-specific samples exceeded their levels in the negative control sample by more than four-fold. Not surprisingly, the Aβ peptide itself was amongst the proteins identified whose enrichment levels in specific versus unspecific affinity capture samples were most pronounced. In contrast, assignments of APP peptides that map to regions outside of Aβ were low scoring (that is, did not pass a 95%
significance threshold) and therefore not credible. The highest levels of B-oAβ1-42 co-enrichment were exhibited by peroxiredoxin-5, r-1A, lactate dehydrogenase, hypoxanthine-guanine phosphoribosyltransferase, ATP synthase subunits beta and gamma, and acyl CoA thioester hydrolase. Several of the Aβ1-42-biotin candidate interactors revealed by this analysis were previously known to interact with Aβ peptides, including ATP synthase (Verdier et al. 2005; Schmidt et al. 2008) and glyceraldehyde-3-phosphate dehydrogenase (Verdier et al. 2005; Verdier et al. 2008), and/or had been shown to be present at altered abundance levels in cells exposed to Aβ (Lovell et al. 2005).

To determine the extent to which the binding of individual Aβ interactors was governed by Aβ aggregation, we next compared directly the interactome of proteins that bind to monomeric versus oligomeric preparations of the Aβ1-42-biotin bait. This experiment revealed a preference amongst the most highly enriched Aβ1-42 candidate interactors for binding to pre-aggregated bait peptides. Exceptions represented the robust and preferred binding of histones H2, H3 and H4 to monomeric Aβ1-42 bait matrices. Moreover, a majority of proteins, which exhibited lower levels of Aβ1-42 co-enrichment in Experiments I and II, were observed to bind preferentially to monomeric Aβ bait peptides.

2.2.2 Pre-aggregated Aβ binds to SST14 but not to its preprosomatostatin precursor through a binding epitope in the N-terminal half of Aβ1-42

When we waived the requirement that peptides had to be at least six amino acids long to be considered for protein identification, a tandem mass spectrum assigned to the five amino acid sequence ‘NFFWK’ came to the fore, which exhibited pronounced preference for binding to C-terminally tethered oAβ1-42-B. A query of human genome databases revealed that this peptide, owing to its unusual composition, and despite its short length, could only have originated from the well-known paralogs preprosomatostatin or preprocortistatin (Figure 2.1A). This conclusion was strengthened by the fact that this peptide is naturally preceded by a tryptic cleavage site and the exquisite match between observed and in silico predicted fragment ions (Figure 2.1C). In light of the high intensity ion counts of fragments observed for this peptide and its robust co-enrichment with oAβ1-42-B in three biological replicates, it first seemed puzzling that the identification of this protein group was not corroborated by other spectra that map to sequences outside of the ‘NFFWK’ sequence stretch. Searching for a plausible explanation, it became
apparent that preprosomatostatin and preprocortistatin give rise to cyclic neuropeptide hormones through a series of posttranslational trimming steps, and the ‘NFFWK’ peptide is the only tryptic peptide derived from the mature hormone that is of sufficient length and distinct sequence to be readily identifiable by MS (Figure 2.1A). Although neither preprosomatostatin nor preprocortistatin had passed stringent filtering criteria required for inclusion in the Aβ interactome data tables (because their identification could not be based on at least three unique peptides with a minimum length of six amino acids), close inspection of Experiment III data under omission of these filters suggested that mature somatostatin was not only present in the dataset but represented the protein, whose levels were most selectively enriched in oAβ1-42-B affinity capture eluates. This conclusion was further corroborated by two additional PSMs that mapped to regions outside the SST14 neuropeptide sequence and one additional five amino acid peptide of the sequence ‘TFTSC’ that could be assigned (albeit not unambiguously) to the mature SST14 neuropeptide sequence itself. More specifically, consistent with the notion that Aβ1-42 interacts separately with the SST14 neuropeptide and preprosomatostatin precursor, the distributions of TMT signature ions derived from tryptic ‘NFFWK’ and ‘TFTSC’ peptides derived from the mature SST14 neuropeptide exhibited identical TMT signature ion profiles patterns that differed fundamentally from the respective TMT profiles of preprosomatostatin peptides which mapped to sequences upstream of the SST14 sequence domain. In agreement with the interpretation that the ‘NFFWK’ had probably originated from SST14, not CST17, no peptides were observed in this or other experiments that could be uniquely assigned to preprocortistatin. Finally, these experiments repeatedly established that the ‘NFFWK’ peptide binds preferentially to oligomeric (pre-aggregated) but not monomeric Aβ1-42- biotin (Figure 2.1B).

2.2.3 SST14 and CST17 delay Aβ1-42 aggregation in a Thioflavin T fluorescence assay

To explore the influence of SST14 on Aβ1-42 more rigorously, a Thioflavin T (ThT) fluorescence assay was applied that incorporated previously reported methodology advancements (Hellstrand et al. 2010), including the removal of residual Aβ1-42 aggregates by size-exclusion chromatography immediately prior to the recording of ThT fluorescence spectra (Figure 2.1D). The application of this method revealed that the presence of 15 mM SST14 in the reaction mix reproducibly extended the lag phase of Aβ1-42 aggregation by more than two hours.
and lowered the signal amplitude during the subsequent stationary phase (Figure 2.1E). No delay was observed when SST14 was replaced by the AVP negative control peptide. Importantly, the SST14-dependent lag phase extension correlated directly with the concentration of SST14 in the reaction mix (Figure 2.1E).

When Aβ1-42, at a concentration of 50 µM, was subjected to similar aggregation conditions in the absence of ThT, analysis by negative stain electron microscopy revealed a reduction in aggregate size when Aβ1-42 was incubated with SST versus Aβ1-42 alone (Figure 2.1F). Strikingly, only oligomeric structures were observed when Aβ1-42 was incubated in the presence of equimolar concentrations of SST, whereas the incubation of Aβ1-42 alone resulted in the appearance of characteristic amyloid fibrils.

2.2.4 Aβ1-42 forms a distinct 50-60 kDa SDS-stable complex in the presence of SST14

When ThT assay fractions, which had been incubated in the presence of SST14 (or CST17) for 18 hours, were analyzed by Western blotting without prior boiling in SDS, 6E10-reactive bands that migrated at 50-60 kDa became apparent (Figure 2.1G, lanes 2 and 3). These bands, which were not seen in samples incubated either without these peptides or with AVP, were even more pronounced when the same samples had been boiled in the presence of SDS (Figure 2.1G, lanes 6, 7, 10, and 11). Interestingly, similar bands were not observed when Aβ1-40 served as the substrate for aggregation (Figure 2.1G, lanes 13-16), suggesting that the two most C-terminal residues of Aβ1-42 confer properties that are essential for the formation of the 50-60 kDa oligomeric assemblies. Only when SDS was present in the sample buffer, low mass bands appeared in the Western blot that migrated at a level expected for the monomeric Aβ peptide, consistent with the interpretation that these peptides were released from larger oligomeric or fibrillar aggregates under denaturing conditions (Figure 2.1G, lanes 5-16). Strikingly, in samples that contained SST14 (or CST17) a fraction of these low mass bands were occasionally (in well-resolved gels) observed to migrate at levels expected for heterodimers of Aβ1-42 and SST14 (or CST17) (Figure 2.1G, lanes 6, 7, 10 and 11). Consistent with the 3-amino acid smaller size of SST14, relative to CST17, the respective heterodimers containing SST14 migrated slightly faster than those containing CST17. A five-fold increase in Aβ1-42 levels in the reaction mix further emphasized the appearance of these low mass heterodimers but also
revealed strong signals migrating at the expected size of Aβ1-42 dimers (Figure 2.1G, lanes 9 to 12). Notably, whereas evidence for heterotrimers composed of two Aβ1-42 molecules and one SST14 (or CST17) molecule was never obtained, we occasionally observed signals that migrated at molecular masses expected for SDS-stable complexes consisting of three Aβ1-42 peptides linked to SST14 (or CST17) (Figure 2.1G, lanes 10 and 11). Corroborating this interpretation was the fact that bands matching this size were never observed in fractions lacking SST14 (or CST17) or containing AVP as a negative control peptide. These biochemical analyses indicated the formation of SDS-stable oligomeric complexes of Aβ1-42 and SST14 (or CST17) made of building blocks comprising one or three Aβ1-42 peptides.
A Preprocortistatin (1) MTFSSDLLEASGATATALPEPQFT--PROSEHCEGAAIRKSS--LLEPFLAWF
Preprosomatostatin (1) -------TSGACDAALHLVLAGSCGTYVEDPLRQFWKLAAAGQELAKHFLAELLE

Signal sequence

B

200 µg synthetic Aβ1-42
gel filtration (Superdex 75)
1 µM monomeric Aβ1-42
96-well microplate

C

E

F

G

H

Figure 2.1 Discovery and validation of SST-Aβ interaction. (A) Sequence alignment of preprocortistatin and preprosomatostatin. The signal sequence and the boundaries of the bioactive cortistatin and somatostatin peptides are indicated by horizontal bars. Identical residues

Preprocortistatin (57) TSCASAGFLEG---------ARVARHQEGFGQORQPRDMPF[β1-42]-biotin
Preprosomatostatin (60) PMCTENDTESLSQAEQDEMRLG1SANNLMAFERRACG[β1-42]-biotin

MW [kDa]

