BETA-SITE APP CLEAVAGE ENZYME 1 DEFICIENCY ENHANCES REACTIVE ASTROCYTE AMYLOID BETA CLEARANCE

by

JOHN ZHOU

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Molecular Medicine

CASE WESTERN RESERVE UNIVERSITY

August, 2022

CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of
John Zhou

candidate for the degree of **Doctor of Philosophy***.

Committee Chair
Xiongwei Zhu, PhD

Committee Member
Riqiang Yan, PhD

Committee Member
Justin Lathia, PhD

Committee Member
James Leverenz, MD

Date of Defense
06/09/2022

*We also certify that written approval has been obtained for any proprietary material contained therein.

Table of Contents

List of figures

List of tables

Acknowledgements

Completing my PhD has been a complex and at times difficult journey. But ultimately, as this phase of my scientific career comes to a close, I feel fulfilled by the work that has been done and grateful to the people that have sponsored me on this path. I write these acknowledgements knowing that words are limited in scope and do not reflect my eternal appreciation.

To my family, I want to thank my mother and father for engendering in me a deep respect for the scientific process. I was partially inspired to pursue sciences, because of childhood, dinner time chats about reagents, experiments, and mice. To my mother, I appreciate your sharing of experiences from your own scientific career and being a useful sounding board to test out my wilder ideas. To my father, I appreciate the enthusiasm you showed toward my research and giving me the confidence to communicate my thoughts effectively and with purpose. To my brother, thanks for making me laugh when I need it the most.

To my lab, I want to thank everyone for their scientific and moral contributions for the past 8 years. To Dr. Riqiang Yan, there are not enough words to convey the appreciation I have for your support. I came out of college directionless and you brought me into a lab environment that has turned me into an excited and motivated scholar. I hope to repay you in the future by continuing to build a scientific career that will make you proud. To Dr. Wanxia He, your activity behind the scenes - buying reagents, dealing with companies, and day-to-day maintenance – has kept this lab running despite all the complications.

Additionally, your caring nature helped me to feel at home in the Yan lab. To Dr. Xiangyu Hu, you have always been the stalwart source of technical and scientific help. I always felt comfortable asking you about the details of some procedure and learned a lot from this process. To To Dr. Golam Sharoar, you taught me so much as a mentor. You gave me the confidence to trust my own scientific instincts, as well as a commitment to lead and guide the next generation of scientists. Dr. Neeraj Singh, we sometimes had difficult times working in parallel with each other, but at the end of the day I always respected your hard work and appreciate you including me in your work. To Marc Benoit and Sarah Palko, your friendships made coming into lab a genuinely fun time. Additionally, your comments and help to prepare for presentations pushed my scientific communication skills to a much higher level. To James Galske, Jacob Hudobenko, and Joe Pathoulas, thank you for being my first group of students and working on the CXCL14 project. To all the other technicians, postdocs, and students of the lab, thank you for your contributions that has made my lab experience one that is productive and stimulating.

To my thesis committee, your guidance and flexibility has turned rough circumstances of a mid-PhD change of institutions into a relatively smooth transition. To Dr. Xiongwei Zhu, as committee chair, you always kept the meetings running without issue. I also appreciate your interest in my projects and the specificity of the advice and questions that you gave. To Dr. James Leverenz, as a Clinical Mentor, you opened my eyes to the clinic and your questions have always helped me to think more widely about the implications of

my studies. To Dr. Justin Lathia, not only do I admire your incisive scientific mind, but I also appreciate your leadership skills. From the small time I spent rotating in your lab, I observed a leadership style that was modern, collaborative, and effective that I hope to model in the future.

To the Molecular Medicine program, thank you for giving me the educational background and learning space for me to grow into a confident scientist. To Dr. Johnathan Smith, your support for all your students is apparent, but I was especially touched by your continuing support after I moved to Connecticut. To my cohort, I will forever cherish our time together in that tiny room in Lerner and spinning the wheel of questions. We've scattered to different parts of the country and different job fields, but those memories have always been a welcome succor. To the other instructors and faculty members both at Cleveland Clinic, Case Western, and University of Connecticut, thank you for being a part of my academic and scholastic community.

To all my friends, especially A.D, A.H., A.W, J.M.R, J.B.I, J.R, M.S., and T.S., thank you for your sorely needed companionship. My obsession for work could be sometimes singular and isolating. As I plunged into the depths of tantalizing possibilities and theories, I was holding my breath without even knowing it. But when I needed it the most, my friends were there with an invitation to live life and breath.

Beta-site APP Cleavage Enzyme 1 Deficiency Enhances Reactive Astrocyte Amyloid Beta Clearance

Abstract

by

JOHN ZHOU

Abnormal accumulation of amyloid beta (Aβ) peptide in the brain is regarded as the possible causative agent of Alzheimer's disease (AD). Increased production of Aβ or impaired clearance of Aβ results in the aggregation of Aβ into plaques, which are surrounded by glia cells. Beta-site APP Cleavage Enzyme 1 (BACE1) is the rate-limiting secretase required for production of Aβ. A recent study by our lab using conditional deletion of BACE1 in an AD mouse during adulthood showed a reversal of previously formed plaques and rescues Aβ-associated behavioral deficits.

One possibility for this phenomenon is that BACE1 deficiency facilitates astrocytic Aβ clearance. Using astrocyte cultures and astrocyte specific knockdown of BACE1 in mice, we found that BACE1 inhibition enhances both astrocytic Aβ uptake and degradation. Furthermore, using scRNA-seq, we found that BACE1 knockout mice reactive astrocytes have transcriptomes distinct from wild-type reactive astrocytes.

We further explored the role of several differentially expressed genes in BACE1 deficient reactive astrocytes, namely clusterin (Clu) and C-X-C motif chemokine 14 (Cxcl14). CLU, a known positive mediator of Aβ clearance, was significantly upregulated in BACE1 deficient astrocytes cultures and siRNA

knockdown of Clu significantly attenuated Aβ clearance. Furthermore, we investigated the role of CXCL14 in the context of AD. CXCL14 is an orphan chemokine that has been well studied in the fields of inflammation and cancer, but a heretofore unknown role in neurodementia and AD. In vitro studies suggest that CXCL14 enhances transcytosis cells critical in the clearance of Aβ, as well as directly protecting neurons from amyloid beta insult.

We propose that increased CLU and CXCL14 changes resulting from BACE1 inhibition were mediated by BACE1 cleavage of astrocytic insulin receptors and downstream MAPK. Together, our study suggests a novel function of BACE1 in astrocytes and Aβ clearance

Chapter 1: **Introduction**

1. Background

The work present in this dissertation describes a novel mechanism for activating astrocytes to enhance amyloid beta (Aβ) clearance for the amelioration of Alzheimer's disease (AD). The presence of Aβ plaques was first discovered by Alois Alzheimers in the first case of AD of Aguste Deter.(Hippius & Neundörfer, 2003) Numerous studies have pointed to Aβ as the primary causative agent of AD leading to the formulation of the "amyloid cascade hypothesis".(Cline, Bicca, Viola, & Klein, 2018) Broadly, the hypothesis describes a model where toxic Aβ is produced by the cleavage of neuronal amyloid precursor protein (APP) by Beta-site APP cleavage enzyme 1 (BACE1).(H. Hampel et al., 2021; Vassar et al., 1999; Yan et al., 1999; Yan & Vassar, 2014) Glia cells, including astrocytes, play an essential role in clearing Aβ by direct uptake, secreting Aβ degrading, and many other biological processes.(Davis et al., 2021; Katsouri et al., 2020; Ries & Sastre, 2016; Tony Wyss-Coray et al., 2003) Therefore, AD therapies should aim to reduce Aβ production and enhance glia clearance. This dissertation will focus on AD, the amyloid cascade hypothesis, BACE1, and the role of astrocytes in AD pathology. Next, a distinct mechanism for enhancing astrocytic clearance of Aβ specific BACE1 deficiency by will be fully explored. Next, a novel chemokine will be examined for its potential role in the amelioration of AD pathology. Finally, future perspectives of enhancing Aβ clearance for the alleviation of AD pathology will be discussed.

2. Alzheimer’s disease

AD is the most common age related neurodementia with approximately 6 million patients in the United States alone.("2021 Alzheimer's disease facts and figures," 2021) As the population of the US ages and without effective disease modifying treatments, the number of AD sufferers is predicted to increase to 15 million patients in the year 2050. Certain rare mutations result in early onset – before the age of 65 – forms of familial AD. The vast majority of late onset AD (LOAD) is clinically defined from other dementias by deficiencies in certain cognitive domains, such as memory, executive function, language skills, and behavior. The severity of these deficiencies increase in these patients and eventually lead to death 5-12 years after symptoms first occur.(Vermunt et al., 2019) Previously, confirmation of AD diagnosis would require the detection of extracellular Aβ plaques and dystrophic neurites in post mortem patient brain. The presence of intracellular tau tangles, reactive gliosis, and neuronal atrophy of the hippocampus and around lateral ventricles are other pathological signs of AD.(Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredici, 2006; Braak & Braak, 1991; Thal, Rub, Orantes, & Braak, 2002) In 2018, the National Institute on Aging and the AD Association (NIA-AA) Working Group introduced the use of imaging and fluid biological markers for the diagnosis of AD, in order to facilitate early detection of AD before the onset of clinical symptoms (Figure 1.1). The NIA-AA developed the Amyloid/Tau/Neurological Injury (A/T/N) framework to categorize existing and developing AD biomarkers as a means to assist AD research and accelerate drug development.(Cummings, 2019; Harald Hampel et

al., 2021) Table 1.1 summarizes a variety of imaging and fluid-based biomarkers fitting within the A/T/N framework.(Gauthier, 2019) A review of AD biomarkers has been published by Zhou, Benoit, and Sharoar in 2021.(Zhou, Benoit, & Sharoar, 2021)

The early presence of amyloid in cerebrospinal fluid (CSF) and the positron emission tomography (PET) imaging before the onset of other biomarkers and clinical symptoms adds additional evidence that Aβ is the major causative agent behind AD i.e. the “amyloid cascade hypothesis”, which will be discussed in the next section.

3. Amyloid cascade hypothesis

The amyloid cascade hypothesis is regarded as the most popular theory behind the case of AD, Aβ although hypothesis is not immune from debate.(Cline et al., 2018; D. J. Selkoe & J. Hardy, 2016) As mentioned previously, the presence of Aβ categorically defines AD, since its first discovery by Alois Alzheimer. In 1991, Braak and Braak developed a staging paradigm to pathologically evaluate the accumulation of Aβ load, which begins in the entorhinal cortex and temporal lobes, then the hippocampus, an area associated with memory, and finally throughout all regions of the brain. But whether or not Aβ plaques were case or consequence of AD was still unknown. Early genetic found a correlation mutations in genes related to amyloid processing were and familial AD suggesting that that early accumulation of Aβ triggers later AD clinical symptoms. Later as neuroimaging and fluid biomarker techniques have become more advanced, Aβ deposition has also been in certain brain regions such at

early stages of preclinical AD, thereby adding additional evidence to the amyloid cascade hypothesis.(Dubois et al., 2014; Villemagne et al., 2012)

Aβ is primarily generated from the cleavage of amyloid precursor protein (APP), a type 1 transmembrane proteins found primarily in neurons, by the endopeptidase BACE1, in the so-called “amyloidgenic pathway”.(Vassar et al., 1999; Yan et al., 1999) BACE1-cleaved APP is then subsequently cleaved by γ-secretase to generate a variable length of 39-43 amino acid Aβ peptide. (Figure 1.2) Once generated, monomeric Aβ peptide undergoes spontaneous conformational changes from monomeric to oligomeric, and then to β-sheet enriched insoluble fibrils. (Finder & Glockshuber, 2007)(Figure 1.3) While fibrillar Aβ is the main component of senile plaques, soluble oligomers is most toxic component. The Aβ with the length of 42 amino acids ($A\beta_{42}$) is considered the more aggregate prone and forms the majority of senile plaques and soluble oligomers.(Haass & Selkoe, 2007) Cleavage of APP by α-secretases occurs within the Aβ region and initiates the “non-amyloidgenic pathway” and subsequent cleavage products are associated with neuronal and synaptic homeostasis. (Figure 1.2)

Homeostatic levels of BACE1 activity and the soluble Aβ are required for normal neuronal function.(Riqiang Yan, 2017) Therefore, the level of Aβ in the brain is regulated by its generation from APP, aggregation, efflux through blood brain barrier (BBB), proteolytic degradation, and glial clearance. Impairment of these regulatory mechanisms results in excessive Aβ accumulation in the brain resulting in cellular injury, as well as synaptic dysfunction eventually leading to

cognitive deficits and AD as reviewed in Yan, Fan, Zhou, and Vassar in 2016.(Yan, Fan, Zhou, & Vassar, 2016)

Evidence from genome wide association studies (GWAS) indicates dysfunction of Aβ glial clearance genes as a probable causes of AD.(Chung et al., 2018; Kunkle et al., 2019) For example, Apolipoprotein E (APOE) is an apolipoprotein produced primarily by astrocytes in the central nervous system that when lipidated binds to extracellular Aβ thereby enhancing the uptake, degradation, and transport of extracellular Aβ.(Schoemaker, Poirier, Escobar, Gauthier, & Pruessner, 2016) GWAS point to ApoE single nucleotide polymorphism (SNP) ε4 as being genetic variant most associated with late onset AD. (Kunkle et al., 2019; Waring & Rosenberg, 2008) (Figure 1.4) The APOE ε4 variant results in a loss of function of Aβ binding and subsequent reduced Aβ clearance. Furthermore, APOE ε4 binds to microglial Triggering Receptor Expressed On Myeloid Cells 2 (TREM2) leads to activation of microglial to an inflammatory, neurotoxic Damage Associated Microglial (DAM) 2 like state with reduced Aβ phagocytic and clearance ability.(Ewers et al., 2020; Krasemann et al., 2017; Nguyen et al., 2020; Parhizkar et al., 2019) Interestingly, Trem2 SNPs have also been identified in AD GWAS.(Kunkle et al., 2019) (Figure 1.4) AD drug development has also targeted glial Aβ clearance pathway. (Figure 1.5) Most prominently, a number of antibodies targeting different forms of Aβ for enhanced clearance have advanced to phase III clinical trials, with two, aducanumab and lecanumab being submitted for FDA approval.(Cummings, Lee, Zhong, Fonseca, & Taghva, 2021; Schneider, 2020; Sevigny et al., 2016)

Because BACE1 is the rate-limiting enzyme of Aβ production, inhibition of BACE1 has also been a major therapeutic target for the prevention of AD. However, challenges have arisen that have dissuaded the pursuance of BACE1 as a drug target.(Egan et al., 2019; McDade, Voytyuk, et al., 2021) Despite this, additional insights into possible benefits from BACE1 inhibition within specific contexts have emerged in an intriguing manner. These challenges and current developments regarding BACE1 as a target for AD will be discussed in the next section.

