THE EFFECTS OF EPHB RECEPTOR TYROSINE KINASES ON LEVELS OF AMYLOID-BETA PROTEIN IN THE HIPPOCAMPUS AND THE PROGRESSION OF ALZHEIMER'S DISEASE

by

Christine Riddle

Submitted in partial fulfillment of the
requirements for Departmental Honors in
the Department of Biology
Texas Christian University
Fort Worth, Texas

May 2, 2014

THE EFFECTS OF EPHB RECEPTOR TYROSINE KINASES ON LEVELS OF AMYLOID-BETA PROTEIN IN THE HIPPOCAMPUS AND THE PROGRESSION OF ALZHEIMER'S DISEASE

Project Approved:

Michael Chumley, Ph.D.
Department of Biology
(Supervising Professor)

Giridhar Akkaraju, Ph.D.
Department of Biology

Gary Boehm, Ph.D.
Department of Psychology

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEGEMENTS

I would like to thank Dr. Chumley for taking me under his wing and inviting me to become a member of his research laboratory as a mere sophomore at TCU. Dr. Chumley's excellent guidance, passion for teaching, and unwavering quest for knowledge is inspiring to everyone who works with him. I have learned so much more than I thought possible during these past few years, and I am so grateful to have had this opportunity.

I would also like to thank my family: my mom and dad, who have supported my goals no matter how challenging they may seem, and my sister, who has shown me the way to pursuing a career in medicine.

Lastly, I dedicate this research to the memory of my grandmother, who passed away of Alzheimer's disease in 2010 yet whose positive influence continues to shine through in her five children. You are missed but never forgotten.

"Decide what to be, and go be it." – The Avett Brothers

INTRODUCTION

The brain, at a molecular level, is composed of an ever-changing sea of neuronal connections. Any increase or decrease in activity between these connections, or synapses, has been shown to directly affect the strength of the synapse, a concept termed synaptic plasticity. Simply put, the more a synapse is used, the stronger it will become. The neurons comprising a normally functioning brain exhibit this characteristic of plasticity, one that is critical for neuronal viability. However, the converse and unfavorable situation can also occur and is well demonstrated in Alzheimer's disease (AD), a progressive brain disorder that largely arises due to the loss of synaptic activity (Shankar and Walsh 2009).

Santiago Ramón y Cajal, an eighteenth century Spanish pathologist and histologist, hypothesized correctly the resulting effects of a loss of synaptic plasticity when he wrote that "dementia could result when synapses between neurons are weakened as a result of a more or less pathological condition, that is, when processes atrophy and no longer form contacts, when cortical mnemonic or association areas suffer partial disorganization" (Cajal 1928). Alzheimer's disease is characterized clinically by a gradual onset of dementia, or disorder of mental processes, including memory loss, personality changes, and impaired cognitive reasoning (Shankar and Walsh 2009). Consequently, this disease places an increasingly heavy emotional and financial burden on patients, families, and society, with an estimated cost of $604 billion in 2010. It is also projected that the number of AD cases, which was around 36 million in 2012, will triple by the year 2050 (Huang and Mucke 2012). Healthcare systems worldwide will be enormously affected by this disease; therefore, it becomes increasingly important to

examine the hypotheses surrounding the etiology of AD in order to formulate possible treatment options.

Pathologically, the Alzheimer's brain is atrophied and degenerated due to decreased synaptic activity (Shankar and Walsh 2009). A prominent hypothesis explaining the source of this loss of activity and subsequent dementia is the "amyloid hypothesis," which is supported by findings of amyloid plaques and neurofibrillary tangles in brains affected by AD. Interestingly, it is not the sheer number of plaques and tangles that correlates directly with severity of dementia, but the extent of synaptic loss (Shankar and Walsh 2009). In light of this data, what, then, is causing this loss?

As aforementioned, numerous amyloid plaques litter AD-affected brains. These plaques are composed of amyloid-β protein (Aβ), the molecule thought to be the cause of AD pathogenesis. Normally, Aβ maintains a steady level in the brain through an equilibrated state of production, degradation, and clearance. An accumulation of Aβ leads to self-association, producing a range of aggregate sizes. Since the amount of amyloid plaques does not correlate with severity of dementia in AD, researchers have hypothesized that it is the non-fibrillar, soluble Aβ assemblies that are responsible for synaptic loss, though the exact composition of these assemblies is unknown. In addition, another current hypothesis is that the plaques act as reservoirs for these diffusible oligomers and thus contribute to synaptic loss to neurons within the immediate vicinity of each plaque (Mucke and Selkoe 2012).

Previous studies done with mouse lines transgenic for human amyloid-precursor protein (hAPP), the molecule that gives rise to Aβ via proteolysis, also support the hypothesis that soluble Aβ causes cognitive deficit through interference with synaptic

function and subsequent aberrant network activity. Transgenic hAPP mice have little neuronal loss and develop synaptic degradation and cognitive deficit before amyloid plaques form in the brain, suggesting that this synaptic degradation is caused by soluble Aβ, not plaques. Additionally, it was shown that manipulations preventing or reversing synaptic destruction correlate directly with cognitive impairment in the mice (Mucke and Selkoe 2012). Transgenic mouse models have also shown that neurofibrillary tangles (NFTs) correlate more closely with dementia than do plaques. However, the main protein component of NFTs, tau, is able to cause neuronal dysfunction independently of the tangles themselves, suggesting instead that NFTs are not key culprits of the synaptic loss seen in AD (Huang and Mucke 2012).

Mutations in the amyloid precursor protein (APP) gene cause early-onset autosomal dominant AD and distinctly exhibit the relationship between soluble Aβ production and cognitive deficit. In this version of the disease, interference with APP processing causes altered, irregular production of Aβ molecules, which, as previously mentioned, are normally maintained at specific levels. Interestingly, the APP gene resides on chromosome 21, so Down's syndrome patients with trisomy 21 have an extra copy of the APP gene. These patients develop early-onset dementia and brains with pathologic hallmarks of AD. However, patients with partial trisomy 21 excluding the APP gene do not develop early-onset AD. Clearly, an inverse relationship between levels of APP gene expression and age of disease onset is demonstrated (Huang and Mucke 2012).

If soluble Aβ is the molecular component to be blamed for the degradation of synapses, how does it mediate this depression? Synaptic activity enhances Aβ production, which increases extracellular Aβ levels and also reduces postsynaptic excitability in a

negative-feedback mechanism (Mucke and Selkoe 2012). It is thought that a pathological over-accumulation of Aβ due to altered APP processing leads to a gain-of-function effect that results in decreased plasticity through suppression of excitatory postsynaptic activity (Shankar and Walsh 2009). The theory of synaptic plasticity often includes the role of N-methyl-D-aspartate (NMDA) glutamate receptors, which convert synaptic activity into a calcium signal that results in downstream effects of long-term potentiation (LTP), the strengthening of the synapses thought to lead to learning and memory formation. In AD, soluble Aβ may block the uptake of glutamate at synapses, leading to chronic activation and desensitization of NMDA receptors, increased Aβ release, plaque formation, and long-term depression (LTD), eventually culminating on impairment of plasticity and consequent neurodegeneration (Texidó et al. 2011).

