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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Cell Biology

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Serotonergic Signaling Pathways that Suppress Amyloid Beta in Mouse Models of AD

by

Jonathan Robert Fisher

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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ABBREVIATIONS

3xTgAD: 3 mutation transgenic Alzheimer's disease mouse line

5-HT: serotonin (5-hydroxytryptamine)

5-HTR: serotonin receptor

5XFAD: 5 mutation transgenic Alzheimer's disease mouse line

Aβ: amyloid beta

AD: Alzheimer's disease

ADAM10: a disintegrin and metallopeptidase 10

ADAM17: a disintegrin and metallopeptidase 17

α-secretase: alpha-secretase

Aph1: anterior pharynx-defective 1

APP: amyloid precursor protein

β-arrestin: beta arrestin

β-secretase: beta-secretase

BACE1: β-site APP cleaving enzyme 1

BSA: bovine serum albumin

CamKII: Calcium-calmodulin dependent kinase two

cAMP: cyclic adenosine monophosphate

cFOS: FBJ osteosarcoma oncogene

CHO cells: Chinese hamster ovary cell line

CNS: central nervous system

CSF: cerebrospinal fluid

D1R: Dopamine 1-like Receptor

ERK: extracellular signal-regulated kinase (mitogen activated protein kinase)

γ-secretase: gamma-secretase

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GPCR: g protein coupled receptor

HEK cells: human embryonic kidney cell line

ISF: interstitial fluid

JNK: c-Jun N-terminal kinase

LRP1: low density lipoprotein receptor 1

MEK: MAPK/ERK kinase (mitogen activated kinase kinase)

MMP2: matrix metallopeptidase 2

MMP9: matrix metallopeptidase 9

M1R: muscarinic acetylcholine 1 receptors

NFT: neurofibrillary tangles

NMDA: N-methyl-D-aspartate

PBS: phosphate buffered saline

pERK: phosphorylated (active) ERK

PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase

PIB: Pittsburg compound B

PKA: protein kinase a

PKC: protein kinase c

PS1: presenilin 1

PSEN2: presenilin enhancer 2

sAPPα: soluble APP alpha-secretase fragment

Src: Src (Sarcoma) tyrosine kinase

SSRI: selective serotonin reuptake inhibitor

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ABSTRACT OF THE DISSERTATION

Serotonergic Signaling Pathways that Suppress Amyloid Beta in Mouse Models of AD

By

Jonathan Robert Fisher

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2014

Professor John Robert Cirrito, Chair

A diagnosis of Alzheimer's disease is one of the most devastating things one can hear. This terrible disease robs people of their ability to remember cherished events as their brains become riddled with beta amyloid plaques. Alzheimer's is especially terrifying because there currently are no effective treatments for slowing or stopping the disease. However, recent research has shown that plaque formation is correlated to concentrations of amyloid beta. This discovery suggests that limiting amyloid beta production could potentially halt the disease. One promising avenue for slowing amyloid beta production is serotonergic signaling.

This dissertation presents evidence for a direct sequence of signaling events from serotonin receptors to reduction of amyloid beta by alpha-secretase. Two serotonin receptors, 5-HT_4R and 5-HT_7R, reduce amyloid beta levels in the brains of transgenic mice that model Alzheimer's disease. These receptors are linked to activation of PKA, and blocking PKA activity increases amyloid levels in mice. PKA leads to activation of ERK, a kinase which acts in both the nucleus and the cytoplasm. We show SSRI antidepressant treatment fails to produce changes in gene expression which suggests ERK acts within the cytoplasm to reduce amyloid beta.

Finally, we show that selective inhibition of ADAM10, the primary alpha-secretase, is unable to block the beneficial effects of SSRI antidepressants in transgenic mice. These discoveries explain the mechanisms regulating amyloid beta reduction by serotonin activity and also offer a more selective therapy for Alzheimer's disease.

Chapter 1: Introduction and Significance

Alzheimer's Disease (AD) is one of the most insidious and damaging conditions menacing mankind today. AD dementia starts relatively benign with minor forgetfulness of every day experiences or mundane appointments. However, it progresses to destroy memories of key experiences, eliminates recognition of close family members, and ultimately annihilates a person's ability to care for oneself as an adult. Even if one ignores the severe mental trauma of family and friends as someone progresses through AD, the economic damage is substantial enough to give anyone pause. More than 5 million Americans are currently estimated to have AD, and the number is expected to triple in the coming decades (Hebert et al., 2013). The cost of care for dementia in the United States was between $157 and $215 billion dollars in 2010 (Hurd et al., 2013). Extrapolation of the costs in coming decades leads one to realize how large a threat AD is to global health and economic stability. Understanding the mechanisms of the disease and developing treatments to combat it are the only way to stop the imminent threat.

AD Pathology and Progression

Although AD was identified over 100 years ago, the mechanisms driving its pathology have only been characterized in the last few decades. Current hypotheses suggest AD is driven by accumulation of two distinct proteins in the brain: amyloid beta (Aβ) and tau. Tau is a microtubule associated protein, but dysregulation of its phosphorylation state leads it to abandon microtubules and accumulate as intracellular neurofibrillary tau tangles (NFTs) (Holtzman et al., 2011). Aβ is produced by sequential cleavage of the membrane-bound amyloid precursor protein (APP) by beta (β) and gamma (γ) secretase enzymes (Seubert et al., 1993; Edbauer et al., 2003). This cleavage results in the release of a 37-42 amino acid peptide into the extracellular space, the interstitial fluid (ISF), and eventually the cerebrospinal fluid (CSF). Various mechanisms in the body help remove Aβ from brain tissue. Several enzymes, such as neprilysin and matrix-

metalloproteinase-9 (MMP9), are produced by glial cells to degrade Aβ (Yan et al., 2006; Yin et al., 2006; Hickman et al., 2008). These enzymes break down Aβ within the brain. Bulk flow of CSF into the blood stream removes large amounts of solutes such as Aβ (Ji et al., 2001; Iliff et al., 2012). However, these Aβ clearance mechanisms eventually become ineffective. Shibata et al showed that Aβ clearance from the brain drops as mice age, and higher concentrations of Aβ in the CSF hindered its clearance (Shibata et al., 2000). When concentrations of Aβ reach a critical threshold, the peptide begins aggregating and precipitates out of solution to form extracellular amyloid plaques (Lomakin et al., 1997; Bero et al., 2011). Although the $A\beta_{40}$ peptide is more prevalent in the brain, the $A\beta_{42}$ peptide is more prone to aggregation (Jarrett et al., 1993; McGowan et al., 2005; Chen and Glabe, 2006). There are two classes of Aβ plaques present in the brain: diffuse and fibrillar. Diffuse plaques contain Aβ in non-beta sheet conformations; these plaques have ill defined borders and are not associated with neurite damage (Iwatsubo et al., 1994; Morris and Price, 2001; Holtzman et al., 2011). Fibrilar plaques contain a dense core of Aβ that is arranged in a beta-sheet conformation, and they are surrounded by dystrophic neurites and activated glial cells (Iwatsubo et al., 1994; Morris and Price, 2001; Holtzman et al., 2011). The increased gliosis and neuropathy around fibrilar Aβ plaques suggests they are more toxic than diffuse plaques. Accumulation of plaques and NFTs leads to loss of function, degeneration, and death of neurons. The neuronal dysfunction and death likely lead to the dementia symptoms characteristic of the disease.

Several studies support the role of Aβ initiating AD. People expressing mutations in APP near β or γ-secretase cleavage sites show early onset of the disease, and the mutations can be passed to progeny in a dominant fashion (Goate et al., 1991; Murrell et al., 1991). Mutations in

γ-secretase subunits can also induce early AD onset and can be passed along to children (Borchelt et al., 1997). These familial AD patients are valuable resources for scientific study. The progression of Down syndrome also supports the idea that Aβ causes AD. People suffering from Down syndrome have three copies of chromosome 21, the chromosome that encodes APP, and these patients always develop AD pathology while young (Holtzman et al., 2011). Postmortem analysis of humans with familial AD mutations showed the presence of NFTs increased with the number of Aβ plaques (Price and Morris, 1999). Interestingly, the same study showed some congnitively normal controls also possessed Aβ plaques suggesting they may have been in the pre-clinical phase of the disease (Price and Morris, 1999). While NFTs are part of AD pathology, they are not considered the initial trigger for the disease. People with mutations in tau develop a separate disease known as frontotemporal dementia where plaques are absent (Ballatore et al., 2007). Also, recent work has shown that changes in CSF Aβ levels preceed changes in tau in humans carrying familial AD mutations which suggests Aβ disregulation is the initial trigger for AD (Bateman et al., 2012). Most importantly, these changes in Aβ levels began decades before signs of AD dementia manifested (Perrin et al., 2009; Holtzman et al., 2011; Bateman et al., 2012). If the pathology begins decades prior to AD symptoms, then there is a large window for potential therapy. Once possible treatment could be targeting the processing of APP into Aβ.

Aβ Processing: APP, secretases, and endocytosis

Production of Aβ requires complex coordination of multiple proteins. As stated previously, APP is an integral membrane protein of unknown function. Knocking out the gene in mice has no deleterious effects (Matrone et al., 2011). The protein is widely expressed

throughout the body. However, APP levels are highest in the brain and can be found across multiple regions (Tanzi et al., 1987). At a sub-cellular level, APP can be found on the plasma membrane, golgi, pre-synaptic vesicles, and endosomes of neurons (Marquez-Sterling et al., 1997; Chyung and Selkoe, 2003). Interestingly, expression of cell surface APP is most prevalent on axons, but it shows diffuse intracellular staining across the entire cell (Marquez-Sterling et al., 1997). This diffuse staining is APP in neuronal vesicles or endosomes. APP expression appears restricted to neurons under normal circumstances. However, activated astrocytes begin expressing APP following brain injury (Siman et al., 1989). Similar results were shown with microglia. APP expression was absent until microglia were activated by spinal cord injury (Banati et al., 1993). Glial activation is observed in AD, but does not occur until plaques achieve fibrilar status. Therefore, the initial deposition of Aβ is most likely due to neurons. Also, there is evidence the potential for Aβ production is lower in glia than neurons. Zhao et al showed that transgenic mice overexpressing mutant APP specifically in astrocytes produced less Aβ than mice that overexpressing mutant APP in neurons (Zhao et al., 1996).

Aβ production begins with β-secretase activity. The putative β-secretase enzyme is known as memampsin2 or BACE1, and it cleaves APP just outside its transmembrane domain at methionine 596 (Seubert et al., 1993; Vassar et al., 1999; Lin et al., 2000). BACE1 can be found in multiple organs, but its highest expression is in the brain and pancreas (Vassar et al., 1999; Lin et al., 2000). BACE1 is a transmembrane protein found in the golgi, vesicles, and endosomes (Vassar et al., 1999). shRNA against BACE1 significantly reduces Aβ levels, and crossing BACE1 knockout mice with mice expressing mutant APP completely blocks plaque formation (Vassar et al., 1999; Luo et al., 2003). Conversely, overexpression of BACE1 increases Aβ production in vitro (Vassar et al., 1999).

The final step of Aβ production requires γ-secretase. γ-secretase is a complex of presenilin-1 (PS1), nicastrin (NCT), anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2) (Edbauer et al., 2003). Following β-secretase activity, γ-secretase cleaves the remaining APP fragment within the transmembrane domain in a stepwise manner to release the $A\beta_{40}$ or $A\beta_{42}$ peptide (Takami et al., 2009). Mutations in PS1 can lead to increased production of the aggregation-prone $A\beta_{42}$ peptide (Borchelt et al., 1997). γ-secretase can be found in the plasma membrane, golgi, and endosomal comparments (Lah and Levey, 2000). However, the enzyme complex is not fully mature until leaving the golgi, and this fact suggests it is active at the plasma membrane or within endosomes (Haass et al., 2012). Knocking out components of γ-secretase stops Aβ production in vitro, but KO animals are embryonic lethal due to defects in Notch signaling (Li et al., 2003).

There is a great deal of evidence supporting the idea that most Aβ processing occurs within endosomes. Mutating the YXNP endocytosis motif or the cytoplasmic domain of APP results in a significant reduction in Aβ (Koo and Squazzo, 1994; Perez et al., 1999; Cam et al., 2005). Blocking endocytosis by lowering potassium or by expressing dominant negative dynamin also results in lower Aβ production (Koo and Squazzo, 1994; Chyung and Selkoe, 2003; Cam et al., 2005; Cirrito et al., 2008; Zhu et al., 2012). BACE1 requires a low pH, such as those in endosomes, to function optimally (Vassar et al., 1999). Interestingly, neuronal BACE1 and APP do not enter the same subcellular compartment until the neurons are stimulated (Das et al., 2013). This colocalization is blocked when endocytosis is inhibited with dynasore (Das et al., 2013). Neuronal activity has been tied to Aβ production in other studies as well. Stimulating activity in mouse hippocampal slices increased Aβ in media, and reducing activity had the

opposite effect (Kamenetz et al., 2003). Aβ plaques in humans and mice appear earliest in the default mode network, a group of brain regions which are active even when the subject is not mentally engaged in a task (Buckner et al., 2005; Bero et al., 2011). Lowered neural activity during sleep also plays a role. Aβ levels are higher when mice are awake and lower as they sleep (Kang et al., 2009). Pharmacological manipulation of neural activity confirms its role in Aβ production. Stimulating seizures in mice increases Aβ levels, and increasing neuronal activity with lactrotoxin or picrotoxin has the same effect (Cirrito et al., 2005, 2008). Inhibiting mouse action potentials with tetrodotoxin causes a reduction in Aβ (Kamenetz et al., 2003; Cirrito et al., 2005). This increase in Aβ by neuronal activity is caused by neurons using endocytosis to recycle membrane components after neurotransmitter release (Cirrito et al., 2008).

There is an alternate path for APP that prevents formation of Aβ. Alpha (α) secretase cleaves APP between the β and γ-secretase sites and prevents the production of Aβ (Vingtdeux and Marambaud, 2012). Production of the α-secretase product sAPPα coincides with a reduction in Aβ (Nitsch et al., 1996). A disintegrin and metalloproteinase 10 (ADAM10) has been shown to be the putative neuronal α-secretase enzyme (Lammich et al., 1999; Kuhn et al., 2010). ADAM10 is highly expressed throughout the mouse and human brain (Yavari et al., 1998). Overexpression of ADAM10 in mice expressing mutant APP reduced the concentrations of Aβ and number of plaques in the brain (Postina et al., 2004; Suh et al., 2013). Interestingly, mice overexpressing a dominant negative ADAM10 had increased plaque load (Postina et al., 2004). Active ADAM10 can be found at the plasma membrane or the trans-golgi network (Lammich et al., 1999; Marcello et al., 2013). However, research suggests that ADAM10 can bind adaptin proteins and be internalized after neuronal activity (Marcello et al., 2013). A similar protein,

ADAM17, can also cleave APP as an α-secretase in vitro (Buxbaum et al., 1998; Vingtdeux and Marambaud, 2012). ADAM17 α-secretase activity is upregulated when cells are stimulated with phorbol esters in vitro (Buxbaum et al., 1998; Kuhn et al., 2010). Current research suggests ADAM10 acts as the constitutive α-secretase under basal conditions, and ADAM17 α-secretase activity is increased by extracellular stimulation (Kuhn et al., 2010). Presumably, increasing α-secretase activity could be a potential therapy for AD. Figure 1.1 shows a model of the different processing pathways for APP.