4. BACE1

BACE1 is a 501-amino acid long, aspartyl protease that, in the central nervous system, is highly expressed in neurons, oligodendrocytes, and astrocytes. (Figure 1.6) BACE1 is first synthesized in the endoplasmic reticulum from proBACE1. Numerous post-translational modification for BACE1 have been reviewed in Yan, Fan, Zhou, and Vassar 2016.(Yan et al., 2016) After maturation, BACE1 is transported along the secretory pathway including the trans-Golgi network and endosome, where the acidic environments promote BACE1 cleavage of substrates, typically Type I transmembrane proteins, such as APP.

Since the identification of BACE1 as the major β-secretase of APP, inhibiting or sequestering BACE1 from APP has been a major therapeutic target.(Yan, 2016; Yan & Vassar, 2014) Initial studies showed that BACE1 deficiency by genetic deletion or small molecule inhibitors in amyloid mouse models did not develop amyloid plaques.(Cai et al., 2001) These early studies also showed that

BACE1 inhibition in mice had relatively few off-target phenotypes compared to γ-secretase inhibition.

Later, numerous studies showed that BACE1-null mice actually displayed many unintended phenotypes, primarily due to BACE1 cleavage of alternative substrates.(H. Hampel et al., 2021; Hu et al., 2010; Wang, Song, Laird, Wong, & Lee, 2008) (Table 1.2) BACE1 interaction with these substrates results in deficits in various physiological functions including synaptic plasticity, synaptic vesicle trafficking, astrogenesis, and remyelination. Furthermore, BACE1 inhibitors in humans have all failed in phase III clinical trials.(Egan et al., 2019; Novak et al., 2020) Notably, development for Verubecestat, a BACE1 and BACE2 inhibitor developed by Merck, was discontinued in 2018. Even though patients treated with Verubecestat did see reduced Aβ plaque load, they also experienced mildly reduced cognitive scoring. Because of similar behavioral deficits between Verubecestat treated patients and BACE1-null mouse models, the off target effects of global BACE1 inhibition pose major barriers to the pursuance of BACE1 inhibition as an AD therapy.

Despite the failure for global BACE1 inhibitors, promising research suggests that targeting BACE1 at a particular stage of AD disease progression might prove beneficial for treating AD. In 2015, Thakker et al. showed that intracerebral delivery of a small molecule inhibitor of BACE1 after plaque formation in the Tg2576 amyloid model saw a slight reversal in previously formed plaques.(Deepak R. Thakker et al., 2015) In 2018, Hu et al. conditionally knocked out BACE1 using a $BACE1^{fl/fl}$;UBC-creERT2 mouse line, resulting in

progression deletion of BACE1 after P30 in this line, with about 50% BACE1 inhibition achieved by P60.(X. Hu, B. Das, H. Hou, W. He, & R. Yan, 2018) (Figure 1.7A) Most interestingly, adult inhibition of BACE1 in a 5xFAD mouse model, a mouse model with rapid amyloid plaque generation starting at P60, ($BACE1^{fl/fl}$; UBC-creERT2;5xFAD) did not just prevent the accumulation of additional Aβ plaques, but completely reversed the presence of previously formed plaques. (Figure 1.7B) Because generally Aβ plaques are thought to be insoluble and Aβ glia clearance is relatively inefficient, this suggests that BACE1 inhibition must somehow also enhance Aβ glia clearance. Furthermore, adult BACE1 inhibition in 5xFAD mice does not result in deficits in contextual memory in a fear conditioning assay, suggesting that adult BACE1 inhibition avoids synaptic deficits caused by germline BACE1 deletion. (Figure 1.7C). Results from this study, suggest that specific timing and dosing BACE1 inhibition might be critical in reducing amyloid load in AD patient, while avoiding off target effects.

In order to fully understand physiological roles for BACE1, researcher have also examined the cell specific effects of BACE1 inhibition. Adult deletion of BACE1 in neuronal cells avoided the majority of symptoms from germline deletion of BACE1, although axonal organization of the hippocampus was somewhat disrupted.(Ou-Yang et al., 2018) Recent papers by our lab, actually show that deletion of BACE1 in microglia after plaque formation in 5xFAD mouse model largely recapitulates the reversal or previously formed plaques, (5xFAD;$Bace\text{-}1^{fl/fl}$;Cx3cr1CreER with tamoxifen [+TAM] compared to [-TAM]). (B. D. Neeraj Singh, John Zhou, Xiangyou Hu and Riqiang Yan 2022; X. H. Neeraj

Singh, Marc Benoit, Brati Das, John Zhou, Jose Davila, Li-Huei Tsai, Manolis Kellis, Riqiang Yan 2022)(Figure 1.8A) Furthermore, single cell RNA-seq (scRNA-seq) revealed that BACE1 deletion in microglia results in a greater proportion of DAM 1 microglia, a previously identified sub population of microglia found to be neuroprotective and have increased phagocytic ability. (Figure 1.8B) Altogether, this suggests that BACE1 inhibition in glia subtypes might be beneficial in enhancing Aβ clearance, while avoiding the cleavage of neuronal substrates important for development or homeostatic processes. Furthermore, this also highlights the importance of investigating particular subtypes of glia cells in the context of BACE1 inhibition and AD.

BACE1 is also highly expressed in both oligodendrocytes and reactive astrocytes.(Bettegazzi et al., 2011; Chacón-Quintero, Pineda-López, Villegas-Lanau, Posada-Duque, & Cardona-Gómez, 2021; Zhao, O'Connor, & Vassar, 2011) However, the role of BACE1 in the function of these cell types has not been elucidated. I chose to investigate the role of BACE1 in reactive astrocytes due to the known presence of BACE1, as well as the known roles of reactive astrocytes in AD, which will be discussed in the next section.

5. Reactive Astrocytes in the AD context

Astrocytes represent the major glia cell types in the brain providing critical roles in homeostasis and in the disease context. In response to various signals, for example Aβ, tau, or cytokines in the context of AD, astrocytes upregulate intermediate filaments such as vimentin (Vim) and glial fibrillary acidic protein (GFAP) and reach a hypertrophic, reactive state.(Clarke et al., 2018; John Zhou,

2022; Liddelow & Barres, 2017; Perez-Nievas & Serrano-Pozo, 2018) Reactive astrocytes are typically found surrounding Aβ plaques in the AD brain.(Braak & Braak, 1991) Whether reactive astrocytes are beneficial or harmful is context dependent, with many subtypes of astrocytes defined morphologically and transcriptionally.

In the context of AD, reactive astrocytes play a critical role in clearing Aβ and ablation of astrocytes is incredibly deleterious to AD pathology. (Davis et al., 2021; Katsouri et al., 2020) Several studies have demonstrated that astrocytes play a direct role in direct uptake and degradation of Aβ both in vitro and in situ.(Katsouri et al., 2020; Nuutinen et al., 2007; Tony Wyss-Coray et al., 2003; Xiao et al., 2014) A study from one group suggests that astrocytic Aβ uptake and degradation may result in production of cytotoxic byproducts in vitro, (Söllvander et al., 2016) for the most part direct astrocytic Aβ clearance is seen as an important mechanism to reduce Aβ load. Furthermore, reactive astrocytes act as the major secretory cell in the context of AD. For example, reactive astrocytes are the primary source for APOE and clusterin (CLU) - apolipoproteins that bind Aβ, prevent aggregation, and enhance uptake – C-C motif ligand 2 (CCL2) – a chemokine that attracts additional glia cells to plaque sites for clearance - and insulin degrading enzyme (IDE) – an enzyme that degrades extracellular Aβ, as well as insulin.(Dorfman et al., 2010; Evangeline M. Foster, Adrià Dangla-Valls, Simon Lovestone, Elena M. Ribe, & Noel J. Buckley, 2019; Maarouf et al., 2018; Nelson, Sagare, & Zlokovic, 2017; Nuutinen et al., 2007; Ries & Sastre, 2016; Vekrellis et al., 2000; A. M. Wojtas et al., 2017) Furthermore, astrocytes play an

important role in maintaining glutamate levels in the synapse during homeostatic conditions, but also mediate glutamate excitotoxicity in the context of AD.(Mahmoud, Gharagozloo, Simard, & Gris, 2019)

As AD pathology progresses however, reactive astrocytes direct clearance of Aβ becomes inefficient.(Guo et al., 2020; S. A. Liddelow et al., 2017; Perez-Nievas & Serrano-Pozo, 2018; Zulfiqar, Garg, & Nieweg, 2019) Furthermore, chronic reactive astrocytes may also contribute to further neuronal cell death by producing pro-inflammatory cytokines. Various markers of reactive astrocytes are elevated in CSF during AD including Chitinase-3-like protein 1 (CHI3L1), also known as YKL-40.(Bonneh-Barkay, Wang, Starkey, Hamilton, & Wiley, 2010; Janelidze et al., 2016; Llorens et al., 2017) Therefore, whether reactive astrocytes are beneficial or harmful in AD is context dependent.

In order to further elucidate the complex heterogeneity of reactive astroctyes, Liddelow et al. transcriptionally defined two subtypes of reactive astrocytes. In response to middle cerebral artery occlusion (MCAO) – a stroke model - A2 reactive astrocytes emerged as beneficial astrocytes with increased phagocytosis of debris.(Clarke et al., 2018; Liddelow & Barres, 2017; S. A. Liddelow et al., 2017) In response to lipopolysaccharide treatment or cyotokines, the A1 subtype was the primary reactive astrocyte subtype. A1 astrocytes were found to upregulate members of the compliment cascade and were neurotoxic. Since then, astrocytes with some A1 subtype reactive astrocyte markers have been discovered in AD retinas. However since the widespread emergence of scRNA-seq and its application to various disease, it is clear that many reactive

astrocytes subtypes exist beyond the A1/A2 paradigm. (Figure 1.9) Therefore, it is critical to discover reactive astrocyte subtypes and understand their response to various disease associated stimuli and contexts.

The role of BACE1 on reactive astrocyte activity is relatively unknown. As mentioned in the above section, BACE1 has also been detected in reactive astrocytes surrounding plaques and blood vessels of AD patients. Zhao et al. specifically showed that treating astrocytes with aggregated forms of Aβ were able to specifically upregulates BACE1 and APP.(Zhao et al., 2011) Furthermore, it was determined that Aβ induced calcium influx and downstream NFAT4/calcineurin signaling pathways to upregulate BACE1 expression in astrocytes.(Sompol & Norris, 2018) BACE1 inhibition also increases astrogenesis and hypertrophy of astrocytes through alteration of Jagged/Notch pathway.(X. Hu, W. He, X. Luo, Katherine E. Tsubota, & R. Yan, 2013) (Figure 1.10)

The research presented thus far suggests that BACE1 may have additional effects on reactive astrocyte function especially in the context of AD. However due to heterogeneity of astrocyte subtypes, it is very possible that BACE1 may only effect a certain population of astrocytes. Therefore, we utilized scRNA-seq to identify mouse astrocyte populations affected by BACE1 inhibition in an unbiased manner. The experimental approach for this particular study will be discussed in the next section.