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

native gel (no SDS) SDS-PAGE Aβ interaction domain

SST-14 SST-28

SSTR1-5 core binding domain

Asn5 Trp8 Phe7 Phe11 Lys9 Thr10 Phe6

Asn5Phe6Phe7Lys9Thr10Phe11

Signal sequence

Aβ interaction domain
are highlighted by black background shading, and peptide sequences observed by mass spectrometry are shown in colored fonts. (B) Expanded view of MS3 spectrum derived from ‘NFFWK’ parent spectrum (shown to the right) in interactome study based on oAβ1–42-biotin baits and mAβ1–42-biotin negative controls. In this view, the relative intensities of tandem mass tag signature ions reflect the relative abundances of the ‘NFFWK’ peptide in side-by-side generated affinity purification eluate fractions, indicating preferential binding of SST to pre-aggregated oAβ1–42. (C) Example tandem MS spectrum supporting the identification of the peptide with amino acid sequence ‘NFFWK’. Fragment masses attributed to B- and Y-ion series are shown in red and blue colors, respectively. (D) Workflow of ThT-based aggregation assay. (E) SST14 delays Aβ1–42 aggregation in ThT fluorescence assay in a SST14 concentration dependent manner. (F) Negative stain electron microscopy of Aβ1–42 and Aβ1–42–SST14 complexes. Top panel: Aβ1–42 was fibrillized in PBS at a concentration of 50 mM. Individual Aβ1–42 amyloid fibrils and small clusters were visualized. Bottom panel: Incubation of equimolar concentrations (50 mM) of Aβ1–42 and SST14 under identical conditions resulted in oligomeric assemblies only. No amyloid fibrils were observed. Magnification bars = 100 nm. (G) Immunoblot analyses with an antibody directed against an N-terminal Aβ epitope (6E10) reveal that CST17 (or SST14) co-assemble with Aβ1–42 into oligomers of 50–60 kDa that withstand boiling (lanes 2 and 3) but partially disintegrate in the presence of SDS. Note bands of 5–6 kDa, consistent with the existence of SDS-resistant heterodimeric complexes of mAβ1–42 and SST14 (or CST17), and the well-defined oligomeric bands of 50 and 55 kDa (lanes 6 and 7) that were observed in samples derived from the co-incubation of SST14 (or CST17) with Aβ1–42, but not Aβ1–40 (compare lanes 6 and 7 with lanes 14 and 15). Note also that signals interpreted to represent trimeric Aβ1–42, but not dimeric Aβ1–42, can be seen to migrate slower in the presence of SST14 (or CST17) but not in the presence of the negative control peptide AVP (compare lanes 9 and 12 with lanes 10 and 11). Black arrowhead labeled with ‘m’, ‘d’, and ‘t’ designate bands interpreted to consist of monomeric, dimeric and trimeric Aβ1–42. Green and red arrowheads were used to label bands interpreted to represent SDS-stable heteromeric building blocks consisting of SST14 (or CST17) bound to monomeric and trimeric Aβ1–42, respectively. (H) Model of SST14, showing the position of its disulfide bridge between cysteine 3 and 14, and the binding domains required for docking to its SST receptors or Aβ.

2.2.5 Tryptophan-8 is critical for SST14-dependent perturbation of Aβ1-42 seeding and fibril growth

To delineate key residues and minimal components required for SST to interact with Aβ1-42, we next had a series of SST-derived peptides custom-synthesized that differed in one or two amino acids from the wild-type SST sequence or were truncated, thereby missing the internal disulfide bridge. Based on prior high-resolution NMR models available for the cyclic peptide and structure-activity relationship data derived from SST-receptor docking studies, we hypothesized that a striking hydrophobic ‘belt’ composed of three phenylalanines and a tryptophan, which is flanked by two lysine side-chains in the 3D rendering of SST (Figure 2.1H), might also be
critical for binding to Aβ1-42. With regard to the juxtaposed phenylalanines, we were in particular interested to learn if the well-known ability of their side-chains to engage in pi stacking plays a role in the SST-Aβ1-42 interaction. Interestingly, the replacement of one or two of the phenylalanines to leucines did not rescue the lag phase extension phenotype in the ThT fluorescence assay, indicating that the aromatic nature of these residues is not essential for the interaction (Figure 2.2A). However, when we replaced the single tryptophan present in position 8 both the lag phase extension and the reduction in total ThT fluorescence observed in the presence of wild-type SST were rescued (Figure 2.2A). This outcome was documented when tryptophan was replaced with alanine, proline, histidine or tyrosine. The fact that even the replacement with tyrosine rescued the lag phase extension phenotype indicates that tryptophan does not merely act in this context by providing an aromatic side-chain but suggests that other structural features present in tryptophan provide the necessary fit for binding to Aβ1-42 in this PBS-based system.

Analogous experiments with truncated SST peptides revealed that a peptide encompassing SST residues 5-11 can replace wild-type SST with regard to its effect on Aβ1-42 (Figure 2.2B), thereby upholding the conclusion that the ability of SST to form its internal disulfide bridge is not essential for this effect. However, further stripping of one amino acid from each end of this peptide resulted in an SST6-10 peptide, which lacked the ability to influence Aβ1-42 aggregation (Figure 2.2B).
Figure 2.2 Epitope mapping experiments reveal that tryptophan-8 in SST14 sequence is essential for lag phase extension of Aβ1-42 in ThT incorporation assay. (A) and (B) Thioflavin T absorbance assay data based on SST point mutants and deletion constructs, respectively. Please see legend for sample compositions.

2.3 Discussion

The current study was conducted with the intent to produce an in-depth inventory of proteins within the human brain that oAβ1-42 binds to. The study made use of a hypothesis-free discovery approach that capitalized on advanced workflows for the high-pressure nanoflow reversed-phase separation and relative quantitation of peptides, as well as recent improvements to mass spectrometry instrumentation. Taken together, these advances afforded an unprecedented depth of analysis of oAβ1-42 interactors in three independent interactome datasets, each undertaken with three biological replicates of samples and controls. More specifically, these analyses facilitated the direct comparisons of binders to oAβ1-42-baits that were tethered to
affinity capture matrices through N- or C-terminal biotin moieties, or had been captured in oligomeric versus monomeric form. The data generated in this study revealed that, despite their small size, oAβ1-42 baits bind reproducibly to more than one hundred proteins in all analyses undertaken and these interactions appear to be dependent on Aβ1-42 having a free C-terminus. The most remarkable finding in this study was the observation that the small cyclic peptide somatostatin, which has previously been implicated in the etiology of AD but had not been shown to interact with Aβ, binds directly to oAβ1-42 in several orthogonal biochemical assays. Furthermore, distinct assemblies of 50-60 kDa were robustly generated in the presence of SST in reaction mixtures with Aβ1-42 but not with Aβ1-40. These assemblies are particularly intriguing since they are reminiscent of the previously reported Aβ*56, an Aβ complex observed in transgenic APP mouse models and human AD brains that correlates with cognitive impairment and toxicity (Lesné et al. 2006; Lesné et al. 2013). These findings may be relevant for Aβ-directed diagnostics and may signify a role of SST14 in the etiology of AD.

2.3.1 SST biology overlaps with AD pathology

Further to our discovery of a direct interaction between SST and Aβ, a number of reports in the literature also indicate that SST may be associated with pathological Aβ-related processes in AD. In a relatively recent study, it was observed that synthetic SST14 spontaneously self-associates into supramolecular nanofibrils under physiological pH and salt conditions (van Grondelle et al. 2007). These in vitro fibrils exhibit green/yellow birefringence upon Congo red dye binding. Attenuated total reflection-FTIR spectroscopy data of SST fibrils were consistent with them acquiring a β-hairpin backbone conformation and cross-β symmetry (van Grondelle et al. 2007), features shared with the fibril structure of Aβ1–42 (Gremer et al. 2017). The dense nature of amyloids offers the cell an elegant solution to a storage challenge. Not only are these structures efficient from a space-management perspective but the absence of water in such amyloid assemblies protects the peptide from degradation and limits undesirable interactions with other molecules (Sawaya et al. 2007). However, this solution also bears a risk, namely the possibility of functional amyloids encountering other peptides, which harbor a propensity to convert to pathogenic amyloid if permissive conditions are met. The fact that we observed a need for either SST or Aβ to be pre-aggregated for a direct interaction to occur raises the intriguing possibility that their interaction may emerge as a rare example of in vivo cross-seeding between functional
and pathologic amyloidogenic peptides.

Although our current data are restricted to in vitro paradigms, there is reason to anticipate that our data may bear significance for AD. In fact, several reports corroborate the notion that spatial proximity of Aβ and SST14 exist in the human brain, particularly in areas relevant to AD. Because both APP and PPSST undergo endoproteolytic cleavages during their passage through the secretory pathway that give rise to Aβ and SST, respectively, a first opportunity for these peptides to interact may already exist on route to their cellular release (LaFerla, Green, and Oddo 2007). With regard to their tissue distribution within the brain, primary areas where SST14 and Aβ overlap include the frontal and parietal cortices, hippocampus, and potentially the hypothalamus and amygdala (Schettini 1991; Gahete et al. 2010). Relative to somatostatin gene products, mRNA levels of cortistatin may be lower in several subareas of the brain (Dalm et al. 2004) and its mature CST17 peptide may target a strongly overlapping, yet distinct, set of receptors (Cordoba-Chacon et al. 2011).