Many previous analyses of the AD brain have shown significant decreases in synaptic density within the hippocampus. A major component of the limbic system, the hippocampus plays an important role in memory consolidation and spatial navigation. The primary mediators of excitatory synaptic transmission in the hippocampus are the NDMA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, both of which have been shown to be essential for induction and maintenance of LTP. In relation to the hippocampus, then, proper function of both receptors is necessary for memory and spatial learning (Simón et al. 2009).

There is a vast kinetic complexity of the pathways involved in synaptic signaling throughout the brain, including the crucial role of the Eph receptor and its ligand, the ephrin. Eph receptors encompass the largest family of cell-surface receptor tyrosine kinases (RTKs), with at least fourteen receptors in two different classes already identified

in mammals (Simón et al. 2009). RTKs transduce extracellular stimuli into cells and trigger signaling cascades. More specifically, Eph receptors are incredibly important in axon guidance during neurogenesis, reorganization of the brain's cytoskeleton, and regulation of plasticity, especially within the hippocampus. Among their many roles are those of maintenance and stabilization of synaptic structure, regulation of excitatory neurotransmission, and regulation of NMDA and AMPA receptor function (Simón et al. 2009).

There is much evidence that Eph receptors also play an important role in the synapse loss seen in AD. A study by Simón et al. (2009) showed significant Eph receptor reduction in the hippocampus before any onset of cognitive deficit appeared in hAPP mice. Interestingly, within the same study, reduced levels of Eph receptors were found in postmortem hippocampal sections from AD patients, strongly suggesting that Eph receptor expression plays a role in AD pathogenesis (Simón et al. 2009).

There are two classes of Eph receptors: A and B. The EphA family preferentially binds to ephrinA ligands, while the EphB family normally binds to ephrinB ligands. The ephrins differ from normal ligands by their attachment to cell membranes, which enables the extracellular domain of Eph receptors to bind to the ligand and activate signaling cascades in the ligand-expressing cells, creating a reverse signal. The Eph/ephrin complex is therefore very versatile due to its bi-directional signaling transduction capability (Chen et al. 2012).

Normally, ephrin binding causes phosphorylation of Eph receptors at synapses, resulting in activation of signal transduction cascades. EphA4, EphB1, EphB2, and EphB3 have all been found localized at excitatory synapses in the hippocampus. Mouse

models with deletions for all three EphB genes lack formation of mature dendritic spines, while single mutant models have relatively normal dendritic spine formation, suggesting that the genes have an additive, redundant effect for correct neurogenesis. The triple mutant mice also exhibit reduced levels of both NMDA and AMPA receptors, molecules critical for proper synaptic function (Chen et al. 2012).

Out of the nine class A and six class B receptors, EphB2 has been extensively studied in regards to AD, because it is found in abundance throughout the hippocampus and helps regulate the NDMA receptor signaling pathway. In AD pathogenesis, Aβ has been found to bind to the extracellular domain of EphB2 and trigger its degradation, decreasing its hippocampal expression. The dentate gyrus, an area of the hippocampus with high rates of neurogenesis that functions in new memory formation, is one of the areas strongly affected by this decrease. Not surprisingly, the decrease in EphB2 results in impaired synaptic plasticity, LTD, and reduced memory formation. However, the opposite effect was seen when hAPP transgenic mice were induced to increase EphB2 expression in the dentate gyrus: the deficits in synaptic function, NMDA receptor signaling, and memory formation were reversed. Cognitive deficits, including those associated with spatial learning and novel object recognition, were also improved in these animals. These results suggested that decreased levels of hippocampal EphB2 correlated strongly with cognitive dysfunction associated with the soluble Aβ seen in AD pathology (Cissé et al. 2010). Additionally, processing of the EphB2 receptor is mediated by γ-secretase, the enzyme highly responsible for cleaving APP into soluble Aβ. Mutations in presenilin 1 (PS1), the core component of γ-secretase, correlate closely with familial AD,

because loss of the PS1 gene leads to a decrease in EphB2 levels and subsequent NMDA receptor deficit (Chen et al. 2012).

It has been shown that ephrinB2 molecules bind synergistically with metabotropic glutamate receptors (mGluRs), cell-surface G-protein coupled receptors that cluster postsynaptically and are important in modulating synaptic function. Interestingly, mGluR activation results in overstimulation of neurons by the neurotransmitter glutamate, leading to neuronal damage and death, a process called excitotoxicity. The ephrinB2 ligand that binds with mGluR also binds a subunit of the NMDA receptor, forming a functional complex, which augments the excitotoxicity and leads to LTD. This data provides evidence that mGluRs are highly dependent on Eph/ephrin signaling. Clearly, synaptic function and neuronal networking are very sensitive to expression levels of Ephs and ephrins, isolating them as key players in the synapse loss seen in AD (Chen et al. 2012).

One of the paradoxes in neurodegenerative disease pathology stems from the fact that NMDA receptor stimulation leads to both survival and death of neurons. The specific outcome depends on the location of the NMDA receptor: synaptic NMDA receptor stimulation, primarily via Ca^{2+}-dependent signaling, leads to neuroprotectivity, while stimulation of extrasynaptic NMDA receptors leads to cell death. When the balance of these two receptor subsets is disrupted, neuronal dysfunction occurs (Hardingham and Bading 2010).

In 2013, a study done by Geng et al. that EphB2 also has protective characteristics for hippocampal neurons, shielding them against the toxicity of soluble Aβ oligomers, and that this neuroprotective effect is achieved by increasing the expression of synaptic

NMDA receptors within the hippocampus. Normally, stimulation of synaptic NMDA receptors drives phosphorylation of the cAMP response element binding (CREB) protein, an important target of Ca^{2+} signaling, resulting in favorable survival events in neurons. Conversely, extrasynaptic NMDA receptor stimulation shuts off the CREB pathway and leads to cell death. In this study, the authors showed that induced EphB2 overexpression in hippocampal neurons protected the cells against the harmful effects of Aβ oligomers. The overexpression of EphB2 created a neuroprotective shield by preventing a decrease of synaptic NMDA receptor expression. Additionally, EphB2 overexpression also prevented downregulation of the CREB pathway by Aβ oligomers, leading to a reduction in cell death (Geng et al. 2013).

Though the mechanisms by which EphB2 protects hippocampal neurons against the toxicity of Aβ oligomers are still unclear, there is evidence that EphB2 binds NMDA receptors to increase Ca^{2+} signaling in developing neurons via CREB phosphorylation. There is also data suggesting that increasing EphB2 expression triggers neurons to deliver more NMDA receptors to synapses. These events both lead to increased neuronal viability in the hippocampus in the presence of soluble Aβ oligomers to defend against neurotoxicity (Geng et al. 2013).

As stated before, AD is an intensely progressive neurodegenerative disease and is affecting more and more of the world's population every year. Currently, no therapies exist to successfully prevent or treat AD. Certainly, a critical therapeutic objective is to identify ways to block events in the Aβ-triggered pathogenic cascade that lead to an aberrant neuronal network. Specifically, either blocking the production of Aβ itself or enhancing its removal seems to be a pragmatic plan of action (Mucke and Selkoe 2012).