6. scRNA-seq Experimental Approach

As mentioned previously, scRNA-seq is a powerful tool to examine astrocyte heterogeneity. To this end we isolated astrocytes from 3 pairs of diencephalon from P60 Bace1$^{-/-}$ and Bace1 $^{+/+}$ mice (two males, one female per genotype). (Figure 1.11) Astrocytes were isolated using magnetic assisted cell sorting (MACS) system (Milltenyi) and immunomagnetic beads for Astrocyte Cell Specific Antigen 2 (Milltenyi) into single cell suspension. By isolating astrocytes alone for RNA seq, we hoped to increased sequencing power and therefore increase resolution for various astrocyte sub types. Furthermore, we chose to use MACS, because it offers a gentler method of astrocyte isolation than flow assisted cell sorting (FACS). Various reports have indicated that high stress induced by FACS microfluidics may result in artificial activation of astrocyte cells. Astrocyte samples were checked for debris and viability (at least 80% live cells), before 12,000 cells per sample were loaded onto Chromium Single Cell chips version 3 chemistry (10x Chromium) at the Jackson Lab Single Cell Genomics facility. Theoretically, within the chip microfluidics each astrocytes is encapsulated with a single bead containing barcoded reverse transcription primers within hydrophilic sphere known as gel emulsion spheres (GEMs). Within these GEMs reverse transcription takes places, resulting in uniquely barcoded cDNA libraries, which can subsequently be submitted for high throughput sequencing (Illumina 2500 or Novaseq). Subsequently, read counts are filtered and analyzed by Cell Ranger, a custom single cell transcriptomics pipeline. Seurat version 3 was used for further analysis, specifically quality

control, data transformation/normalization (SCTransform), and Uniform Manifold Approximation and Projection (UMAP) method was used for dimensional reduction and clustering.

Our belief is that this approach allows us to transcriptionally identify a larger variety of astrocyte subtypes in a robust and unbiased manner. Furthermore, this method using MACS to enrich astrocyte cell solution in a gentle matter, does not result in artificial reactivity. Therefore, transcriptomes and behaviours of astrocytes submitted for scRNAseq might recapitulate the original transcriptomes and behaviors of Bace1$^{-/-}$ and Bace1 $^{+/+}$ astrocyte in vitro and in vivo.

7. Outstanding Questions

The role of BACE1 in neuronal maintenance and Aβ generation has been well covered. However, the role of BACE1 in glia cell population is currently unknown and under investigation. Previous research indicates that adult BACE1 inhibition may reverse Aβ plaque deposition, most likely through enhancing glia Aβ clearance. Furthermore, reactive astrocytes, a population know to contribute to Aβ clearance, express BACE1 at high levels. Therefore, our goal was to investigate the role of BACE1 inhibition on reactive astrocyte behavior, specifically Aβ clearance. Our overall hypothesis was that BACE1 inhibition enhances astrocytic Aβ clearance for the purposes of developing effective and specific therapies for AD that would avoid neuronal side effects.

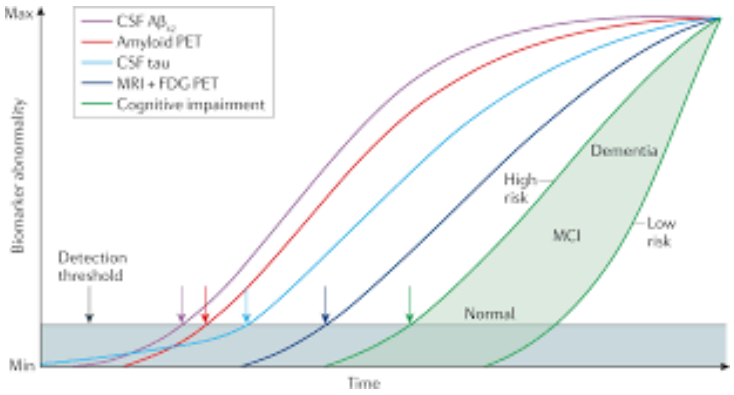
8. Research Objective

To investigate the role of BACE1 in astrocytes function and Aβ clearance, I used scRNA seq to identify astrocyte populations with enhanced differentially expressed genes related to Aβ clearance. In chapter 2, I discovered that BACE1 inhibition in mice results in a reactive astrocyte population with enhanced Aβ clearance genes including ApoE, Clu, and C-x-c motif ligand 14 (Cxcl14). I confirmed that BACE1 null astrocytes had enhanced Aβ clearance function and that increased CLU played a critical role in this phenotype. Furthermore, I found that BACE1 mediated the astrocytic insulin receptor pathway and that this pathway was responsible for enhancing CLU and CXCL14 levels. In chapter 3, I will further investigate the role of CXCL14 in the context of inflammation and AD. Specifically, I will investigate the role of CXCL14 on microglia clearance Aβ and

its neuroprotective role in neurons. In chapter 4, I will describe the implications of my work and possible future directions.

9. Figures and figure legends

Figure 1.1 Schematic diagram of A/T/N biomarker levels across AD progression



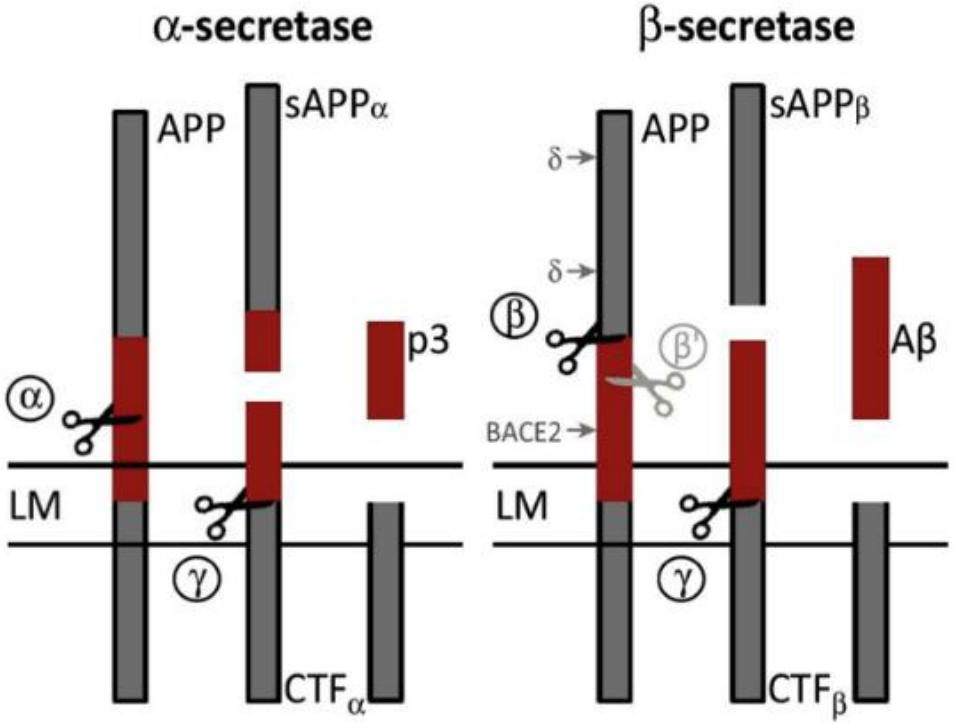
Taken from (Harald Hampel et al., 2021).

Table 1.1 A/T/N biomarkers from various sources

Factor	Imaging	CSF	Blood
Amyloid-β load	[^{11}C]-PIB	Amyloid-β (1-42)	APP 699-711
	[^{18}F]-NAV4694		Amyloid-β (1-42)
	[^{18}F]-florbetapir		Amyloid-β (1-40)
	[^{18}F]-florbetaben		
	[^{18}F]-flutemetamol		
Neurofibrillary tangles	[^{18}F]-Ro948	Phosphorylated tau	The association of serum phosphorylated tau with tangles is unclear
	[^{18}F]-AV1451		
	[^{18}F]-MK6240		
	[^{18}F]-PI2620		
	[^{11}C]-PBBB3		
Neurodegeneration	MRI	Total tau	Neurofilament light chain (NFL)
	[^{18}F]-FDG	Neurofilament light chain (NFL)	
		Neurogranin (Ng)	
		Synaptosomal-associated protein 25 (SNAP-25)	
		Neuron-specific enolase (NSE); heart fatty acid binding protein (HFABP)	
Vascular load	MRI	CSF albumin:plasma albumin ratio	α-synuclein
Lewy body load	N/A	α-synuclein	N/A
Neuroinflammation	Microglial activation:	Microglial activation:	Microglial activation:
	[^{11}C]PK11195	Chitinase-3-like protein 1 (YKL-40)	Chitinase-3-like protein 1 (YKL-40)
	[^{11}C]PBR28	Soluble TREM2 (sTREM2)	Cytokines:
	[^{11}C]DAA1106	Cytokines: TNF-α, IL-6, IL-1β	TNF-α, IL-1 β,
	[^{18}F]DPA714	Chemokines:	Chemokines:
	[^{11}C]DPA713	Monocyte chemotactic protein 1 [MCP-1]	Monocyte chemotactic protein 1
	[^{18}F]ER176		
	[^{18}F]GE180		
	[^{11}C]L-des-deprenyl		

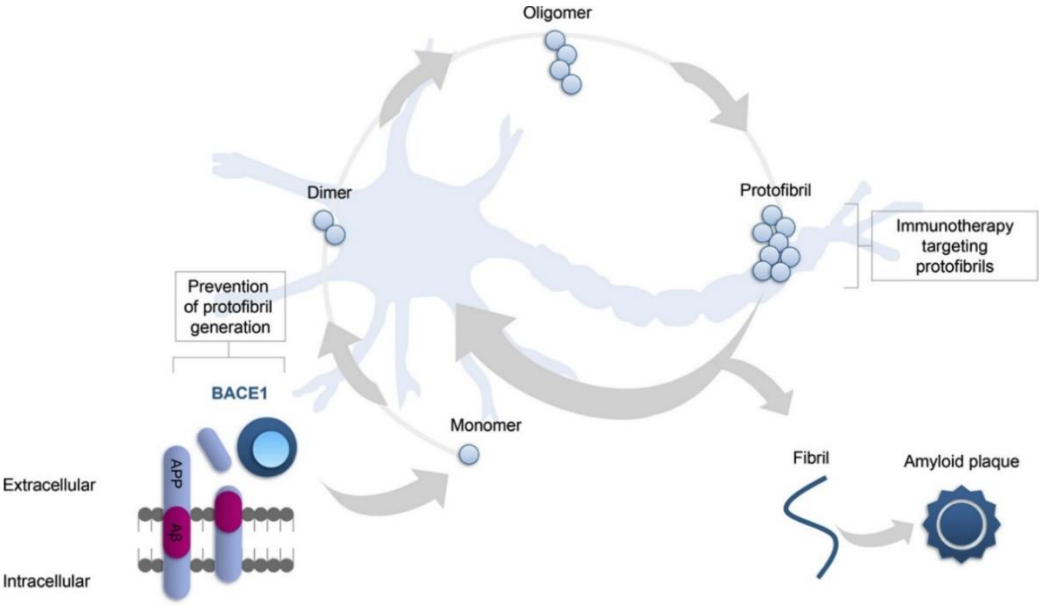
Taken from (Gauthier, 2019)

Figure 1.2 Schematic diagram of amyloid precursor protein and α/β secretase pathways



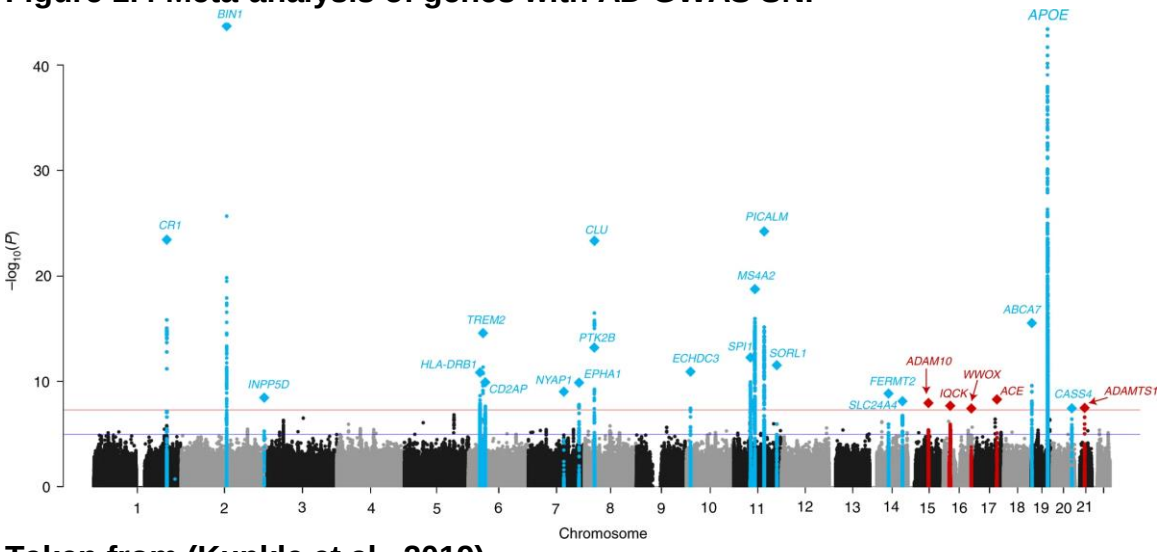
Modified from (H. Hampel et al., 2021)