AD mouse models

Multiple transgenic mouse models of AD have been created to investigate the mechanisms of the disease. The first mouse model was developed in 1995 by expressing human APP with a familial AD mutation (Games et al., 1995). These mice developed Aβ plaques, gliosis, and neuropathy in their CNS similar to human AD. Many other mouse models were produced in subsequent years with different gene promoters or different human proteins. Most of these models over-express human APP or an Aβ processing gene with one or more familial AD mutations. Unfortunately, no single mouse model perfectly recreates the entire suite of AD pathologies or recreates the normal human progression of the disease. However, the models have been integral for examining methods of Aβ production, plaque formation, and brain clearance.

The mouse model system we use is known as the APP/PS1 line. These mice overexpress human APP with the Swedish mutation (APPswe), an amino acid substitution where Lysine 670 becomes Asparagine and Methionine 671 becomes Leucine. This familial AD mutation occurs adjacent to the β-secretase cleavage site of APP and enhances processing into Aβ. APP/PS1 mice also express a version of PS1 found in familial AD that lacks its ninth exon (PS1ΔE9).

Expression of both mutant proteins accelerates the process of Aβ aggregation, and the mice develop plaques at 5 months of age (Borchelt et al., 1997; Jankowsky et al., 2004). The APP/PS1 line is not a perfect model of human AD because the mice do not develop tau neurofibrillary tangles or display neuron death. However, their production of Aβ is helpful for examining its metabolism. We use the APP/PS1 mice on a C3H/B6 background and use the *APP/PS1* $^{+/-}$ offspring for experiments; hemizygous mice produce equal amounts of γ-secretase products as homozygous mice (Savonenko et al., 2003; Jankowsky et al., 2004). We use two to three month old mice for all experiments; the dynamics of ISF Aβ change as plaques accumulate in the brain (Cirrito et al., 2003). Using young mice allows us to examine regulatory mechanisms Aβ metabolism under normal physiological conditions.

In vivo microdialysis

We use in vivo microdialysis to monitor cellular processes that modulate brain interstitial fluid (ISF) Aβ levels in APP/PS1 mice. Microdialysis is a method which allows us to sample Aβ on an hourly basis from awake, freely mobile mice. We use a surgical stereotax to place a guide cannula within the left posterior hippocampus of the mouse. The cannula is cemented in place to prevent any movement which could damage the probe. A 2 mm probe with a 38 kilodalton (kDa) molecular weight cutoff dialysis membrane is inserted through this cannula. Proteins under 38 kDa, like the 4.4 kDa Aβ peptide, can freely diffuse across this membrane. A microdialysis buffer solution of artificial CSF containing 0.15-2% bovine serum albumin is pumped through this probe. The artificial CSF mimics the ionic profile of the CSF and ISF; the bovine serum albumin protein helps prevent Aβ from sticking to the plastic tubing that links the syringe pump, microdialysis probe, and fraction collector. Diffused protein samples are collected in a cooled

fraction collector in hourly increments. In order to prevent tangling of the tubing lines, mice are housed in automated RaTurn cages. The cages will rotate in the opposite direction of the animal's movement as directed by a sensor arm attached to a collar on the mouse. These cages allow animals to move freely without the need for restraints to protect the microdialysis probe or lines. Figure 1.2 demonstrates the principles of microdialysis. We perform a sandwich ELISA to measure amounts of $A\beta_{x-40}$ or $A\beta_{x-42}$ in each collected sample. We establish a mean baseline concentration of Aβ for each mouse for 6 hours before treating with pharmacological agents. Most agents we used are small molecules that can freely cross the microdialysis membrane. We can dissolve agents in microdialysis buffer and pump them through the microdialysis probe in a process called reverse microdialysis. This process allows us to deliver drugs specifically to the hippocampus to examine local neuronal effects. Reverse microdialysis also allows us to circumvent the exclusive nature of the blood brain barrier; we can treat mice with compounds that normally would not enter the brain.

Measuring Aβ: CSF vs ISF

There can be some discrepancy between researchers as to which pool of Aβ, CSF or ISF, is preferrential for analysis. The CSF pool is currently the preferred method for Aβ sampling in humans; a spinal tap allows for evaluation of Aβ or other brain metabolites with a minimally invasive procedure. However, spinal taps are applied at the lumbar spine, a site several feet from the brain. Also, the CSF bathes the entire central nervous system. These facts suggest spinal taps may not faithfully represent contents of a specific brain region. Also, the slow six hour turnover rate and lack of active pumping means CSF components may be several hours old when drawn

from the lumbar spine (Cserr et al., 1992). Therefore, CSF sampling for Aβ is best for analyzing global changes in the CNS over longer periods of time.

The ISF pool produces an accurate and fast readout for Aβ metabolism in a specific brain region. Changes in ISF Aβ reflect changes in localized neuronal activity and APP processing (Cirrito et al., 2003). The downside of using the ISF is the level of invasion. Sampling ISF Aβ requires surgical implantation of a microdialysis probe directly into the brain. This probe allows for diffusion of small molecules, such as Aβ, across the membrane. A solution of artificial CSF flows through the probe and diffused proteins are collected for analysis by ELISA. These microdialysis probes allow for hourly collection and measurement of Aβ from a specified brain region (Cirrito et al., 2003). Even though they are more precise, the microdialysis measurements of ISF Aβ are most often used only in research animals.

Current AD Therapies and the Link to Depression

One of the reasons AD is so terrifying is its lack of effective treatments. The only two treatments currently available are acetylcholinesterase inhibitors or NMDA antagonists. However, these drugs only focus on treating the dementia symptoms instead of stopping the disease (Finkel, 2004; Shen et al., 2011). Innoculating mice with Aβ to stimulate the immune system showed promise in slowing the disease. However, clinical trials innoculating humans with aggregated Aβ showed lower efficacy and were stopped due to severe side effects (Hock et al., 2003; Orgogozo et al., 2003; Serrano-Pozo et al., 2010). A different route of therapy attempted to infuse an Aβ antibody, solenezumab, into AD patients. Solenezumab was well tolerated in a Phase II clinical trial, but there was no evidence of improved cognitive ability in patients (Farlow et al., 2012). Another avenue for combating the disease would be to reduce γ-

secretase activity. Unfortunately, this method of therapy was ineffective. A recent clinical trial showed that semagacestat, a γ-secretase inhibitor, had no protective effect in AD patients and caused severe side effects like skin lesions and cancers (Doody et al., 2013). A alternative therapy target is needed that can effectively slow the disease without killing the patient.

Facing an AD diagnosis is a sad event, but several studies have shown a link to depressive disorders and AD. Meta-analysis of human AD studies show many patients exhibit signs of depression one year before AD diagnosis (Green et al., 2003). However, this result is confounded by the fact early AD symptoms can manifest as depression (Lopez et al., 2003). If the analysis is continued further back, however, there still is a strong correlation of developing AD even if the depression occcurred decades before disease onset (Green et al., 2003). Other analyses of human studies show that a single episode of depression increases chances of AD later in life (Geerlings et al., 2008; Geda, 2010). Studying the link between AD and depression led to a fortiutous discovery utilizing a novel Aβ imaging technique. Pittsburg compound B (PIB) was developed in 2006, and this radiotracer labeled Aβ plaques so they could be read with PET scans in living people (Mintun et al., 2006). Retroactive analysis of elderly depressed patients with PIB revealed that those who had been treated with selective serotonin reuptake inhibitor (SSRI) compounds in the five years preceeding the study had less Aβ signal than those without SSRI therapy (Cirrito et al., 2011). Increasing serotonin levels had an negative effect on Aβ deposition.

Serotonin in the CNS

Serotonin (5-hydroxytryptamine or 5-HT) is one of the most prominent neurotransmitters in the brain. Initially discovered in the gut in the 1930s, 5-HT was shown to constrict blood vessels, and this discovery defined its name (Hannon and Hoyer, 2008). 5-HT is highly

conserved in the animal kingdom. 5-HT receptors (5-HTRs) can be found in lower species such as flat worms, *C. elegans*, and *Drosophila* as well as higher organisms like man. Serotonergic signaling is widespread throughout the brain. Cell bodies of serotonergic neurons reside within the median raphe nuclei of the brain stem (Steinbusch, 1981). Projections from these neurons travel to the nearly all regions of the CNS. Serotonergic fibers innervate the thalamus, hypothalamus, septum, caudate-putamen, and the hippocampus; all regions of the cortex show serotonergic input as do the cerebellum and spinal cord (Steinbusch, 1981).

The widespread influence of 5-HT throughout the brain means it regulates a variety of important brain functions. Projections to the hypothalamus and limbic system regulate food intake and body temperature (Rodríguez et al., 2012). Serotonergic signaling regulates pain sensation in spinal cord nuclei (Dogrul et al., 2009). 5-HT signaling in the cortex regulates impulse control, general cognition, and decision making in response to threats (Cools et al., 2008). The widespread knowledge of SSRIs clearly displays the impact of 5-HT on emotional regulation. Serotonergic input also plays a role in adult neurogenesis. The subgranular layer of the hippocampus is one of the few places were adults generate new neurons. Selectively killing 5-HT neurons innervating this region or blocking their ability to produce 5-HT significantly reduced BrdU staining in the hippocampus (Brezun and Daszuta, 1999). Neurogenesis in this region is hypothesized to be important to memory formation. There are a myriad of studies linking 5-HT to short and long term memory in the hippocampus and other brain regions (Buhot et al., 2000). These links between 5-HT and memory make the neurotransmitter increasingly interesting with regard to dementia disorders with memory loss like AD.

<u>Serotonin Receptor Subtypes</u>

The longevity of 5-HT as a signaling molecule in the animal kingdom led to a diverse number of 5-HT receptors (5-HTRs). There are at least 14 different 5-HTRs expressed in the body each of which produce multiple isoforms (Hannon and Hoyer, 2008). These receptors are categorized into 7 classes by their pharmacological activity, downstream signaling pathways, and protein structure. The majority of these classes are G-protein coupled receptors (GPCRs), but 5-HT_3Rs are ion channels. I will describe the major classes below.

5-HT_1Rs and 5-HT_5Rs are coupled to $G_{i/o}$ proteins. Activation of these receptors leads to Protein Kinase C (PKC) activation and inhibition of adenylate cyclase (Francken et al., 2000; Leone et al., 2000; Adayev et al., 2003). There are five different 5-HT_1R subtypes, but they show similar expression profiles in the brain. 5-HT_1Rs can be found presynaptically as autoreceptors on serotonergic neurons as well as post-synaptically in the cortex, caudate, hippocampus, and amygdala (Hannon and Hoyer, 2008). There are two subtypes of 5-HT_5Rs, but only 5-HT_{5A}R is expressed in humans (Francken et al., 2000). 5-HT_{5A}R expression is high in the human cortex, hippocampus, and cerebellum (Pasqualetti et al., 1998). Expression of 5-HT_1R is high in the mouse hippocampus, but 5-HT_5R expression is quite low in comparison to other classes (Tanaka et al., 2012).

5-HT_2Rs preferentially bind G_q proteins and activate Calcium-calmodulin activated kinase 2 (CaMKII) (Hannon and Hoyer, 2008; Lairez et al., 2013). There are three 5-HT_2R subtypes with varying expression profiles. 5-HT_{2A}R shows strong signal in the cortex and medulla, but weak hippocampal staining (Hannon and Hoyer, 2008). 5-HT_{2B}R can be found throughout peripheral tissues, but its expression is low in the brains of humans (Kursar et al., 1994; Tanaka et al., 2012). 5-HT_{2C}R is found in the hippocampus, amygdala, and thalamus

(Hannon and Hoyer, 2008). 5-HT_{2A}R and 5-HT_{2C}R show moderate expression in the mouse hippocampus, but are less prevalent than 5-HT_1Rs (Tanaka et al., 2012).

5-HT_3Rs are unique in the fact they form ligand-gated ion channels instead of acting as GPCRs. The two subtypes form heteromeric pentamer complexes that trigger Ca^{2+} and Na^+ influx to depolarize the cells (Hannon and Hoyer, 2008). Expression of 5-HT_3R in the brain is relatively low compared to other 5-HT receptors. However, there is higher 5-HT_3R signal in the hippocampus of most species (Hannon and Hoyer, 2008). Expression of 5-HT_3R is slightly lower than 5-HT_2Rs in the mouse hippocampus (Tanaka et al., 2012)

5-HT_4R, 5-HT_6R, and 5-HT_7R preferentially bind G_s proteins, increase cyclic AMP (cAMP) levels via adenylate cyclase, and activate Protein Kinase A (PKA) (Robert et al., 2001; Norum et al., 2003; Hannon and Hoyer, 2008). Splicing variation produces at least nine different 5-HT_4R isoforms, and their expression is widespread throughout the brain. 5-HT_4R can be found in the septum, striatum, hippocampus, substantia nigra, and amygdala (Hannon and Hoyer, 2008). Expression levels of mouse 5-HT_4R are second only to 5-HT_1R in the hippocampus (Tanaka et al., 2012). 5-HT_6R is expressed in human striatum, amygdala, hippocampus, and cortex (Hannon and Hoyer, 2008). Expression in the rat brain is quite strong in all areas of the hippocampus (Ballaz et al., 2007). Alternative splicing of 5-HT_7R gives rise to 4 different isoforms with similar pharmacological profiles (Hannon and Hoyer, 2008). 5-HT_7R expression in the rat brain is strong in the hippocampus as well as within the thalamus (Ballaz et al., 2007).

Serotonin and AD

Several studies have linked reduced serotonergic activity with the progression of AD. Post mortem analysis of human AD brains showed a reduction in overall 5-HT levels and

reduction in 5-HT$_4$R (Reynolds et al., 1995; Nelson et al., 2007; Rodríguez et al., 2012). These reductions in 5-HTR were still present even when accounting for neuronal death from the disease. Numbers of serotonergic neurons in the dorsal raphe nuclei were shown to decline in human AD patients (Hendricksen et al., 2004). Similar effects are seen in AD animal models. APP$_{swe}$/PS1$_{\Delta E9}$ mice express mutant forms of APP and PS1 that were found in familial AD patients. Studies have shown that serotonergic projections degenerate near Aβ plaques and overall projection count declines with age in APP$_{swe}$/PS1$_{\Delta E9}$ mice (Liu et al., 2008). Levels of serotonergic somas in the raphe nuclei were reduced by 50% in 18 month old mice while other neuronal types were unaffected.

The connection between 5-HT and AD became more prominent when 5-HT activity was shown to reduce Aβ production. Stimulating 5-HT$_4$R in CHO cells increases α-secretase processing of APP to rapidly increase sAPPα (Robert et al., 2001). Activation of 5-HT$_2$R with serotonin in fibroblasts led to an increase in sAPPα as well (Nitsch et al., 1996). Similar effects have been seen in vivo. Treating guinea pigs with 5-HTR agonist reduced Aβ levels and increased sAPPα in the CSF (Arjona et al., 2002). Interestingly, diets high in tryptophan, the amino acid base for 5-HT synthesis, were shown to reduce Aβ plaque loads in the hippocampi of APP transgenic mice by 17% (Noristani et al., 2012). Further analysis showed that the high tryptophan diet increased serotonergic activity in multiple brain regions. Chronic SSRI treatment in 3xTgAD mice, a mouse model of AD, reduced memory deficits and reduced Aβ levels in the brain (Nelson et al., 2007). SSRI modulates Aβ in acute phases as well. Treatment with several SSRIs in the APP/PS1 mouse model showed a 25% reduction in ISF Aβ within a matter of hours (Cirrito et al., 2011) (Figure 1.3). Chronic treatment over the course of several months caused a

50% decrease in plaque load in these mice. A single treatment with 5-HT or 5-HT_4R agonist increased sAPPα and reduced Aβ in the CSF of 5XFAD mice (Giannoni et al., 2013). These impressive results appear to occur in humans as well. Young adult humans given the SSRI citalopram showed a reduction in CSF Aβ in a matter of hours (Sheline et al., 2014).