In light of the data discussed previously, increasing EphB2 levels or function could be beneficial in prevention of AD, because Eph/ephrin signaling is clearly very important in the creation of functional synapses. It is known that EphB2 levels in the hippocampus are significantly decreased in AD brains. If it is found that EphB2 depletions contribute to deficits in regions of the brain other than the hippocampus as well, pharmacological treatments could be used to increase EphB2 expression or modulate its activity in order to induce neuroprotectivity throughout the brain (Cissé et al. 2010; Chen et al. 2012).

The data presented in this paper was done in collaboration with research pursued by Dr. Mark Henkemeyer at University of Texas Southwestern (UTSW) Medical Center in Dallas. This collaboration aims to present additional evidence supporting Dr. Henkemeyer's preliminary research, which suggests that EphB receptors not only exhibit neuroprotective effects on hippocampal neurons but also bind to, degrade, and eliminate the Aβ oligomers that build up in synapses.

As previously stated, it has been shown that the EphB family binds to NMDA receptors to increase Ca^{2+} influx and signaling cascades, strengthening synapses through the process of LTP. Specifically, this binding occurs through the extracellular domain of the EphB receptor to the NR1 subunit of the NMDA receptor. This recruits and clusters the receptor to the synapse of excitatory neurons, resulting in neuroprotectivity and increased synaptic plasticity. Mutant animals lacking EphB receptors exhibit diminished hippocampal-based learning and impaired memory formation. Additionally, the extracellular domain of the EphB2 receptor has been shown to bind and internalize Aβ oligomers, triggering degradation of the EphB2:Aβ complex by proteasomes.

The hypothesis presented by Henkemeyer, which states, "EphB receptors play an important role in regulating the balance between Aβ production and clearance in the brain", stems from novel data collected at UTSW using EphB knockout (KO) mice. In EphB1/B3 double KO and EphB1/B2/B3 triple KO mice aged to 14 months, it was found that the CA1 region of the hippocampus, a hotspot for early plaque formation in AD, contained granular plaques as seen with a modified Bielschowsky silver stain (Figure 1). The plaques were also shown to be immunoreactive for Aβ using a 6E10 monoclonal antibody that binds to murine Aβ when present at high concentrations (Figure 2). All of the mutant mouse brains analyzed exhibited larges, numerous granular plaques, while none of the wild-type (WT) mice with identical backgrounds and ages had brains showing this pathology. In addition, the triple mutants had more plaque clusters on average than the double mutants, suggesting that the three EphB receptors work in tandem to prevent plaque formation. This is the first KO mouse model exhibiting an accelerated age-related Aβ plaque formation in the absence of any dominant AD transgene, usually the initial cause of excess Aβ production.

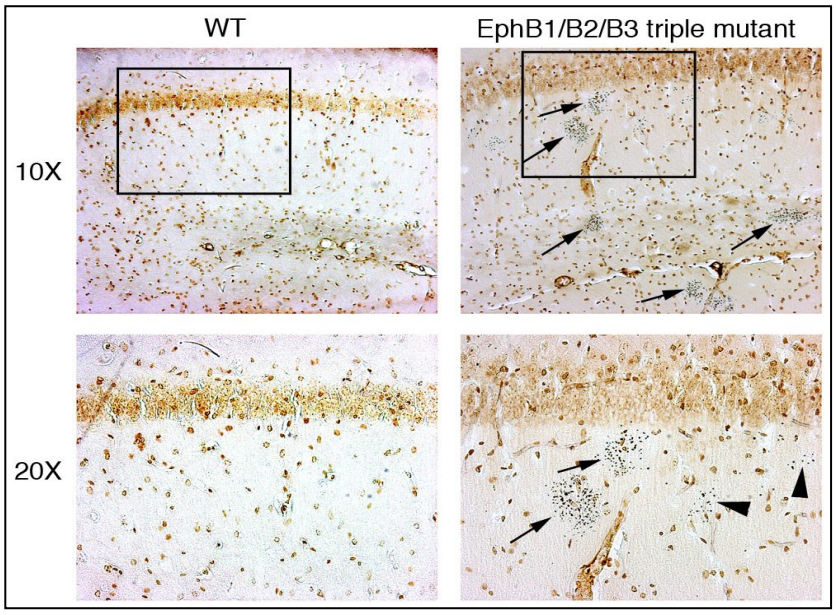

Figure 1. Modified Bielschowsky silver stain shows granular argyrophilic plaques in the hippocampal CA1 region of 14 month old *EphB* mutants (arrows and arrowheads), but not in age and background matched WT mice.

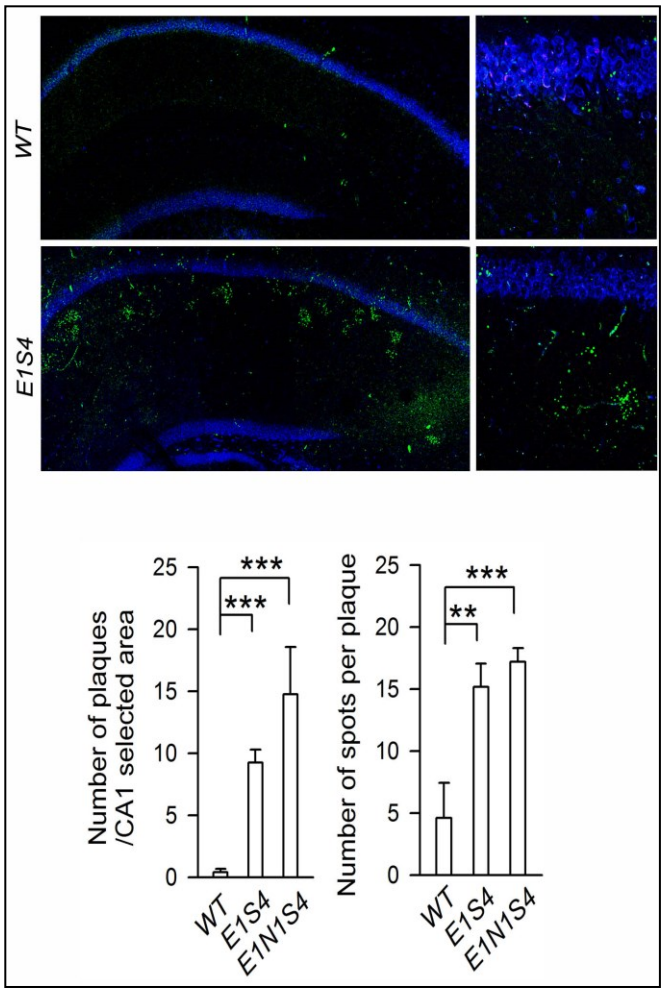

Figure 2. Abeta immunofluorescence in hippocampal sections of 14 month old WT, *EphB1EphB3* double and *EphB1EphB2 EphB3* triple KO mice also labels granular plaque clusters mainly restricted to the nerve fiber rich CA1. Quantification of the Abeta-positive spots indicates the double (E1S4, N=3) and triple (E1N1S4, N=3) mutants exhibit highly significant increases in the number of plaques (defined as containing 4 or more granular spots) per a defined area of CA1 (left bar graph) and in the actual number of granular spots per plaque (right bar graph). Note that in the 12 sections of the WT mice analyzed (N=3), only three plaques that contained 4 or more granular spots were identified, and they averaged only 5 spots.