Figure 1.3 Schematic diagram of amyloid generation and aggregated isoforms



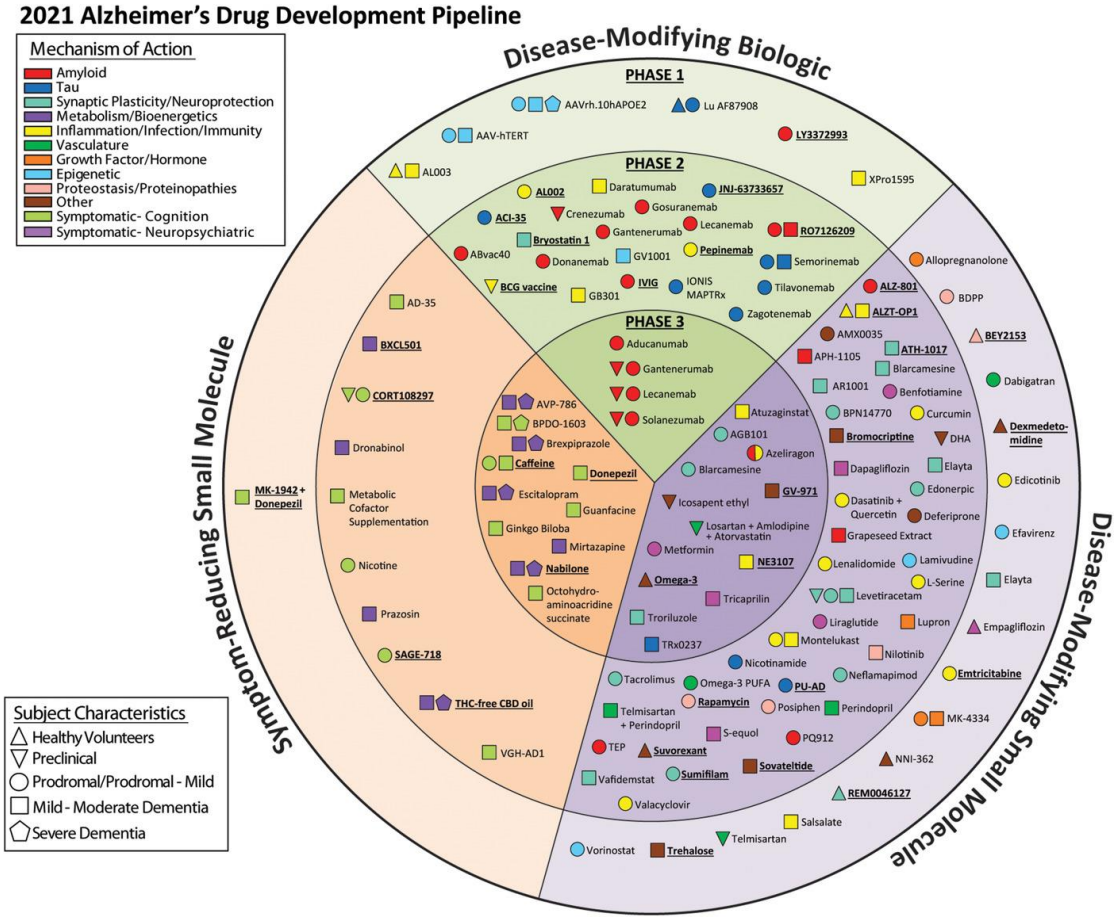
Taken from (H. Hampel et al., 2021)

Figure 1.4 Meta-analysis of genes with AD GWAS SNP



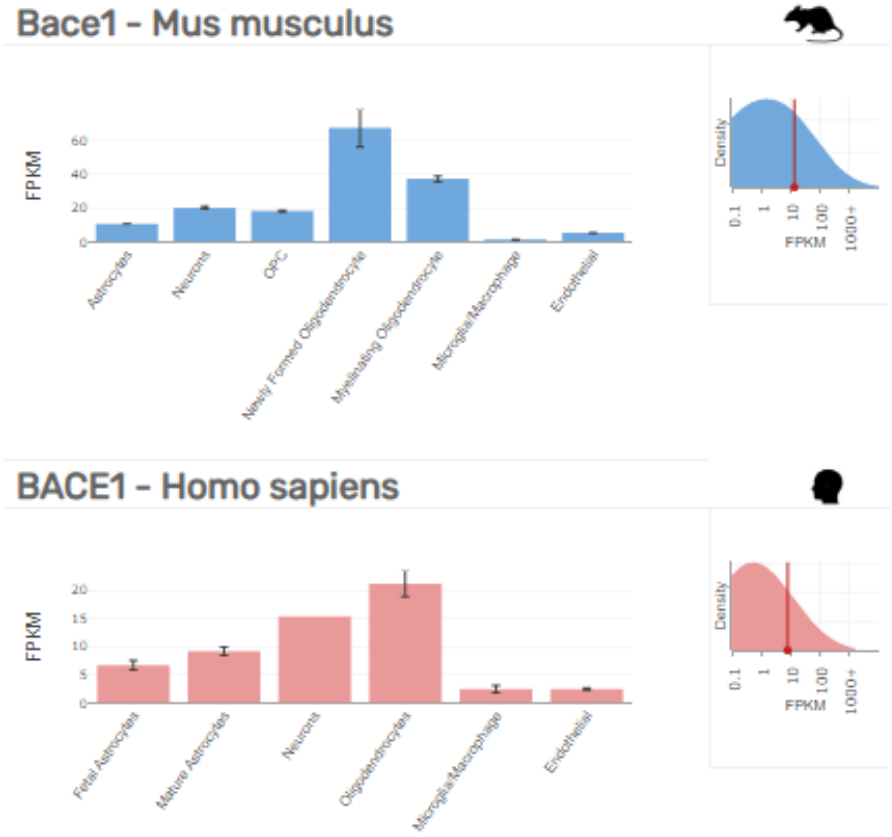
Taken from (Kunkle et al., 2019)

Figure 1.5 Diagram of AD clinical trials at different phases of development and under various categories



Taken from (Cummings et al., 2021)

Figure 1.6 RNA-seq of BACE1 gene expression in various brain cell types



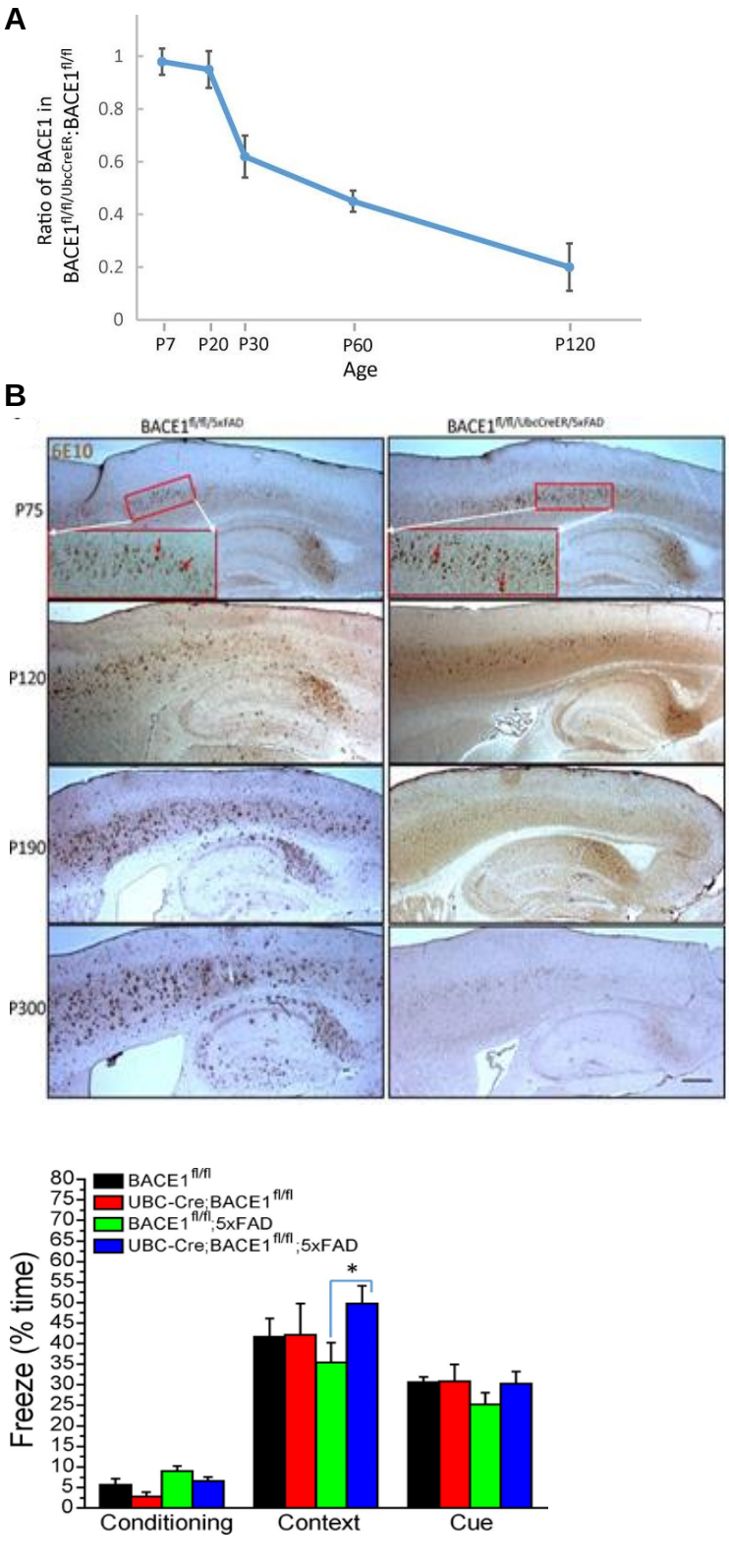
Taken from (Y. Zhang et al., 2014; Y. Zhang et al., 2016)

Table 1.2 Summary of verified BACE1 substrates and cleavage sites

Protein substrate	The substrate recognition site
APP	KM↓DAEFRHDSGY↓EVHHQK^{V}LVFFAEDVGSNK-*TM*
CHL-l	WGDNDSIFQ↓DVIETRGRETAGLDDISTG-*TM*
Delta-1	GYVCECARGYGGPNCQFLLPELPPGPAVVDL↓TEKLEGQGG
IL-1R2	PVTREDLHMDFKCVVHNTLSF↓QTLRTTVKE-*TM*
Jag1	KE↓ITDKIIDLVSKRDGNSSLIA↓AVAE^{V}VRVQRRPLKNR-*TM*
Jag2	LIQGAAHAIVAAITQRGNSSLLL↓AVTE^{V}VKVETVVTGGS-*TM*
Navβ2	IMNPPDRHRGHGKIHL↓QVL↓MEEPPERDST-*TM*
Nrg1 (type I and III-β1α)	GDRCQNYVMASF^{V}YKHLGIEF↓MEAEELYQKR-*TM*
Nrg1 (type III-β1α)	TTETNL↓QTAPKLSTSTSTTGT-*EGF-like domain*
Nrg3	FLPKTDSILSDPTDHLGIEF↓MESEEVYQRQ-*TM*
PSGL-1	VTHKGIPMAASNL↓SVNYPVGAPDHISVKQC-*TM*
Sez6	AASLDGFYNGRSL↓DVAKAPASSALDAAH-*TM*
Sez6L	ICKVNQDSFEHALEA↓EAAAESSLEGGNMA-*TM*
ST6Gal 1	SGMAVKEQSKPMQFEKAQ↓LTLAEYDSGKK-*TM*

Taken from (R. Yan, 2017b)

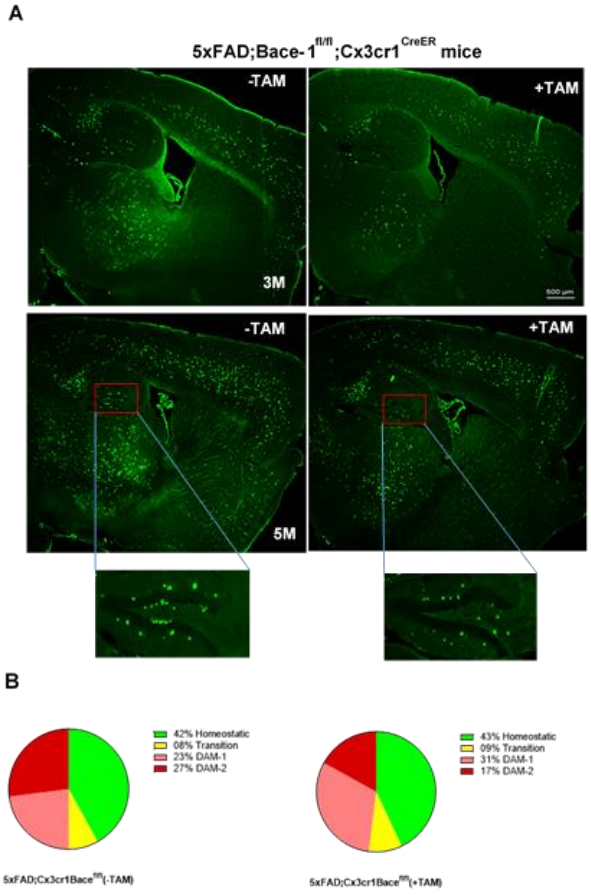
Figure 1.7 Adult BACE1 inhibition reverses preformed Aβ plaques without affecting memory



(A) Quantification of BACE1 expression from Western blot of total brain lysate from BACE1$^{fl/fl}$;UBC-Cre normalized to BACE1$^{fl/fl}$. (B) Representative 6E10 (APP and Aβ marker) immunohistochemistry of BACE1$^{fl/fl}$; 5xFAD BACE1$^{fl/fl}$ and UBC-Cre; 5xFAD across different ages. Red arrow and insets show higher magnification images for Aβ plaques. (C) Quantification of fear conditioning results showing freezing times under cue/context conditions for BACE1$^{fl/fl}$;, BACE1$^{fl/fl}$;UBC-Cre, BACE1$^{fl/fl}$; 5xFAD, and BACE1$^{fl/fl}$;UBC-Cre; 5xFAD mice.