Levels of SST have been observed to decline gradually throughout adult life. In fact, somatostatin mRNA was amongst a few dozen gene products whose levels in the frontal cortex correlated inversely with age (Lu et al. 2004). Interestingly, the natural decline in SST levels is further accentuated in AD (Gahete et al. 2010), and most likely correlates with an approximately 50% reduction in the number of somatostatinergic neurons observed in various brain areas (Saiz-Sanchez et al. 2015; Saiz-Sanchez et al. 2010). In fact, one of the earliest biochemical changes documented in the cerebral cortex of AD patients was an accelerated reduction in SST immunoreactivity (Davies, Katzman, and Terry 1980). The first indication that SST may have a more intimate role in the AD pathogenesis emerged in histochemical studies, which detected processes of somatostatinergic neurons in immediate proximity to neuritic plaques in the cingulate, frontal and temporal cortices (Morrison et al. 1985), as well as in the amygdala and hippocampus (Armstrong et al. 1985). The central conclusion from these reports has withstood the test of time and was corroborated by a recent study, which revealed that morphologically well-conserved somatostatinergic cells colocalized to a high percentage with Aβ in the olfactory (43%) and piriform cortex (65%) (Saiz-Sanchez et al. 2015). Notably, the same study reported that virtually all cell debris of somatostatinergic neurons colocalized with senile plaques. With regard to the tau pathobiology of AD, early data suggested that somatostatinergic neurons not
only exhibited pronounced morphological changes but also were prone to exhibit neurofibrillary tangles (NFTs). However, more recent work exploring the vulnerability of different types of neurons to this second pathological AD hallmark found only weak evidence for an association of neurofibrillary tangles with somatostatinergic neurons (Saiz-Sanchez et al. 2015). There is robust evidence that SST can regulate the catabolism of Aβ through its interaction with a family of SST receptors. More specifically, it has been shown that binding of the SST ligand triggers an intracellular event cascade that modulates the expression of neprilysin, a membrane metalloendopeptidase involved in Aβ degradation (Saito et al. 2005). Lastly, two independent genome-wide association studies, using Finnish and Chinese patient cohorts, identified the SST gene as a genomic region with the potential to modulate the risk of acquiring AD (Vepsalainen et al. 2007; Xue, Jia, and Jia 2009).

### 2.3.2 Evidence for interactions between other amyloidogenic peptides

In considering the possible significance of the SST-Aβ interaction in the AD context, it is useful to also take into account prior reports of interactions between amyloidogenic peptides with an influence on amyloid aggregation kinetics. There is no shortage of reports documenting the widespread existence of this phenomenon (Sarell, Stockley, and Radford 2013). For example, amyloid formation was promoted in vitro when Aβ was mixed with α-synuclein (Ono et al. 2012). A closer look revealed that Aβ and α-synuclein formed hybrid, pore-like oligomers, which could embed in the cell membrane, resulting in abnormal ion conductance (Tsigelny et al. 2008). Importantly, this phenomenon can also manifest in vivo, as was elegantly shown when animal models of Aβ amyloidosis were inoculated with PrPSc prions (Morales et al. 2010).

Several parameters need to be met for a co-incubation of two amyloidogenic peptides to promote amyloid formation. It has been proposed that critical amongst these is a need for conditions that are conducive to increasing the amyloidogenic potential of at least one of the co-incubated peptides, and to work with peptides that exhibit compatible structural and post-translational features, as well as share sequence characteristics (Sarell, Stockley, and Radford 2013). Close examination of SST and Aβ sequences reveals that the ‘NFFWK’ core Aβ binding epitope within SST bears resemblance to the ‘LVFFA’ segment within Aβ (residues 17–21). The latter is considered a critical determinant for Aβ fibrillogenesis and has served as a template for derivatizing effective β-sheet breaker peptides (Soto et al. 1996). It is likely that cross-seeding of the kind observed for pathogenic amyloidogenic peptides extends to functional amyloids. Indeed,
the first examples for this phenomenon were recently reported: exposure of aged rats to bacteria expressing the amyloidogenic protein curli resulted in enhanced brain α-synuclein aggregation (Chen et al. 2016). Moreover, co-expression of the functional amyloid protein Orb2 (an ortholog to human CPEB) with the pathogenic Huntingtin protein revealed that these two proteins co-aggregate (Hervas et al. 2016). This example is particularly pertinent, as it provides a precedent for an interaction between a functional and pathogenic amyloid that might modulate memory consolidation.

2.3.3 Model of possible involvement of functional amyloids in AD

Based on the various SST-related observations made in the aforementioned studies, a model has been proposed whereby declining levels of SST, observed during aging, may be responsible for reduced clearance of Aβ, leading to its net accumulation and, eventually, Aβ-induced cell death in AD (Hama and Saido 2005). In this context, monomeric SST is expected to act in a protective manner by inducing the release of Aβ degrading enzymes. Our data indicate that monomeric SST may also be therapeutic due to its ability to interfere with Aβ fibrillization. The model is incomplete, however, because it provides no explanation for why, among all types of neurons, somatostatinergic neurons were repeatedly observed at the ‘epicenter’ of the AD pathobiology, as characterized by their proximity to senile plaques. This spatial overlap would be paradoxical given that the SST-releasing neurons would be particularly well suited to induce the release of neprilysin. Elevated neprilysin levels, in turn, on the basis of their Aβ cleavage capacity, would be expected to reduce the risk to have an amyloid plaque forming. It is possible that a dichotomy of outcomes exist following exposure to monomeric versus aggregated SST, with monomeric SST being protective and aggregated SST potentially being harmful. Such a scenario would account for the aforementioned paradox.

In light of the existence of highly condensed amyloid stores of SST (Maji et al. 2009) and our new SST-Aβ binding data, the following revised model is emerging (Figure 2.3): as levels of SST transcripts decline naturally with aging, the Aβ clearance mechanisms in nearby neurons may become less effective, leading to a net increase in Aβ. Near the synaptic SST-release sites of the remaining SST-positive neurons, micro-zones must exist that contain high concentrations of residual SST amyloids. Although the post-release steps are not fully understood (Anoop et al. 2014), there is sufficient evidence that the disassembly of amyloids proceeds in time-scales of
minutes to hours (Maji et al. 2008), which may lead to sustained elevated concentrations of their building blocks nearby. Even higher concentrations of SST amyloid may spill out of neuritic processes of dying somatostatinergic neurons. The co-occurrence of elevated levels of SST aggregates and elevated levels of Aβ may form an environment conducive to the formation of mixed oligomers, with some assemblies being toxic towards the SST-releasing neurons closest to their formation, and others being innocuous or even protective. Under certain conditions, the formation of toxic assemblies is favored and tau hyperphosphorylation is induced in nearby SST-positive cells, a phenomenon observed in primary hippocampal neurons (Wang et al. 2017). As these cells, and others in the vicinity, are increasingly compromised, brain levels of SST would be expected to continue to decline and would not be available to interfere with Aβ aggregation, or to indirectly reduce Aβ levels by neprilysin induction (Saito et al. 2005). Consequently, Aβ levels would increase relative to SST levels, and Aβ aggregation pathways could undergo a shift to fibrillar Aβ aggregation, thereby setting the stage for senile plaque formation. In order to truly determine the validity of this model, SST knockout mice could be crossed with an Aβ amyloidosis mouse model. The analysis of the distribution of Aβ plaques and other Aβ-related pathology in the intercrossed line could then indicate whether SST is protective or harmful in AD.

Naturally, given their widespread and overlapping distributions, it is possible that SST (or CST) and Aβ also interact and influence each other in a healthy brain. Because the Aβ binding epitope within these cyclic neuropeptide hormones comprises the ‘FWKT’ motif required for their binding to human SST receptors (SSTRs) (Veber et al. 1979) (Figure 2.1H), levels of extracellular Aβ may modulate the canonical intracellular signaling pathways emanating from SSTRs. This may lead to a range of outcomes, including changes to memory and cognition modulated by somatostatinergic neurons in hippocampal and cortical networks (Epelbaum et al. 2009).
Figure 2.3 Model of SST influencing AD pathobiology. Concentrations of bioactive SST (indicated with green background shading) in the brain are highest in proximity to SST release sites or in areas where SST amyloid can spill out of damaged neuritic processes of somatostatinergic neurons. Where these sites overlap with regions of relatively high Aβ production (indicated by red background shading), micro-zones can exist that may comprise mixed aggregates of both peptides. (1) Monomeric SST induces a signaling cascade that leads to the transcriptional activation of neprilysin expression. (2) Neprilysin protein contributes to the destruction of Aβ. (3) The presence of mixed Aβ-SST conformers may initially favor the formation of oligomeric species that can induce tau hyperphosphorylation and induce cell death in nearby neurons (4) As the relative amounts of SST decline, these oligomeric species may become dominated by Aβ and may seed the formation of senile plaques. Note that while only one neuron is depicted here for simplicity, it is suggested that SST-dependent effects may not only manifest in an autocrine manner but may also act on nearby neurons in a paracrine mode.
2.4 Conclusions

