S100 INHIBITORS: A NEW PARADIGM FOR TREATING ALZHEIMER'S DISEASE

A Senior Scholars Thesis

by

DAVID MATHAI

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Biochemistry and Genetics

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ABSTRACT

S100 Inhibitors: A New Paradigm for Treating Alzheimer's Disease. (April 2011)

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Alzheimer's disease (AD) is the most common form of dementia, a neurological condition characterized by cognitive impairment and the inability to perform daily tasks. Neuritic protein aggregates in the brain, known as plaques, are a hallmark of AD pathology. These plaques are deposits of the amyloid-β (Aβ) peptide, which is derived from a larger molecule known as the amyloid precursor protein (APP). APP can be processed in one of two ways: in the non-amyloidogenic pathway, APP cleavage generates a variety of fragments that are thought to promote neuronal growth and survival; in the amyloidogenic pathway, APP cleavage leads to the production of the neurotoxic Aβ peptide. Our lab has recently demonstrated that inhibition of two members of the S100 family of calcium binding proteins, S100A1 and S100B, synergistically reverses cognitive decline in the PSAPP mouse model of AD. However, the molecular underpinnings of this process are not known. This study tests the hypothesis that improved cognition, which is accompanied by a reduction in plaque load, is due to increased non-amyloidogenic APP processing. Western blotting was used to

quantify the levels of full-length APP and Aβ in brain lysates from 12-14 month old PSAPP and PSAPP/S100 knockout mice. APP and Aβ levels were similar in PSAPP/S100 knockout and PSAPP mice. These data suggest that the decreased pathology observed in PSAPP/S100 knockout mice is not attributable to alternative processing of APP. Future directions of study include an examination of other possible mechanisms that may be responsible for observed differences in pathology. The molecular processes of plaque buildup and Aβ clearance have been proposed as targets for further study in S100 knockout mice. Once identified, this mechanistic information can be used toward the development of S100 inhibitors that improve memory loss in patients. The availability of novel pharmacological interventions for AD will significantly reduce the monetary and societal impacts of this debilitating disease.

DEDICATION

This thesis is dedicated to the loving memory of Rajiv “Vato” Paranadni. Among several other great lessons, he taught me that life is too brief to take so seriously, that it is never too late to learn something new, and that Longhorns are not so bad after all.

ACKNOWLEDGMENTS

My appreciation for the accomplishment of this thesis is directed toward Dr. Danna Zimmer for encouragement after the most stringent critical readings of the manuscript and also for the vision to facilitate without giving me anything less than an ambitious challenge. I am also indebted to Emily Roltsch for her revisions and tireless defense to my daily onslaught of inquiries, be it questions concerning RAGE receptor dynamics or queries concerning where I could find free food in the Vet school on any given day of the week. I would like to thank Katie Albers for technical assistance and Ilka Maza for being a wall to bounce ideas, data, and frustration off of, as the other member of our Undergraduate Research Team. I also thank my family and friends for pretending to be interested enough in my research pursuits to guide me through the days of torn gels and forgotten incubations. Finally, I would like to acknowledge my puppy, Maya, who made the last few days of writing so much harder than the rest. Thank you.

NOMENCLATURE

AD	Alzheimer's disease
Aβ	Amyloid beta
APP	Amyloid precursor protein
CTF	Carboxy-terminal fragment
PSAPP ABKO	PSAPP/S100A1$^{-/-}$/S100B$^{-/-}$

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CHAPTER I

INTRODUCTION

Due to the increasing life-expectancy of the American population and the decline of heart disease, cancer, and stroke, Alzheimer's disease (AD) will soon be the leading cause of death in America (Alzheimer's Association, 2011). AD is the most common form of dementia, a neurological condition characterized by cognitive impairment and the inability to perform daily tasks. About 5.4 million Americans are estimated to have AD, 5.2 million of whom are age 65 or older (Alzheimer's Association, 2011). In addition to the societal impact of the disease, AD puts a significant financial burden on the economy. In 2011 alone, America will spend $183 billion on AD-related health care, aside from the loss of productivity and related care-giving expenses incurred (Alzheimer's Association, 2011). The indirect per-patient cost of Alzheimer's taken on by primary caregivers is estimated to exceed $8,000 a year (Turró-Garriga et al., 2010). The extensive impact of this debilitating disease can largely be attributed to a lack of effective treatment; existing medications slow the worsening of symptoms in only some patients and, even then, only for a limited time. As such, the identification of novel therapeutics that target the underlying neuronal dysfunction in AD is a high priority. It is thus critical to further delineate the biochemical basis of the disease pathology.