Taken from (Xiangyou Hu, Brati Das, Hailong Hou, Wanxia He, & Riqiang Yan, 2018)

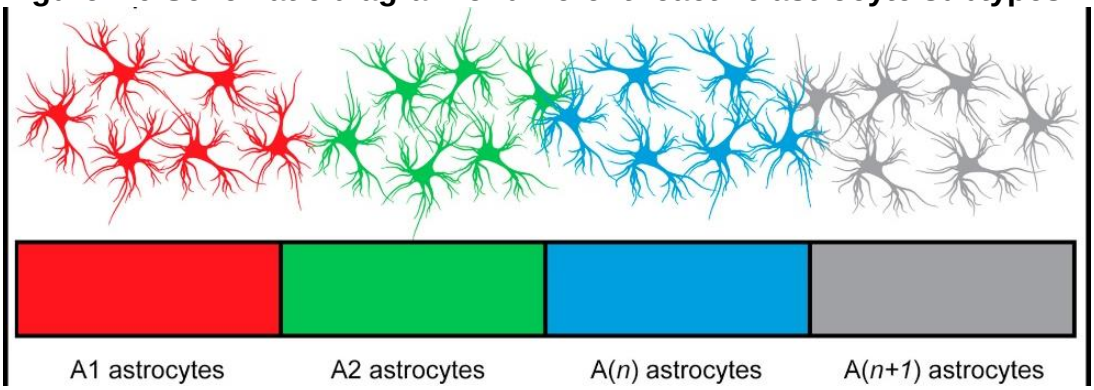
Figure 1.8 Microglial BACE1 deletion reverses preformed plaques Aβ plaques by increasing proportion of DAM1 microglia



(A) Imaging of Thioflavin –S (green) stained Aβ plaques from Bace1fl/fl; CX3CL1-CreERT2 injected with and without tamoxifen (+/- TAM). Injections began at 3 months to delete BACE1 in microglia after plaque depositions. Reduction in Aβ plaque load was observed at 6 months in Bace1fl/fl; CX3CL1-CreERT2 + TAM. Inset focuses on plaque deposition in the hippocampus. (B) Proportion of homeostatic, DAM1, and DAM2 microglia as defined by scRNA-seq from Bace1fl/fl; CX3CL1-CreERT2 with and without TAM.

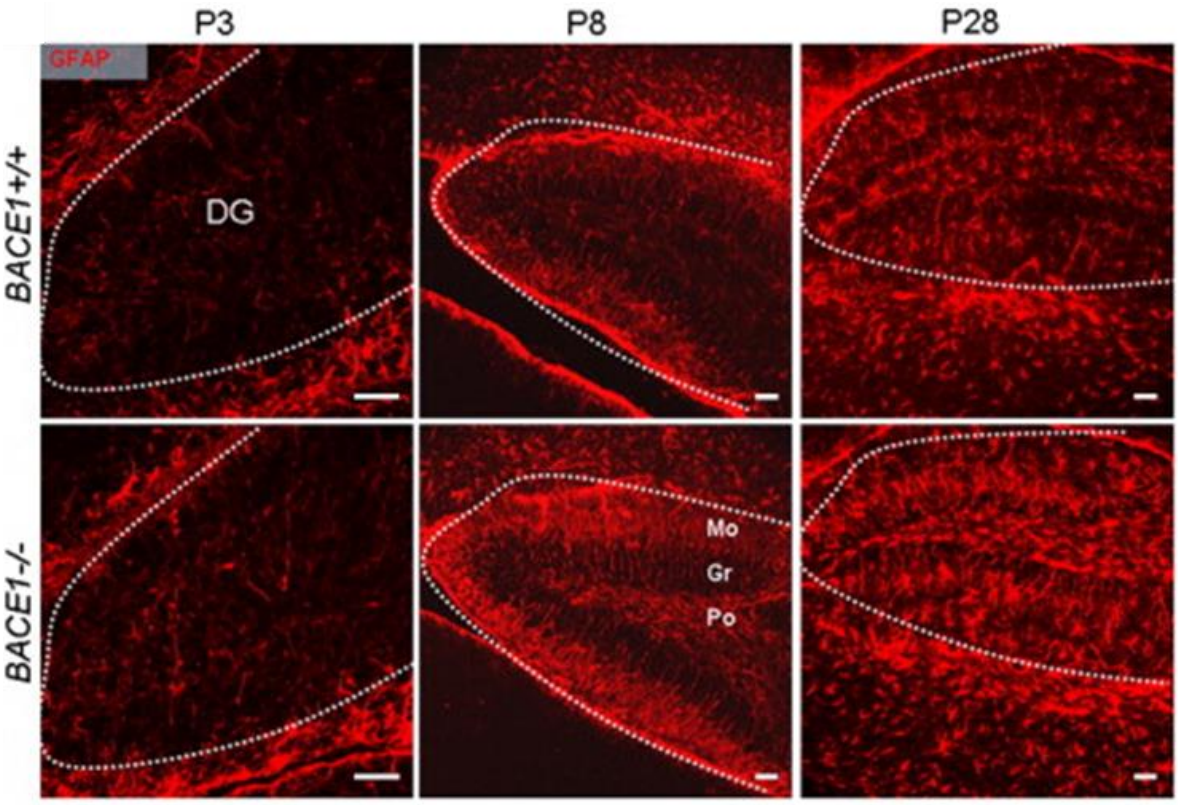
Taken from (B. D. Neeraj Singh, John Zhou, Xiangyou Hu and Riqiang Yan 2022; X. H. Neeraj Singh, Marc Benoit, Brati Das, John Zhou, Jose Davila, Li-Huei Tsai, Manolis Kellis, Riqiang Yan 2022)

Figure 1.9 Schematic diagram of different reactive astrocyte subtypes



Taken from (Liddelow & Barres, 2017)

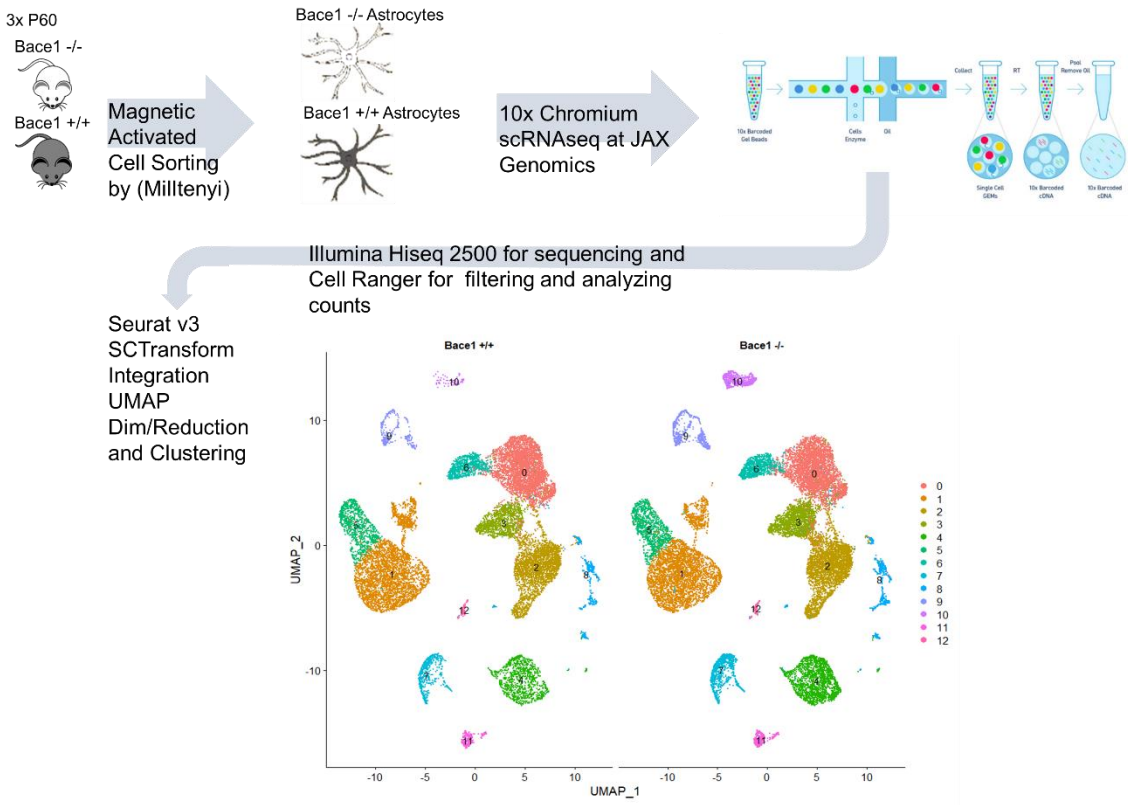
Figure 1.10 BACE1 deletion enhances astrogenesis



Representative immunohistochemistry of reactive astrocytes stained with GFAP (red) in from dentate gyrus (DG) of P8 BACE1-/- and BACE1+/+ mice. Increase of GFAP positive astrocytes in BACE1-/-.

Taken from (Xiangyou Hu et al., 2013)

Figure 1.11 Workflow of scRNAseq approach for this study



Astrocytes from P60 Bace1 +/+ and Bace1-/- are extracted by ACSA2+ immune beads and MACS then submitted for 10x scRNAseq genomic sequencing. Within microfluidic chamber cells are incorporated into GEMs and uniquely barcoded. (Adapted from UConn Genomics Core) cDNA libraries are recovered then submitted for sequencing before being trimmed, aligned, and prepared for downstream analysis.

Chapter 2: **BACE1 Deficiency Enhances Astrocytic Aβ Clearance by Increasing Clusterin**

1. Introduction

Typical clinical symptoms of Alzheimer's disease (AD) are gradual loss of memory and cognitive ability, likely resulted from amyloid deposition and neurofibrillary tangles in the patients' brains (Corriveau et al., 2017; DeTure & Dickson, 2019; Dubois et al., 2016; Guo et al., 2020). BACE1 is identified as Alzheimer's β-secretase for initiating production of β-amyloid peptides (Aβ) from amyloid precursor protein (APP), and oligomeric or aggregated Aβ is viewed as the major pathogenic protein causing amyloid deposition and the senile plaques (Jack et al., 2018). Inhibition of BACE1 is, therefore, pursued for treating AD patients (Yan & Vassar, 2014). As demonstrated in clinical trials, BACE1 inhibition is effective for reducing brain Aβ levels (McDade, Voytyuk, et al., 2021). However, BACE1 inhibitors have failed in the trials due to lack of cognitive benefit (Egan et al., 2019; Novak et al., 2020; Sur et al., 2020). This is in line with reports that reveal critical roles of BACE1 in the control of synaptic transmission (Das et al., 2021), proper organization of hippocampal mossy fiber infrapyramidal bundle (Ou-Yang et al., 2018), regulation of synaptic plasticity such as long term potentiation (LTP) from Schaffer collateral-CA1 synapses and in mossy fiber-CA3 synapses (X. Hu et al., 2018; Lombardo et al., 2019; Wang et al., 2008; Wang et al., 2010; Zhu, Peters, Filser, & Herms, 2018). BACE1-null mice also exhibit deficits in myelination and neurogenesis, as well as schizophrenia-like behaviors and seizures (H. Hampel et al., 2021).

Considering the fact that abnormal accumulation of Aβ leads to amyloid deposition, which is associated with pathological events including the

intraneuronal tau hyperphsophorylation and formation of neurofibrillary tangles (McDade, Llibre-Guerra, Holtzman, Morris, & Bateman, 2021; D.J. Selkoe & J. Hardy, 2016), it becomes important to understand how to properly inhibit BACE1 activity and to avoid unwanted side effects. We have previously shown that deletion of BACE1 in the adult mice removes pre-existing amyloid plaques (X. Hu et al., 2018), implying the potential upregulation of clearance machinery, which is most likely mediated by glia cells. BACE1 is expressed by astrocytes, and germline BACE1 deficiency induces astrogenesis during mouse early development (X. Hu, W. He, X. Luo, K. E. Tsubota, & R. Yan, 2013). Hence, we aimed to determine the role of BACE1 in astrocytes by utilizing the generated BACE1-null and conditional knockout mice.

We purified astrocytes from 2-month old BACE1-null mouse brains and conducted single cell RNAseq experiment. Deletion of BACE1 in astrocytes increased the percentage of reactive astrocytes when compared to wild type (WT) littermates. Genes related to Aβ clearance, including Apolipoprotein E (ApoE) and Clusterin (Clu), were significantly increased in BACE1-deleted astrocytes. We confirmed that this upregulation of CLU in BACE1-null astrocytes also resulted in an increase at the protein level in astrocytes from global and astrocyte-specific deletion of BACE1. Downregulation of CLU partially reversed the effect of BACE1 deletion on astrocytic clearance of Aβ. Mechanistically, we show that astrocytic BACE1 deficiency results in an increase in insulin receptor signaling and downstream p38 and ERK1/2 signaling pathways, which may regulate transcription of the Aβ clearance-related genes. Together, this suggests that

astrocytic deletion of BACE1 may contribute to a reduction in amyloid plaques and might provide an alternative therapeutic pathway that avoids off-target effects associated with significant inhibition of neuronal BACE1.