Despite a wealth of data that connected SST to the etiology of AD for over 30 years, it has only now emerged that direct interactions between SST and the Aβ peptide may exist in the brain. Collectively, our results, which documented an influence of SST on Aβ aggregation kinetics, in the context of prior pertinent knowledge paint a scenario whereby SST14 may not just be a passive bystander in AD. Urgently needed are studies that explore whether the presence or absence of SST affects Aβ amyloidosis in animal models. As so often, a first round of experiments may not yet be conclusive. For example, the presence of CST, which appears to interact similarly with Aβ, could compensate for the loss of SST and mask a critical contribution of these peptides to amyloid plaque pathogenesis. In the absence of conclusive data on the physiological significance of the Aβ-SST interaction, a second line of investigation could aim to characterize if the 50–60 kDa SDS-resistant core is merely composed of Aβ or represents a co-assembly product of the two peptides. The latter is suggested by the existence of heteromer bands we sometimes observed when co-incubating the two peptides. The ability to enrich such intermediate aggregation products may, in turn, facilitate the generation of antibodies, which could be used to evaluate if similar aggregation products exist in brain sections or cerebrospinal fluid samples obtained from AD cases and controls. Finally, there is no reason to assume that Aβ is the only pathogenic amyloid that can interact with functional amyloids. A rigorous evaluation of the scenario painted in this perspective is needed to determine its broader significance for neurodegenerative diseases.
Chapter 3: The Human Somatostatin Interactome and the Identification of the P-Type ATPases as Candidate Interactors of SST

Please note that the majority of this chapter, with minor revisions, is in preparation for publication in the following manuscript:


Candidate’s role: Performed all affinity capture experiments, western blot analyses, functional assays, and contributed to the preparation of samples for interactome analysis.

Summary: Based on our previous Aβ-binding data, which identified SST as the most selective interactor of oAβ and validated the ability of SST to interfere with Aβ fibrillization, we were interested to learn more about SST’s own binding partners and molecular environment. In particular, we were intrigued at the possibility that SST may interact with other amyloidogenic proteins, particularly those involved in the various neurodegenerative diseases. While the principal binding partners of SST, the SSTRs, have been individually isolated and extensively investigated, to our knowledge, there has been no large-scale analysis of SST-interacting proteins. In order to address this gap in the literature, we have performed an in-depth search for SST-binding proteins, using biotinylated SST peptides as baits and human frontal lobe extract as the biological source for SST-binding candidates. Making use of a previously optimized workflow that includes isobaric tagging for the relative and absolute quantitation of proteins, we report here on a selective interaction between SST and members of the superfamily of P-type ATPases. Our data reveal that SST14 and SST28, but not similar neuropeptides, are both capable of engaging in this interaction, which may rely on SST features shared with the binding epitope necessary for binding to the SSTRs and Aβ. Functional analyses in three different cell lines indicate that SST may negatively modulate the K⁺ uptake rate of the Na⁺/K⁺-ATPase in a cell type-specific manner.
3.1 Materials and methods

3.1.1 Peptides

Synthetic human peptides, including SST14 (AS-24277), SST28 (AS-22902), and VIP (AS-22873) were purchased from Anaspec, Inc. (Fremont, CA, USA). Additional peptides, including biotinylated SST peptides (biotin-SST14, biotin-SST28, and biotin-SST11) and the truncated/mutant SST14 peptides (SST14-W8P, SST5-11, SST6-10) were custom synthesized by LifeTein LLC (Hillsborough, NJ, USA).

3.1.2 Antibodies

The anti-sodium/potassium alpha 1 ATPase antibody (ab7671; Abcam Inc., Toronto, ON, Canada) and the anti-sodium/potassium beta 1 ATPase antibody (GTX113390; GeneTex Inc., Irvine, CA, USA) were used in this study for immunoblot analyses.

3.1.3 Western blot, Coomassie, and silver staining

For SDS-PAGE analysis, samples were mixed with Bolt LDS sample buffer (B0007; Thermo Fisher Scientific, Burlington, ON, Canada) containing 2.5% 2-mercaptoethanol and boiled for 10 minutes at 60°C before loading. The samples were separated on Bolt 10% Bis-Tris Plus gels (NW00102BOX; Thermo Fisher Scientific, Burlington, ON, Canada) in MES SDS Running Buffer (NP0002; Thermo Fisher Scientific, Burlington, ON, Canada) for 1 to 1.5 hours at 120 V. For immunoblot analyses, peptides were transferred to polyvinylidene difluoride (PVDF) membranes at 50 V in Tris-glycine buffer containing 10-20% methanol for 2 hours. Membrane blocking steps were done in standard Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% fat-free milk and incubated overnight with the appropriate primary antibodies. Following three washes with TBST, membranes were incubated for 1 hour with 1: 2000 diluted anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (170-6516, 170-6515; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The band signals were visualized using enhanced chemiluminescence reagents (4500875; GE Health Care Canada, Inc., Mississauga, ON, Canada) and X-ray films or a LI-COR Odyssey Fc digital imaging system (LI-COR Biosciences, NE, USA). Where indicated, Coomassie and/or silver staining were performed to visualize all proteins present in the sample.
3.1.4 Affinity capture of SST14- and SST28-binding proteins from human frontal lobe extracts

Streptavidin UltraLink Resin beads (53114; Thermo Fisher Scientific, Burlington ON, Canada) were chosen as the affinity capture matrix in this study. The biotinylated SST14, SST28, and SST11 peptides were captured on the resin by 2 hour incubation in PBS at room temperature while undergoing continuous agitation on a slow-moving turning wheel. The biological source material for the interactome analysis consisted of human frontal lobe tissue samples from individuals (3 males) who died of non-dementia related causes at ages of 74, 76 and 82 years. The brains were obtained from the tissue biobank at the Tanz Centre for Research in Neurodegenerative Diseases, where they had been stored in -80°C freezers. 150 mg pieces of each brain were used per experimental condition and were homogenized in lysis buffer containing cOmplete Protease Inhibitor Cocktail (11836170001; Roche, Mississauga, ON, Canada) and PhosStop phosphatase inhibitor tablets (04906837001; Roche, Mississauga, ON, Canada) and solubilized using 0.6% CHAPS detergent (C3023; Sigma-Aldrich, Oakville, ON, Canada). For follow-up validation experiments, 30 mg pieces of brain sample were used. Following centrifugation at 21,000 g for 1 hour to remove insoluble material, the protein concentration was normalized across all samples. The brain lysate was subsequently added to the pre-saturated affinity capture beads (20 µL per biological replicate) and incubated overnight at 4°C. The affinity capture beads were then extensively washed during three wash steps with a total of 60 mL of lysis buffer containing 150-500 mM NaCl. Prior to elution, the affinity matrix was subjected to a pre-elution wash with 15 mL of 10 mM HEPES that served the purpose to reduce detergent and salt levels. Captured proteins were eluted either by rapid acidification in a solution containing 0.2% trifluoro-acetic acid and 20% acetonitrile in deionized water (pH 1.9) (for mass spectrometry) or by boiling for 10 minutes at 60°C in Bolt LDS sample buffer (B0007; Thermo Fisher Scientific, Burlington, ON, Canada) containing of 2.5% 2-mercaptoethanol (for Western blot analyses).

3.1.5 Cell culture

Human neural progenitor (ReN VM) cells (SCC008; Millipore Sigma, Etobicoke, ON, Canada) were cultured according to the manufacturer’s instructions. Mouse neuroblastoma Neuro-2a (N2a) cells (CCL-131; American Type Culture Collection, Manassas, VA, USA) and human embryonic kidney (HEK293) cells (CRL-1573; American Type Culture Collection, Manassas,
VA, USA) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS) (catalog number 12484028; Invitrogen Canada, Burlington, ON, Canada), 1% GlutaMAX (catalog number 35050061; Invitrogen Canada), and antibiotics in a 24 well format.

### 3.1.6 \(^{86}\text{Rb} \) uptake assay

Assay procedures were more or less based on previously established protocols (Larre et al. 2010; Koltsova et al. 2012; Kleene et al. 2007). More specifically, three hours before each experiment, the cell culture medium was replaced with DMEM containing 0.2% FBS. Following serum deprivation, the cells were treated with ouabain (2 mM) (O3125; Sigma-Aldrich, Oakville, ON, Canada) and/or SST14 (50 \(\mu\)M) or with vehicle (control). After incubation for 15 minutes at room temperature, 2 \(\mu\)Ci \(^{86}\text{RbCl} \) in water (NEZ072; PerkinElmer, Woodbridge, ON, Canada) was added to each well and the cells were incubated for another 10 minutes at 37°C. The supernatants were then removed and cells were washed four times with 1 mL ice-cold wash buffer (100 mM MgCl\(_2\), 10 mM HEPES, pH 7.4) before lysis in 500 \(\mu\)L buffer containing 1% NP40, 150 mM Tris (pH 8.3), and 150 mM NaCl. 250 \(\mu\)L aliquots of the cell lysates were transferred to vials containing 10 mL Ultima Gold liquid scintillation cocktail (6013326; PerkinElmer, Woodbridge, ON, Canada) and assayed using a liquid scintillation counter (LS6500; Beckman Coulter; Mississauga, ON, Canada). Additional 10 \(\mu\)L aliquots were used for protein concentration determination using BCA reagents (23228 and 1859078; Thermo Fisher Scientific, Burlington, ON, Canada).