This thesis follows the style of The Journal of Neuroscience.

The amyloid cascade hypothesis is the prevalent theory for the intricate AD pathophysiology that includes the development of neuritic protein aggregates known as plaques and twisted neurofibrillary fibers known as tangles (Simón et al., 2010). This hypothesis states that dysregulation of amyloid precursor protein (APP) processing ultimately leads to dementia in AD patients. There are two main forms of AD that progress similarly. In early-onset AD, symptoms begin to appear in an individual's 40s and 50s and are usually caused by genetic mutations within families that lead to the accumulation of amyloid-β (Aβ), a toxic protein fragment (Alzheimer's Association, 2010). However, these account for only a small percent of diagnoses. Over 95% of AD cases are classified as late-onset; these incidences affect individuals in their 60s and older and usually occur sporadically, without a direct genetic basis for inheritance (Tanzi and Bertram, 2005). The inheritance of sporadic AD is less certain and has been attributed to a combination of genetic, lifestyle, and environmental factors (Blennow et al., 2006). In these cases, failure of normal clearance mechanisms contributes to Aβ peptide accumulation (Revesz et al., 2002). Nevertheless, in both forms of AD, the increase in Aβ levels is recognized as the initiating event that induces neurodegeneration and synaptic loss that result in characteristic cognitive decline (Thinakaran and Koo, 2008).

The neurotoxic peptide, Aβ, is derived from APP, which can be processed in one of two ways (Fig. 1 *A*). In the non-amyloidogenic pathway, the proteolytic enzymes α-secretase and γ-secretase serially cleave APP, generating a variety of protein fragments. The initial

α -secretase cleavage generates a long secreted form of amino-terminal APP (sAPPα) and a C-terminal fragment known as APP-CTF83 (CTF83). Subsequent cleavage by γ-