Immunofluorescent staining of Abeta plaques using 6E10 antibody (Covance) and Alexa-488 secondary antibody (Jackson Immunoresearch) produces green staining where Abeta aggregates are located, and a neuronal nuclear counter stain (Molecular Probes) to show the Dentate Gyrus and CA1 regions of the hippocampus.

The data also confirmed that both EphB1 and EphB2 bind to Aβ using both a sensitive bead-bound, proximity-based chemiluminescent AlphaScreen assay and a cell-based receptor clustering assay. The AlphaScreen assay showed that the EphB1 and EphB2 extracellular domains bind to human Aβ 1-42 oligomers (Figure 3). The clustering assay demonstrated that human APP/Aβ binds to and clusters EphB1 and EphB2 receptors (Figure 4). These results support the idea that EphB receptors are neuroprotective and suggest that the mechanism by which they function might be one of a molecular sponge that binds to and targets Aβ oligomers for proteolytic degradation.

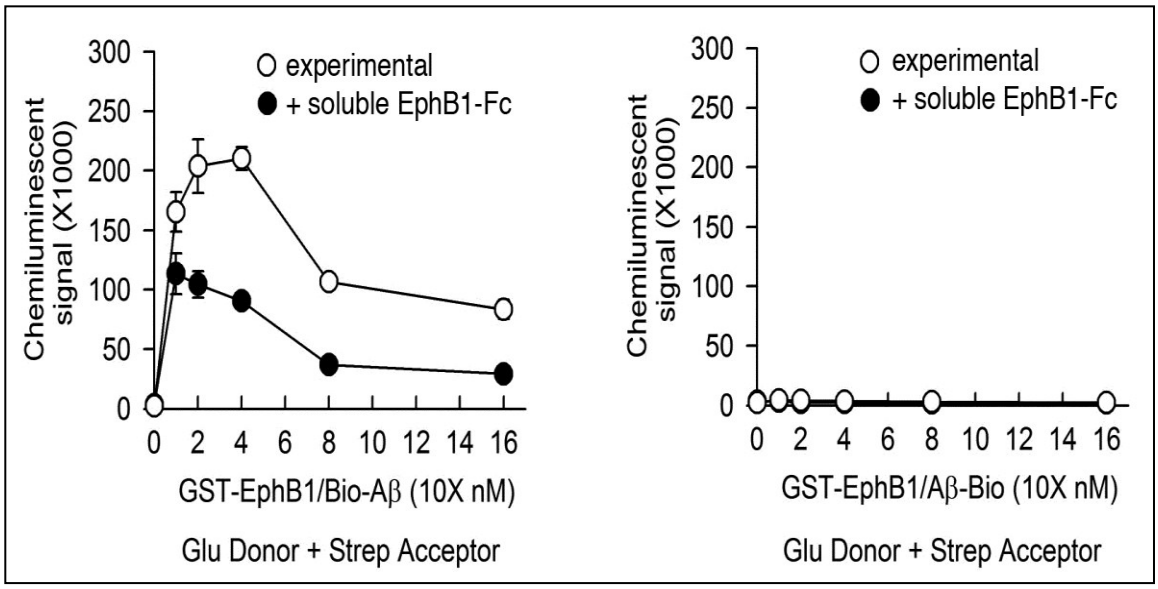

Figure 3. Plots of chemiluminescent reads from initial AlphaScreen assay using 0-160 nM equal concentrations of GST-EphB1 and either N-terminal Bio-Abeta (left) or C-terminal Abeta-Bio (right) peptides. Only the N-terminal tagged Bio-Abeta peptide exhibited binding to EphB1 (open circles), which was strongly reduced in the presence of 4X molar excess soluble EphB1-Fc competitor (closed circles). The C-terminal Abeta-Bio tag is presumably masked/buried upon binding to EphB1 such that it is not able to bind the streptavidin acceptor beads or perhaps the Abeta-Bio peptide is not properly presented to EphB1 when bound to the acceptor beads. Shown are the mean (+-SD) from 4 identically treated wells per reaction condition taken after an O/N incubation/equilibration at RT. Similar reads were also obtained at 3 hr.

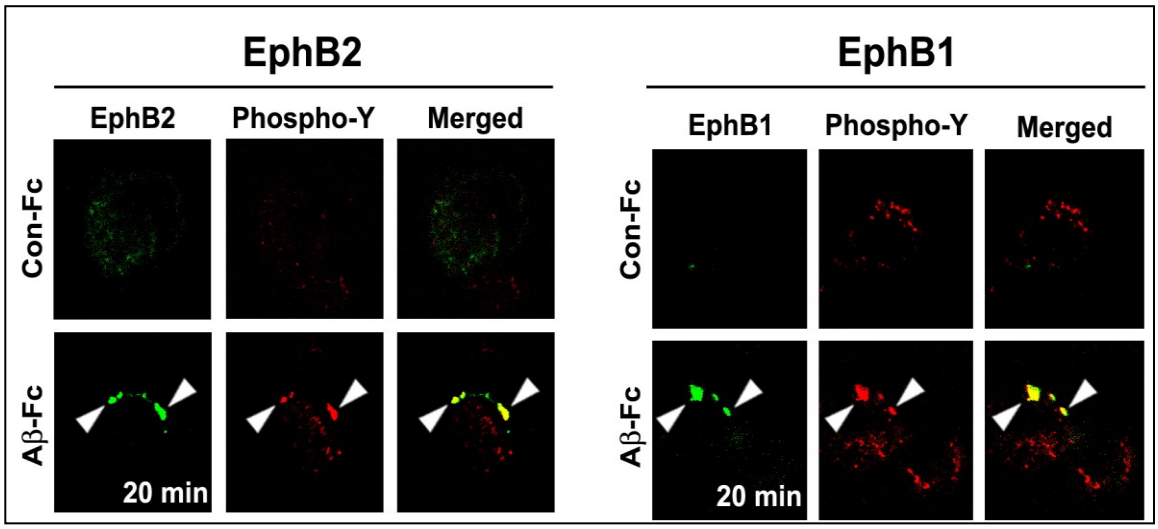

Figure 4. APP/Abeta-Fc is able to bind and cluster EphB2 and EphB1 receptors. BHK cells transfected with HA-EphB1 or HA-EphB2 were exposed to 2 ug/ml soluble preclustered APP/Abeta-Fc. Cells were then fixed and stained with anti-HA and anti-phospho-tyrosine antibodies. 5 ug/ml preclustered unconjugated Fc was used for a negative control.