2. Materials and Methods

Adult mouse astrocyte isolation

Astrocytes were isolated from mouse brains using adult brain dissociation kit (MiltenyiBiotec, cat.no. 130-107-677) for single-RNA sequencing (see below), as well as isolating astrocytes from Bace1$^{fl/fl}$; Gfap-cre and Bace1$^{fl/fl}$ Briefly, mice were perfused with 25 mL of PBS and forebrains were isolated and transferred to gentleMACS Dissosciator (Milltenyi) to form a single-cell suspension. Subsequently, myelin, cell debris, and erythrocytes were removed and remaining cells were immunolabelled with ACSA2 immunomagnetic beads (cat. No. and 130-097-678). The cell suspension was passed though magnetic columns and QuadroMACS separators, which retained ACSA2-positive cells on the column.

Single-cell RNA sequencing

For single cell RNA-sequencing, ACSA2 positive astrocytes from littermate controlled 2-month old BACE1-null and WT mice. 3 separate samples per group (2 males, 1 female) were submitted for downstream analysis. Astrocytes from 3 pooled brains from 10-month old Bace1$^{fl/fl}$; UBC-cre;5xFAD and Bace1$^{fl/fl}$; 5xFAD mice were also submitted for scRNA seq. Astrocytes were checked for cell number and viability. Samples with little debris and >80% viability were then used for single cell RNA sequencing (Univeristy of Connecticut Single Cell Genomics). 12,000 cells of each sample type were loaded onto 10x Chromium Single Cell RNA

sequencing chips. Sample mRNAs were barcoded and subsequently converted into cDNA. After cDNA library quality inspection, libraries were sequenced using an Illumina Nextseq 5000 sequencer. CellRanger pipeline (Univeristy of Connecticut Single Cell Genomics) provided initial UMI counts and tSNE clustering.

For secondary analysis, the Seurat packaged in R (Satija lab) was used for to determine differential gene expression using normalized read counts for identifiable genes, reclustering, displaying violin plots of gene expression, and mapping gene expression in cells.

Quality controls parameters were set to only include cells with greater than 1,000 identifiable genes, less than 25,000 read counts, and less than 20% mitochondrial RNA. Read counts were normalized and transformed using SCTransform. PCA dimensional reduction was followed by UMAP clustering.

Primary perinatal astrocyte culture and isolation

Primary astrocytes cultures were prepared from littermate controlled perinatal mouse pups resulting from Bace1+/- hetero breeding. Pups were genotyped at P0 (see below) and then primary mixed glia were prepared from P1-3as described previously (Chen et al., 2010; Schildge, Bohrer, Beck, & Schachtrup, 2013). Briefly, brain tissue was isolated from skulls, bisected, and then incubated with 0.25% TriZol at 37°C for 15 minutes, with occasional gently swirling. Afterwards, tissue solution was gently triturated with fire-polished glass pipettes and passed through a 70 micron filter to form a single-cell suspension. The mixed glia culture was cultured in a T75 flask in DMEM-F12 containing 10% heat-inactivated fetal bovine

serum, 2mM L-glutamine, and 1% penicillin/streptomycin (Life Technologies) for 12-14 days. Mixed glia cultures were split twice with 4-5 day intervals to achieve a pure mature astrocyte culture.

Astrocytes were seeded 1x10^6 for 6 well plates for Western blot and 1x10^4 for 8 well chamber sides and media was switched to DMEM-F12 medium containing 1% G5 astrocyte media supplement (Gibco) for 12-24 hours before treatment. Cells were maintained and grown in a humidified atmosphere of 5% CO2 at 37°C.

Preparation of aggregated $A\beta_{42}$

Non-tagged $A\beta_{42}$ peptide (ThermoFisher) and fluorescent Hilyte 555 tagged-$A\beta_{42}$ (Anaspec) were prepared according to previous reports (Stine, Jungbauer, Yu, & LaDu, 2011). Briefly, peptides were solubilized in 0.1% NH4OH containing 0.01% (w/v) NaN3, and further re-suspended in DMEMF12 (pH 7.4) and stored at -20°C. Just prior to use, peptides were oligomerized by incubation with constant rotation for 24 hours at 4°C. Formation of beta-sheets was detected by Thioflavin-T fluorescence indicating the formation of aggregated $A\beta_{42}$ (data not shown). Cells were treated with 2 uM of aggregated $A\beta_{42}$ for 36 hours for most experiments, except for Western blotting of phosphorylated signaling pathways, in which cells were treated with $A\beta_{42}$ for 4 hours or otherwise indicated.

Clusterin siRNA knockdown

Knockdown Clu in astrocyte cultures was tested using Clusterin (Santa Cruz Biotechnology, catalog no. sc-43689) or control scrambled siRNA (Santa Cruz Biotechnology, catalog no. sc-37007) according to manufacturer's instructions.

Briefly, siRNA plasmids were combined with siRNA transfection medium and duplexed with siRNA transfection media. Astrocyte specific G-5 media was removed and replaced with siRNA duplexed transfection solution for 6 hours. Afterwards transfection solution was replaced with astrocyte specific G-5 media.

For determining correct dosage, cells were incubated with 10, 20, 40, 80 pmol of Clu siRNA or 80 pmol of Ctrl siRNA, before switching to astrocyte media. After 72 hours in astrocyte media, cells were lysed and probed for Clu expression. For determining effects of Clu knockdown on astrocyte $A\beta_{42}$ clearance, cells were treated with 80 pmol of Clu or Ctrl siRNA before treatment with aggregated $A\beta_{42}$ either untagged (Western blot) or tagged with HiLyte 555 (Cytochemistry imaging).

Western blotting

Astrocytes from perinatal culture or acutely isolated from adult mice were washed twice with ice-cold PBS and lysed on in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml aprotinin, for 10-30 minutes at 4°C with rotation. The lysate was collected and further bath sonicated on ice for 30 s on and off cycles at 60 hz for 5 minutes and then centrifuged at 15 000 × g for 30 minutes at 4°C. Protein concentrations were determined using a BCA assay kit (Pierce). Equal amounts of protein from each sample were loaded and electrophoretically resolved on 4%–12% SDS-PAGE (NuPAGE system, Life Technologies) gels. After electrophoresis, proteins were transferred to nitrocellulose membranes at 100V for 2 h. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hr at room temperature. The

membranes were probed with primary antibody (see table for antibody list and dilution), followed by incubation with appropriate secondary HRP-conjugated antibody (1:2,000). The antibody-bound proteins were detected by an iBright 1500 imaging system (Invitrogen). To ensure equal loading, the blots were reprobed with monoclonal anti-actin (1:10,000) or calnexin (1:1,000). For quantification purposes, band intensities of immunoblots were analyzed using image J software.

Cytochemistry imaging

After treatments with HiLyte Fluor-555-labeled $A\beta_{42}$ (Anaspec, Fremont, CA) , Astrocytes were washed with 3 times with PBS for 5 mins, before fixation with 4% formaldehyde for 10 minutes at room temperature, and permeabilization with 0.1% TritonX-100 for 5 minutes. After further washing, cells were incubated with 100 nM of Alexaflour 488 phalloidin (Thermofisher A12379) and Topro3 (Thermofisher T3605) diluted in 1% BSA/PBS for 10 minutes at room temperature. Cells were washed then mounted on coverslips with 90% glycerol, 20 mM Tris-HCL (1M, pH 8.8), and 0.5% (w/v) p-Phenylenediamine (CSHB Protocol). Images were captured with either a Leica TCS-SP8-AOBS or Zeiss LSM 800 Confocal microscope.

Images were quantified with ImageJ software. Integrated density of HiLyte Fluor-555-labeled $A\beta_{42}$ fluorescence intensity with the bounds of a mask based upon phalloidin defined maximum threshold was measured and normalized to phalloidin mask area and nuclei marked by ToPro3.

Mouse strains and breeding strategy

Astrocyte specific Bace-1 deletion was achieved by breeding Gfap specific Cre expressing mice, Tg-Gfap-cre 73.12Mvs, (JAX stock#012886, the Jackson Laboratory) with Bace-1 conditional mice (Bace-$1^{fl/fl}$) carrying loxP-flanked genes as previously described (X. Hu et al., 2018). The resultant $BACE1^{fl/+;}$Gfap-cre were crossed with Bace-$1^{fl/fl}$ to obtain a colony with the following genotype: Bace-$1^{fl/fl}$; Gfap-cre. Bace-$1^{fl/fl}$; Gfap-cre were maintained by breeding with Bace-$1^{fl/fl}$.

Adult conditional knockout $Bace1^{fl/fl}$;UbcCreER and $Bace1^{fl/fl}$;UbcCreER;5xFAD were bred and maintained as previously described (X. Hu et al., 2018). $Bace1^{fl/fl}$ mice were bred with Tg (UBC-Cre/ERT2) mice (JAX stock#0007001; Jackson Laboratory) to obtain mice heterozygous for the transgenic Cre and homozygous for the floxed Bace1 ($Bace1^{fl/fl}$/UbcCreER) mice. $Bace1^{fl/fl}$ mice were bred with Tg (APPSwFlLon,PSEN1*M146L*L286V) mice (5xFAD; Jackson Laboratory) to obtain mice heterozygous for the transgenic 5xFAD and homozygous for floxed Bace1 ($Bace1^{fl/fl}$/5xFAD) mice. All lines were routinely backcrossed with C57BL/6J mice for at least five generations to ensure consistent genetic background for phenotypic analyses. BACE1-null mice were achieved by heterozygous breeding of Bace1+/- males and females (Jax stock# 004714, Jacksonlab) as previously described.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Lerner Research Institute and University of Connecticut Health Center in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory.Animals.

Genotyping primers

Primer Target	Sequence (5'->3')
APPSwFlLon – common	ACC CCC ATG TCA GAG TTC
APPSwFlLon – mutant reverse	CGG GCC TCT TCG CTA TTA C
APPSwFlLon – wild type reverse	TAT ACA ACC TTG GGG GAT GG
Bace1 - forward	AGG CAG CTT TGT GGA GAT GGT G
Bace1 mutant	TGG ATG TGG AAT GTG TGC GAG
Bace1 - reverse	CGG GAA ATG GAA AGG CTA CTC C
Gfap-cre forward	TCC ATA AAG GCC CTG ACA TC
Gfap-cre reverse	TGC GAA CCT CAT CAC TCG T
Loxp forward	TCTGACGATGGCACACATAAGC
Loxp reverse	TGCTAGTGTTTCCTGTCACCTG
UBC-creERt2 forward	GAC GTC ACC CGT TCT GTT G
UBC-creERt2 reverse	AGG CAA ATT TTG GTG TAC GG

Antibody list used for the study

Antibody Name	Catalog no.	RRID:	Manufacturer
Actin (Clone AC-15)	A5441	AB_476744	Sigma
ApoE (F-9)	sc-390925	AB_2892618	Santa Cruz
Amyloid Beta (Oligomer), A11	AHB0052	AB_2536236	ThermoFisher Scientific
BACE1 (D10E5)	5606	AB_1903900	Cell Signaling
Caln	C4731	AB_476845	Cell Signaling

cJun (60A8)	9165	AB_2130165	Cell Signaling
Clusterin-a (B-5)	sc-5289	AB_673566	Santa Cruz
CXCL14	ab137541		Abcam
IRα	sc-57344	AB_782041	Santa Cruz
IRβ (CT-3)	sc-57342	AB_784102	Santa Cruz
p38 (D13E1)	8690	AB_10999090	Cell Signaling
pp38 (Thr180/Tyr182)	4511	AB_2139682	Cell Signaling
pIRβ (10C3)	sc-81500	AB_1125642	Santa Cruz
pcJun(Ser63)	9261	AB_2130162	Cell Signaling
pErk1/2 (Thr202/Tyr204)	4370	AB_2315112	Cell Signaling
pJNK (Thr183/Tyr185)	9251	AB_331659	Cell Signaling
Tubulin	sc-5274	AB_2288090	Santa Cruz

Statistical analysis

Bar graph results are expressed as mean±SD. Violin plots are displayed as medians with interquartile ranges. Fold change values and p-values for scRNA seq comparison were based on Wilcoxon Ranked Sum test generated by Seurat. The statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego). Student's t-tests were used to compare between 2 groups. Two-way ANOVAs were used to compare multiple groups, post-test of Sidak or Bonferonni was used to compare between different groups. Differences with *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$ were considered significant.

3. Results

Deletion of BACE1 in astrocytes alters transcriptomic profiles favoring reactive population

To understand the role of BACE1 in astrocytes, we enriched astrocytic cell population from 3 brains of wild-type (WT) and BACE1-null mice at the age of 2-month old BACE1-null mice (2 male, 1 female) using astrocyte-cell specific antigen 2A (ACSA2) immunomagnetic beads. Purified astrocytes were subjected to 10x Genomic single cell RNA sequencing and initial read count analysis by Cell Ranger (10x Genomics). The sequencing results were analyzed and clustered into UMAP-defined clusters using Seurat R package (Satija lab). (Figure 2.1A) Quality control of parameters were set so further analysis only included cells containing >1,000 identified genes, <25,000 read counts, and <20% mitochondrial RNA. Approximately 17,000 cells were identified for each genotypes.