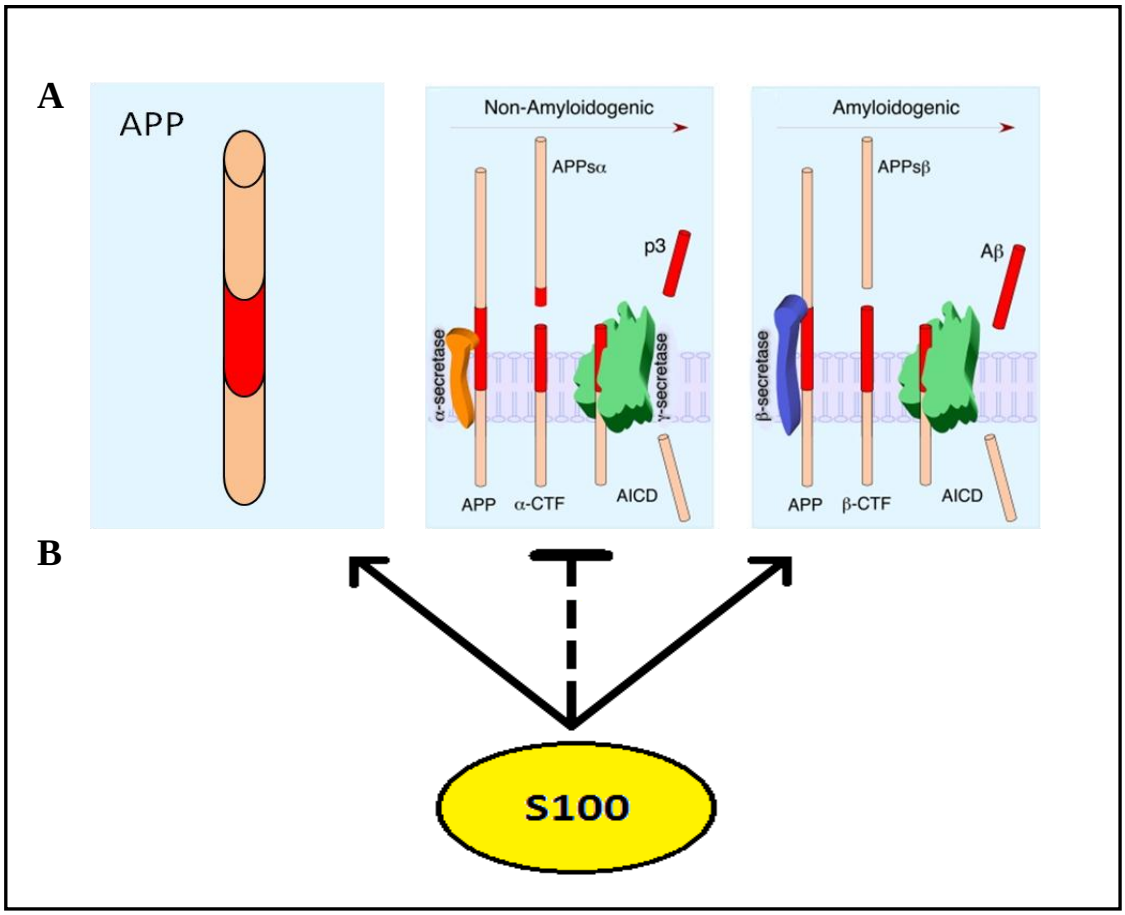

Figure 1. *The role of S100 proteins in APP processing*. ***A,*** APP can be cleaved via an amyloidogenic or a nonamyloidogenic processing pathway, generating a variety of protein fragments. Amyloidogenic processing gives rise to toxic Aβ. ***B,*** Certain S100 proteins are suspected to act at a molecular level by increasing total APP levels and amyloidogenic processing (solid arrows) and by inhibiting nonamyloidogenic processing.

secretase releases the APP intracellular domain (AICD50) and the P3 fragment. These nonamyloidogenic fragments are thought to promote neuronal growth, survival, and differentiation (Chow et al., 2010). In the amyloidogenic pathway, β-secretase (β-site cleaving enzyme, BACE1) cleaves first instead of α-secretase, generating sAPPβ and

CTF99. The presence of multiple APP cleavage sites leads to the production of a variety of peptide isoforms; for instance, CTF89 is often found along with CTF99 following β-secretase action. Further processing by γ-secretase results in the formation of AICD50 and the Aβ peptides responsible for AD pathology. As before, the presence of multiple proteolytic sites results in a variety of fragment lengths: $A\beta_{1-40}$ is the most common isoform, and the more toxic $A\beta_{1-42}$ peptide is normally found in lower amounts. In AD pathology, mutations in APP or γ-secretase lead to the increased prevalence of $A\beta_{1-42}$ after processing (Tanzi and Bertram, 2005). $A\beta_{1-42}$ aggregates readily and accumulates as both soluble oligomeric aggregates and insoluble amyloid plaques. The relative levels of CTF83 and CTF89/99 can also be used to quantify amyloidogenic and nonamyloidogenic APP processing. Agents that decrease amyloidogenic processing and/or increase nonamyloidogenic processing could be used in patients to slow disease progression.

Recent findings suggest that several members of the S100 protein family may be implicated in APP processing (Mori et al., 2010). The S100 Ca^{2+} binding family is comprised of over 21 members, and many of these proteins are known to play a role in various neurodegenerative disorders (Hoyaux et al., 2000). The AD brain is known to exhibit disrupted calcium homeostasis and elevated levels of several S100 proteins (Supnet and Bezprozvanny, 2010). S100 proteins are suspected to mediate APP processing at several points along its metabolic pathway (Fig. 1 *B*). Overexpression of one family member, S100B, increases pathogenesis and total Aβ levels in a transgenic