The significance of this preliminary data is two-fold, including the creation of a KO animal model with which to study plaque formation as seen in early-onset AD and the isolation of EphB receptors as key molecules that protect the brain by preventing early Aβ deposition. In the absence of EphB receptors, Aβ plaques build up as the brain ages, probably due to the reduction of clearance of Aβ. Overall, these ideas are consistent with theories discussed previously, which suggest that soluble Aβ oligomers are the culprits causing the synaptic dysfunction seen in neurodegenerative diseases like AD. Since the amino acid sequences of the mouse and human EphB1, EphB2, and EphB3 receptors are almost identical, it is reasonable to suggest that understanding these receptors' function in mice is critical to discovering and understanding their function in the aging human brain.

Because of the EphB receptor's critical role of helping to maintain a state of homeostasis in the brain by targeting Aβ for clearance, there should be higher levels of Aβ oligomers in animals lacking EphB receptors. The research detailed in the remainder of this paper aims to support this hypothesis with data collected in the form of Western blots, dot blots, and enzyme-linked immunosorbent assays (ELISAs).

METHODS

Animals

All animals were housed and cared for in the Texas Christian University Vivarium. The room had a light/dark cycle of 12 hours. The animals were kept in standard cages (12.5 cm x 15 cm x 15 cm) in groups of 3 or 4. Daily vivarium checks for good health and sanitation were performed. Animals were given free access to food and

water at all times throughout the day. The Institutional Animal Care and Use Committee at Texas Christian University approved all experiments.

Littermates weaned at three weeks of age were separated based on gender and had a small 0.5 cm piece of tail removed for genotyping. The animals used in this research study were of two different strains: EphB2$^{-/-}$ (knockout for EphB2 receptor) and EphB2$^{-/-}$;EphB3$^{-/-}$ (compound knockout for EphB2 and EphB3 receptors). All strains were of a CD1 background. The control animal used was a 5XFAD model (transgenic for five known human mutant genes that lead to human AD-like pathology).

Aβ ELISA

Mice were euthanized by CO_2 asphyxiation, brains removed, and hippocampus dissected. Removed tissues were homogenized in a lysis buffer consisting of 25mM Tris @ pH 7.5, 150mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 1x protease inhibitor (IBI Scientific, Peosta, IA) and allowed to lyse and freeze overnight.

Protein concentration was determined by DC protein assay, which utilizes a reagent that is commonly used with detergent-based lysis buffers. A 1.52µg/µl protein standard serially diluted in lysis buffer down to 0.2µg/µl provided the standard curve with which to determine the concentration of the unknowns. 5µl of unknown protein were pipetted into a 96-well plate, followed by the addition of 25µl of reagent A' and 200µl of reagent B. After 15 minutes of incubation in the dark, the absorbance of each well was read at 750nm in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC).

The BetaMark Aβ $_{x-42}$ ELISA (Covance Research Products, Dedham, Massachusetts) is a 48-hour procedure that utilizes a 96-well antibody coated plate, into

which both samples and standards of known concentrations are placed. To perform this ELISA, a preparation of working incubation buffer, 1X wash buffer and standard intermediates were made prior to the start of the assay. Next, the Standard Diluent was used to reconstitute the Aβ standard and to prepare the standard curve. The samples were diluted 2:1 with working incubation buffer, which includes the HRP-labeled detection antibody. 100µl of each dilution of the standard curve, in duplicate, and 100µl of each unknown sample in triplicate to the plate and was incubated over night at 2–8 degrees Celsius. On the following day, the wells were washed 5 times with the 1X wash buffer. After the washes, 200 µl of TMB, a substrate for the HRP enzyme, was added to each well. The plate was then incubated for 45 minutes at room temperature in the dark. After this incubation period, the plate was read at the optical density of 620nm.

Aβ Western Blot

After determining the concentration of the stock solutions of protein, samples were prepared for loading by mixing proper amounts of protein sample, lysis buffer, and sample buffer to equal a concentration of 1µg/µl. Proteins were denatured by boiling for 5 minutes in a dry bath (Labnet Intl., Woodbridge, NJ), with as the presence of β-mercaptoethanol (Sigma, St. Louis, MO) in the sample buffer to reduce intramolecular disulphide bonds.

Boiled samples were loaded into 12% Tris-Tricine acrylamide gels at 25ul per well. All Western blot hardware, including gels, gel cassettes, gel box, and transfer cells, is a product of Bio-Rad, Hercules, CA. Current was applied to the gels at 200 watts for 50 minutes. Proteins were transferred (Bio-Rad Trans-blot SD, Bio-Rad, Hercules, CA) from the polyacrylamide gel electrophoresis (PAGE) gel to a polyvynilidene fluoride (PVDF)

membrane (Millipore, Billerica, MA). The current applied through the transfer cell was conducted through filter papers (Kaysville, UT) soaked in electrolytic buffer as per the instructions provided with our transblotter. The transfer of protein from gel to membrane required 0.15 amps per gel, for 40 minutes.

The membrane was then blocked in 5% non-fat, blotting grade milk in PBST with 0.02% azide (Sigma, St. Louis, MO) for 45 minutes. Primary antibody, mouse anti-Aβ (6E10; from Covance), was mixed at 1:500 dilution in 5% non-fat, blotting grade milk in PBST. Membranes were incubated in primary antibodies at 4^{o}C overnight.

After the removal of primary antibody, the membranes were washed in several washes of PBST, followed by the addition of a peroxidase-linked donkey anti-rabbit secondary antibody at a concentration of 1:5000 (Jackson ImmunoResearch, West Grove, PA). Membranes were incubated with secondary antibody for one hour.

After removal of secondary antibody, the membranes were washed in several washes of PBS. They were then placed on transparencies and coated in SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). An image of the resulting chemiluminescence was acquired using a Syngene G:Box (Syngene, Frederick, MD). Denitometric analysis was done using GeneTools software, with β-actin as the loading control.

Dot Blot

Mice were euthanized by CO_2 asphyxiation, brains removed, and hippocampus dissected. Removed tissues were homogenized in a lysis buffer consisting of 25mM Tris @ pH 7.5, 150mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% sodium dodecyl

sulfate, and 1x protease inhibitor (IBI Scientific, Peosta, IA) and allowed to lyse and freeze overnight.

Lysates were centrifuged (13,000 g for 20 minutes). The lysate was removed and saved. The insoluble material (pellet) was resuspended in lysis buffer and sheared through a 27-gauge needle.

Membranes were spotted with dots of either lysate or pellet. Dots were left on membranes for 3 minutes. Membranes were blocked and Aβ detected as previously mentioned in Western Blot section. Both blots used a primary antibody of mouse anti-Aβ (6E10; from Covance). The first blot was spotted with dots of equal volume of 10 microliters lysate or pellet. The second blot was normalized and spotted such that each dot held 0.269 micrograms of total protein.