There already is evidence that SSRI antidepressants may be protective against AD. A recent population analysis in Denmark charaterized depressed citizens, their depression therapy, and their incidence of dementia. Patients taking anti-depressants showed a modest reduction in developing AD (Kessing et al., 2011). Many AD patients present depression symptoms, and they are treated with anti-depressants in addition to their AD medication. A recent study compared AD patients taking SSRI antidepressants and acetylcholinesterase inhibitors to people only taking the acetylchoinesterase inhibitors. Patients taking both classes of drugs showed improved cognitive scores and were able to perform more daily tasks than those people taking AD therapy alone (Mowla et al., 2007). These studies suggest SSRI antidepressants have potential to slow or prevent AD.

Modulating 5-HT is a very promising treatment for AD. SSRIs have been cleared for safe use in humans and are already prescribed to millions to fight depression. Although SSRIs can produce side effects, they are tolerable enough that 85% of patients will continue therapy despite discomfort (Hamon and Blier, 2013). However, SSRIs are not specific for a 5-HT receptor; they merely prolong the amount of time 5-HT stays in the synapse. Identifying which of the 14 different 5-HT receptors are responsible for reducing Aβ would allow for more precise treatment of AD and would mitigate unpleasant side effects of broad SSRI therapy. Elucidating the downstream signaling cascades and effector proteins could also provide more therapeutic targets.

Figure 1.4 diagrams the multiple 5-HTRs, their downstream kinases, and possible mechanisms for reducing Aβ.

Using pharmacology we have shown the 5-HTRs that stimulate G_s proteins can reduce ISF Aβ as well as SSRI. Blocking downstream PKA signaling activity of these receptors completely abolishes the effects of SSRI. We also provide evidence the reduction in Aβ is not mediated by changes in gene expression, but is dependent on changes in α-secretase activity. These discoveries narrow the future search for AD treatment to a select group of proteins.

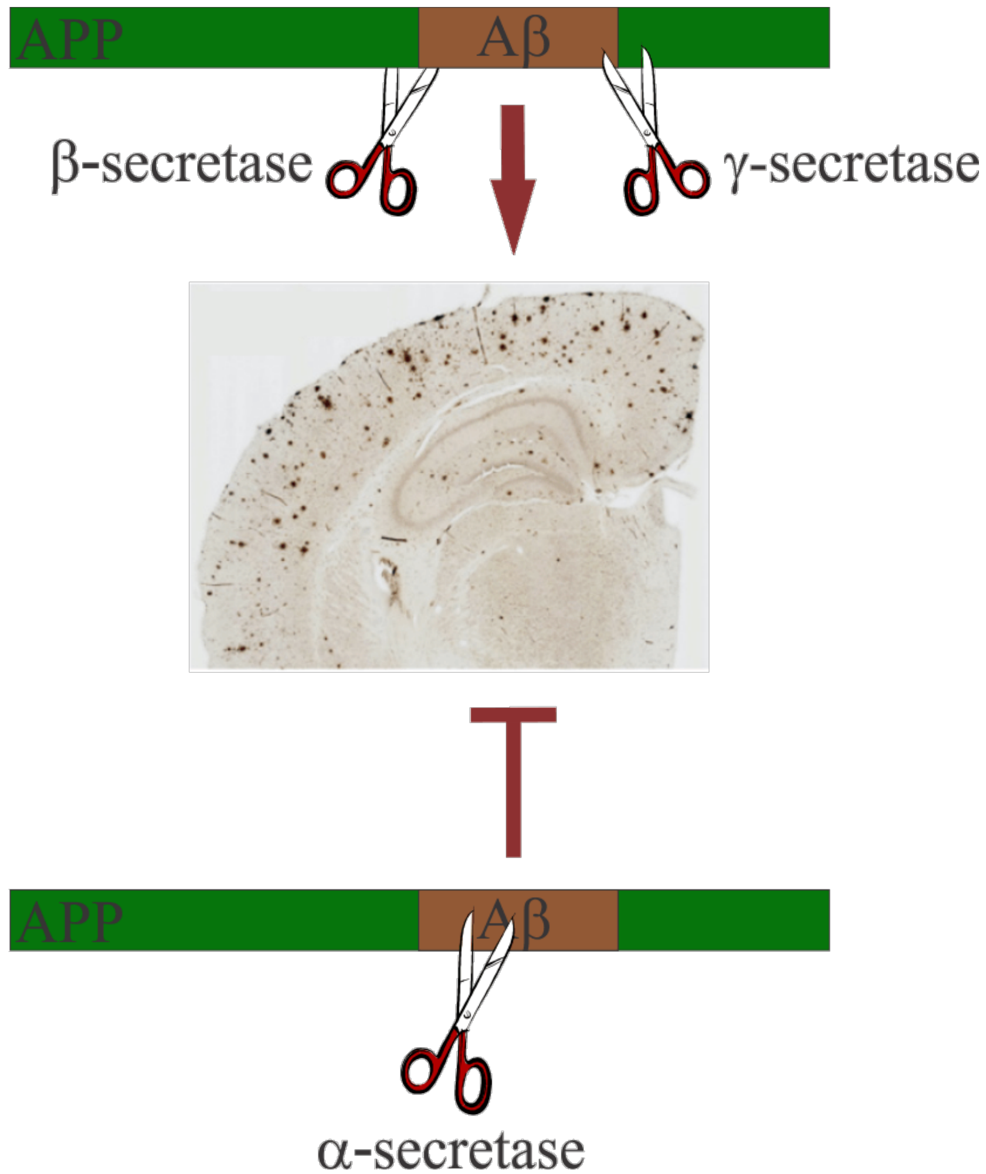

Figure 1.1: Model for separate APP processing pathways. APP can be cleaved by β and γ-secretase to release the Aβ peptide. This peptide accumulates in the brain as the amyloid plaques characteristic of AD. The alternative path for APP is cleavage by α-secretase. Cleavage by this enzyme precludes the formation of Aβ.

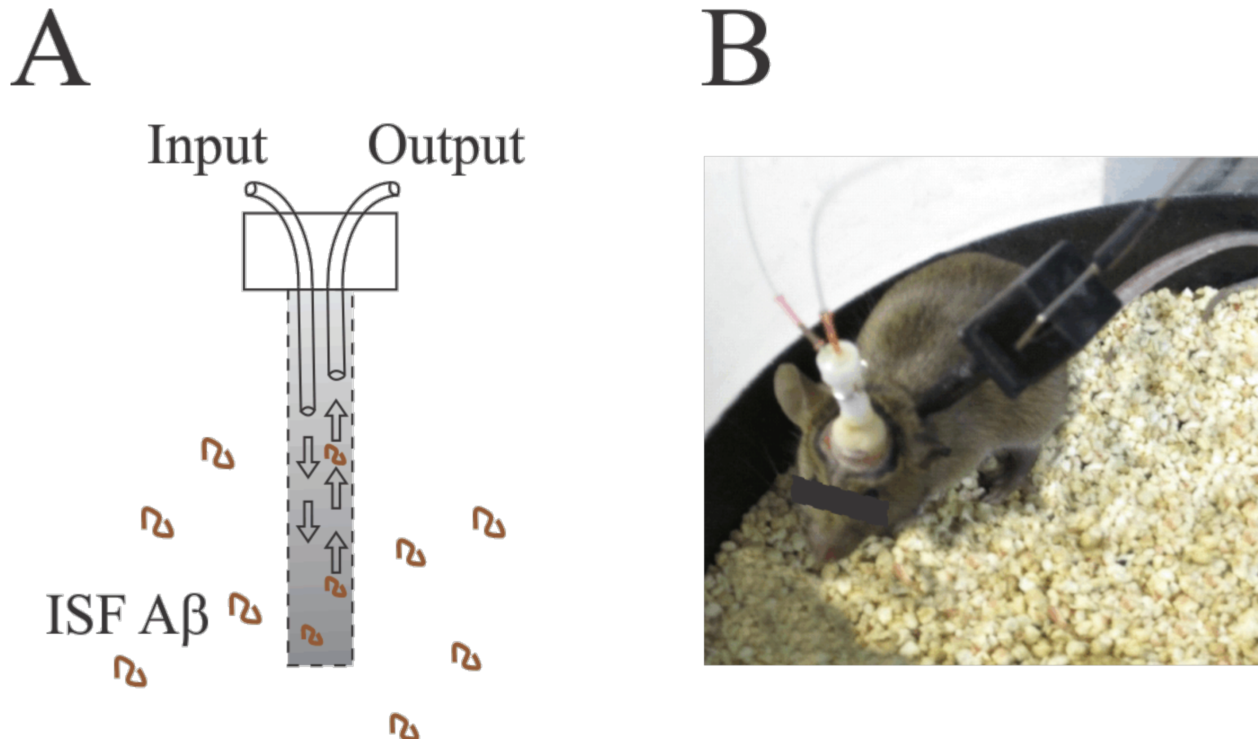

Figure 1.2: In vivo microdialysis allows for measurement of ISF proteins in awake mice. A. Schematic of the microdialysis: the microdialysis probe is inserted into the mouse hippocampus. A syringe pump forces microdialysis buffer into the probe via the input tubing line. Any proteins in the brain ISF that are under 38 kDa, such as Aβ, can cross the semi-permeable probe membrane. Dialyzed proteins exit via the output line to the cooled fraction collector. **B.** Mice are freely moving during microdialysis. The cannula (white) is cemented directly to the mouse skull. Input and output tubing lines are visible. A collar around the mouse's neck is connected to a sensory lever (black) on the round RaTurn cage. As the mouse moves its head, the cage rotates to prevent the mouse from tangling the lines.

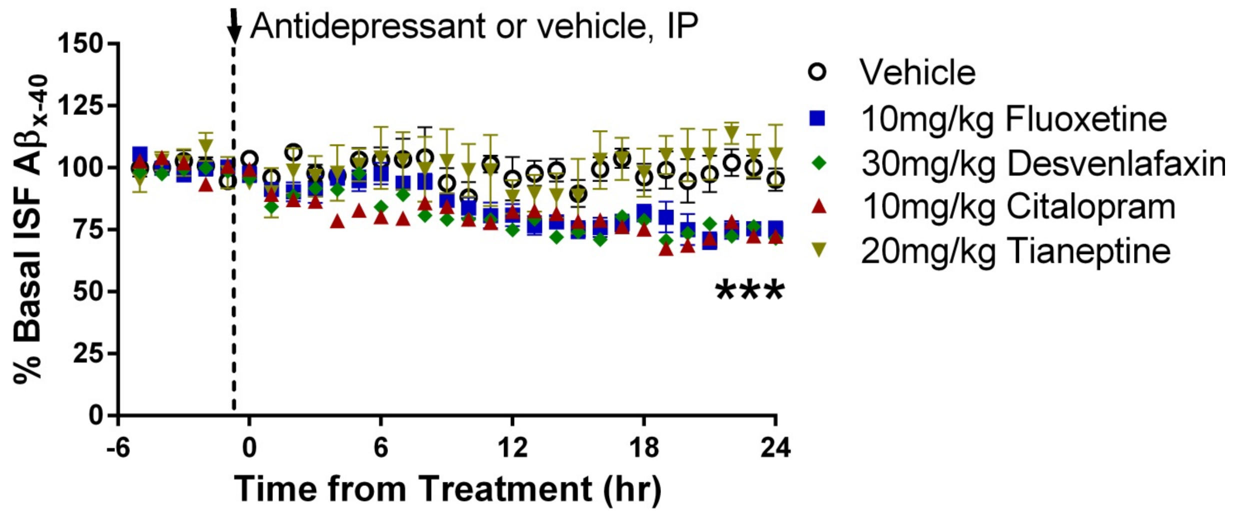

Figure 1.3: Acute SSRI treatment reduces ISF Aβ in APP/PS1 mice. 3 month old APP/PS1 mice were treated with a single dose of anti-depressant drugs. The SSRI compounds Citalopram, Desvenlafaxin, and Fluoxetine reduced ISF Aβ by 25%. However, the non-SSRI agent Tianeptine had no effect on Aβ levels (Cirrito et al., 2011).

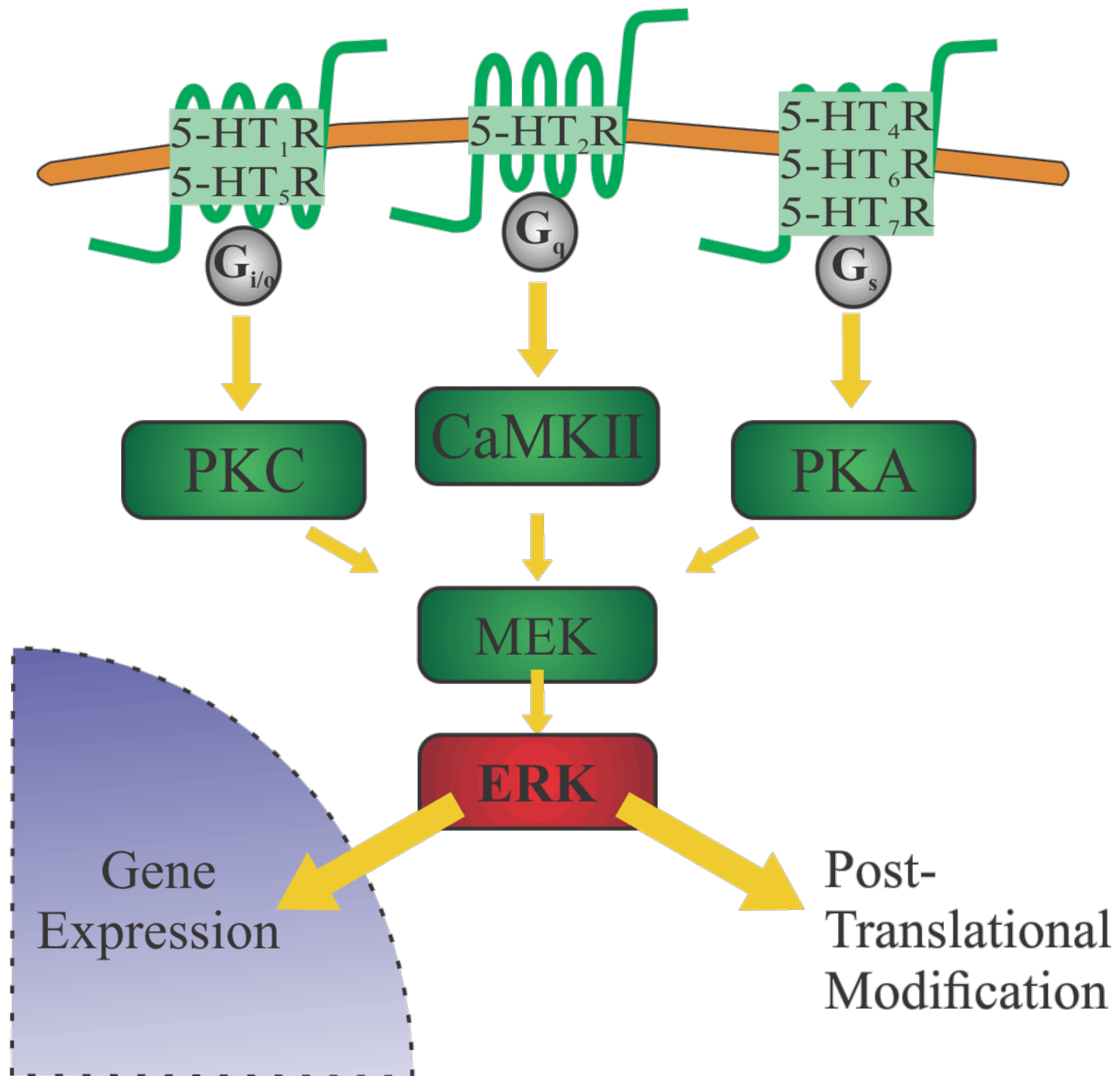

Figure 1.4: 5-HTRs can trigger several intracellular signal cascades. $G_{i/o}$ linked 5-HTRs activate PKC, G_q linked 5-HTRs activate CaMKII, and G_s linked 5-HTRs activate PKA. All these signal kinases lead to MEK and ERK activation. The final targets of activated ERK after serotonergic activity are unknown. ERK can translocate into the nucleus to change gene expression or act on proteins within the cytoplasm.