AD mouse model (Mori et al., 2010). In the same experiment, elevated CTF99 and sAPPβ products were detected, suggesting that the increase in Aβ was due to increased amyloidogenic processing; this conclusion was further supported by a concurrently observed increase in β-secretase expression and activity. Conversely, AD progression is mitigated when S100B synthesis is blocked chemically (Mori et al., 2006). The interactions of S100B and its target, the receptor for advanced glycation endproducts (RAGE) have been suggested as critical areas for future AD study (Leclerc et al., 2010). In addition, studies show that ablation of S100A1 in neuronal cells reduces intracellular APP levels (Zimmer et al, 2005). Our lab has also recently demonstrated that ablation of both S100A1 and S100B synergistically reduces Aβ-load (unpublished observations). My proposal will test the central hypothesis that reduced Aβ-load in the S100A1/S100B double knockout mouse model of AD (PSAPP/S100A1$^{-/-}$/ S100B$^{-/-}$) is due to decreased amyloidogenic processing and increased nonamyloidogenic processing of APP. Western blotting will be used to achieve the following specific aims:

(1) Quantify levels of full-length APP

(2) Quantify levels of amyloidogenic Aβ

We expect that these studies will be the first to demonstrate whether or not inhibition of S100A1/S100B has a role in APP processing. If S100 proteins are implicated in processing, these results would suggest that S100 inhibitors could be used in patients to decrease levels of Aβ and improve cognitive function. Otherwise, several alternative mechanisms of regulation would be considered for therapeutic potential. Nevertheless, the development of new, effective pharmacological interventions is critical for

combating the staggering impact that AD has on individuals living with the disease, caregivers, and the economy.

CHAPTER II

METHODS

Experimental methods consisted of the development of mouse lines, brain lysates, western blotting, and quantification. A schematic of experimental design can be found in Figure 2.

Mice

The PSAPP line used was generated by crossing Tg2576 and 6.2 mouse lines. Tg2576 mice express double missense mutations (K670N/M671L) that cause a dramatic increase in brain transgenic APP compared to endogenous APP and also result in increased levels of Aβ in the brain (Hsiao et al., 1996). The 6.2 line is characterized by a mutation (M146L) in Presenilin-1, a protein which is known to be involved in γ-secretase cleavage of APP (Duff et al., 1996). The double transgenic PSAPP mouse model resulting from the cross consequently recapitulates AD-like processing and pathology found in humans (Holcomb et al., 1998). The PSAPP/S100A1$^{-/-}$/S100B$^{-/-}$ double knockout line (PSAPP ABKO) was generated by crossing PSAPP double transgenic mice with S100A1$^{-/-}$/S100B$^{-/-}$ mice and subsequent interbreeding of the heterozygous offspring. PSAPP littermates were used as controls to ensure that any measured differences were not attributable to differences in genetic background. All procedures were performed with approval from the Texas A&M University Institutional Animal

Care and Use committee in accordance with guidelines from the *NIH Guide for the Care and Use of Laboratory Animals.*