RESULTS

Aβ ELISA

An ELISA (Figure 5) was performed in order to quantify the amount of Aβ protein in the hippocampal tissues of each of four mouse models: wild-type (WT), 5XFAD, EphB2$^{-/-}$, and EphB2$^{-/-}$;EphB3$^{-/-}$. The WT tissue, used as the normal control, showed approximately 100 picograms per milligram (pg/mg) of Aβ. The 5XFAD tissue, used as the abnormal control due to its forced production of high levels of Aβ like those seen in AD, showed a significant increase in Aβ: around 560 pg/mg. Neither the EphB2$^{-/-}$ nor the EphB2$^{-/-}$;EphB3$^{-/-}$ showed a significant increase in Aβ, with detection around 130 and 150 pg/mg, respectively.

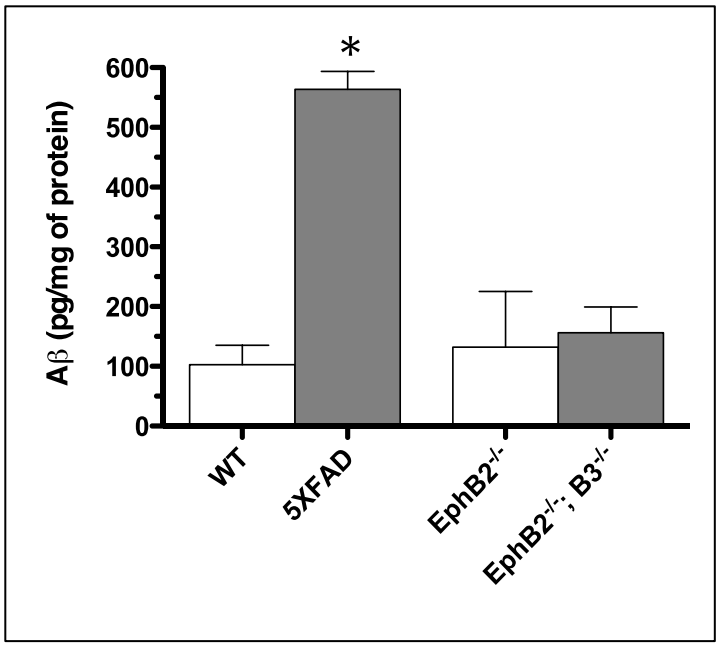

Figure 5. Levels of Aβ in the hippocampus of four different mouse genotypes. A significant main effect of genotype on Aβ level was discovered ($F(3,36)=15.18$, $p=<0.0001$). Bonferoni post-hoc analysis shows only 5XFAD is significantly different from all other genotypes ($p<0.05$) thus indicating that the lack of EphB2 or EphB2 and EphB3 does not increase levels of Aβ.

Aβ Western Blot

In order to detect the presence of Aβ protein in the hippocampal tissues of the mouse models, a Western blot was performed (Figure 6). The 5XFAD model was used as the control in this experiment, and the dark line underneath its label indeed represents a positive detection of Aβ. Similar to the ELISA results, tissue lysates from the EphB2$^{-/-}$ KO and the EphB2$^{-/-}$;EphB3$^{-/-}$ double KO showed no Aβ protein.

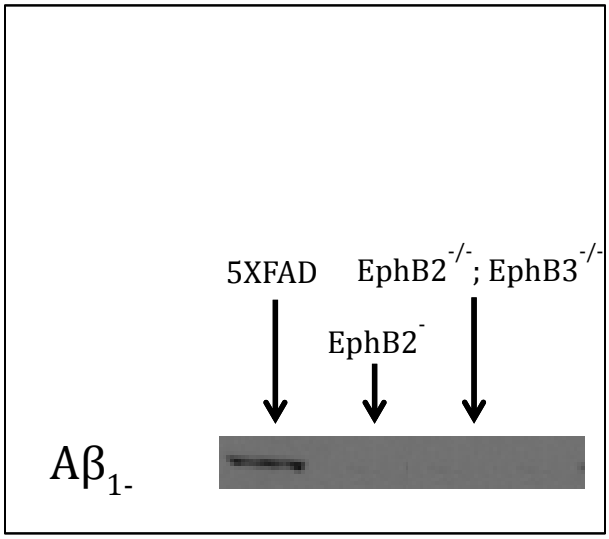

Figure 6. Representative western blot for $Aβ_{1-42}$ in hippocampal lysates from 5XFAD transgenic mice, as well as EphB2$^{-/-}$ and EphB2$^{-/-}$; EphB3$^{-/-}$ compound null animals.

Dot Blot

Dot blots were performed to test all possible sites of accumulation of Aβ protein in the hippocampal tissue, including the soluble lysates (which were tested previously with the ELISA and Western blot) and insoluble pellets (which included the remainder of the tissue).

Figure 7 shows the first dot blot test, which used 6E10 mouse anti-Aβ as the primary antibody. Lysates were dotted on the left side and pellets were dotted on the right side of the membrane. There was strong immunofluorescent signal from the Aβ transgenic mouse control (5XFAD) lysate as expected. Neither the WT or EphB2$^{-/-}$;EphB3$^{-/-}$ lysates produced any fluorescent signal. All three dots of pellet material produced a somewhat weaker signal than the control lysate, with the WT slightly brighter than the control and KO.

Figure 8 shows the second dot blot test, which also used 6E10 mouse anti-Aβ as the primary antibody. Lysates and pellets were dotted as in the first test. However, the total amount of protein was controlled for in this experiment, with 0.269 micrograms in each dot. In this procedure, only the 5XFAD lysate control produced any discernable fluorescent signal. No signal from any other tissue sample was detected, suggesting that if Aβ is present as seen in the pellets from Figure 7, the total amount of Aβ must be very small.

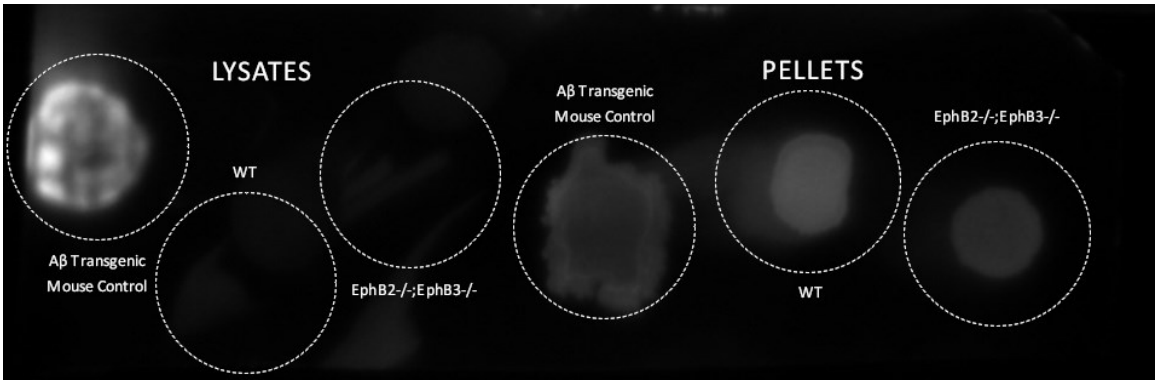

Figure 7. Dot Blot. 10 µl of whole lysate from 5XFAD, WT, and EphB2-/-;EphB3-/- hippocampus was loaded onto a PVDF membrane and probed using the 6E10 antibody from Covance. Pelleted material from the lysates were then boiled in sample buffer and also loaded.