### 3.1.7 Sample preparation for interactome analysis

Processing of the affinity-capture eluates followed previously described protocols (Wang et al. 2017; Jeon and Schmitt-Ulms 2012). First, the organic solvent was removed from the samples using a centrifugal evaporator. The acidity of the sample was reduced by the addition and continuous evaporation of an additional three volumes of water. Two volumes of 9 M urea were then added to denature one volume of protein mixtures at a final concentration of 6 M urea within 10 minutes at room temperature. The pH was further adjusted to pH 8.0 by the addition of 100 mM HEPES (pH 8.0). Following reduction for 30 minutes at 60°C in the presence of 5 mM Tris (2-carboxyethyl) phosphine (TCEP), proteins were alkylated for 1 hour at room temperature in the presence of 10 mM 4-vinylpyridine (4-VP). Protein mixtures were diluted with 50 mM
tetraethylammonium bromide (TEAB; pH 8.0) to a total volume of 100 mL to ensure that urea concentrations were not in excess of 1.5 M. Digestion of samples with side-chain-modified porcine trypsin (90057; Thermo Fisher Scientific, Burlington, ON, Canada) proceeded overnight at 37°C. Primary amines were covalently modified with isobaric tagging for relative and absolute quantitation (iTRAQ) reagents (4381663; SCIEX, Concord, ON) by following the manufacturer’s instructions. The labeled digests were then pooled into a master mixture and purified with C18 (A5700310; Agilent Technologies, Inc., Mississauga, ON) or SCX (A5700410; Agilent Technologies, Inc., Mississauga, ON, Canada) Bond Elut OMIX tips, again following the manufacturer’s instructions. Finally, upon reconstitution in 0.1% formic acid, peptides were analyzed by tandem mass spectrometry on an Orbitrap Fusion Tribrid instrument using previously described parameters (Wang et al. 2017).

3.1.8 Post-acquisition data analyses

The post-acquisition data analyses of interactome data sets was conducted against the human international protein index (IPI) database (Version 3.87) which was queried with Mascot (Version 2.4; Matrix Science Ltd, London, UK) and Sequest HT search engines within Proteome Discoverer software (Version 1.4; Thermo Fisher Scientific, Burlington, ON, Canada). Spectra exceeding a stringent false discovery rate (FDR) target of ΔCn of 0.05 for input data and a FDR of 0.01 for the decoy database search were detected and removed by the Percolator algorithm (Käll et al. 2007) as described before (Gunawardana et al. 2015). PEAKS Studio software (Version 6.0; Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used to assess the reproducibility of nano-HPLC separations. A maximum of two missed tryptic cleavages and naturally occurring variable phosphorylations of serines, threonines and tyrosines were considered. Other posttranslational modifications considered were carbamylations, oxidation of methionines and deamidation of glutamines or asparagines.
3.2 Results

3.2.1 Workflow of SST interactome analysis

The purpose of this study was to generate an in-depth inventory of human brain proteins that are capable of binding to SST using an unbiased in vitro discovery approach based on affinity capture mass spectrometry. To generate the bait matrices for these analyses, N-terminally biotinylated SST14 or SST28 were pre-bound to commercial beads composed of streptavidin conjugated to agarose (Figure 3.1A). Human frontal lobe extracts derived from three postmortem brains of individuals that had died of non-dementia causes served as the biological source material. Although SST14 is the predominant form of the peptide, analyses were extended to SST28 on the basis of concerns that the small size of SST14 may preclude binding of potential interactors due to steric hindrances caused by the affinity matrix (Figure 3.1B). Other investigators had before similarly taken advantage of the 14-amino acid N-terminal extension of SST28 as a natural ‘spacer’ between the biotinyl group and the receptor binding sequence (Eppler et al. 1992; Schonbrunn, Lee, and Brown 1993) (in this prior work, the SST ligands were, however, added to cultured cells prior to their solubilization in the presence of detergent, an approach that is precluded when dealing with human brain homogenates). As an additional, negative control served an N-terminally biotinylated SST derivative (hereafter referred to as SST11) with the amino acid sequence AGCKNFAFTSC, which lacks three of the four aforementioned critical residues for SST14 binding to its known SSTR receptors (Figure 3.1A).

Sample groups were analyzed side-by-side in triplicates. The zwitterionic detergent 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) was used to solubilize membrane proteins in human frontal lobe samples, a choice based on previous reports that established the ability of this detergent to extract the SSTRs (Eppler et al. 1992; He et al. 1989). Following overnight incubation of the brain extracts with affinity matrices, bound proteins were eluted by rapid acidification, denatured in 9 M urea, reduced, alkylated and trypsinized. To avoid inadvertent variances between samples, which are notoriously observed when mass spectrometry analyzes are undertaken consecutively, individual peptide mixtures were labeled with distinct iTRAQ labels in an eight-plex format and then combined. Following a ZipTip-based cleanup procedure, using strong cation exchange (SCX) and reversed phase (RP) chromatography media, the iTRAQ-labeled peptide mixtures were subjected to four-hour split-free RP nanospray separations coupled online to an Orbitrap Fusion Tribrid mass spectrometer, which was...
configured to run an MS/MS/MS (MS3) analysis method. Using Sequest HT and Mascot (Version 2.4) search algorithms, tandem MS spectra were matched to peptide sequences by referencing the human international protein index database (IPI; Version 3.87). The relative levels of individual peptides in the eight samples were determined by comparing the intensity ratios of the corresponding low mass iTRAQ signature ions in MS3 fragment spectra.

**Figure 3.1 Design of SST interactome experiment.** (A) Schematic representation of the bait peptides used in affinity capture experiments. (B) Workflow of SST interactome analysis designed to compare binders of N-terminally biotinylated-SST28 (x) and SST14 (y) to a truncated mutant peptide, SST11 (z).
3.2.2 The SST interactome

The combined SST interactome analyses from three biological replicates yielded the assignment of 13,843 mass spectra, which could be matched to 402 protein groups, comprising a total of 879 unique proteins, with a false discovery rate (FDR) of 3.6% (Figure 3.2A). Stringent filtering on the basis of the quality of relative quantitation data, i.e., requiring proteins to be quantified by a minimum of three iTRAQ reporter ion profiles and to exhibit low levels (< 30%) of deviations in their relative levels between biological replicates, reduced the list of SST candidate interactors to 88 proteins (please see Table 3.1 for a truncated list of proteins identified, sorted according to their average enrichment in the three biotin-SST28 affinity capture eluates, and Table S1 for a full list of candidate interactors, sorted alphabetically). Propionyl-CoA carboxylase, a protein ubiquitously expressed in eukaryotic cells that is naturally biotinylated (Kaziro, Leone, and Ochoa 1960), thereby allowing it to bind to the streptavidin affinity capture matrix independently of the SST bait peptides, served as a useful internal control (Figure 3.2B; Table S1). Its even protein levels across all eight eluate fractions provided assurance that lysates contained similar levels of brain proteins and that capture conditions were indeed comparable across all samples.

Amongst all SST candidate interactors, the alpha-1 subunit of the Na⁺/K⁺-ATPase (ATP1A1) displayed the highest level of SST co-enrichment (Table 3.1), ranging from 6.0 to 8.0 times the levels observed in SST11 negative control eluates (Figure 3.2B). As a first step in the characterization of candidate SST-binders, the 50 proteins that exhibited the highest levels of enrichment on SST28 capture matrices were subjected to a Gene Ontology (GO) analysis. This analysis revealed that the dataset was significantly enriched in proteins with annotations linked to ion transport. More specifically, ‘cation-transporting ATPase activity’, ‘active transmembrane transporter activity,’ and ‘ATPase activity, coupled to transmembrane movement of substances’ emerged as highly significantly associated ‘Molecular Functions’ (Figure 3.2C). Similarly, an analysis of the ‘Biological Processes’ associated with these proteins revealed that ‘ATP hydrolysis coupled cation transmembrane transport,’ ‘ion transport,’ and ‘proton transmembrane transport’ were top-listed, highly significant annotations in this category (Figure 3.2C). Indeed, several of the candidate binders, including Na⁺/K⁺-transporting ATPase, excitatory amino acid transporter, V-type proton ATPase, and ADP/ATP translocase, have known roles in accordance with these molecular functions and biological processes (Table 3.1). In addition to these transport proteins, a number of candidate binders were identified that belong
to the SNARE family of proteins, including syntaxin, synaptosomal-associated protein 25 (SNAP25), and synaptotagmin, which are involved in mediating the fusion of synaptic vesicles with their target membranes. Other candidate SST28 interactors include the catalytic (serine/threonine-protein phosphatase 2B) and regulatory (calcineurin subunit B) subunits of the calcium-dependent protein phosphatase, calcineurin. SST28s themselves, however, were not observed in the dataset. Taken together, these results were rather unexpected and pointed toward an unappreciated interaction of SST with members of the family of P-type ATPases and SNARE protein complexes.