According to the unique gene signature in different cell clusters as previously described (Tasic et al., 2018), we marked major populations of nonreactive astrocytes (indicated by Atp1b2 expression or and low vimentin [Vim] expression), reactive astrocytes (high Aqp4 and high Vim), microglia (Cluster of differentiation 68 [CD68]), oligodendrocytes (myelin and lymphocyte protein [Mal]), oligodendrocyte precursor cells (OPC) (platelet-derived growth factor receptor A [Pdgfr2]), and endothelial cells Chondroitin sulfate proteoglycan 4 [CSPG4]) (Figure 2.1B). Thus, cells from UMAP-defined clusters 0, 2, 3, 6 were non-reactive astrocytes (labeled as “Astrocytes”); clusters 4, 8, 10 were reactive

astrocytes, (labeled as “R astrocytes”); clusters 1, 5 were oligodendrocytes; cluster 7, 12 were OPCs; cluster 9 were microglia; cluster 11 were endothelial cells; and were the remaining were mixed or undefined (Figure 2.1C). We noted that nonreactive astrocytes (in salmon) were and reactive astrocytes (green) represented the majority of cells. Approximately 20% of cells were actually identified as oligodendrocytes (ochre), while microglia (purple), endothelial cells (blue), and OPC (pink) accounted for a much smaller population, indicating a mostly effective enrichment of astrocytes. (Figure 2.1D) Reactive astrocytes comprised approximately 20% of total cells in BACE1$^{-/-}$ compared to only 10% reactive astrocyte population in BACE1$^{+/+}$.(Figure 2.1D) Consistently, a significant increase in GFAP protein level was evident when lysates from BACE1-null and WT primary astrocyte cultures were examined by Western blotting experiments, indicating that BACE1 deletion promotes astrocytes in the reactive states. (Figure 2.1E).

Astrocytes and reactive astrocytes were further sub clustered and compared. (Figure 2.2A) Comparing the transcriptomes of pan reactive astrocytes from BACE1-null and WT mice using, 37,658 unique gene signatures were identified, and found 213 genes that were significantly differentially expressed genes (DEGs) ($p < 0.05$, $\log2FC > 0.2$). Figure 2.2B represents these most significant of DEGs, with green dots highlighting specific genes of interest suspected related to clearance of β-amyloid peptides and yellow dots highlighting genes part of the AP1 transcription family. Among these genes, we found several genes known to have roles in clearance of β-amyloid peptides (Aβ),

glutamate homeostasis, metabolism, and hippocampal synaptogenesis. Clu and C-X-C Motif Chemokine Ligand 14 (Cxcl14) expression levels were significantly elevated in BACE1-null reactive astrocyte gene clusters (Figure 2.2C and D). Western blot analysis showed that both CLU and CXCL14 protein levels were increased in BACE1-null primary astrocytes, both basally and when stimulated with Aβ, compared to WT astrocytes (Figure 2.2E and F). There was also a small increase in ApoE mRNA transcripts BACE1 null reactive astrocyte when treated with Aβ, however this did not translate to the protein level.

Deletion of BACE1 in 5xFAD mice enhances expression of Clu and Cxcl14

As previously mentioned, conditional knockout of BACE1 in the adult reverses previously formed amyloid plaques in 5xFAD;Bace1$^{fl/fl}$;UBCcre mice compared to non-BACE1 deleted 5xFAD;Bace1$^{fl/fl}$ mice (Hu et al., 2018). To understand the role that adult BACE1 deficiency might play on astrocyte transcriptomes in the 5xFAD background, we compared scRNA-Seq of astrocyte enriched cells from 14-month old 5xFAD;Bace1$^{fl/fl}$;UBCcre and 5xFAD;Bace1$^{fl/fl}$ mice. In this case cells from 3 brains were pooled into one sample per genotype. About 4,000 astrocytes were recovered from these mice after magnetic activated cell sorting (MACS) and cell type filtration. When comparing reactive astrocyte population transcriptomes from 5xFAD;Bace1$^{fl/fl}$;UBCcre and 5xFAD;Bace1$^{fl/fl}$ mice, 1,412 DEGs were found. Among these DEGs, insulin degrading enzyme (IDE) was upregulated in the 5xFAD;Bace1$^{fl/fl}$;UBCcre compared to 5xFAD;Bace1$^{fl/fl}$ mice. Furthermore, we once again found significantly elevated

Clu and Cxcl14 gene expression in the reactive astrocyte population in the case of 5xFAD;Bace1$^{fl/fl}$;UBCcre mice (Figure 2.3A and B).

Our data suggest that both germline and adult BACE1 deficiency increases a group of astrocytes that commonly express more Clu and CXCL14. Likely, these Clu^{high} and Cxcl14^{high} cells are capable of clearing more β-amyloid peptides (Aβ) as higher levels of Clu are known to increase Aβ clearance (Bettens et al., 2015; Long and Holtzman, 2019).

BACE1 deficiency increases uptake and degradation of Aβ by astrocytes

Since BACE1 deficiency altered astrocytic transcriptomic profiles, we then asked whether astrocytes with BACE1 deficiency would enhance Aβ clearance, which might contribute to the reversal of pre-formed amyloid plaques in 5xFAD mice with deletion of BACE1 in the adult as previously reported (Hu et al., 2018). We cultured primary astrocytes from WT and BACE1-null mice, and then treated these cultured astrocytes with aggregated oligomeric human Aβ42 according to the published procedure (Stine et al., 2011). We found that BACE1-deleted astrocytes had visibly increased uptake of more HiLyte Fluor-555-labeled Aβ4242 (Anaspec, Fremont, CA) after 12 hrs incubation around 12 hrs compared to WT controls (Figure 2.4A). High magnification images show that the majority of the HiLyte Fluor-555-labeled Aβ42 signal was found within astrocyte cell bodies (Figure 2.4A, inset). Quantification showed that enhanced uptake of aggregated Aβ4242 began around 6 hrs post incubation and plateaued around 12 hrs, while it took about 36 hrs for WT astrocytes to uptake about the same amount of HiLyte Fluor-555-labeled Aβ4242 (Figure 2.4B).

We also examined Aβ levels by western blotting experiments. Although the levels of oligomeric Aβ4242 in BACE1-null astrocytes were higher than that in WT controls during initial incubation (12 to 36 hrs), significantly less Aβ4242 was found at the time of 72 hrs (Figure 2.4C). This was further confirmed by multiple replication experiments (Figure 2.4D), indicating that BACE1 deletion likely also enhanced degradation of oligomeric Aβ4242. In line with previous reports (Bettegazzi et al., 2011; Chacón-Quintero et al., 2021; Zhao et al., 2011), astrocytes treated with Aβ42 appeared to enhanced expression of BACE1 (Figure 2.4C), suggesting that elevated BACE1 in AD brains might have vicious cycle inhibitory effect on astrocytic Aβ clearance.

Clusterin contributes to enhanced Aβ uptake in BACE1 null astrocytes

Because, CLU is known to regulate Aβ clearance and degradation and elevation of the CLU protein level was evident, we chose to examine whether elevated CLU levels would contribute to enhanced Aβ uptake and degradation in BACE1-null astrocytes. To this end, we identified that one well characterized siRNA (Santa Cruz Biotechnology) was able to reduce CLU protein levels in primary astrocytes in a dose dependent manner, with the 80 pmol of Clu siRNA down-regulated about 48.6% expression of CLU (Figure 2.5A and B).

We then pretreated BACE1-null and WT astrocytes with 80 pmol of control or Clu siRNA before treating astrocytes with aggregated Aβ42, and examined intracellular Aβ levels by Western blot (Figure 2.5C and D) and fluorescently tagged Aβ by confocal imaging (Figure 2.5E). We showed that knocking down

CLU in BACE1-null astrocytes reduced the uptake of Aβ42, when compared to BACE1-null astrocytes treated with control siRNA (Figure 2.5C and EC).

Further quantification showed a clear shift in the uptake pattern (Figure 2.5D): slowed uptake of Aβ42 by astrocytes when CLU levels were reduced, and treatment with Clu siRNA reduced total levels of Aβ42 in both BACE1-null and WT astrocytes from 0.5 to 36 hours compared to control siRNA treated astrocyte (Figure 2.5C and D). Aβ42 levels were increased in CL siRNA treated BACE1-null astrocyte, when compared to their WT counterparts at 72 hours, suggesting that Clu siRNA only delayed Aβ uptake in BACE1-null astrocytes, but did not entirely disrupt endocytosis of Aβ42 (Figure 2.5C and D). Confocal images also showed delaying uptake of Aβ42 when comparing BACE-null astrocytes with and without specific Clu siRNA treatment (Figure 2.5E).

Altogether, these results add evidence that Clu does indeed play a role in direct astrocytic clearance of Aβ. Furthermore, it suggests that increased Clu levels underlies the increased uptake of Aβ in BACE1-null astrocytes. However, the increased level of Aβ at the 72-hour time point indicates that other factors in addition to Clu levels might explain the enhanced uptake in BACE1-null astrocytes.

BACE1 deficiency increases p38, JNK1/2ERK1/2 and cJun activity

In order to investigate signaling pathways responsible for the increased expression of Clu and other aforementioned DEGs in BACE1 deficient astrocytes, we performed a database search using BioMart in order to determine common transcription factors for these DEGs. Members of the activated protein

1 (AP1) transcription family were the most commonly found elements to be related to DEGs of BACE1 deficient astrocytes, including Cxcl14 and Clu (Table 1). Furthermore, some members of the AP1 family themselves were DEGs upregulated in BACE1 deficient reactive astrocytes (Figure 2.2B) AP1 activation is known to be active by the upstream molecules such as pP38, JNK1/2, and ERK1/2, and MAPK and MAPK (Herdegen and Waetzig, 2001; Raivich and Behrens, 2006). We found that the levels of phosphorylated JNK were not significantly changed, but levels of phosphorylated p38, cJun and ERK1/2 were significantly elevated in BACE1-null astrocyte cultures (Figure 2.6A). Quantification of replicated results were are shown in Figure 2.6B.

Our results imply that BACE1 deficiency in astrocytes results in an increase of pP38 and JNK1ERK1/2 activation, which then phosphorylates and activates downstream cJun/Ap1 mediated transcription, including ApoE and Clu transcription, which are related to astrocytic clearance of Aβ.

BACE1 deficiency increases astrocytic insulin receptor signaling

We further explored how BACE1 deficiency would activate above the signaling molecules in astrocytes discussed above. It was previously reported that insulin receptor (IR) is a BACE1 cleavage substrate in the liver (Meakin et al., 2018), and insulin signaling regulates both p38 and ERK1/2 MAPK activity. IR is composed of a heterodimer dimer, α and β subunits, and the membrane anchored β subunit is identified as a BACE1 substrate. When BACE1 is deleted, IRβ subunit is no longer cleaved and more preserved IRβ subunit might be available for enhancing p38 and ERK1/2 activity. We therefore investigated

whether BACE1 deficiency might also enhance astrocytic insulin receptor availability.

Western blot revealed that BACE1 deficiency significantly reduced levels of BACE1-cleaved IRβ C-terminal fragment (CTF) in BACE1-null astrocytes compared to their WT controls (Figure 2.7A), consistent with prior results that BACE1 deficiency abrogated cleavage of IRβ in astrocytes. An increase of total mature IRα and IRβ levels was also noted, while as well as a much larger increase in phosphorylated IRβ (pIRβ) subunit (Figure 2.7A), which is required for downstream activation for pP38 and ERK1/2 MAPK. Quantification of replicated results confirmed changes of these proteins (Figure 2.7B). Altogether, this suggests that astrocytic BACE1 regulated IR receptor availability by cleavage in a matter similar to liver BACE1 and IR.

We further explored the relationship between IR We tested whether insulin signaling might be responsible for the elevated Clu and CXCL14 levels in BACE1-null astrocytes by adding using BMS-754807 (SelleckChem), a potent IR tyrosine kinase phosphorylation inhibitor (Figure 7C and D). BMS-754807 treatment resulted in a potent reduction of both Clu and CXCL14 levels in BACE1-null (Figure 2.7C). Quantification of replicated results confirmed changes of these proteins (Figure 2.7D). This suggests that elevated Clu and CXCL14 levels in BACE1-null astrocytes is dependent on IR signaling.

Altogether, this suggests that astrocytic BACE1 regulated IR availability by cleavage in a matter similar to liver BACE1 and IR and that IR signaling may play a role in reactive astrocyte amyloid clearance.

Targeted deletion of BACE1 in astrocytes increases astrocytic levels of IR, CLU and CXCL14

To investigate whether in vivo astrocytic BACE1 deficiency results in increased IR, CLU, and CXCL14, we bred Bace1fl/fl mice with Gfap-cre [Tg-Gfap-cre 73.12Mvs, the Jackson lab] and examined Bace1fl/fl;GFAP-Cre mice, which conditionally deleted BACE1 mostly in astrocytes due to expression of Cre recombinase by the mouse GFAP promoter (Garcia et al., 2004). To avoid potential effects from Gfap-expressing lineage cells, we isolated astrocytes from 2-month old BACE1fl/fl;GFAP-Cre mice using ACSA2+ immunobeads and the purified ACSA2+ astrocytes were used for Western blot (Figure 2.8).

We found that astrocytes from BACE1fl/fl/GFAP-Cre mice had an increased amount of mature IRβ and reduced IRβ CTF compared to BACE1fl/fl astrocytes in a manner similar to above in vitro conditions (comparing Figure 2.7 and 8). We also found a significant increase in Clu and CXCL14 protein levels while ApoE levels were comparable to BACE1fl/fl mouse astrocytes (Figure 2.8). These changes in IR β subunit, Clu, and CXCL14 were not seen in BACE1KO whole brain lysate or the non-astrocyte fraction from BACE1fl/fl/GFAP-Cre GFAP-cre; BACE1fl/fl mice (data not shown). Altogether, this suggests that BACE1 deficiency increases astrocytic IR signaling and its downstream molecule Clu and CXCL14 in a mostly a cell autonomous manner both in vivo and in vitro.