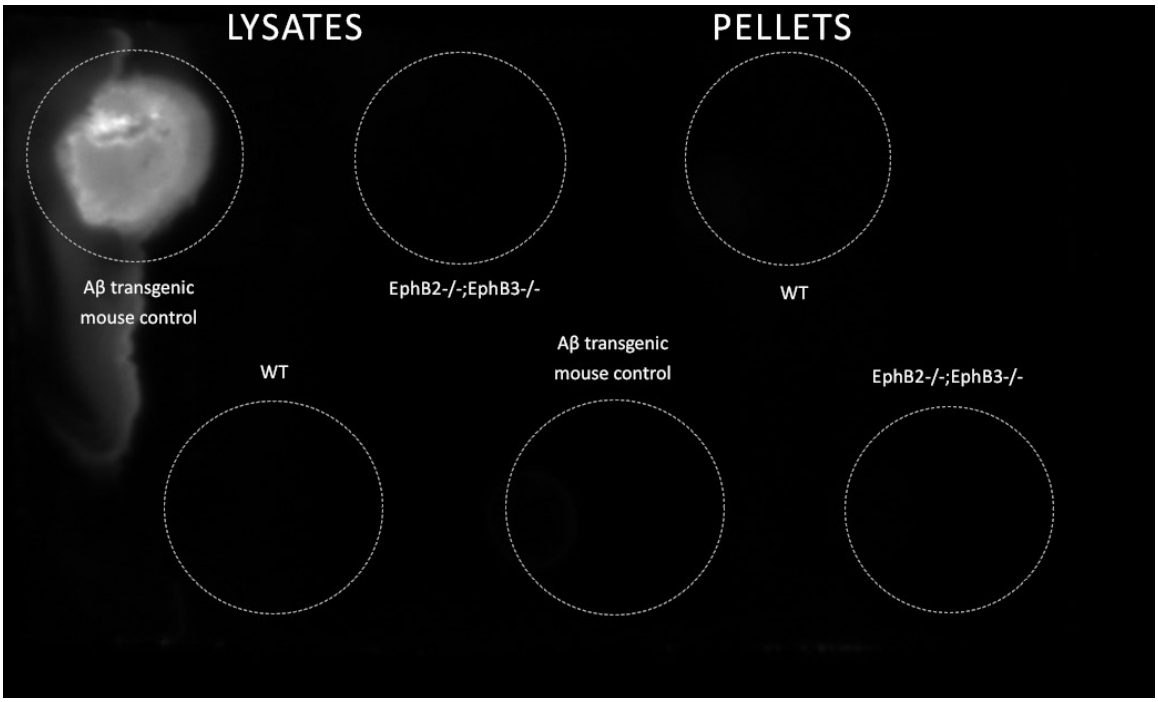

Figure 8. Dot Blot. 0.269 µg of protein from 5XFAD, WT, and EphB2-/-;EphB3-/- hippocampus lysate and insoluble pellet was loaded onto a PVDF membrane and probed using the 6E10 antibody from Covance.

DISCUSSION

The results of the experiment described above were unexpected in light of the data already acquired from Dr. Henkemeyer's laboratory. The goal of this project was to gain additional data to support the hypothesis that there should be an increase in Aβ protein in the hippocampal tissue of animals lacking critical EphB receptors. If EphB receptors do indeed have a role in removal of Aβ from the synapse, the lack of EphB receptors should lead to an increase in Aβ similar to that seen in AD. The results obtained in the ELISA, Western blot, and dot blots do not support this postulation.

In the ELISA, only the hippocampal tissue from the 5XFAD transgenic control mouse exhibited significantly higher levels of Aβ than the wild-type tissue. This was surprising, because it was predicted that both the EphB2$^{-/-}$ and EphB2$^{-/-}$;EphB3$^{-/-}$ lysates would also contain significantly elevated levels of Aβ. These results indicate that the lack of EphB2 or EphB2 and EphB3 receptors does not increase levels of Aβ protein. However, previously completed research from the Henkemeyer lab showed that the hippocampi from EphB KO mice contained granular plaques immunoreactive for Aβ, contradicting the data presented in this paper.

Results from the Western blot also did not agree with predicted outcomes. It was expected that the hippocampal lysates from the EphB2$^{-/-}$ and EphB2$^{-/-}$;EphB3$^{-/-}$ animals would demonstrate a positive detection of Aβ, but the opposite result was seen, again contrasting the data previously acquired in the Henkemeyer project.

Therefore, a third procedure was devised in order to fully test the presence of Aβ in the tissue samples. Since Aβ is known to aggregate in complexes with metal ions, it is possible that it could be found bound in a complex in the insoluble part, or the pellet, of

the hippocampal tissue. The dot blots attempted to detect the presence of Aβ in these pellets. With the original hypothesis in mind, it was expected that the blot would show strong signals from the 5XFAD and KO lysates. However, an explanation for the ELISA and Western blot results in this study to comply with the original hypothesis would be that the Aβ was accumulating in the pellet and not in the lysates. The dot blot in this case would show strong signals from the 5XFAD lysate and pellet as well as strong signals from the KO pellet, indicating the presence of elevated levels of Aβ in the KO animals.

The first dot blot, done with equal volumes of lysate and pellets, only revealed a strong signal from the 5XFAD control lysate. Though there was some signal (and therefore some Aβ present) from the three dots of pellet, these results could not be taken as fully accurate, because the volumes of lysate and pellet used naturally contained different concentrations of total protein. Therefore, the lysates and pellets needed to be normalized in order to compare the results more accurately.

Following normalization, the dots of lysates and pellets used for the second dot blot test contained equal amounts of total protein. The predictions made for the first test still applied to the second one. However, the only dot that had any fluorescent signal to indicated the presence of Aβ was the 5XFAD lysate. Again, these results indicate that the lack of EphB receptor does not lead to elevated levels of Aβ, challenging the original hypothesis and data already acquired by Henkemeyer.

The results presented in this paper beg the question: Why did these KO animals not exhibit high levels of Aβ protein like those seen in AD? Frankly, the answer to this puzzle is unquestionably complex and still largely unknown. One possible explanation is

that the hippocampal tissue from these animals actually does contain elevated levels of Aβ, and the methods used in this study were merely not sufficient to detect the protein.

In the normalized dot blot, each dot of either lysate or pellet contained only 0.269 micrograms of total protein. Subsequently, the amount of Aβ in each sample, if present, would be even smaller. Even with an antibody specific for Aβ, it would be difficult to detect such small amounts of protein. Therefore, it cannot be concluded that there is no presence of Aβ in these samples, and it is possible that the method of detection was insufficient due to such a small amount of total protein following normalization.

The control mouse used in this study is a transgenic model in which the mouse expresses two human genes with 5 mutations known to lead to an accumulation of Aβ and AD-like pathology. This also means that the Aβ it produces is human. The primary antibody used in this study, 6E10 mouse anti-Aβ, was created to bind to human Aβ. In fact, all of the antibodies used in recent AD studies have been produced to bind to human Aβ. Additionally, the KO animals used in this study produced rodent Aβ, not human Aβ. Though the 6E10 antibody is supposed to cross-react with rodent Aβ, it is possible that it does not bind as well to rodent Aβ as it does to human Aβ. This could explain why the detection of Aβ in the 5XFAD tissue was so strong while there was little to no detection of Aβ in the KO tissues.