Table 3.1 Top-listed 30 interactors of SST28. Proteins are listed according to their average enrichment in the three biotin-SST28 eluates. This list was derived from Table S1 when a filter was applied that excluded proteins that displayed greater than 50% variation in enrichment between the two control conditions.
Figure 3.2 Benchmarks of SST interactome analysis and preliminary data. (A) Chart illustrating the number of peptide-spectrum matches (PSMs) versus the false discovery rate (FDR) in the interactome dataset. (B) Relative quantitations of the proteins propionyl-CoA carboxylase (left), which displays similar levels of enrichment across all samples, and Na\(^+\)/K\(^+\)-transporting ATPase subunit alpha-1 (right), which is highly enriched in biotin-SST28 affinity capture.
eluates. The box plot depicts the enrichment ratios (R) of individual propionyl-CoA carboxylase peptides used for quantitation in log2 space, in addition to the median peptide ratio and Inter Quartile Range (IQR). A subset of peptides (red circles) was eliminated from the quantitation due to redundancy or failure to pass stringency thresholds. Relative protein levels are depicted as ratios, with the ion intensities corresponding to the heaviest isobaric labels acting as the reference. (C) ‘Cellular Component’ and ‘Molecular Function’ Gene Ontology analyses of the shortlisted proteins that displayed the highest enrichment in biotin-SST28 affinity capture eluates.

3.2.3 SST28 binds to multiple members of the P-type ATPase superfamily

Among the P-type ATPases that showed the strongest levels of SST-dependent enrichment were various subunits of the Na+/K+-ATPase (alpha-1, alpha-2, alpha-3, beta-1) and isoforms 1 and 2 of the plasma membrane Ca²⁺-transporting ATPase. These proteins were enriched in biotin-SST28 affinity capture eluates but not in biotin-SST14 eluates. To determine if steric hindrances caused by tethering SST14 to the affinity matrix precluded binding of ATP1A1, affinity capture experiments were repeated using a design in which free SST14 was assessed for its ability to compete for binding to the biotin-SST28 bait matrix (Figure 3.3A). The subsequent immunoblot assessment of assay fractions validated that ATP1A1 is, indeed, captured by biotin-SST28 (Figure 3.3B). Moreover, pre-incubation of the brain lysate with free SST14 diminished the capture of ATP1A1 by biotin-SST28, consistent with the interpretation that SST14 can also bind to the Na+/K+-ATPase when it is not sterically restrained. In light of the well-known relative abundance of Na+/K+-ATPases, the concern arose whether this interaction was indeed specific or merely reflected the high relative protein levels of these P-type pumps in the brain. Further analyses of affinity capture eluate fractions by silver staining revealed that the stringent washing steps applied to the capture matrix had removed many of the most abundant proteins (Figure 3.3B). In fact, only a small subset of proteins visible in the input samples was retained by the SST28 matrix. Collectively, these findings established that both SST28 and SST14 interact selectively with Na+/K+-ATPases.
Figure 3.3 Identification of ATP1A1 as a candidate interactor of SST28/14. (A) Workflow of competitive binding experiments undertaken to validate the SST-Na⁺/K⁺-transporting ATPase subunit alpha-1 interaction. (B) SDS-PAGE analysis of SST28 affinity capture eluates visualized by immunoblot and silver stain. Immunoblot against ATP1A1 reveals that binding of ATP1A1 to biotin-SST28 can be blocked by pre-incubation of the brain lysate with free SST14 (50 µM; lane 4). The prominent bands in the eluates corresponding to ATP1A1 are absent when the same samples are analyzed by silver stain (lanes 7 and 8). Note the presence of intense bands in the input and unbound fractions (lanes 5 and 6) that are absent from SST28 affinity capture eluates, indicating that SST is not inclined to interact with other proteins simply based on their abundance. We interpret the bands at approximately 50, 14, and 4 kDa in the eluate samples to correspond to streptavidin subunits and biotin-SST28 that leached off the affinity capture resin during the elution step.
3.2.4 Characterization of SST binding to the Na⁺/K⁺-ATPase

To begin to delineate the binding epitope within SST required for its binding to the Na⁺/K⁺-ATPase, we next performed a series of competitive binding experiments based on the aforementioned design (Figure 3.3A) and various SST-derived peptides. More specifically, again using biotin-SST28 or biotin-SST11 as baits, human brain lysates were pre-incubated with either SST14 (50 uM) or a mutated version of the peptide, SST14-W8P (50 uM), which contains a tryptophan to proline substitution in the previously determined SSTR binding epitope. In addition to ATP1A1, immunoblot analyses validated binding of SST28 to ATP1B1, the beta-1 subunit of the Na⁺/K⁺-ATPase. As expected, both interactions could be blocked by pre-incubation of the brain lysate with SST14 (Figure 3.4A). Additionally, these experiments revealed that the single amino acid substitution in the receptor binding site of SST prevented its binding to these proteins. This inference was based on the observation that, pre-incubation with SST14-W8P did not impair the interaction of ATP1A1 or ATP1B1 with biotinylated-SST28 (Figure 3.4A). Analogous competition experiments, which made use of the truncated SST14 derivatives SST5-11 or SST6-10 lacking N- and C-terminal residues as blocking peptides, indicated that the latter peptides, although comprising the critical tryptophan-8 residue, did not compete with Na⁺/K⁺-ATPase binding to SST28 (Figure 3.4B). In other words, the core FWKT sequence segment within SST required for binding to the SSTRs is essential but not sufficient for binding to the Na⁺/K⁺-ATPase.

To further assess the specificity of the interaction between SST and P-type ATPases, the binding competition analyses were extended to vasoactive intestinal peptide (VIP), another 28-amino acid neuropeptide whose tissue expression overlaps with SST (Epelbaum et al. 1979). Pre-incubation of human brain extract with either SST28 or VIP revealed that SST28, but not VIP, was able to block the binding of biotin-SST28 bait to ATP1A1 (Figure 3.4C), further corroborating the conclusion that there is specificity to the interaction between SST and the Na⁺/K⁺-ATPase.
Figure 3.4 Validation of SST binding to Na⁺/K⁺-ATPase alpha and beta subunits. (A) Immunoblot analysis of competitive binding experiment with SST14 and the mutant peptide SST14-W8P (left) and quantification of Western blot data (right). Capture of ATP1A1 and ATP1B1 by biotin-SST28 can be blocked by pre-incubation of the brain lysate with free SST14 (50 µM; lanes 5-7), but not with a mutant SST14 with an amino acid substitution (W8P) in the receptor-binding site (50 µM; lanes 8-10). Note that the negative control bait peptide, biotin-SST11, failed to capture any detectable ATP1A1 (lanes 11-13). Coomassie staining of the same blot confirms that an equal amount of protein was loaded in each well, using the streptavidin.
subunits released from the affinity matrix as a loading control. (B) Capture of ATP1A1 by biotin-SST28 can be blocked by pre-incubation of the brain lysate with free SST28 (50 µM; lane 6) or SST14 (50 µM; lane 7), but not with SST14-W8P (50 µM; lane 8). Truncated versions of SST14 also fail to block capture of ATP1A1 (50 µM; lanes 9 and 10), despite containing the receptor binding sequence (FWKT). Coomassie staining confirms that protein loading was consistent across the gel. (C) Capture of ATP1A1 by biotin SST-28 can be blocked by pre-incubation of the brain lysate with free SST28 (25 µM or 50 µM; lanes 3 and 5) but not the similar neuropeptide, VIP (25 µM or 50 µM; lanes 4 and 6).

3.2.5 SST inhibits $^{86}$Rb uptake by the Na$^+$/K$^+$-ATPase

In order to address whether the interaction between SST and the Na$^+$/K$^+$-ATPase has an effect on the activity of the pump, we undertook an in vitro $^{86}$Rb uptake assay in human neural progenitor (ReN), mouse neuroblastoma (N2a) and human embryonic kidney (HEK293) cell lines, which was adapted from previously established protocols (Larre et al. 2010; Koltsova et al. 2012; Kleene et al. 2007). Experiments were performed in the absence and presence of ouabain, a potent inhibitor of the Na$^+$/K$^+$-ATPase, to discern the Na$^+$/K$^+$-ATPase-dependent and -independent uptake of $^{86}$Rb, a radionuclide which pharmacologically mimics K$^+$. In the presence of SST14 (50 µM), total $^{86}$Rb uptake was reduced to 91.5%, 53.6% and 74.4% in ReN, N2a and HEK293 cells, respectively, compared to controls (Figure 3.5). In contrast, total $^{86}$Rb uptake was reduced to 50.7% in ReN cells, 8.9% in N2a cells and 54.3% in HEK293 cells following treatment with ouabain (2 mM). The ouabain-mediated reduction of $^{86}$Rb uptake was not potentiated by the treatment of cells with both ouabain (2 mM) and SST14 (50 µM), consistent with the interpretation that the inhibitory effect of SST14 on $^{86}$Rb uptake is mediated by the Na$^+$/K$^+$-ATPase. These findings indicate that SST14 reduced the activity of the Na$^+$/K$^+$-ATPase in a cell type-specific manner, with N2a cells being particularly susceptible to SST-mediated inhibition, HEK293 cells being partially susceptible, and ReN cells displaying some resistance to SST-mediated Na$^+$/K$^+$-ATPase inhibition.
Figure 3.5. SST14 regulates $^{86}$Rb uptake by the Na$^+$/K$^+$-ATPase. The addition of SST14 (50 µM) to ReN, N2a and HEK293 cells reduces the uptake of $^{86}$Rb to 91.5%, 53.6% and 74.4%, respectively, compared to controls. Following treatment with ouabain (2 mM) the uptake of $^{86}$Rb is reduced to 50.7% in ReN cells, 8.9% in N2a cells and 54.3% in HEK293 cells, respectively. Note that the addition of SST14 to ouabain treated cells does not further reduce the uptake of $^{86}$Rb compared to cells treated with ouabain alone. Mean values and standard deviation were calculated from three biological replicates. *$p < 0.05$.