Chapter 2: 5-HT_4R and 5-HT_7R activate PKA to reduce ISF Aβ

ABSTRACT

Methods that reduce the production rate of Aβ show promise as a therapy for AD. Previous research has shown that single dose treatment with an SSRI or 5-HT can reduce ISF Aβ by 20-25%. However, there are at least 14 different 5-HTRs currently identified by science, and SSRI drugs potentially act on all 5-HTRs by raising 5-HT levels in the synapse. We tested the hypothesis that a subset of 5-HTR subclasses were responsible for this reduction in Aβ. We pharmacologically stimulated individual receptor subtypes with selective agonists via reverse microdialysis and examined the effects on ISF Aβ in the APP/PS1 mouse model of AD. We discovered that G_s linked receptors (5-HT_4R, 5-HT_6R, and 5-HT_7R) could reduce ISF Aβ while other classes had no significant effect. We also found that combinatorial inhibition of 5-HT_4R and 5-HT_7R could significantly raise ISF Aβ when blocking a single receptor had no effect. G_s linked receptors are known to activate PKA activity via adenlyate cyclase and cAMP. Inhibition of PKA activity completely abolished the effects of SSRI and led to an increase in ISF Aβ levels. Stimulating PKA activity with a selective D1R agonist led to a 25% reduction in ISF Aβ. These results suggest that 5-HT acts through the G_s linked 5-HTRs and PKA to reduce ISF Aβ.

INTRODUCTION

Many lines of research suggest that accumulation of Aβ in plaques in the brain is the trigger for AD. Now that its role in the disease is known, studies are examining methods for limiting Aβ production as a potential method of therapy. Research has shown that several neurotransmitters have been able to accomplish this feat in vivo. Stimulating muscarinic M1 receptors reduced Aβ pathology and increased sAPPα levels in 3xTgAD mice (Caccamo et al., 2006). Blocking activity of this receptor or its downstream signaling kinases showed the opposite effects. High doses of NMDA induced a 50% reduction in ISF Aβ in APP/PS1 mice (Verges et al., 2011). Our lab recently showed a 25% reduction in ISF Aβ with a single dose of SSRI or 5-HT in the same animal model (Cirrito et al., 2011). This effect appears to transfer to humans. CSF Aβ dropped after a single treatment with the SSRI citalopram in healthy young adults (Sheline et al., 2014). Identifying the mechanisms underlying these neurotransmitter-mediated reductions in Aβ could produce therapies for AD.

5-HT is a singularly interesting candidate for AD therapy because SSRI drugs are already approved for use in humans for treating disease. Millions across the globe take SSRIs to combat major depressive disorders. Now that it is known 5-HT can reduce Aβ in mice and humans, it is possible that clinical trials can be arranged to test SSRI therapy in AD patients. However, understanding the underlying mechanisms that produce this effect would allow for more targeted therapy. SSRIs are generally well-tolerated, but side effects are known to occur. Gastrointestinal discomfort is most common, but sexual and cardiovascular effects are also prevalent. Identifying the effective 5-HTRs and their downstream signaling components could produce more targeted therapy and abrogate some side effects of SSRIs.

The various 5-HTRs segregate into four major groupings based on their downstream intracellular signaling pathways (Hannon and Hoyer, 2008). $5\text{-}HT_3R$ acts as a cation channel allowing for calcium mediated signaling within the neurons. $5\text{-}HT_1R$ and $5\text{-}HT_5R$ are linked to $G_{i/o}$ proteins and PKC signaling. However, only one isoform of $5\text{-}HT_5R$ is expressed in humans. $5\text{-}HT_2R$ is G_q linked and has been shown to activate CamKII. $5\text{-}HT_4R$, $5\text{-}HT_6R$, and $5\text{-}HT_7R$ activate G_s proteins and PKA signaling cascades. Isolating and identifying the effects of each receptor subclass was difficult in the past as 5-HT binds to each receptor with equal affinity. Fortunately, the success of SSRI therapy has led to pharmaceutical development of selective agonists for individual 5-HTR classes. These agonists are selective for only one 5-HTR subtype. By using these selective agonists, we could identify which 5-HTRs lead to reductions in ISF Aβ.

METHODS AND MATERIALS

Animals

All experiment protocols using animals were performed in accordance to the guidelines proposed by the Animal Safety Committee at Washington University. We bred *APP/PS1ΔE9* (APP/PS1) hemizygous mice (The Jackson Laboratory) (Savonenko et al., 2003) to wildtype C3H/B6 mice and aged the *APP/PS1*$^{+/-}$ offspring to 2-3.5 months for experiments. Mice were screened for APP/PS1 transgenes by PCR from tail DNA.

Compounds

All pharmaceutical compounds were ordered from Tocris Biosciences unless otherwise noted. All compounds delivered by reverse microdialysis were diluted in microdialysis buffer consisting of artificial cerebrospinal fluid (aCSF) with 0.15-2% BSA (Sigma-Aldritch); see Table 2.1 for concentrations used for each experiment. Unfortunately, the absolute concentration of drug that enters the brain is unknown due to possible differences in efflux from the probe, the diffusion of drug within the brain ISF, and the distinct pharmaco-kinetics of each agent. We used a starting concentration ten times stronger than the pharmacological efficacy (IC_{50} for inhibitors and K_a for agonists) of each drug with the assumption that only 10% will cross the microdialysis membrane. Citalopram hydrobromide (Toronto Research Chemicals) was diluted in PBS and injected intraperitoneally (IP) at 10 mg/kg.

In vivo microdialysis

In vivo microdialysis to measure brain ISF Aβ in the hippocampus of freely moving APP/PS1 mice was performed as previously described (Cirrito et al., 2003, 2008, 2011). This

method captures soluble molecules the extracellular fluid that are below the 30 kDa molecular weight cutoff of the probes. Under volatile isoflurane anesthetic, guide cannula (BR style; Bioanalytical systems) were cemented into the left hippocampus (3.1 mm behind Bregma, 2.5 mm lateral to midline, and 1.2 mm below dura at a 12° angle). Two mm microdialysis probes were inserted through the guides so their membranes were completely contained in the hippocampus (BR-2, 30 kDa molecular weight cut-off membrane; Bioanalytical systems). Microdialysis buffer was aCSF (perfusion buffer in mM: 1.3 $CaCl_2$, 1.2 $MgSO_4$, 3 KCl, 0.4 KH_2PO_4, and 122 NaCl, pH 7.35) containing 2% BSA (Sigma) that was filtered through a 0.1 μM membrane. The flow rate was 1.0 μL/min. Samples were collected every 60 or 90 minutes with a refrigerated fraction collector in polypropylene tubes and assessed for $A\beta_{x\text{-}40}$ or $A\beta_{x\text{-}42}$ by ELISA. Basal ISF Aβ levels were defined as the mean concentration of Aβ over the 6 hours preceding drug administration. Aβ levels were normalized to the basal Aβ concentration for each animal. After establishing baseline ISF Aβ, pharmaceutical agents (5-HTR agonists, 5-HTR antagonists, PKA inhibitors) were diluted in microdialysis buffer and infused directly into the hippocampus by reverse microdialysis (see Table 2.1 for concentrations). Citalopram, an SSRI, was intraperitoneally injected 8 hours after drug treatment at 10 mg/kg when testing drug efficacy against SSRI. Statistical significance was assayed using the student T-test method in the GraphPad Prism 6 software.

Aβ Sandwich ELISA

ISF Aβ levels were measured using sandwich ELISAs as described (Cirrito et al., 2011). A mouse anti- $A\beta_{40}$ antibody (mHJ2) or mouse anti- $A\beta_{42}$ antibody (mHJ7.4) was used to capture and a biotinylated central domain antibody (mJH5.1) was used to detect followed by

streptavidin poly-HRP-40 (Fitzgerald Industries). All ELISAs were developed using Super Slow ELISA TMB (Sigma) and absorbance read on a Bio-Tek Epoch plate reader at 650 nm. The standard curves for each assay used synthetic human $A\beta_{1-40}$ or $A\beta_{1-42}$ peptide (American Peptide).

RESULTS

Selective activation of a set of 5-HTRs can reduce ISF Aβ

We administered selective 5-HTR agonists by reverse microdialysis in young APP/PS1 mice to test their efficacy in reducing ISF Aβ. Starting concentrations for each compound are listed in Table 2.1. Special care was given to ensure the estimated doses that crossed the membrane did not exceed concentrations where the compounds lose their selectivity for a given 5-HTR. Treating mice with selective agonists for 5-HT_4R and 5-HT_7R reduced ISF Aβ by 23% and 25% respectively (Figure 2.1 A and B). Interestingly, the reduction in Aβ mediated by activity of a single receptor is equivalent to effects induced by 5-HT itself (Figure 2.1 B). 5-HT_6R agonist treatment was also able to reduce Aβ. However, the 11% reduction was not statistically significant (Figure 2.1A and B). However, this ISF Aβ reduction did not occur for all tested 5-HTRs. Treatment with agonists selective for 5-HT_1R or 5-HT_2R did not produce significant changes in ISF Aβ (Figure 2.1C and D). We chose not to analyze 5-HT_5R because there are no selective agonist compounds for this receptor. Avoiding 5-HT_5R is no loss as its expression is quite low in the mouse hippocampus compared to other receptor subtypes. These results suggest that the G_s linked subset of 5-HTRs is responsible for the reduction in ISF Aβ by SSRI.

Simultaneous inhibition of 5-HT_4R and 5-HT_7R increases ISF Aβ

If 5-HT_4R and 5-HT_7R mediate the reduction in ISF Aβ, then blocking their activity should have the opposite effect. To test this hypothesis, we administered GR113808 and SB258719, selective antagonists for 5-HT_4R and 5-HT_7R respectively, via reverse microdialysis.

We treated the mice with a single antagonist, SB258719, for 8 hours before adding the second. Control mice were given vehicle for 8 hours before treatment with both antagonists. As shown in Figure 2.2A, antagonizing a single receptor did not significantly change Aβ levels at time point 8. However, blocking both receptors for 16 hours induced a significant increase of 32% in ISF Aβ (Figure 2.2B). These findings reinforce the idea that 5-HT_4R and 5-HT_7R are responsible for the reduction in ISF Aβ by serotonin.

PKA Activity Modulates ISF Aβ Levels

The strong effects of 5-HT_4R and 5-HT_7R led us to examine downstream signaling pathways. 5-HT_4R and 5-HT_7R activate G_s proteins and cause PKA activation. To test the effects of PKA in 5-HT mediated ISF Aβ reduction, we administered the selective small molecule PKA inhibitor KT5720 via reverse microdialysis. Treatment with KT5720 led to a modest increase in ISF Aβ levels (Figure 2.3A). After eight hours of treatment with KT5720, we gave mice a 10 mg/kg dose of the SSRI citalopram by IP injection. Interestingly, KT5720 treatment completely blocked the effect of SSRI and increased ISF Aβ concentrations by 36% (Figure 2.3B). To confirm the specificity of the results, we repeated the experiment with a PKA specific peptide inhibitor called PKI 12-22 amide (Dalton et al., 2005). PKI was infused by reverse microdialysis for eight hours, followed by an IP injection of citalopram. Once again, PKA inhibition significantly increased ISF Aβ by 54% despite the presence of SSRI (Figure 2.3B). These results suggest PKA activity is required for ISF Aβ reduction by 5-HT signaling.

If inhibition of PKA increases in ISF Aβ, then PKA stimulation could create the opposite effect. Serotonin is not the only neurotransmitter that can activate PKA; dopamine 1 receptors (D1R) can increase cAMP as well as phosphoinositide hydrolysis (O'Sullivan et al., 2004;

Waddington et al., 2005). To test if PKA activation is sufficient to reduce ISF Aβ levels, we administered SKF 83822, a selective agonist for D1R that only stimulates cAMP production, by reverse microdialysis. Thirty-six hour treatment with SKF 83822 reduced ISF Aβ 24% compared to vehicle control (Figure 2.3C and D). This result suggests activation of PKA is key to reducing ISF Aβ.

DISCUSSION

Previous research has shown that SSRI treatment can reduce brain ISF Aβ in mice. Defining the specific mechanisms responsible for this reduction is quite complex. The fourteen different 5-HTR subtypes separate into multiple signaling cascades, and their downstream effectors can interact with each other in neurons. Also, each 5-HTR has its own unique expression pattern within the hippocampus. This fact creates more difficulties when trying to understand the roles of individual receptors in overall brain physiology. In this report, we use pharmacology to identify which 5-HTRs were responsible for lowering ISF Aβ in the mouse hippocampus.

Selective stimulation of 5-HT_4R and 5-HT_7R could significantly lower ISF Aβ while agonists for 5-HT_1R, 5-HT_2R, and 5-HT_6R could not (Figure 2.1). 5-HT_5R was not examined because there are no selective agonists for this receptor subtype that are commercially available. While we did not see any effect with other selective 5-HTR agonists, we did not assay drug activity in the brain. However, we are confident in our results because we calculated the dosage using IC_{50} values and concentrations used in previous literature in the field. The 25% Aβ reduction by 5-HT_4R and 5-HT_7R activity was equal to the reduction induced by serotonin. This finding suggests that activity of these two receptors could be responsible for the majority, if not the entirety, of the reduction in ISF Aβ by 5-HT. The importance of these receptors is illustrated by treating mice with their antagonists. Simultaneously blocking activity of 5-HT_4R and 5-HT_7R significantly increased ISF Aβ (Figure 2.2). These results suggest that basal activity of 5-HT_4R and 5-HT_7R regulates normal Aβ metabolism. Recent literature supports the idea that activity of a single 5-HTR could change Aβ metabolism in the brain. Tesseur et al showed one month

treatment with a 5-HT_4R selective agonist could reduce soluble $A\beta_{40}$ and plaque load in the hippocampus (Tesseur et al., 2013). Also, Gianonni et al showed therapy with a weak 5-HT_4R agonist could increase concentrations of the α-secretase product and reduce plaque load in 5XFAD mice (Giannoni et al., 2013). Basal 5-HT activity may keep Aβ production to a minimum in healthy individuals, but this process may be perturbed as animals age or become ill.

There is a clinical connection between 5-HT receptors and human AD. Research has shown that 5-HT_4R levels are reduced in human AD brains (Reynolds et al., 1995). Numbers of serotonergic neurons in the dorsal raphe nuclei were shown to decline in human AD patients (Hendricksen et al., 2004). Loss of these neurons would reduce serotonergic activity throughout the brain. As we show in this document, loss of serotonergic activity would result in increased Aβ production. This process could accelerate plaque deposition and enhance AD progression.