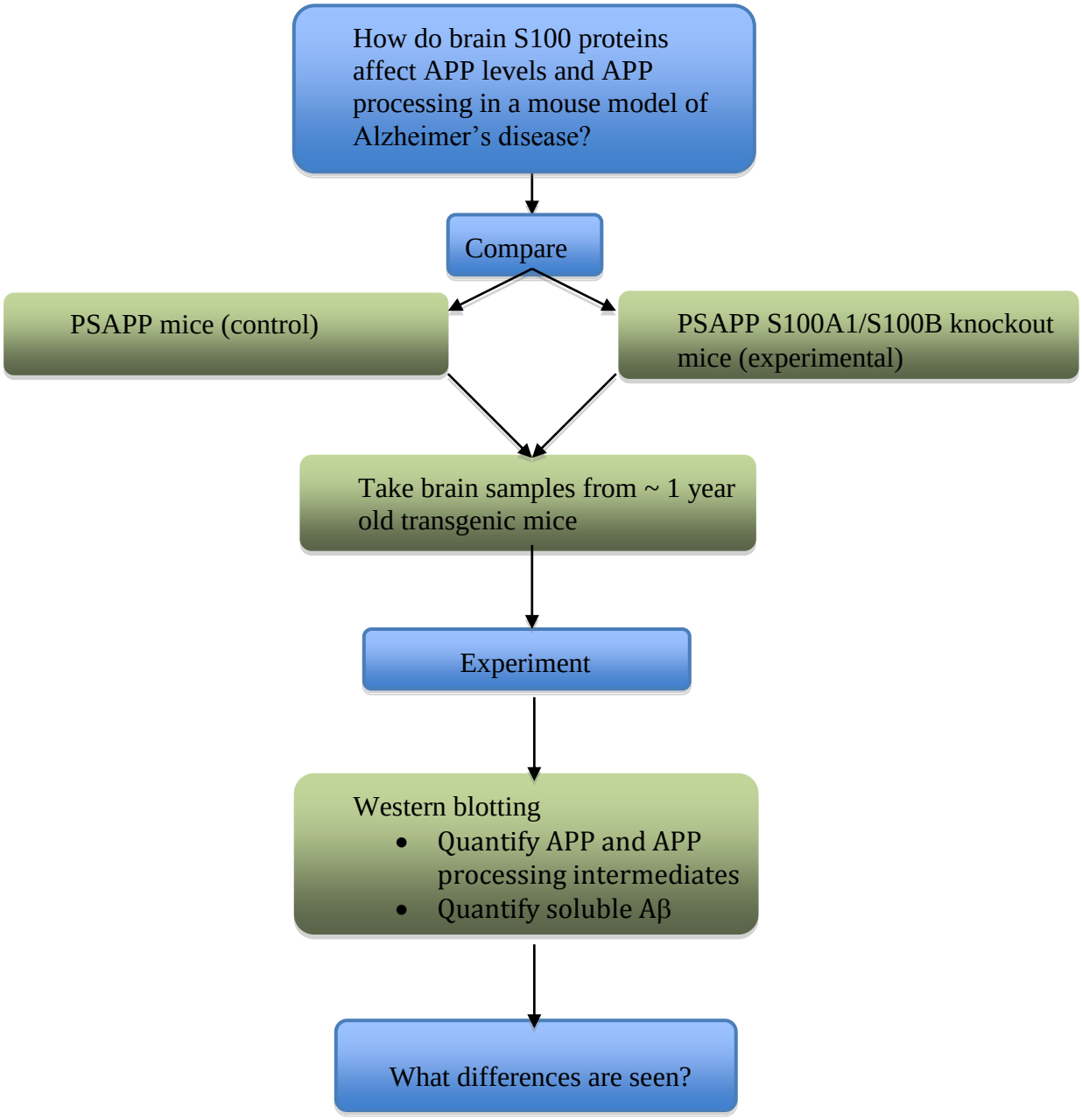

Figure 2. *Flow diagram of experimental design.*

Tissue preparation

PSAPP (*n* = 5) and PSAPP ABKO (*n* = 5) mice aged twelve to fourteen months old were anesthetized, and brains removed, dissected, and frozen. Lysates were prepared by homogenizing 0.05g tissue with 1mL TPER containing 10 mM EDTA and 3X HALT inhibitor (Thermo Scientific, Rockford, IL). Soluble and insoluble fractions were prepared by centrifugation at 14000 rpm for 1 min at 4°C. All samples were stored at -80°C until use. Protein concentrations were determined by the Bradford method (Bradford, 1976).

Western blot

Western blot analysis was used to quantify the levels of full-length APP and APP processing intermediates. Samples of 10-20ug total protein in sample buffer (4% w/v SDS, 1 mM DTT, 0.075 glycerol, 1.2 mg/mL Bromophenol blue, 40 mM Tris-HCL, pH 6.8) were then size-fractionated on 4-20% gradient SDS polyacrylamide gels (Bio-Rad, Hercules, CA). Precision Plus Protein Western C Standards (Bio-Rad) were used as molecular weight markers. After gel electrophoresis, proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA) at 30 V for 60 min at 4°C. Following transfer, membranes were blocked in non-fat dry milk [5% w/v non-fat dry milk, 0.1% v/v Tween 20, Tris-buffered saline (TBS) (200 mM NaCl, 50 mM tris, pH 7.5)] for 60 min. After blocking, membranes were incubated with primary antibodies in non-fat dry milk: rabbit polyclonal C-terminal APP antibody (1:500 dilution of A8717; Sigma-Aldrich, St. Louis, MO), mouse monoclonal N-terminal APP antibody (clone