Another possible explanation lies too in the realm of methodical error. The pellets used in the dot blots included all of the insoluble material from the hippocampal tissue and were sheared through a small needle in order to be dotted onto the membranes. It is possible that the pellets were not sheared enough such that the Aβ was still contained inside of an insoluble complex and not dotted onto the membranes.

A strict interpretation of the results presented in this study leads to the conclusion that the EphB2$^{-/-}$ and EphB2$^{-/-}$;EphB3$^{-/-}$ animals do not have elevated levels of Aβ in their hippocampal tissue. If this is indeed the case, why did the Henkemeyer lab produce such different results? One explanation is that the animals used in this study only had one or two EphB receptors knocked-out. In the Henkemeyer project, the KO animals lacked either two or three EphB receptors, and the animals lacking three had more plaque build-up than those lacking only two. This suggests that the EphB receptors have a cumulative effect on regulation and balance of Aβ in the brain. Therefore, it is possible that the lack of two EphB receptors may not be enough to cause a large Aβ accumulation, but the lack of three EphB receptors could lead to an exponential build-up detectable by ELISAs, Western blotting, or dot blotting. This would explain the data in this paper as well as agree with the original hypothesis presented by Henkemeyer.

CONCLUSIONS

"The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory." (Cajal 1906) Though the amount of research on the world that is the brain is increasing, there still remains so much that scientists do not yet know. Much of what is considered knowledge is merely speculation. Indeed, even with the extensive research on Alzheimer's disease, no effective treatment or cure has been formulated, whilst the number of AD patients continues to grow.

Eph receptors are known to have critical roles in neurogenesis and regulation of synaptic transmission. Like the mysteries of the brain, however, scientists still are far from understanding everything about the roles that Eph receptors play in the plaque formation seen in AD. Additionally, research has shown that these receptors interact with

numerous complex pathways in the brain, making the task of discovering the importance of Eph receptors even more challenging.

More studies need to be performed before scientists can speculate possible AD therapy with Eph receptors. In order to fully test the potential cognitive deficit of mice lacking critical Eph receptors, future experiments could include behavioral studies with hippocampal-based learning tasks as well as hippocampal lysate and pellet protein analysis of KO mice for three or more EphB receptors. Larger samples could be used so that the dot blots included in these studies would have a higher amount of total protein following normalization. It is possible that these revisions of methods to detect Aβ and measure cognitive deficit could lead to results that support the original hypothesis stated in this paper.

Overall, it remains important that research continues to be done to explore the pathology and chemistry of Alzheimer's disease. If the complex biological pathways of AD are better understood, the possibility of discovering treatment options or even a cure becomes reality.

REFERENCES

Cajal Ry. 1991. Degeneration and Regeneration of the Nervous System. Edition 1. USA: Oxford University Press. 976 p.

Cajal Ry. 1906 Dec 12. The Structure and Connexions of Neurons. Nobel Lecture. Sweden.

Chen Y, Fu A, Ip N. 2012. Eph Receptors at synapses: Implications in neurodegenerative diseases. Cellular Signaling. 24:606-611.

Cissé M, Halabisky B, Harris J, Devidze N, Dubal D, Sun B, Orr A, Lotz G, Kim D, Hamto P, et al. 2010. Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. Nature. 000:1-6.

Geng D, Kang L, Su Y, Jia J, Ma J, Li S, Du J, Cui H. 2013. Protective effects of EphB2 on $A\beta_{1-42}$ oligomer-induced neurotoxicity and synaptic NMDA receptor signaling in hippocampal neurons. Neurochemistry International. 63:283-290.

Hardingham G, Bading H. 2010. Synaptic versus extrasynaptic NMDA receptor signaling: implications for neurodegenerative disorders. Nature Reviews Neuroscience. 11:682-696.

Huang Y, Mucke L. 2012. Alzheimer Mechanisms and Therapeutic Strategies. Cell. 148:1204-1222.

Mucke L, Selkoe D. 2012. Neurotoxicity of Amyloid β-Protein: Synaptic and Network Dysfunction. Cold Spring Harbor Perspectives in Medicine [Internet]. [cited 2013 Oct 11]. Available from: http://perspectivesinmedicine.cshlp.org/content/2/7/a006338.short?rss=1 doi: 10.1101/cshperspect.a006338

Shankar G, Walsh D. 2009. Alzheimer's disease: synaptic dysfunction and Aβ. Molecular Neurodegeneration [Internet]. [cited 2013 Oct 11]; 4(48). Available from: http://molecularneurodegeneration.com/content/4/1/48 doi: 10.1186/1750-1326-4-48

Simón A, López de Maturana R, Ricobaraza A, Escribano L, Schiapparelli L, Cuadrado-Tejedor M, Pérez-Mediavilla A, Avila J, Del Río J, Frechilla D. 2009. Early Changes in Hippocampal Eph Receptors Precede the Onset of Memory Decline in Mouse Models of Alzheimer's Disease. Journal of Alzheimer's Disease. 17:773-786.

Texidó L, Martín-Satué M, Alberdi E, Solsona C, Matute C. 2011. Amyloid β peptide oligomers directly activate NMDA receptors. Cell Calcium. 49(3):184-190.

ABSTRACT

Alzheimer's disease is a progressive brain disorder characterized by a gradual onset of dementia. A prominent hypothesis of the cause of AD is the loss of synaptic plasticity due to amyloid-beta plaque formation in the brain. Research has shown that soluble Aβ molecules, not plaques, are responsible for disruption of synaptic function via altered processing of the amyloid precursor protein, overproduction of Aβ, suppression of postsynaptic activity through blockage of NMDA glutamate receptors, and long-term depression. Eph receptors are a family of receptor tyrosine kinases important in neurogenesis and regulation of synaptic plasticity whose levels of expression play a critical role in synaptic loss in AD. Reduction of EphB receptors results in decreased expression of NMDA receptors, increased Aβ levels, and cognitive deficit; increased EphB receptor expression has a neuroprotective effect by helping clear Aβ from the brain. This paper hypothesizes that increased levels of Aβ should be seen in hippocampal tissues of rodents lacking EphB receptors. ELISA, Western blot, and dot blots were performed to measure levels of Aβ in KO mice for EphB2 and EphB2/B3. None of the results showed the significant increase in Aβ that was hypothesized. However, it remains possible that Aβ was present in amounts too low to detect with the methods used or that Eph receptors have a cumulative effect such that knocking out more than two produces exponentially higher levels of Aβ formation. Future studies should include KO rodents for three or more Eph receptors as well as behavioral studies to assess cognitive deficit. With the number of AD cases projected to triple by 2050 and understanding of Eph receptors still largely unknown, it is important that AD research continues to be a priority in order to understand the disease and formulate treatments.