Stimulating 5-HT_4R and 5-HT_7R leads to activation of PKA. We showed that blocking PKA activity with two selective inhibitors could significantly raise Aβ and abolish the effects of SSRI (Figure 2.3). Activation of PKA appears to have the opposite effect. A selective agonist for D1R that specifically activates PKA significantly reduced ISF Aβ (Figure 2.3C). The time course for the reduction in ISF Aβ was slightly slower for the D1R agonist than 5-HTR agonists. This result is not surprising as different receptors will have different kinetics for binding substrates and initiating downstream signaling. Different receptors are also under different regulatory mechanisms; perhaps D1R is internalized to stop signaling more quickly than 5-HTRs. PKA has been tied to AD pathology in the past, but the focus has been neuronal responses after exposure to Aβ. PKA inhibition reduced phosphorylation of Tau after exposing neurons to Aβ in vitro (Wang et al., 2013). However, overexpression of the beta-secretase enzyme BACE1

reduced cAMP and PKA activity in mice (Chen et al., 2012). Our findings are the first to show PKA activity can reduce Aβ production in vivo and blocking its activity has the opposite effect.

Our results implicating PKA are contradictory to a recent report from the DeStrooper group. Their research suggests cAMP and PKA are not necessary for 5-HT$_4$R mediated regulation of APP processing; they suggest Src and phospholipase C are responsible (Pimenova et al., 2014). These differing results are probably due to the different systems used in each paper. Pimenova used SH-SY5Y neuroblastoma cells for all their experiments while our projects were performed in live mice. SH-SY5Y cells are excellent for quickly examining cellular processes, but they cannot fully recreate the physiology of a living brain. Also, the conflicting results could be the product of using different agents for stimulating or blocking kinase activity. Each pharmacological drug will have different kinetics with regards to half-life and target affinity.

Surprisingly, 5-HT$_6$R failed to significantly reduce ISF Aβ in our experiments. This result is interesting because 5-HT$_6$R is G$_s$ linked and can activate PKA. This discrepancy could be due differences in downstream signaling regulation of each receptor subtype. 5-HTRs have been shown to be endocytosed to end their signaling activity, and this process is mediated in part by β-arrestin proteins (Ahn et al., 2004; Schmid et al., 2008; Barthet et al., 2009). Perhaps 5-HT$_6$R is more rapidly endocytosed and silenced than the other G$_s$ linked 5-HTRs. Conversely, β-arrestins have also been linked to initiating signaling cascades independently of G proteins. βeta-arrestin can act as a scaffolding protein for multiple signal cascades like the ERK, JNK, and PI3K pathways (DeWire et al., 2007; DeFea, 2011). Perhaps β-arrestins trigger these signaling pathways when internalizing 5-HT$_4$R and 5-HT$_7$R but not when internalizing 5-HT$_6$R. Our lab is currently investigating the role of β-arrestin in serotonin mediated reduction of ISF Aβ.

In conclusion, we have shown that stimulating 5-HT_4R or 5-HT_7R can reduce ISF Aβ to a similar extent to 5-HT itself, and this reduction is mediated by PKA activity. These results suggest that targeting these individual receptors could be a potentially ground-breaking therapy for AD. While SSRIs are showing promise as a potential AD treatment, they are prone to gastrointestinal side effects (Rosenzweig-Lipson et al., 2007). This fact is not surprising as 5-HTR expression is quite high throughout the gut (Bard et al., 1993; Kursar et al., 1994; Prins et al., 2000). Other organ system side effects are reported with SSRI therapy. Stimulation of 5-HT_2R in vascular tissue can lead to constriction of blood vessels and erectile problems(Watts et al., 2001; Rosenzweig-Lipson et al., 2007). Narrowing AD therapy to a single 5-HTR could mitigate some of these unpleasant side effects. This targeted therapy could finally provide a way to slow or stop the progression of this terrible disease.

Compound	Target	Active Concentration	Dilution	Concentration Across Membrane	Selectivity Limit
Agonists					
Ipsapirone	5-HT$_1$R	10 nM	1 μM	100 nM	Unknown
WAY161503 Hydrochloride	5-HT$_2$R	4 nM	400 nM	40 nM	233 nM
ML10302 Hydrochloride	5-HT$_4$R	4 nM	400 nM	40 nM	700 nM
ST1936 Oxalate	5-HT$_6$R	13 nM	1.3 μM	130 nM	168 nM
AS19	5-HT$_7$R	0.83 nM	83 nM	8.3 nM	6 nM
SKF 83822 Hydrobromide	D1-like R	3.2 nM	50 nM	5 nM	66 nM
Antagonists					
GR 113808	5-HT$_4$R	0.02 nM	100 nM	10 nM	10 μM
SB 258719 Hydrochloride	5-HT$_7$R	31.6 nM	3.16 μM	316 nM	316 nM
Inhibitor					
GI254023X	ADAM10	5.3 nM	530 nM	53 nM	531 nM
KT5720	PKA	60 nM	6 μM	60 nM	2 μM
PKI 14-22 Amide Myristoylated	PKA	36 nM	3.6 μM	360 nM	15 μM

Table 2.1: Pharmacological agents used for reverse microdialysis. All agents were chosen for their selectivity for their targets. We assumed that only 10% of the compounds would cross the membrane into the brain parenchema, and this amount would be further diluted in the ISF. The concentration for AS19 was over technically over the selectivity limit. However, the 5-HTR that it cross activates was not expressed in the hippocampus (Tanaka et al., 2012).

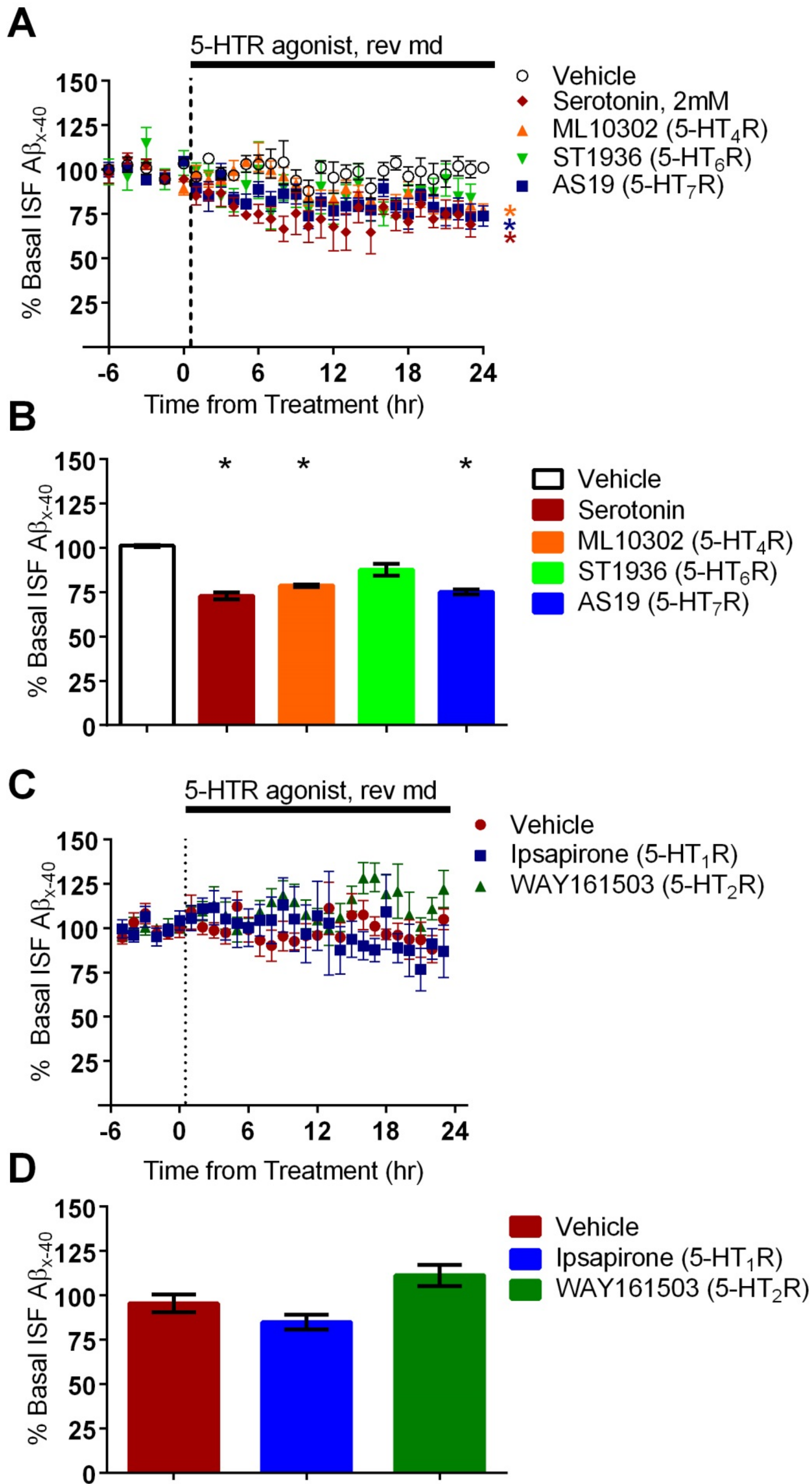
A
5-HTR agonist, rev md
Vehicle
Serotonin, 2mM
ML10302 (5-HT4R)
ST1936 (5-HT6R)
AS19 (5-HT7R)
% Basal ISF Aβx-40
Time from Treatment (hr)
B
Vehicle
Serotonin
ML10302 (5-HT4R)
ST1936 (5-HT6R)
AS19 (5-HT7R)
% Basal ISF Aβx-40
C
5-HTR agonist, rev md
Vehicle
Ipsapirone (5-HT1R)
WAY161503 (5-HT2R)
% Basal ISF Aβx-40
Time from Treatment (hr)
D
Vehicle
Ipsapirone (5-HT1R)
WAY161503 (5-HT2R)
% Basal ISF Aβx-40

Figure 2.1: Selective subset of 5-HT receptors reduce ISF Aβ in vivo. Selective agonists for individual 5-HT receptors or vehicle (DMSO) were infused via reverse microdialysis in 2-3 month old APP/PS1 hemizygous mice. **A.** As assessed by microdialysis, agonist treatment for $5\text{-}HT_4R$ (ML10302, 400 nM) and $5\text{-}HT_7R$ (AS19, 83 nM) cause a significant reduction in ISF Aβ while $5\text{-}HT_6R$ agonist (ST1936, 1.3 μM) reduction was more moderate. **B.** After 23 hours of continuous treatment, ML10302 reduced ISF $A\beta_{x\text{-}40}$ to 78.6% +/- 2.9 (P = 0.0015; n = 5) and AS19 reduced ISF $A\beta_{x\text{-}40}$ 75.1% +/- 6.06 (P = 0.007; n = 7) , but ST1936 only reduced ISF $A\beta_{x\text{-}40}$ to 89.3% +/-8.2 (P = 0.28; n = 6). **C.** Agonist treatment for $5\text{-}HT_1R$ (Ispapirone , 1 μM) or $5\text{-}HT_2R$ (WAY161503, 0.4 μM) showed no significant reduction of ISF Aβ (n = 6-8 animals). **D.** After 23 hours of continuous treatment, Ipsapirone reduced ISF $A\beta_{x\text{-}40}$ to 84.9% +/- 10.3 (P = 0.39; n = 6) and WAY161503 increased ISF $A\beta_{x\text{-}40}$ 111.2% +/- 6.3 (P = 0.11; n = 8). Data represented as mean +/- SEM. (* marks P value < 0.05).

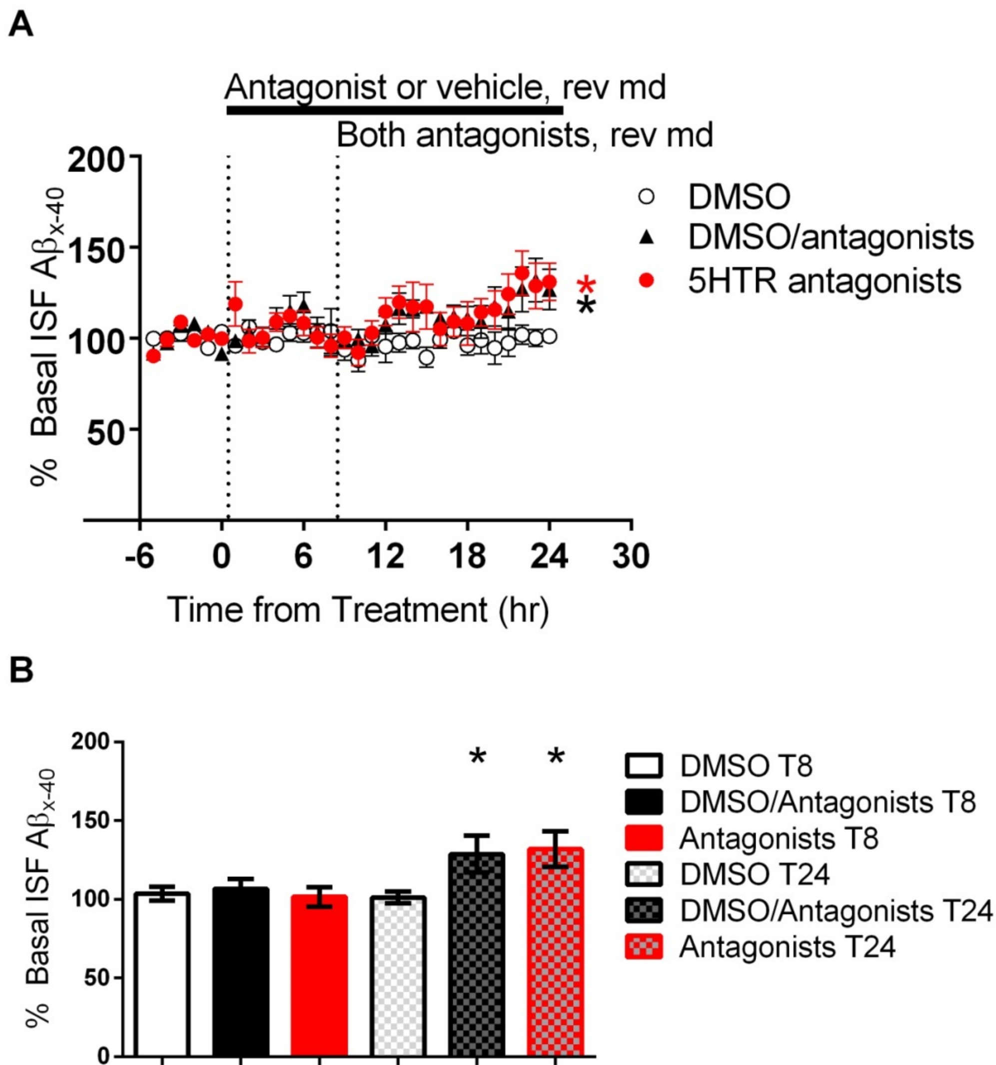

Figure 2.2: Inhibiting 5-HT$_4$R and 5-HT$_7$R simultaneously increases ISF Aβ. Selective 5-HT$_4$R antagonist GR113808 (100 nM) or DMSO vehicle were infused via reverse microdialysis in 2-3 month old APP/PS1 hemizygous mice for eight hours. A mixture of GR113808 and selective 5-HT$_7$R antagonist SB258719 hydrochloride (3.16 mM) were then administered for the next sixteen hours. **A.** As assayed by microdialysis, treatment with a single antagonist has no obvious effect on ISF $A\beta_{x-40}$. Treatment with both antagonists increases ISF $A\beta_{x-40}$. **B.** After eight hours of single antagonist treatment, there was no significant change in ISF $A\beta_{x-40}$. Sixteen hours of treatment with both antagonists increased ISF $A\beta_{x-40}$to 128.7% +/-11.7 (P= 0.05, n=5) and 132% +/- 11.2 (P= 0.04, n=7) for DMSO/Antagonists and Antagonists respectively. Data represented as mean +/- SEM. * marks P value < 0.05.