3.3 Discussion

The goal of the current study was to generate an inventory of human brain proteins that SST binds to. 88 proteins were robustly enriched in biotin-SST affinity capture eluates on the basis of a minimum of 3 confident PSMs that were associated with robust quantitations. Among the top SST candidate interactors were multiple members of the P-type ATPase superfamily. Follow-up experiments, which centered on the Na$^+$/K$^+$-ATPase, validated that both SST28 and SST14 can bind to this pump. We then characterized the specificity of this interaction and observed that binding exhibits selectivity in regards to both the SST bait peptides, when compared to other cyclic peptides, and the P-type ATPase prey, when compared to other abundant brain proteins. Moreover, we identified a tryptophan residue within SST as critical for binding to the Na$^+$/K$^+$-
ATPase. Interestingly, this tryptophan is embedded within the core FWKT sequence motif known to also mediate binding of SST to its cognate SSTR receptors. Finally, we observed that SST has an inhibitory effect on the activity of the Na\(^+\)/K\(^+\)-ATPase in cell type-specific manner.

Our study took advantage of recent improvements to mass spectrometry instrumentation and advanced workflows that incorporated isobaric labeling for relative quantitation. Since we sought to identify any protein that might interact with SST in the brain, a rather generic affinity capture approach was applied. In hindsight we realize that this strategy disfavored the purification of canonical SSTRs which, as members of the GPCR protein family, become unstable and lose their ligand-binding ability when removed from their native environment (Grisshammer 2009; Jamshad et al. 2015), leading investigators to apply cross-linking methods and modified affinity-capture protocols to stabilize the interaction of SST with its canonical receptors (Schonbrunn, Lee, and Brown 1993; Knuhtsen et al. 1990; Brown and Schonbrunn 1993).

A PubMed query that combined the search terms ‘somatostatin’ and ‘mass spectrometry’, although producing more than 150 hits, revealed no prior study that pursued the objective to capture and identify SST interacting proteins by mass spectrometry. Close examination of the pertinent literature suggests that this striking omission may at least in part reflect the fact that the discovery of canonical SSTRs in 1992 predated technology developments underlying modern protein mass spectrometry. Consequently, the first two canonical SSTRs (SSTR1 and 2) were identified through a genomic hybridization strategy, which specifically targeted GPCRs of pancreatic islet cells because some of the earlier research had suggested SSTRs to be members of this receptor family (Brown et al. 1990). The subsequent discoveries of SSTR3, 4 and 5 followed in short order, and were based on conceptually analogous genomic hybridization screens (Bruno et al. 1992; Yasuda et al. 1992; O’Carroll et al. 1992). Thus, whereas until just before that time, the focus in the pertinent literature had been on the purification of SST receptors by biochemical means, this line of research was largely abandoned after 1992. The omission to revisit this question, once mass spectrometry became the method of choice for protein interaction studies, could reflect a view that the known SSTRs are sufficient to explain existing SST-related phenomena, or could have more mundane explanations, including a reality whereby the SST field had more than enough to do trying to dissect the complex molecular underpinnings of SST binding to already five SSTRs. When SSTR1 and SSTR2 were expressed in CHO cells, binding experiments with radiolabeled SST probes revealed that approximately 90% of binding depended
on the presence of the heterologous SSTRs (Yamada et al. 1992). These and similar results obtained with the other SSTR paralogs may have contributed to a lack of motivation to look any further, although they were not sufficient to rule out the existence of additional physiological SST interactors. Consistent with the view that the known SSTRs may not account for all SST interactions, leading up to 1992, the molecular masses of SST candidate proteins were reported to range from 27 to 228 kDa (Rens Domiano and Reisine 1992), and the masses of canonical SSTRs of approximately 40 kDa would have been difficult to reconcile with data of several investigators in the field.

The most striking finding from the current study was the discovery of a selective interaction between SST and members of the P-type ATPase superfamily. In addition to P-type pumps, our interactome study revealed several other novel candidate SST interactors. Some of these had been indirectly linked to SST. For example, both subunits of the heterodimeric protein calcineurin, i.e., the catalytic serine/threonine-protein phosphatase 2B (PPP3CA) and its regulatory subunit (PPP3R1), emerged in this work as candidate SST binders. Calcineurin had previously been proposed to operate as a downstream effector of SSTRs (Renström et al. 1996). Similar levels of enrichment were observed for members of the synaptic vesicle fusion complex, including syntaxin-1A/B, synaptosomal-associated protein 25 (SNAP25), synaptotagmin, and synaptic vesicle glycoprotein 2A. This finding could reflect a reality whereby SST, which is itself stored and released through regulated vesicle release mediated by SNARE proteins, might harbor an intrinsic ability to interact with this fusion complex machinery, or could point toward a broader role of SST in modulating the release of other synaptic vesicles at the synaptic cleft.

Focusing on the Na⁺/K⁺-ATPase for validation studies, we demonstrated that both SST28 and SST14 can bind to this pump through a binding epitope that overlaps with the SSTR binding sequence FWKT. This shared characteristic is noteworthy, as it could have masked the binding of SST to the sodium-potassium pump in prior peptide competition assays. Even more intriguingly, we previously reported that this region of SST is also critical for its binding to oligomeric Aβ (Wang et al. 2017; Solarski et al. 2018), an observation that triggered our interest in other SST interactors in the first place. Indicative of some specificity of this interaction, the VIP peptide of similar mass and characteristics as SST failed to block the capture of ATP1A1 by biotin-SST28. Although this is the first report of a direct binding between SST and P-type ATPases, prior to the discovery of SSTRs multiple authors identified proteins that bound
selectively to SST analogs with molecular weights suspiciously similar to the alpha subunit of the Na$^+$/K$^+$-ATPase (~100 kDa) (Knuhtsen et al. 1990; Sakamoto et al. 1988; Kimura, Hayafuji, and Kimura 1989). Also, at around the same time SST was observed to modulate plasma membrane conductance in various cell types (Yamashita, Shibuya, and Ogata 1986; Mollard et al. 1988; Koch and Schonbrunn 1988; Yamashita, Shibuya, and Ogata 1988; Sims, Lussier, and Kraicer 1991). The primary method by which this was proposed to occur was through binding to GPCRs, which can hyperpolarize the membrane by opening K$^+$ channels and by lowering intracellular Ca$^{2+}$ levels (Ben-Shlomo and Melmed 2010). For example, SST was reported to activate inwardly rectifying K$^+$ channels and inhibit voltage gated Ca$^{2+}$ entry through the action of different G-proteins (Mollard et al. 1988; Koch and Schonbrunn 1988; Yamashita, Shibuya, and Ogata 1988; Sims, Lussier, and Kraicer 1991; White et al. 1993; Yang and Chen 2007; Yang et al. 2005) leading to hyperpolarization of the cell membrane (Yoshimoto et al. 1999). It will be of interest to explore if SST additionally mediates its effects on membrane conductance by directly binding to and activating P-type ATPases. We have already shown here that SST exposure of cells leads to a partial inhibition of the Na$^+$/K$^+$-transporting ATPase in certain cell types. Similarly, SST may bind to and inhibit the Ca$^{2+}$-transporting ATPase, another P-type ATPase we co-isolated, which would be expected to have a modulatory effect on intracellular Ca$^{2+}$ levels.

SST may also influence membrane conductance indirectly: Binding of SST to its cognate receptors is best understood to activate G$\alpha_{\text{ii}}$, a subunit of heterotrimeric G proteins. G$\alpha_{\text{ii}}$ inhibits adenylate cyclase, thereby reducing cAMP production which, in turn, leads in several cell models to a decrease in the levels and activity of the Na$^+$/K$^+$-ATPase (Schmitz et al. 2014; Shahidullah, Mandal, and Delamere 2017). Thus, if direct binding of SST to the Na$^+$/K$^+$-ATPase inhibits the pump, it could constitute an elegant signal amplification mechanism, whereby direct binding of SST to the Na$^+$/K$^+$-ATPase works synergistically with SSTR activation to decrease the levels and activity of the Na$^+$/K$^+$-ATPase. Although this interpretation may recommend itself as the most parsimonious model, more work is needed to establish if this model holds up under close scrutiny. Moreover, unresolved remains why the degree to which SST interfered with ion transport differed across cell types, and whether the SST-Na$^+$/K$^+$-ATPase interaction modulates other signaling activities that have been attributed to the pump.
A related consideration is the role of SST in controlling vascular tone. Na\(^+\)/K\(^+\)-ATPases are a primary pharmacological target for the treatment of hypertension using cardiotonic glycosides (Dostanic-Larson et al. 2005). Multiple lines of evidence suggest that SST also has vasoactive properties, and generally acts as a hypertensive agent. In fact, for many years, somatostatin analogs have been used in the treatment of portal hypertension by inducing vasoconstriction of the splanchnic vasculature (Bosch 1985; Miñano and Garcia-Tsao 2010; Reynaert and Geerts 2003). In rare incidences, these drugs have been observed to induce severe systemic hypertension (Pop-Busui, Chey, and Stevens 2000). Recently, a poly-T repeat sequence in the SST gene promoter was also found to be associated with significant variations in blood pressure (Tremblay, Brisson, and Gaudet 2016). The latter is just one of several reports that linked SST to effects on blood pressure, yet the mechanism by which SST modulates cardiovascular biology has remained elusive. Some investigators proposed that SST indirectly induces vasoconstriction by inhibiting the release of vasodilatory agents, such as glucagon, while others suggest that SST acts locally at the level of the arterial wall (Dimech et al. 1995; Torrecillas et al. 1999; Chatila et al. 2003; Wiest, Tsai, and Groszmann 2001). The identification of a selective interaction between SST and the Na\(^+\)/K\(^+\)-ATPase might be pertinent in this context, with potential implications for administering SST analogs in the clinic.
3.4 Conclusions