22c11; 1:10,000 dilution of MAB438; Millipore), mouse monoclonal C-terminal APP/Aβ isoform antibody (clone 6E10; 1:2,500 dilution of 39320; Covance, Princeton, NJ), and mouse monoclonal β-actin antibody (clone AC15; 1:3,000 dilution of A1978; Sigma-Aldrich). After washing in TBS-T (1X TBS, 0.1 v/v % Tween 20), membranes were incubated in species specific HRP-labeled Goat anti-rabbit antibody (1:10,000 dilution of A120-101P; Bethyl, Montgomery, TX) or HRP-labeled Goat anti-mouse IgG (1:10,000 dilution of 074- 1806, KPL) in non-fat dry milk and Precision Protein StrepTactin-HRP conjugate (Bio-Rad) to visualize molecular weight markers. After washing again in TBS-T, blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Quantification

Antibody binding was visualized on ImageQuant LAS 4000 mini (GE Healthcare) and quantified using ImageQuant TL 7.0 Analysis Software (GE Healthcare). The programmed rolling ball method was used to eliminate background and set a signal baseline. β-actin was used to normalize measured band pixel areas and account for variability and loading error. Following normalization, total APP and Aβ levels were expressed as mean ratios of pixel area $\pm$ the standard error of the mean (SEM). An independent samples t-test was used to determine significance of measured differences (SPSS Inc., Chicago, IL).

CHAPTER III

RESULTS

Western blot optimization

To ensure the best possible signal to noise ratio in the Western blot immunoassay, it was necessary to test for optimal experimental conditions. To assess which transfer membrane provided maximum chemiluminescence signal with minimal background contribution, serial dilutions (1/1000 to 1/10000) of a total brain lysate were blotted onto polyvinylidene fluoride (PVDF) and nitrocellulose membranes and compared. To optimize chemiluminescence, the following development kits were used: Amersham, Amersham Basic, Amersham Advanced, SuperSignal Pico, and SuperSignal Femto. The optimal signal to noise ratios were obtained with the SuperSignal Femto kit and the nitrocellulose membrane. This combination was used to compare APP and Aβ levels in PSAPP and PSAPP ABKO mice.

To assess antibody specificity, the optimized dot blotting procedure was applied to samples of PSAPP and wild-type homogenate for primary antibody (22c11, 6E10, C-terminal, and β-actin) and secondary antibody (HRP-labeled Goat anti-rabbit, HRP labeled Goat anti-mouse). Once the antibodies were found to bind and induce signal, conditions were again optimized for the Western blotting procedure that would be implemented. A comparison of sodium bicarbonate running buffer solution and a tris-glycine based buffer showed that the tris-glycine buffer allowed for better separation of

bands during gel electrophoresis, when 10 ug of protein was loaded per gel lane. The working dilution of β-actin, which would be used for normalization, was established to be 1-3000. Since 22c11 and C-terminal primary antibodies bound to their target proteins with limited success and a high degree of variance, *6E10* was chosen instead for APP/Aβ

Table 1. *Variability of APP and Aβ detection methods with 6E10.* Relative levels of APP and Aβ in PSAPP and PSAPP ABKO mice ran on two different days. Mice were distinguished by a unique identification number, shown in Column 2. Multiple samples of each mouse lysate were run per day, normalized to β-actin, and then averaged prior to further analysis.