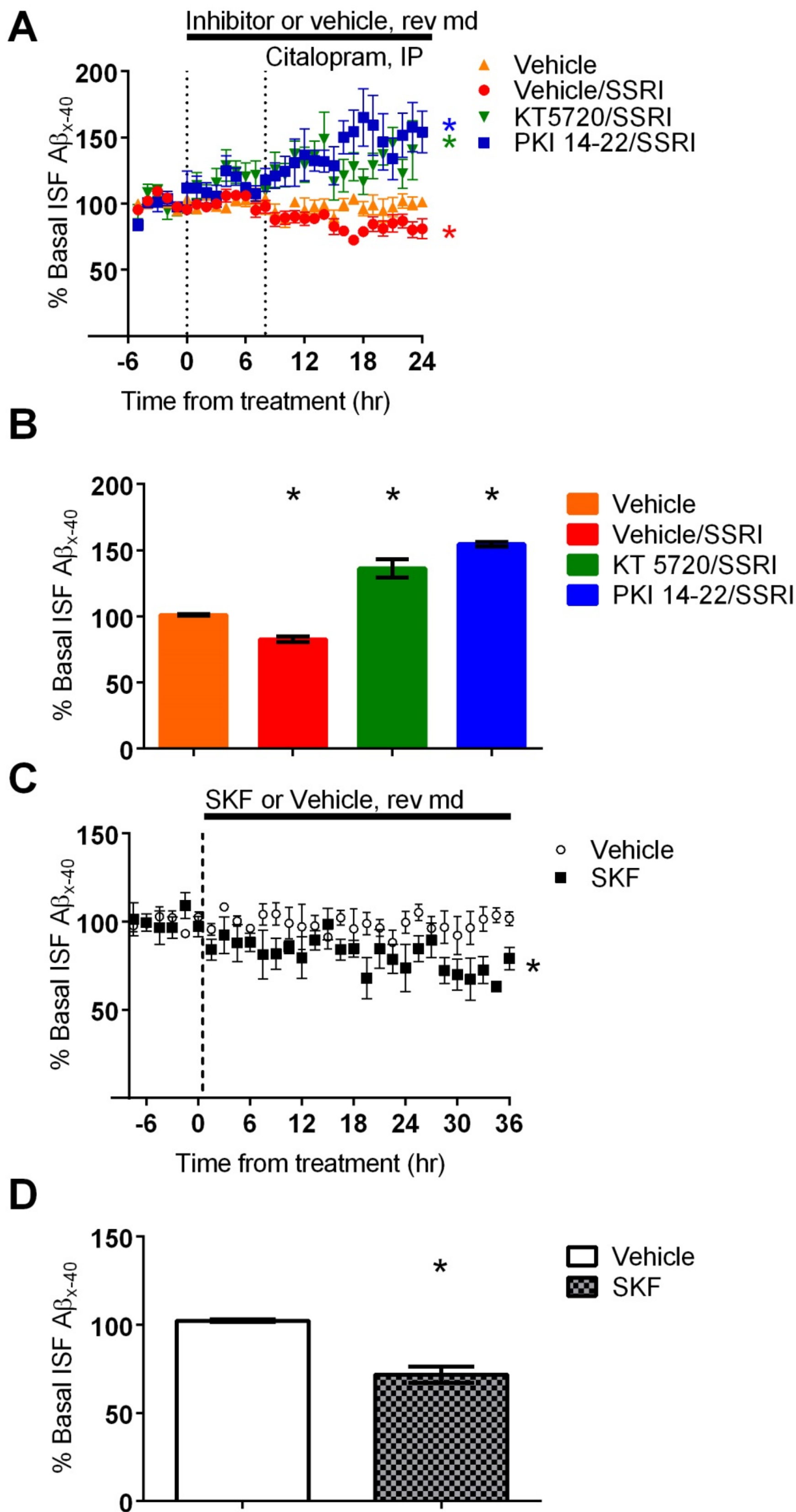
A
Inhibitor or vehicle, rev md
Citalopram, IP
% Basal ISF Aβx-40
Time from treatment (hr)
Vehicle
Vehicle/SSRI
KT5720/SSRI
PKI 14-22/SSRI
B
% Basal ISF Aβx-40
Vehicle
Vehicle/SSRI
KT 5720/SSRI
PKI 14-22/SSRI
C
SKF or Vehicle, rev md
% Basal ISF Aβx-40
Time from treatment (hr)
Vehicle
SKF
D
% Basal ISF Aβx-40
Vehicle
SKF

Figure 2.3: PKA activity modulates production of ISF Aβ. Selective small molecule inhibitor KT5720 (6 μM), peptide inhibitor PKI 14-22 amide, myristolyated (3.6 μM), or vehicle (DMSO and acetonitrile, respectively) were infused via reverse microdialysis in 2-3 month old APP/PS1 hemizygous mice. Eight hours later, the SSRI citalopram was administered by IP injection at 10 mg/kg. **A.** As assessed by microdialysis, inhibiting PKA with two separate compounds increases ISF $A\beta_{x-40}$ levels and negates the effect of SSRI. **B.** After 23 hours of continuous treatment, KT5720 significantly increases ISF $A\beta_{x-40}$ to 136.2% +/-9.1 (P= 0.0091, n=6), PKI 14-22 increases ISF $A\beta_{x-40}$ to 154.5% +/-16.6 (P= 0.019, n=6). Treating vehicle controls with SSRI significantly decreases ISF $A\beta_{x-40}$ to 82.2% +/-4.7 (P= 0.024, n=11). To test if PKA activation is sufficient to reduce Aβ, selective D1R agonist SKF 83822 (50 nM) or Vehicle (DMSO) was infused by reverse microdialysis. **C.** Microdialysis analysis shows significant reduction in ISF $A\beta_{x-40}$. **D.** After 24 hours of continuous treatment, SKF reduced ISF $A\beta_{x-40}$ to 76.13% +/-8.9 (P= 0.015, n=5). Data represented as mean +/- SEM. * marks P value < 0.05.

Chapter 3: ERK mediates 5-HT effects in the cytosol by changing α-secretase activity

ABSTRACT

Methods of reducing Aβ production show promise as therapy for AD. 5-HT or SSRI drugs can reduce ISF Aβ within a few hours in transgenic mice. A similar effect has been shown by selective stimulation of $5\text{-}HT_4R$ and $5\text{-}HT_7R$. Serotonergic activity has been shown to activate the ERK signaling pathway. ERK can modify cell behavior by changing gene expression in the nucleus or by post-translational modification of proteins in the cytoplasm. In this report, we show that acute SSRI treatment does not significantly change gene expression of any genes tied to Aβ metabolism. We also show that inhibition of ADAM10, the constitutive α-secretase enzyme, is unable to block the effects of SSRI. These results suggest that 5-HTR activation of ERK changes the activity of another α-secretase enzyme by post-translational modification.

INTRODUCTION

Discovering a method for slowing or stopping progression of AD is a major motivation for multitudes of researchers. As we have shown in Chapter 2, serotonergic signaling can reduce ISF Aβ levels in the brains of transgenic mice, and this effect is mediated by PKA activity. However, the ultimate effectors of this signaling activity are still unknown. In this report, we provide evidence that ERK mediates the effects of 5-HT by acting on α-secretase in the cytoplasm.

The ERK signaling plays a significant role in numerous cellular functions. ERK has been linked to cell cycle progression, differentiation, stress response, and apoptosis (Wortzel and Seger, 2011). There are two different ERK proteins found in mammals. ERK1 and ERK2 show 75% sequence similarity, and both kinases are expressed throughout the body (Pouysségur et al., 2002). ERKs are proline-targeted serine/threonine kinases; their substrate target site is proline-X-serine/threonine-proline (Gonzalez et al., 1991). ERK is a highly promiscuous kinase that can act within the nucleus or the cytoplasm. There are more than 200 currently known ERK substrates ranging from cytoskeletal elements to transcription factors (Wortzel and Seger, 2011). ERK becomes activated after double phosphorylation by its upstream kinase MEK. Careful regulation of ERK activity by scaffolding proteins and binding partners directs the levels of ERK activation and its ultimate cellular targets. β-arrestin acts as a signaling scaffold for Ras, MEK, and ERK. Knocking down beta-arrestin with shRNA reduced ERK activation in vitro, but overexpression of β-arrestin increased activated ERK (Tohgo et al., 2002; Ahn et al., 2004).

There are several links between ERK, AD, and 5-HT. Human brain tissue from AD patients showed increased levels of activated ERK (pERK) when compared to age matched

controls (Perry et al., 1999). ERK can phosphorylate tau proteins in vitro, and its levels in the CSF of AD patients correlates well with levels of tau (Klafki et al., 2009). Activation of 5-HTRs has been shown to increase ERK activity. Stimulating 5-HT_1R, 5-HT_2R, 5-HT_4R, or 5-HT_7R with selective agonists or 5-HT induces an increase in pERK in vitro (Launay et al., 1996; Leone et al., 2000; Errico et al., 2001; Watts et al., 2001; Adayev et al., 2003; Johnson-Farley et al., 2005). Blocking ERK activity has a direct effect on Aβ production in vivo. Treating APP/PS1 mice with inhibitors for MEK or ERK by reverse microdialysis increased ISF Aβ and completely abolished the effects of SSRI antidepressants (Cirrito et al., 2011; Verges et al., 2011). This result suggests that ERK mediates the effects of 5-HT on Aβ production, but the actual mechanism still needs to be determined.

Regulation of secretase enzymes is a promising explanation for the effects of ERK and 5-HT on Aβ. APP is cleaved by β- and γ-secretase enzymes to produce the Aβ peptide, but α-secretase cleaves APP in such a way that prevents Aβ production. Increasing α-secretase activity or reducing other secretase activity should create a reduction in Aβ. Interestingly, this effect can be induced by stimulating neurotransmitter receptors. Activating muscarinic acetylcholine 1 receptors (M1R) reduced Aβ while increasing sAPPα in HEK cells (Hung et al., 1993). Similar effects were seen in vivo; chronic treatment of 3xTgAD mice with M1R agonist reduced Aβ levels, reduced plaque load, and increased sAPPα (Caccamo et al., 2006). 5-HT is another neurotransmitter that can shift secretase activity in vivo. Acute SSRI treatment of APP/PS1 mice increased α-secretase activity, but β-secretase activity was unchanged (Cirrito et al., 2011). This increase in α-secretase activity could be the result of ERK activation; we have shown that MEK and ERK activity were still increased 8 hours after SSRI treatment in APP/PS1 mice (Cirrito et

al., 2011). Previous research has shown that ERK regulates secretase activity. Activation of ERK increases phosphorylation of ADAM17, the regulated α-secretase enzyme, and increases its activity in vitro (Cissé et al., 2011). Other researchers showed that ERK and ADAM17 can bind to each other (Yin and Yu, 2009). Members of the γ-secretase complex also are ERK substrates. Kim et al showed nicastrin was phosphorylated by ERK, and this phosphorylation event reduced γ-secretase activity (Kim et al., 2006). Similar reductions in γ-secretase activity were seen when treating HEK cells with ERK agonists (Tung et al., 2008).

These discoveries linking 5-HT, ERK, and secretase led us to examine how ERK mediates the reduction in ISF Aβ. We report in this document that activation of ERK by SSRI is not changing gene expression to mediate the reduction in ISF Aβ. Finally, we show that inhibition of ADAM10 has no effect on the reduction in ISF Aβ by 5-HT; this result suggests another α-secretase enzyme is the true effector.

METHODS AND MATERIALS

Quantitative Real Time PCR (qPCR)

2.5 month old APP/PS1 mice were given 10 mg/kg intraperitoneal injections of citalopram in PBS. Mice were sacrificed 16 hours later, and their hippocampi were removed. Total RNA were extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Invitrogen). Individual primers were designed by the Harvard Medical School Primer Bank website http://pga.mgh.harvard.edu/primerbank/index.html (Wang and Seed, 2003; Spandidos et al., 2008, 2010). Any primers that were not available on Primer Bank were designed by hand. Table 3.1 lists the primer pairs used for each gene we analyzed. qPCR was performed using the Fast SYBR Green Master Mix (Applied Biosystems) in ABI 7900HT (Applied Biosystems) with the default thermal cycling program. Dissociation curves were analyzed following qPCR assay to confirm primer specificity. Endogenous mouse GAPDH was used as a normalization reference. Relative mRNA levels were calculated by comparative Ct method using the ABI 7900HT Sequence Detection Systems and GenEx 5 (MultiD analyses). One way ANOVA with multiple comparisions was used to analyze significant changes between genes using the GraphPad Prism 6 software.

In vivo microdialysis

In vivo microdialysis to measure brain ISF Aβ in the hippocampus of freely moving APP/PS1 mice was performed as previously described (Cirrito et al., 2003, 2008, 2011). This method captures soluble molecules the extracellular fluid that are below the 30 kDa molecular weight cutoff of the probes. Under volatile isoflurane anesthetic, guide cannula (BR style;

Bioanalytical systems) were cemented into the left hippocampus (3.1 mm behind Bregma, 2.5 mm lateral to midline, and 1.2 mm below dura at a 12° angle). Two mm microdialysis probes were inserted through the guides so their membranes were completely contained in the hippocampus (BR-2, 30 kDa molecular weight cut-off membrane; Bioanalytical systems). Microdialysis buffer was aCSF (perfusion buffer in mM: 1.3 $CaCl_2$, 1.2 $MgSO_4$, 3 KCl, 0.4 KH_2PO_4, and 122 NaCl, pH 7.35) containing 2% BSA (Sigma) that was filtered through a 0.1 μM membrane. The flow rate was 1.0 μL/min. Samples were collected every 60 or 90 minutes with a refrigerated fraction collector in polypropylene tubes and assessed for $A\beta_{x\text{-}40}$ or $A\beta_{x\text{-}42}$ by ELISA. Basal ISF Aβ levels were defined as the mean concentration of Aβ over the 6 hours preceding drug administration. Aβ levels were normalized to the basal Aβ concentration for each animal. After establishing baseline ISF Aβ, ADAM10 inhibitor GI254023X was diluted to a concentration of 530 nM in microdialysis buffer and infused directly into the hippocampus by reverse microdialysis. Citalopram was intraperitoneally injected 8 hours after drug treatment at 10 mg/kg when testing drug efficacy against SSRI. Results were compared to mice that received vehicle instead of GI254023X. Statistical significance was assayed using the student T-test method in the GraphPad Prism 6 software.

Aβ Sandwich ELISA

ISF Aβ levels were measured using sandwich ELISAs as described (Cirrito et al., 2011). A mouse anti- $A\beta_{40}$ antibody (mHJ2) or mouse anti- $A\beta_{42}$ antibody (mHJ7.4) was used to capture and a biotinylated central domain antibody (mJH5.1) was used to detect followed by streptavidin poly-HRP-40 (Fitzgerald Industries). All ELISAs were developed using Super Slow ELISA TMB (Sigma) and absorbance read on a Bio-Tek Epoch plate reader at 650 nm. The

standard curves for each assay used synthetic human $A\beta_{1-40}$ or $A\beta_{1-42}$ peptide (American Peptide).