In an unbiased search for SST binding proteins in the human brain, we identified several novel candidate SST interactors, including various components of the SNARE complex and members of the P-type ATPase superfamily. The top-listed proteins in our SST interactome were multiple subunits of the Na⁺/K⁺-ATPase. While we were originally interested in identifying SST interactors pertinent to the pathogenesis of AD or other neurodegenerative diseases, the results of this study are potentially more far reaching in terms of significance. If future studies elucidate a mechanism by which SST modulates the function of the Na⁺/K⁺-ATPase, this interaction could represent a novel mechanism by which SST exerts its biological effect. The fact that SST, upon its regulated release from intracellular dense core vesicles, exists for up to several hours in highly concentrated form (Maji et al. 2008), is consistent with a scenario whereby nearby P-type ATPases can be exposed to in vivo concentrations of this peptide that we observed to facilitate in vitro binding. Given the role of the Na⁺/K⁺-ATPase in critical biological functions such as the maintenance of the resting membrane potential and blood pressure regulation, the repurposing of clinically approved SST analogs, such as octreotide and lanreotide, may offer a novel approach whereby these mechanisms can be pharmacologically targeted. In any case, we hope that the data generated in this study will stimulate further investigation of the significance of SST’s molecular interactions and facilitate groundbreaking discoveries of clinical relevance.
Chapter 4: Future Directions in SST Biology in Light of the Current Research

Summary: This chapter describes the impact of the research presented in this thesis and proposes follow-up experiments to further explore the role of SST in health and disease.

The previous two chapters have summarized recent discoveries in the fields of neurodegenerative disease research and SST biology. Chapter 2 highlighted findings from our comprehensive analysis of the Aβ interactome, which identified SST as a selective binder of oAβ with the capacity to modulate Aβ aggregation kinetics and promote the formation of distinct oligomeric Aβ assemblies. These findings were placed in the context of a growing list of observations that implicate SST in the pathogenesis of AD. In Chapter 3, the discovery of a selective interaction between SST and the P-type superfamily of ATPases was presented and discussed, which may reflect a previously overlooked mechanism by which SST exerts its physiological effects. The data derived from both studies argue for a much broader biological role for this small neuropeptide, which was originally viewed simply as a negative regulator of growth hormone release from the pituitary gland.

An intriguing question that remains to be answered is whether the Aβ-binding capacity of SST can be harnessed to develop a novel treatment for AD. Indeed, the inhibitory effect of SST on Aβ aggregation observed in our kinetic aggregation studies suggests that SST-derived agents could be used as therapeutics to block Aβ fibrillization and inhibit plaque formation. Such a scenario would align with observations that SST is also able to induce the transcription of various Aβ-degrading enzymes (Saito et al. 2005; Tundo et al. 2012). However, it is possible that by preventing the formation of higher-ordered Aβ assemblies, which are now viewed as innocuous sinks of Aβ, the pool of Aβ in the brain may become enriched in smaller soluble oligomers that are significantly more toxic. Such oligomers may include the 50-60 kDa assembly observed in our co-incubation studies of Aβ and SST (and CST). In this context, a more effective approach to develop therapeutics for AD would be to generate antibodies against this distinct 50-60 kDa assembly, given its resemblance to Aβ*56 (Lesné et al. 2006; Lesné et al. 2013). Since Aβ*56 is thought to be an oligomer that predominates in early stages of AD, such an antibody could also be used in diagnostic screens for prodromal AD. Indeed, ongoing efforts in our lab are
aimed at isolating and further characterizing this unique oligomer with the goal of using it to develop research and/or therapeutic agents to probe for oAβ in the brain or biofluids.

While our work has clearly demonstrated that SST and Aβ can interact in vitro, a logical next step is to address the significance of this interaction in a living organism. To explore this interaction in vivo we have obtained SST knockout mice and have begun to cross them with APP<sup>NL-F/NL-F</sup> mice, a robust model for studying Aβ-related AD pathology. APP<sup>NL-F/NL-F</sup> mice express APP with a humanized Aβ sequence harbouring two pathogenic mutations: the Swedish (KM670:671NL) and the Iberian (I716F), which elevate total Aβ and Aβ1-42 levels, respectively (Saito et al. 2014). As a result, these mice recapitulate several characteristics of AD pathology, including Aβ deposition in the cortex and hippocampus, neuroinflammation, and synaptic loss. By studying the consequences of SST deficiency in the context of elevated levels of Aβ we will ultimately be able to conclude whether SST is an inert bystander in the etiology of AD or a factor that aggravates Aβ-related pathology. If it appears that SST is essential for AD pathology, a disease intervention strategy could therefore be developed that aims to reduce SST levels. On the other hand, if the lack of SST exacerbates AD pathology, the administration of SST or SST analogs may be able to interfere with the progression of the disease. Either of these outcomes will provide valuable information regarding the biological significance of this interaction.

With regards to the interaction between SST and the P-type ATPases, it is currently unknown to what extent SST is capable of modulating the activity of these ion pumps. While the data from our ⁸⁶Rb uptake assay are consistent with an inhibitory action of SST on the Na<sup>+</sup>/K<sup>+</sup>-ATPase, more work is needed to understand the discrepancies we observed between cell types. It is likely that ATPase binding reflects only a minor mechanism involved in SST signalling, since a relatively high concentration of free SST (50 µM) in solution was required to block the affinity capture of ATP1A1 in our competitive binding experiments and reduce ⁸⁶Rb uptake in our functional assays. This characteristic poses a challenge to the design of in vitro experiments aimed at determining the effect of SST binding on ATPase function, since the SSTRs, which bind SST with high affinity, might mask the effects of ATPase binding. In order to overcome this impediment, it may be necessary to develop SSTR knockout cell lines that can be used to study the SST-ATPase interaction in the absence of background SST binding.
A logical extension of insights gained in this thesis project, that would bridge the model of SST in AD described in Chapter 2 with the SST-interactome data reported in Chapter 3, might explore the impact of SST on the reported interaction between Aβ amylospheroids (ASPDs) and the Na+/K+-ATPase α3 subunit (ATP1A3) (Ohnishi et al. 2015; Petrushanko et al. 2016; DiChiara et al. 2017). In a simple proof-of-principle experiment, the ability of ASPDs to bind to ATP1A3 in the presence of SST could be assessed in a competitive binding experiment to determine if these interactions rely on the same epitope within ATP1A3. Such a scenario is not entirely implausible due to sequence similarities between the central hydrophobic SST motif, which we observed to be essential for binding to the Na+/K+-ATPase, and the amyloid core sequence, LVFFA, present in Aβ (residues 17-21) (Soto et al. 1996). Should SST be capable of blocking this neurotoxic interaction, it would be of great interest to determine if it can prevent the calcium dyshomeostasis, tau abnormalities, and neurodegeneration observed in neurons treated with patient-derived ASPDs (Ohnishi et al. 2015). If the data from such a study are promising, the blockade of the ATP1A3-ASPD interaction by SST-based peptide-mimetics might reflect a strategy to prevent the neurotoxicity caused by these Aβ assemblies.

In addition to the potential future applications that may arise as a result of our SST-related discoveries, it is important to note that our findings may already have a more short-term impact. In particular, since SST analogs are a widely prescribed family of medications for the treatment of various neuroendocrine and digestive disorders, our data are likely of interest to pharmacists and physicians who must consider potential adverse reactions associated with these drugs (de Herder and Lamberts 2003). It is possible that some side effects of these agents, such as systemic hypertension (Pop-Busui, Chey, and Stevens 2000), may be a result of their interaction with the Na+/K+-ATPase or other P-type ATPases. Such a scenario is plausible given that the SST analog, octreotide, contains the core FWKT binding region, which we demonstrated to be essential for Na+/K+-ATPase binding. Additionally, since radiolabelled analogs of SST are used as positron emission tomography probes for various tumour types, it will be of interest to learn if binding to the P-type ATPases or other proteins identified in our interactome study accounts for background noise or false-positive results in SST scintigraphy studies (Kwekkeboom, Krenning, and de Jong 2000). Ultimately, we hope that our data will contribute to informing decisions made by clinicians administering such medications with the objective to achieve the best possible outcomes for the patients receiving these drugs.
Overall, the data presented in this thesis identify potentially novel roles for the small peptide, SST, in human health and disease. Continued work in our lab aims to further elucidate SST’s involvement in Alzheimer’s disease and determine the significance of SST’s interaction with the P-type family of ATPases. We hope that our findings will stimulate the interest of those in the field of SST biology, thereby expediting further knowledge regarding the molecular actions of this multi-faceted neuropeptide.
References


## Table S1. Human brain SST interactome list

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<th>p-Value</th>
<th>Enrichment</th>
<th>q-Value</th>
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### Protein level ratios color bar
- **Red** indicates upregulation
- **Blue** indicates downregulation
- **Green** indicates no change