APP Quantification	I.D.	*Day 1*	*Day 2*
	PSAPP		
	1894	0.258183472	0.258183472
	24	0.868717765	0.815585502
	PSAPP ABKO		
	1925	0.183923831	1.144750539
Aβ Quantification			
	PSAPP		
	1894	0.068236378	0.071164
	24	0.144885488	0.174851
	PSAPP ABKO		
	1925	0.048146112	0.068137

quantification at a working concentration of 1-2500. Furthermore, since issues with C-terminal antibody resolution prevented effective separation and measurement of the CTF89 and CTF99 APP fragments, and it was decided that the immunoassay would focus on a comparison of APP and Aβ levels, rather than amyloidogenic and nonamyloidogenic CTF fragments. To allow for ideal separation and visualization of

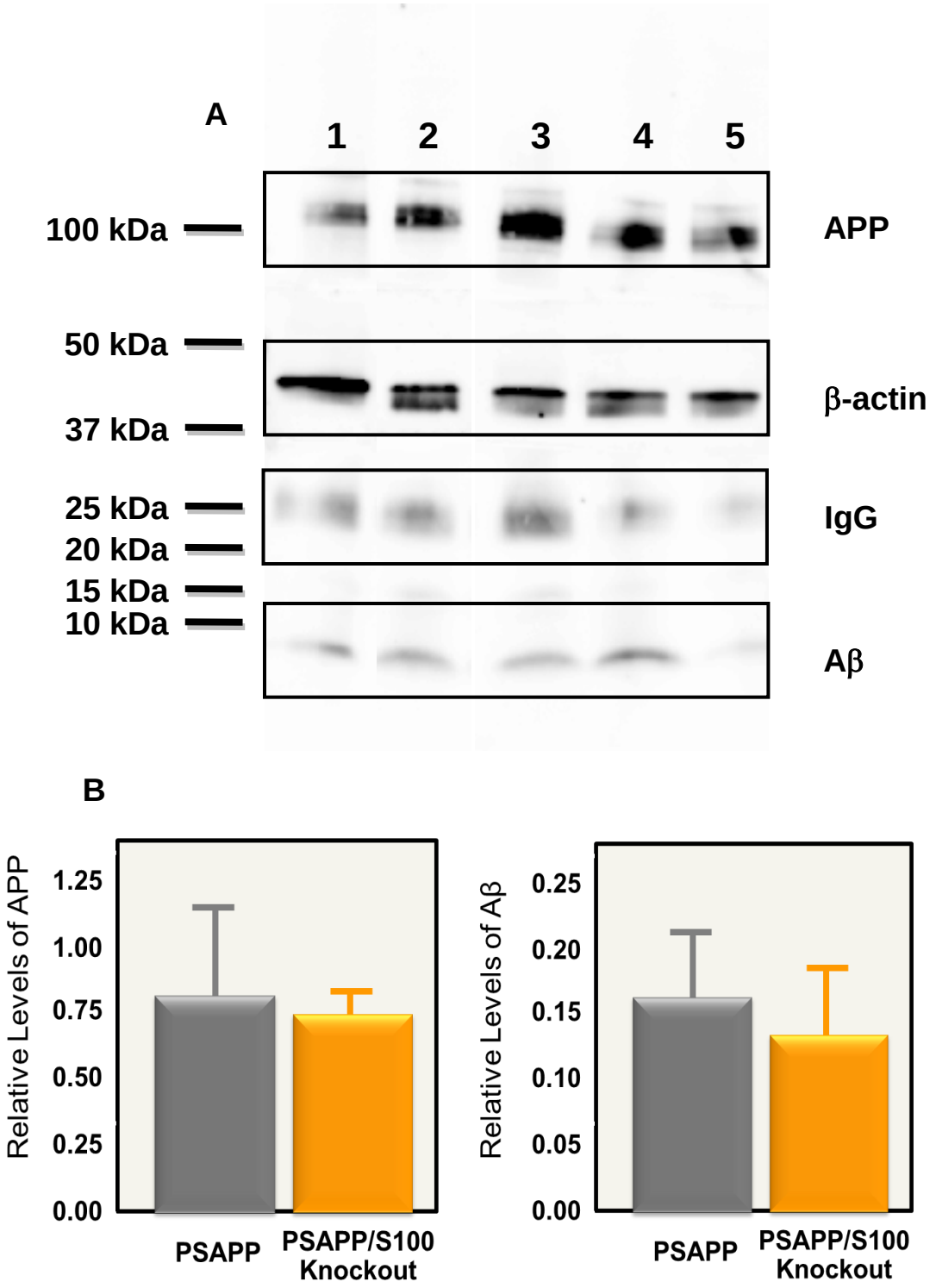

Figure 3. *Quantification of APP and Aβ levels.* ***A***, Representative western blot image. Lanes correspond to lysates as follows, with mouse ID and genotype in parentheses: **1** (1894, PSAPP), **2** (24, PSAPP), **3** (2001, PSAPP S100ABKO), **4** (1893, PSAPP), **5** (1976, PSAPP S100ABKO). Molecular weights of detected bands were determined by calibrating to standard markers, shown here indicating size in kilo Daltons (kDa). ***B***, The histograms depict cumulative data on APP and Aβ levels ± SEM in PSAPP (gray bars, n = 5) and PSAPP S100 ABKO (orange bars, n = 5) mice. Values were expressed as relative amounts normalized to β-actin within gels and were also normalized across gels.

APP and Aβ bands, gels ran for 70 minutes (100 V) and were transferred for 1 hour (30 V). For 6E10 and B-actin primary antibodies, species specific HRP labeled Goat anti-mouse secondary was used at concentrations of 1-10000 and 1-15000 respectively.

APP/Aβ quantification

Membranes were cut horizontally along reference bands in the standard lane corresponding to molecular weights of 50 and 25 kDa to allow for separate development of APP (~ 100 kDa), β-actin (~42 kDa), and Aβ (~6 kDa) bands. It was determined that additional bands present at 110, 50, 25 kDa were due to IgG binding and did not influence quantification of the other bands. Samples from PSAPP (n = 5) and PSAPP ABKO (n = 5) mice were quantified to determine amounts of APP and Aβ present in brain cortex tissue. APP and Aβ levels were expressed as relative ratios of respective protein area to β-actin area, to account for loading error (Tab. 1). The coefficient of variation between gels for PSAPP sample 1894 was used as a normalization factor. Average levels of APP and Aβ normalized to β-actin have been displayed along with standard error of the mean (Fig. 3). The two-tailed P value for mean differences in PSAPP and PSAPP ABKO mice was 0.7979 for the APP comparison and provided a P value of 0.7042 for the Aβ comparison, well above the 0.05 threshold set to assess significance. Thus, there is no difference in levels of APP and Aβ in PSAPP and PSAPP ABKO mice.