RESULTS

SSRI treatment creates no significant change in expression of Aβ related mRNA

ERK carries out its signaling function by translocating into the nucleus to change gene expression or acting in the cytosol to modify enzyme activity by post-translational modification. In order to test if ERK was acting in the nucleus, we measured expression of genes involved in Aβ metabolism following treatment with citalopram. 2-3 month old APP/PS1 mice were treated with 10 mg/kg of citalopram or PBS by IP injection. Animals were sacrificed 16 hours later. We chose 16 hours because that time point showed a significant reduction in ISF Aβ from a single dose of SSRI (Cirrito et al., 2011). We removed the hippocampi, extracted RNA, and performed qPCR on 34 genes involved in Aβ metabolism. The immediate early gene cFOS showed reduced mRNA levels within 24 hours of citalopram treatment (Gąska et al., 2012). This fact made cFOS an excellent positive control for this experiment.

Figure 3.1 shows results from our qPCR analysis. Expression of α-secretases ADAM10 and ADAM17 were not significantly affected by SSRI (Figure 3.1A). APP expression did not change after SSRI treatment (Figure 3.1A). There was a trend in reduction of genes that clear Aβ from the brain. However, the reductions in mRNA for LRP1, MMP2, MMP9, and neprilysin were not statistically significant (Figure 3.1A). There was no significant change in expression in BACE1 or components of γ-secretase (Figure 3.1A). The absence of significant changes in expression of Aβ processing genes forced us to extend our search. We expanded expression analysis to include members of major intracellular signaling cascades. There was no significant change in major upstream signaling genes such as PKA, PKC, or CaMKII (Figure 3.1B). Expression of downstream kinases like Raf, MEK, or ERK was not significantly changed. There

was a reduction in JNK3 kinase expression, but it was not significant (Figure 3.1B). β-arrestin acts as a scaffold for numerous signaling cascades, but there was no change in its expression after citalopram treatment (Figure 3.1B). We also measured expression of 5-HTRs after SSRI treatment. Expression of serotonin receptors was not significantly changed following SSRI (Figure 3.1C). The widespread absence of significant expression changes of genes involved in Aβ metabolism suggests that ERK is not acting in the nucleus to reduce Aβ levels in the mouse brain. This finding suggests ERK is acting on proteins in the cytosol.

ADAM10 does not mediate the effects of SSRI of ISF Aβ

We previously showed that α-secretase activity was increased during SSRI treatment (Cirrito et al., 2011). ADAM10 is the putative α-secretase protein in the brain (Lammich et al., 1999; Kuhn et al., 2010; Vingtdeux and Marambaud, 2012). These facts led us to test if ADAM10 is the target of ERK that mediates the reduction in ISF Aβ by SSRI. We administered GI254023X, a selective ADAM10 inhibitor, by reverse microdialysis at a concentration of 530 nM. After 8 hours, we injected the mice with 10 mg/kg of citalopram. As shown in Figure 3.2A, treatment with GI254023X induces a slight rise in ISF Aβ levels but cannot block the effects of SSRI. ISF Aβ increased 6% over baseline with GI254023X at T8, but levels were comparable to controls at T24 (Figure 3.2B). These results suggest ADAM10 is not mediating the effects of SSRI on ISF Aβ; another α-secretase must be responsible.

DISCUSSION

Previous work has shown that ERK inhibition raises Aβ and abolishes the effects of SSRI in APP/PS1 transgenic mice (Cirrito et al., 2011). ERK can translocate into the nucleus to modify gene expression or act within the cytoplasm to change protein activity. In this report, we provide evidence that ERK activity is restricted to the cytosol and acts on a different α-secretase than ADAM10 to mediate reductions in ISF Aβ. If ERK was acting within the nucleus, then one would expect to see changes in gene expression. However, we observed no significant changes in expression in any genes related to Aβ metabolism, serotonin receptors, or signaling pathways by qPCR of brain tissue following acute SSRI treatment (Figure 3.1). Previous studies have shown reductions in secretase genes after SSRI therapy (Cirrito et al., 2011; Tesseur et al., 2013). However, those reductions were seen after chronic treatment lasting for months whereas these data were obtained after only a few hours. Perhaps chronic down-regulation of Aβ production leads to reduced gene expression over time; only future experiments with several treatment time protocols can determine if this hypothesis is correct. The rapid reduction in ISF Aβ we observed suggests a post-translational response over the slower acting changes in gene expression. These results suggest ERK is acting outside to nucleus to regulate Aβ.

α-secretase was the most likely target of ERK after serotonergic activity. Previous work by our group showed that α-secretase activity was increased after SSRI treatment, but β-secretase activity was unchanged (Cirrito et al., 2011). ADAM10 is the primary α-secretase protein, so we tested the effects of inhibiting this enzyme in the hippocampus. Our results showed that inhibiting ADAM10 could slightly increase ISF Aβ levels, but citalopram treatment still reduced Aβ in the presence of the inhibitor (Figure 3.2). Even though we did not assay drug

activity in the brain after treatment, we are confident in our findings. We calculated the dosage using established IC_{50} values and concentrations used in previous literature in the field. This result was fairly surprising as ADAM10 is accepted as the primary neuronal α-secretase. However, ADAM17 has also been shown to cleave APP as α-secretase and is strongly expressed in the hippocampus (Vingtdeux and Marambaud, 2012). Perhaps ADAM17 can compensate for ADAM10 when its activity was blocked. Another possibility is the idea of a division of labor between the two secretases. ADAM10 has been shown to constitutively produce sAPPα under basal conditions, but ADAM17 produces sAPPα when cells are stimulated. Treating cells with the M1R agonist carbachol increases sAPPα production in vitro, but this effect is absent in fibroblasts that lack ADAM17 (Buxbaum et al., 1998). Knocking out ADAM10 with siRNA reduced basal sAPPα production by 90% in cultured cells; ADAM17 knockdown had significantly less effect on basal sAPPα secretion (Kuhn et al., 2010). However, the opposite pattern emerged when cells were stimulated. ADAM17 siRNA completely abolished increased sAPPα after stimulation with phorbol-12-myristate-13-acetate while ADAM10 siRNA had no effect (Kuhn et al., 2010). A similar situation could be occurring in our experiments with citalopram; stimulation of 5-HTRs could increase α-secretase activity through ADAM17 instead of ADAM10. Unfortunately, there are no commercially available inhibitors that are selective for ADAM17. As a result, we are unable to selectively inhibit ADAM17 in vivo. However, recent research in Italy suggests a selective compound may be available shortly (Nuti et al., 2013). Future work will examine if blocking ADAM17 can block the effects of SSRI.

The mechanism for increasing α-secretase activity after SSRI is most likely direct phosphorylation by ERK. ERK activation has been shown to increase activity of ADAM17 in

vitro. ADAM17 cleavage of the prion precursor protein was increased by ERK activation, but mutants lacking the ERK target site did not show increased activity (Cisse et al., 2011). This form of post-translational regulation could occur with ADAM10 as well; the mammalian ADAM10 polypeptide possesses the proline-X-serine-proline sequence that forms the ERK phosphorylation site (Yavari et al., 1998). Future work will determine if ERK directly phosphorylates ADAM10 or ADAM17.

Direct phosphorylation of α-secretase is not the only way ERK may modify activity. ERK could phosphorylate a shuttling protein to change its cellular location and increase cleavage of APP. β-arrestin is a scaffolding protein that has been shown to bind 5-HTRs and lead to their endocytosis from the plasma membrane (Gelber et al., 1999). β-arrestin also can directly bind to ERK, and this interaction is increased after stimulation of GPCRs (Tohgo et al., 2002, 2003). β-arrestin can move into different subcellular areas after GPCR activity. Stimulating angiotensin receptors induced a shift of β-arrestin into endosomes, and this movement increased co-localization with pERK signal (Ahn et al., 2004). Similar endosomal patterns for β-arrestin appear after stimulating 5-HTRs in HEK cells (Schmid et al., 2008). Perhaps serotonergic activity brings β-arrestin, ERK, APP, and α-secretase together in endosomes, and this interaction is responsible for the reduction of ISF Aβ. The idea of a scaffolding protein interacting with secretase enzymes is not far-fetched. Recent work has shown β-arrestin 2 can bind components of the γ-secretase complex (Thathiah et al., 2013). Future experiments will elucidate the interactions, if any, between these proteins.

This report has provided evidence that reduction in Aβ caused by serotonergic activity is mediated by ERK acting on α-secretase in the cytoplasm. Now that evidence suggests ERK is

not acting within the nucleus, we can devote more attention to its roles in the cytoplasm with regards to Aβ metabolism. Unfortunately, ERK has too many cellular roles to be a direct target for AD therapy. However, manipulating secretase activity via 5-HT signaling demonstrates great therapeutic potential for AD. Future work will determine how ERK is changing α-secretase activity.

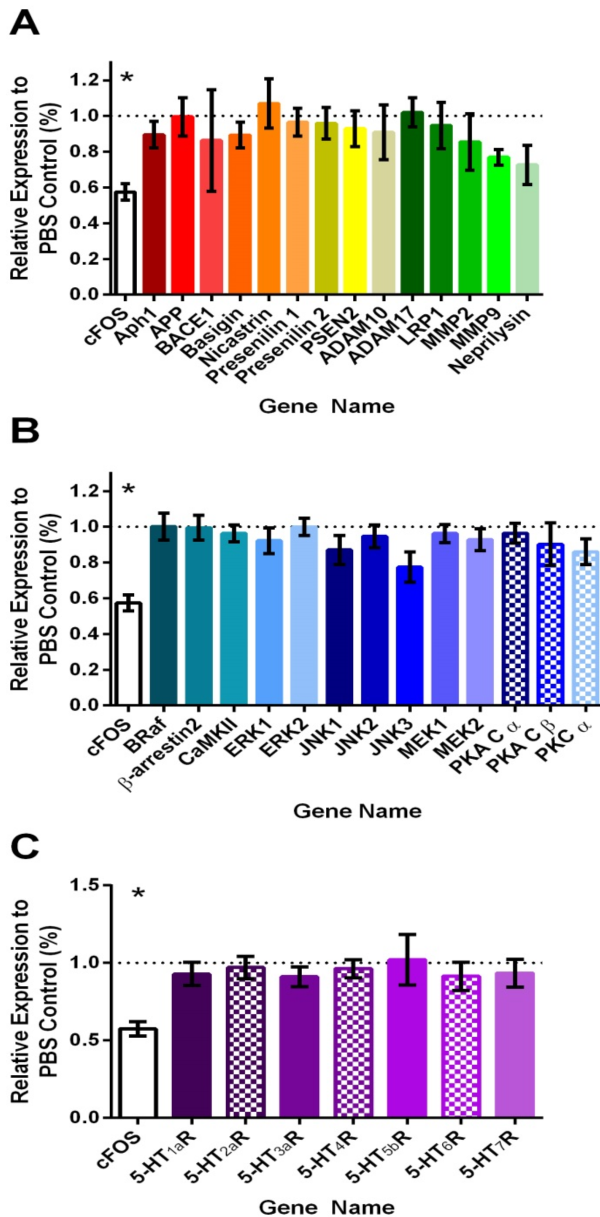

Figure 3.1: ERK activation following SSRI treatment has no significant effect on gene expression for Aβ processing genes. 2-3 month old APP/PS1 hemizygous mice were treated with 10mg/kg citalopram by IP injection. Sixteen hours later brains were removed for qPCR analysis. **A.** qPCR analysis for genes involved in Aβ processing or clearance showed no significant changes in expression. Positive control cFOS was significantly reduced to 57.5% +/- 0.04 (P=0.02). **B.** qPCR analysis for genes encoding signaling proteins downstream of 5-HTR showed no significant changes in expression. **C.** qPCR analysis for 5-HTR genes showed no significant changes in expression. Data are presented as mean +/-SEM. Values are normalized to mean level in PBS injected controls. (n=6). Asterisks mark P values <0.05.

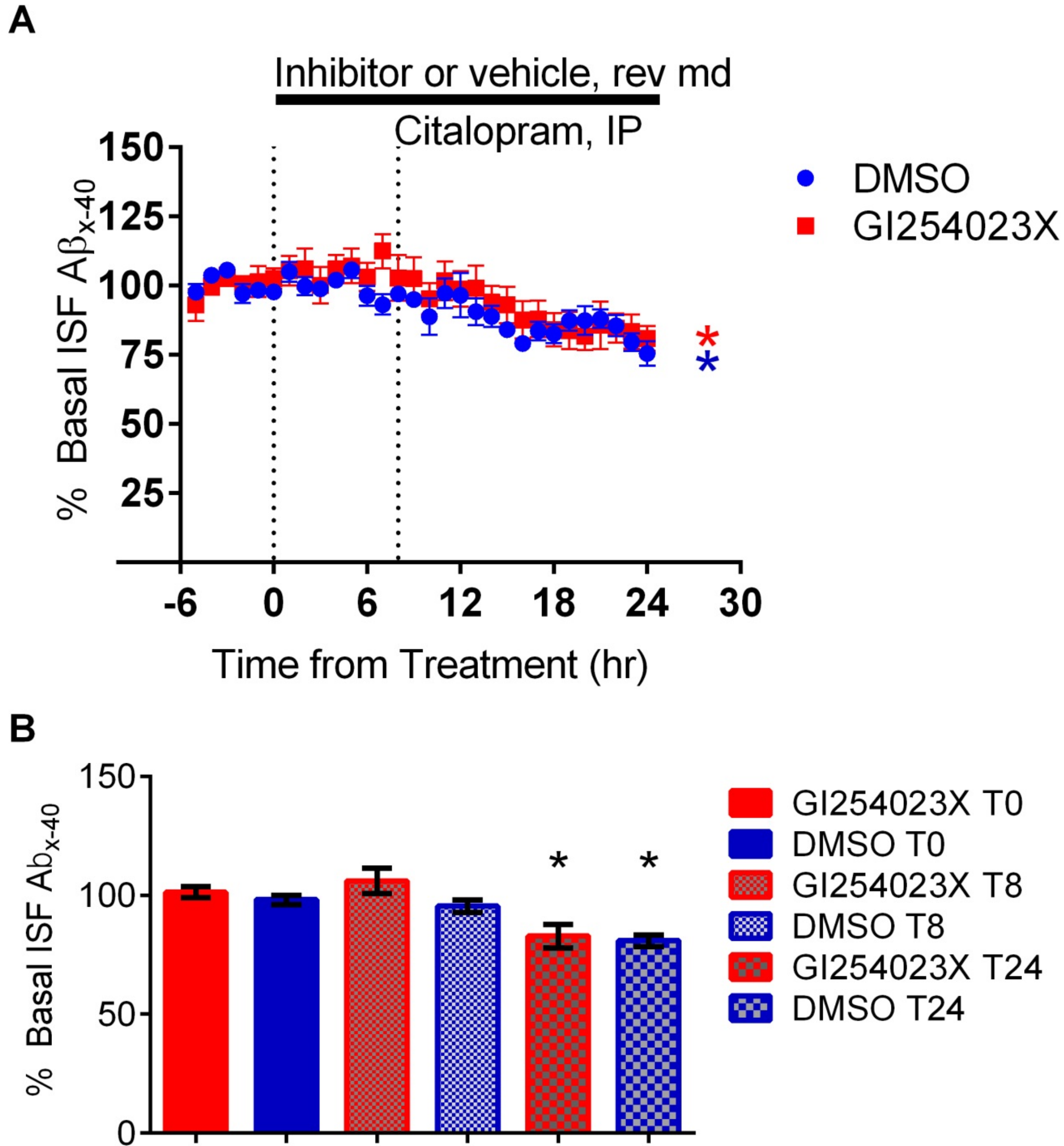

Figure 3.2: Inhibition of ADAM10 cannot block effects of citalopram. Selective ADAM10 inhibitor GI254023X (530 nM) or DMSO vehicle were administered for 8 hours by reverse microdialysis. After 8 hours, mice were injected with 10 mg/kg citalopram by IP injection. **A.** As assessed by microdialysis, GI254023X caused a slight increase in ISF $A\beta_{x\text{-}40}$ levels alone, but was unable to block the effects of citalopram. **B.** After 8 hours of ADAM10 inhibition, there was no significant change in ISF $A\beta_{x\text{-}40}$. 16 hours after citalopram treatment, GI254023X ISF $A\beta_{x\text{-}40}$ levels were reduced to 82.9% +/- 4.9 (P=0.0069, N=6). DMSO ISF $A\beta_{x\text{-}40}$ levels were also significantly reduced to 81.5% +/-1.8 (P=0.0017, N=4). Data are presented as mean +/-SEM. Asterisks mark P values <0.05.