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In summary, the Western blotting results demonstrated that respective APP and Aβ levels in PSAPP and PSAPP/S100A1$^{-/-}$/ S100B$^{-/-}$ mice were indistinguishable. Although Tg2576 mice overexpressing S100B demonstrate increased levels of Aβ and amyloidogenic processing (Mori et al., 2010), PSAPP ABKO mice do not demonstrate the converse effect. This may be attributed to compensatory mechanisms that act to share the role of the absent S100 proteins. Since S100s have highly homologous protein structures, it is possible that the function of other similar proteins account for the similarities in APP and Aβ levels in control and experimental mice.

Future areas of study include the mechanisms of plaque buildup and Aβ clearance. S100A1 and S100B may work to regulate the relative production of Aβ isoforms. This effect may resemble the activity of certain mutations in familial AD that cause the more aggregate-prone $A\beta_{1\text{-}42}$ variant to predominate over $A\beta_{1\text{-}40}$ and thereby contributing to differences in plaque load. The current experimental conditions were directed toward quantifying soluble fractions of brain lysates and thus could not account for differences in plaque composition. Nevertheless, insoluble Aβ fractions could be treated with formic acid and then analyzed for isoform differences. The Enzyme-linked immunosorbent assay (ELISA) could also be used in measurements due to the high resolution ability of the technique.

S100 proteins may also influence plaque load at the level of molecular buildup or clearance. S100 interactions with the RAGE receptor may mediate transport of Aβ across the blood brain barrier. In the absence of S100A1 or B, it is possible that there is a shift in relative transport of Aβ in and out of systemic circulation and a consequent reduction in plaque load. In conclusion, our data suggest that the decline in pathology found in PSAPP/S100A1$^{-/-}$/ S100B$^{-/-}$ mice is not induced by changes in APP processing, and point toward several other possible mechanisms of action.

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