Table 3.1: Primer pairs for qPCR analysis. The majority of primers were collected from the Harvard Primer bank. Primer sets with N/A were designed by hand.

Gene		Sequence	Primerbank ID
ADAM10	F	GTGCCAAACGAGCAGTCTCA	150378457c2
	R	ATTCGTAGGTTGAACTGTCTTCC	
ADAM17	F	AGGACGTAATTGAGCGATTTTGG	34328548a1
	R	TGTTATCTGCCAGAAACTTCCC	
APP	F	TGCAGAATTCCGACATGACT	N/A
	R	GCCTTTGTTTGAACCCACAT	
Aph-1	F	CCGCGCTCGCTCTTTATGT	146198522c1
	R	TGTACTGGTCCATCTCTGTTGT	
BACE1	F	CAGTGGGACCACCAACCTTC	31981412a1
	R	GCTGCCTTGATGGACTTGAC	
Basigin	F	GTGGCGTTGACATCGTTGG	2808468a1
	R	CTATGTACTTCGTATGCAGGTCG	
B-Raf	F	AATTTGGTGGAGAGCATAACCC	7271247a1
	R	ACGGTGTCCATTGATGCAGAG	
β-arrestin 2	F	GGCAAGCGCGACTTTGTAG	21703856a1
	R	GTGAGGGTCACGAACACTTTC	
CaMKII	F	TGGAGACTTTGAGTCCTACACG	161086916c1
	R	CCGGGACCACAGGTTTTCA	
cFOS	F	CGGGTTTCAACGCCGACTA	6753894a1
	R	TTGGCACTAGAGACGGACAGA	
ERK1	F	ACCACATTCTAGGTATCTTGGGT	93102422c3
	R	AGTTTCGGGCCTTCATGTTAAT	
ERK2	F	GGTTGTTCCCAAATGCTGACT	33090821a1
	R	CAACTTCAATCCTCTTGTGAGGG	
GAPDH	F	AGGTCGGTGTGAACGGATTTG	6679937a1
	R	TGTAGACCATGTAGTTGAGGTCA	

JNK1	F	AGCAGAAGCAAACGTGACAAC	7710060a1
	R	GCTGCACACACTATTCCTTGAG	
JNK2	F	TCAGTGGGTTGCATCATGGG	26327765a1
	R	GGATGGTGTTCCTAGCTGTTCA	
JNK3	F	CCATGTCTGTGTTCTTTCTCACG	28892799a1
	R	TTGGTTCCAACTGTGAAGAGTC	
LRP1	F	ACTATGGATGCCCCTAAAACTTG	6678720a1
	R	GCAATCTCTTTCACCGTCACA	
MEK1	F	AAGGTGGGGGAACTGAAGGAT	6678794a1
	R	CGGATTGCGGGTTTGATCTC	
MEK2	F	GTTACCGGCACTCACTATCAAC	31560267a1
	R	CCTCCAGCCGCTTCCTTTG	
MMP2	F	CCTGGACCCTGAAACCGTG	47271505c2
	R	TCCCCATCATGGATTCGAGAA	
MMP9	F	GGACCCGAAGCGGACATTG	N/A
	R	GAAGGGATACCCGTCTCCGT	
Neprilysin	F	CTCTCTGTGCTTGTCTTGCTC	31543255a1
	R	GACGTTGCGTTTCAACCAGC	
Nicastrin	F	TCCGTGGTACTGGCAGGATT	31981205a1
	R	CCCCTGTATCCCCACTAATTGA	
PKA Cα	F	AGATCGTCCTGACCTTTGAGT	7110693a1
	R	GGCAAAACCGAAGTCTGTCAC	
PKA Cβ	F	AGGGCAGGACATGGACATTG	255958317c3
	R	CGCCTTATTGTAACCCTTGCTG	
PKC α	F	GTTTACCCGGCCAACGACT	6755078a1
	R	GGGCGATGAATTTGTGGTCTT	
Presenilin1	F	GGTGGCTGTTTTATGTCCCAA	6679493a1
	R	CAACCACACCATTGTTGAGGA	
Presenilin2	F	TGCCTGTCACGCTGTGTATG	N/A
	R	GTTAAGCACGGAGTTGAGGAG	

PS enhancer2	F	ATCTTGGTGGATTTGCGTTCC	N/A
	R	GCGCCAAACATAGCCTTTGATTT	
5-HT_{1a}R	F	CATCGCGCTAGACAGGTACTG	162135953c3
	R	CAATGAGCCAAGTGAGCGAGA	
5-HT_{2a}R	F	TAATGCAATTAGGTGACGACTCG	27753985a1
	R	GCAGGAGAGGTTGGTTCTGTTT	
5-HT_{3a}R	F	TGTGTACGTGCATCATCGAGG	153791844c2
	R	GCACATCAAAGGGGAAGTTGTAG	
5-HT_{4}R	F	AGTTCCAACGAGGGTTTCAGG	6680325a1
	R	CAGCAGGTTGCCCAAGATG	
5-HT_{5b}R	F	TTGCTGATCGCTGCCACTTT	6754260a1
	R	GTCGAGGCCACCAAGTTATGT	
5-HT_{6}R	F	GCATAGCTCAGGCCGTATGTG	118130478c3
	R	CGCATGAAGAGGGGATAGATGA	
5-HT_{7}R	F	CCTTACCTCCTCTCTTCGGATG	113865997c3
	R	TGGAGTAGATCGTGTAGCCAAA	

Chapter 4: Summary and Future Directions

SUMMARY

Serotonin-mediated therapy for Alzheimer's disease has immense potential to revolutionize treatment, but an understanding of its underlying mechanisms is essential if we are to maximize its effectiveness. The aim of this dissertation was to map the key components of the downstream signaling pathways that mediate serotonin induced reduction in Aβ. We have shown a small set of 5-HTRs can reduce ISF Aβ, traced their signaling through PKA, and shown that ADAM10 is not the ultimate effector protein. This work suggests a serotonin targeted therapy could be beneficial for AD and opens avenues for further research.

The shotgun approach of using SSRI agents may prove effective in treating AD, but our work suggests similar efficacy could be produced simply by activating one or two of the 14 different 5-HT receptors. We have shown in this report that selective agonists for 5-HT_4R and 5-HT_7R could reduce ISF Aβ by 25%. This remarkable ability was not shared by the other classes of 5-HTRs that we tested. Current evidence suggests that the driving factor for Aβ aggregation is the steady-state concentration in the ISF. Even a modest reduction of 25% could prevent formation and growth of Aβ plaques mice (Cirrito et al., 2011). A targeted 5-HT_4R or 5-HT_7R therapy may reduce some of the unpleasant side effects that are common with SSRIs. A therapeutic plan with fewer side effects is more likely to be followed and more effective overall. These selective 5-HTR agonists should be entered in clinical trials for human safety immediately.

Our research suggests that a basal level of serotonergic activity helps keep Aβ production in check. Simultaneously blocking activity of 5-HT_4R and 5-HT_7R produced significant increases in ISF Aβ. However, inhibition of a single receptor had no effect. This result is not

surprising because 5-HT_4R and 5-HT_7R were equally effective at reducing Aβ; one receptor can still maintain Aβ at normal levels if the other is inactive. This maintenance of Aβ levels by basal serotonergic activity could help explain the progressive nature of AD. AD patients show degeneration of serotonergic projections across the brain, and these projections appear especially susceptible to damage by Aβ plaques. Neuropathy of these fibers would probably inhibit 5-HT activity around plaques. Loss of 5-HT activity would increase Aβ production and increase growth of plaques. Larger plaques would damage more serotonergic fibers and initiate a progressive cycle of neuropathy. We could test this hypothesis by examining if 5-HTR agonist treatment can preserve serotonergic projections in brains of transgenic mice. Alternatively, serotonergic projections to a specific brain region could be destroyed, and plaque load in that region could be compared to uninjured controls.

PKA activation appears to be the first signaling step involved in the reduction of Aβ by 5-HT. We showed that blocking activity of PKA by two different methods could completely abolish the effects of SSRI in transgenic mice. We also showed that stimulating PKA activity with a selective dopamine 1 receptor agonist could reduce Aβ to a significant degree. While this is compelling evidence for the role of PKA, there is still more work to be done. The connection between PKA and ERK still needs to be determined. B-raf looks like a promising target, and there are selective inhibitor compounds available from pharmaceutical companies. We could administer these B-Raf inhibitors via reverse microdialysis and monitor ISF Aβ levels. Other experiments could examine other signaling pathways related to cAMP such as Epac or Src.

We were able to provide evidence that activated ERK works within the cytoplasm to reduce Aβ after SSRI treatment. We found no significant change in expression in over 30

different genes that could be linked to Aβ; secretase genes, signaling genes, clearance genes, and receptor genes were unaffected 16 hours after SSRI treatment. The rapid reduction of ISF Aβ by SSRI was a clue that ERK was not changing gene expression in our system. Gene expression changes require more time than post-translational modification of cytosolic proteins. Transcription of mRNA, translation of proteins, and trafficking of proteins to their destinations all require a great deal of time. This fact is compounded by the arborized shapes of neurons; dendritic projections of CNS neurons can be quite long when compared to the soma. A considerable amount of time would be necessary for pERK to travel from a post-synaptic density to the neuronal soma to change gene expression. Our work does not completely eliminate the possibility of pERK acting in the nucleus. Several labs, including our own, have shown changes in secretase genes after chronic treatment with agents that modify 5-HT signaling. Our work merely suggests the rapid reduction in ISF Aβ after 5-HT stimulation is mediated by acute post-translational modification in the cytosol. Future time course experiments could determine when these changes in secretase expression actually occur with chronic 5-HT treatment. Other experiments could determine if pERK stays near synapses or is transported along neurites towards the soma.

We were able to complete the sequence of events that reduce Aβ after serotonergic activity by examining ADAM10. Stopping ADAM10 activity with a selective inhibitor was unable to block the effects of SSRI in transgenic APP/PS1 mice. This result suggests that ADAM10 is not the α-secretase enzyme that is active after SSRI treatment. This finding suggests that ADAM17 is mediating the reduction of ISF Aβ. ADAM17 has been shown to be required for stimulated α-secretase activity in vitro (Kuhn et al., 2010). We have requested a compound that selectively inhibits ADAM17 from the lab that discovered it last year (Nuti et al., 2013).

Using this compound, we will test if selectively inhibiting ADAM17 can block the effects of SSRI. Alternatively, we can administer both the ADAM10 and ADAM17 inhibitors to test if the enzymes are functionally redundant after SSRI. Our results suggest the model presented in Figure 4.1. Serotonin binds to 5-HT_4R and 5-HT_7R to activate PKA. PKA signaling activates MEK and ERK. Activated ERK increases α-secretase activity to reduce ISF Aβ. Future work will determine if ERK directly phosphorylates ADAM10/ADAM17 or changes its cellular localization via interactions with proteins like β-arrestin.

Immunization with aggregated Aβ and treatment with γ-secretase inhibitors have been proven to be generally toxic for human patients even at low to moderate doses. Since AD pathology begins to accumulate decades before symptoms appear, a successful AD therapy must be safe for patients to use for many years. Perhaps a selective 5-HTR agonist can provide this safer long-term strategy for reducing Aβ levels and plaques.

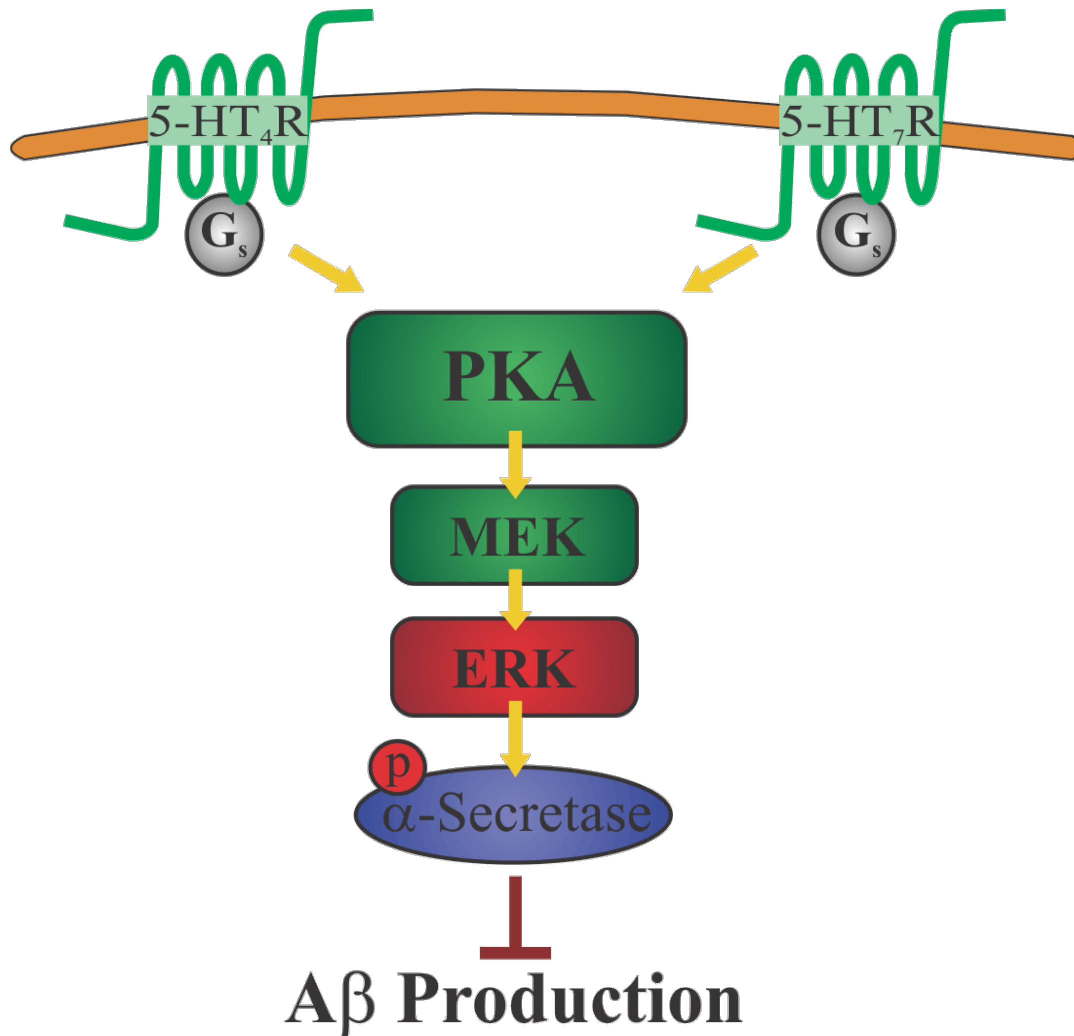

Figure 4.1: Model of Aβ reduction by serotonergic signaling. Serotonin binding to 5-HT_4R and 5-HT_7R leads to the activation of PKA. PKA subsequently activates MEK and ERK. Activated ERK remains in the cytoplasm and phosphorylates α-secretase to increase its activity and reduce Aβ production.

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