4. Discussion

BACE1 is a type I transmembrane aspartyl protease, which mainly cleaves membrane bound proteins such as amyloid precursor protein (APP), type I and

type III neuregulin and Sez6L (Dislich et al., 2015; Hampel et al., 2020). Since its discovery, BACE1 studies have mostly focused on BACE1 role in neurons including amyloid production, myelination, and synaptic function (Das et al., 2021; Hampel et al., 2020; Lombardo et al., 2019; Ou-Yang et al., 2018; Wang et al., 2008; Yan & Vassar, 2014; Zhu et al., 2018). Furthermore, BACE1 inhibitors used in clinical trials have largely failed due to lack of efficacy and because of neuronal side effect of global BACE1 inhibition (Das et al., 2021; Das & Yan, 2019; R. Yan, 2017a; Yan & Vassar, 2014). In contrast, relatively few studies reveal the unique role of BACE1 on astrocytes behavior, despite known expression of BACE1 in reactive astrocytes and its contribution to astrocyte development (X. Hu et al., 2013). Adult conditional knockout of BACE1(X. Hu et al., 2018), as well as chemical inhibition of BACE1(Das & Yan, 2019; D.R. Thakker et al., 2015), reverse previously formed amyloid plaques, suggesting that BACE1 inhibition may also play a role in enhancing glial amyloid clearance. In this study, we conducted unbiased single cell RNAseq experiments and compared transcriptomic profiles in mouse brain astrocytes, with or without BACE1. We provided evidence that BACE1 deficiency increase a population of reactive astrocytes expressing higher levels of genes such as Clu, which are known to enhance clearance of aggregated Aβ. Cultured astrocytes with BACE1 deficiency showed increase Aβ clearance and degradation, suggesting that targeted inhibition of BACE1 or specific downstream pathways in astrocytes may likely be beneficial for AD treatment. Furthermore, we found evidence that astrocytic IR, a potential BACE1 cleavage substrate, and downstream pathways might contribute to astrocytic expression of

astrocytic clearance genes both in vitro and in astrocyte specific BACE1 deficiency mouse model.

In Alzheimer's brains, astrocytes are activated and reactive astrocytes are often found surrounding amyloid plaques. Reactive astrocytes contribute to amyloid clearance directly, by endocytosis (T. Wyss-Coray et al., 2003) or producing amyloid degrading enzymes (Dorfman et al., 2010; Vekrellis et al., 2000)}, and indirectly, by preventing amyloid aggregation (MATSUBARA, SOTO, GOVERNALE, FRANGIONE, & GHISO, 1996; Nuutinen et al., 2007) or enhancing clearance by other cell types (Nelson et al., 2017; Ries & Sastre, 2016). However, as AD pathology progresses, reactive astrocytes not only experience a loss of amyloid clearance efficiency, but may also play a role in promoting neuroinflammation and toxicity (Perez-Nievas & Serrano-Pozo, 2018). Furthermore, genetic descriptions of various disease-related astrocyte subtypes (Boisvert, Erikson, Shokhirev, & Allen, 2018; S.A. Liddelow et al., 2017; Soreq et al., 2017) have made it clear that an unbiased, single cell genetic approach is key to understanding how astrocyte subtypes contribute to neurological diseases. To this end, we used single cell RNA-seq to compare various astrocyte clusters from BACE1 deficient mouse in the case of germline deletion (BACE1-null compare to wild type). In the case of BACE1-null and wild type mice, we found an increase in reactive astrocyte clusters, which is line with the previous observation showing increased astrocyte genesis and reactivity in the BACE1-null mice (X. Hu et al., 2013). When comparing the transcriptomes of individual UMAP defined astrocyte clusters between BACE1-null and wild type mice, a variety of known AD-related

DEGs were noted. Among these upregulated DEGs from BACE1-null reactive astrocytes, ApoE and Clu gained our particular attentions because they are highly expressed in astrocytes, have known roles in clearance of aggregated Aβ as well as are top GWAS risk factors for the development of AD (E. M. Foster, A. Dangla-Valls, S. Lovestone, E. M. Ribe, & N. J. Buckley, 2019{Harold, 2009 #3899; Lambert et al., 2009)}. Cxcl14 was the second most highly expressed gene on the list. It is a chemokine with known roles in peripheral inflammation but was less explored in the CNS or in the field of neurodegeneration. We extend our study of CXCL14 and its dual role in microglial Aβ clearance and neuronal survival in the next chapter. We also discovered upregulation of numerous members of the AP1 transcription family in BACE1-null astrocytes. Although total protein of these AP1 transcription factors was not increased in BACE1-null astrocytes, interestingly we found increased phosphorylation of AP1 transcription machinery.(Figure 2.6)

We found that CLU and CXCL14 protein levels were also increased in cultured BACE1-null primary astrocytes compared to WT controls (Figure 2.2D). Intriguingly, CLU and CXCL14 levels appeared to be increased with $A\beta_{42}$ treatment (Figure 2.2D) and in in the case of adult conditional knockout of BACE1 in an amyloid background (Bace1fl/fl;UBC-cre;5xFAD compared to Bace1fl/fl;5xFAD) (Figure 3A), likely due to an increase in the reactive astrocytic signature. Moreover, targeted deletion of BACE1 in $Bace1^{fl/fl;}$GFAP-Cre mice also showed increase of CLU and CXCL14 protein levels (Figure 2.8A). On the other hand, the increase of ApoE protein levels was not evident, although small elevation in the mRNA levels was detected.

To understand the effect of BACE1 deficiency on amyloid clearance, we compared astrocytes cultured from WT and BACE1-null mice to avoid the inference from other cells types. An increase of fluorescence-labeled $A\beta_{42}$ uptake by BACE1-null astrocytes for the first 12 hours was clearly more obvious than control astrocytes . With the consistently detected elevation of CLU in BACE1-null astrocytes, we asked whether CLU levels would affect uptake of the fluorescence-labeled $A\beta_{42}$ in BACE1-null astrocytes. CLU knockdown resulted in decreased uptake by both BACE1-null and WT astrocytes, suggesting that higher level of CLU correlates with more uptake of $A\beta_{42}$. Interestingly, Clu siRNA treatment in BACE1-null astrocytes still had slightly more uptake of $A\beta_{42}$ than Clu siRNA-treated WT astrocytes, and this observation indicates that other BACE1- regulated genes in astrocytes may also contribute to enhanced uptake of $A\beta_{42}$.

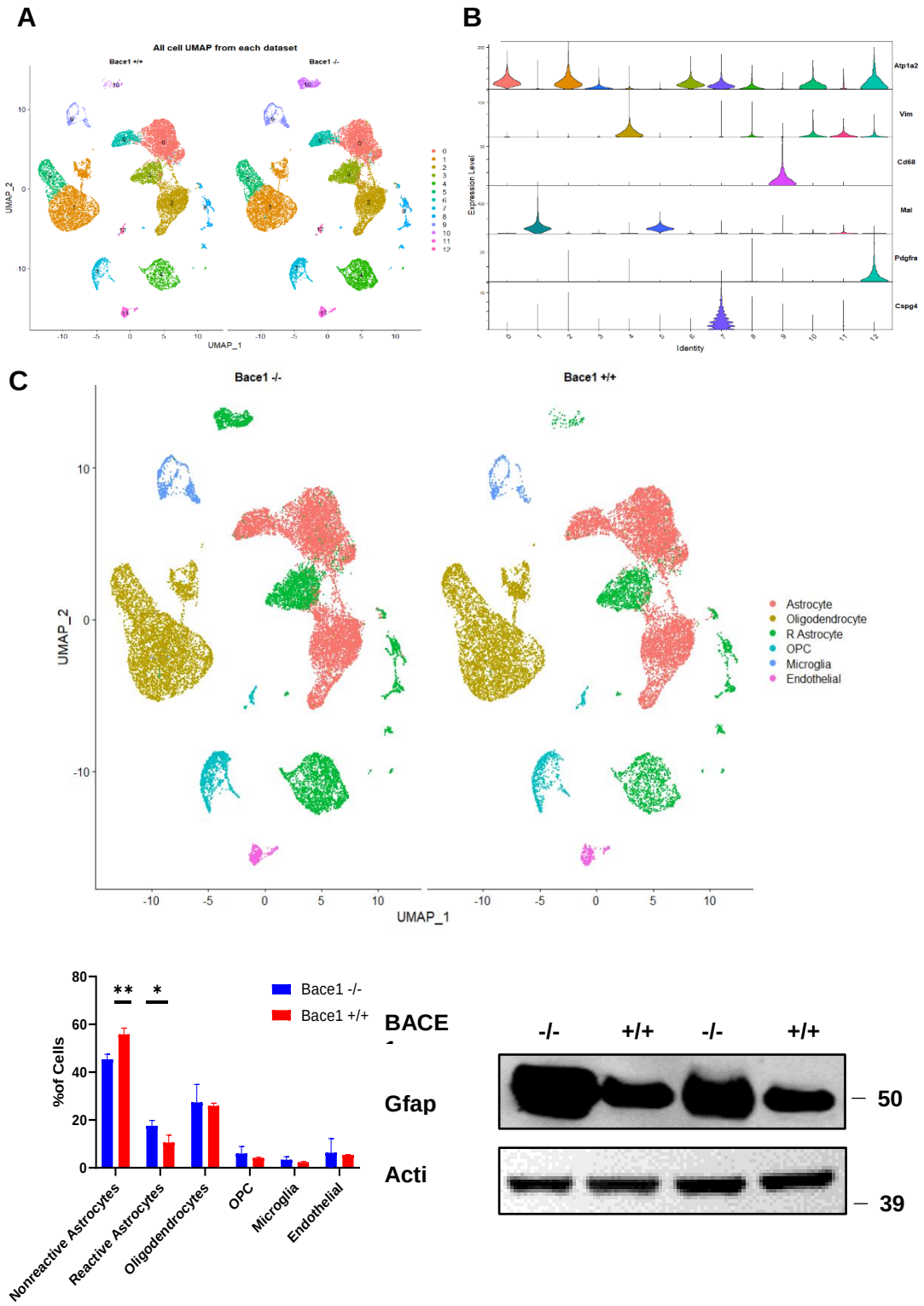
Our validated finding is in line with the published observation regarding the beneficial effect of CLU on astrocytic uptake and degradation of $A\beta_{42}$ (Bettens et al., 2015{Wojtas, 2020 #3895)}. C LU is also found to enhance brain endothelial vascular clearance of $A\beta_{42}$ by protecting against AD associated metabolic stress, glutamate toxicity, and synaptic dysfunction (A. M. Wojtas et al., 2017). We further explored how BACE1 deficiency alter C LU levels, and our mechanistic study implied that C LU elevation is likely related to astrocytic insulin receptor (IR) signaling. IR has α and β subunits and its β subunit (IRβ) was previously identified as BACE1 substrate in the liver (Meakin et al., 2018). We found that BACE1-cleaved C-terminal fragment (CTF) of IRβ was reduced on the western blot by BACE1 deficiency. In Bace1-/-astrocytes, IRβ CTF is consistently higher both in

cultured astrocytes and with astrocyte specific knockout for BACE1, detectable even without γ-secretase inhibition. This elevation of full length IRβ and reduced IRβ CTF is attributable to abrogated cleavage by BACE1 (Figure 2.8). The availability of more functional IRs on the surface of astrocytes of BACE1 deficient astrocytes would most likely explain the elevation of phosphorylated IRβ, its downstream signaling molecules p38, ERK1/2, and cJun(Figure 2.6). Consequently, these transmitted signals will enhance AP1 mediated transcription of Clu. Intriguingly, IDE was also found to be increased by Bace1fl/fl;UBC-cre;5xFAD reactive astrocytes, which in addition to enhancing amyloid clearance, may act in a self-regulatory way to modulate insulin signaling in BACE1 deficient astrocytes. Furthermore, the enhancement of insulin signaling has been pursued in AD clinical trials for purported benefits in adult neurogenesis and synaptic function as well.

In summary, we describe a model of BACE1 inhibition that enhances astrocytic clearance of Aβ, most likely in a cell-specific and autonomous manner, by increasing insulin signaling, which in turn to upregulate expression of Aβ clearance genes such as Clu. (Figure 2.9) Increasing BACE1 levels in AD reactive astrocytes are likely to cause a vicious cycle that may block an efficient astrocytic clearance of Aβ. Hence, specific inhibition of BACE1 in astrocytes may be an alternative strategy for reducing Aβ in human AD therapy that is worthy of further exploration in future.

5. Figures and figure legends

Figure 2.1 BACE1 deficiency enhances reactive astrocyte population.



(A) Total UMAP cluster representation of ACSA2+ positive cells from derived from 2-month old BACE1-null and WT mice via single-cell RNA-seq, N=3 samples, ~8,500 cells per genotype. Clusters are labeled by cell type. (B) Violin plot representation of log2 fold change gene expression of known cell type gene markers and correlated with labeled clusters. (C) UMAP clusters labeled by known cell type comparing samples from BACE1-null or wild-type mice. (D) Proportion of indicated cell types. Comparison of average proportion of each cell types from each samples genotype. * indicates p-value <0.05. Student T-test. each cell type were measured and compared to (E) Gfap Western blot of primary astrocytes cultures lysate derived from BACE1-null and WT mice to confirm increase in reactive astrocytes from BACE1-null mice.

Figure 2.2 Germline BACE1 deficiency results in unique reactive astrocyte transcriptomes.

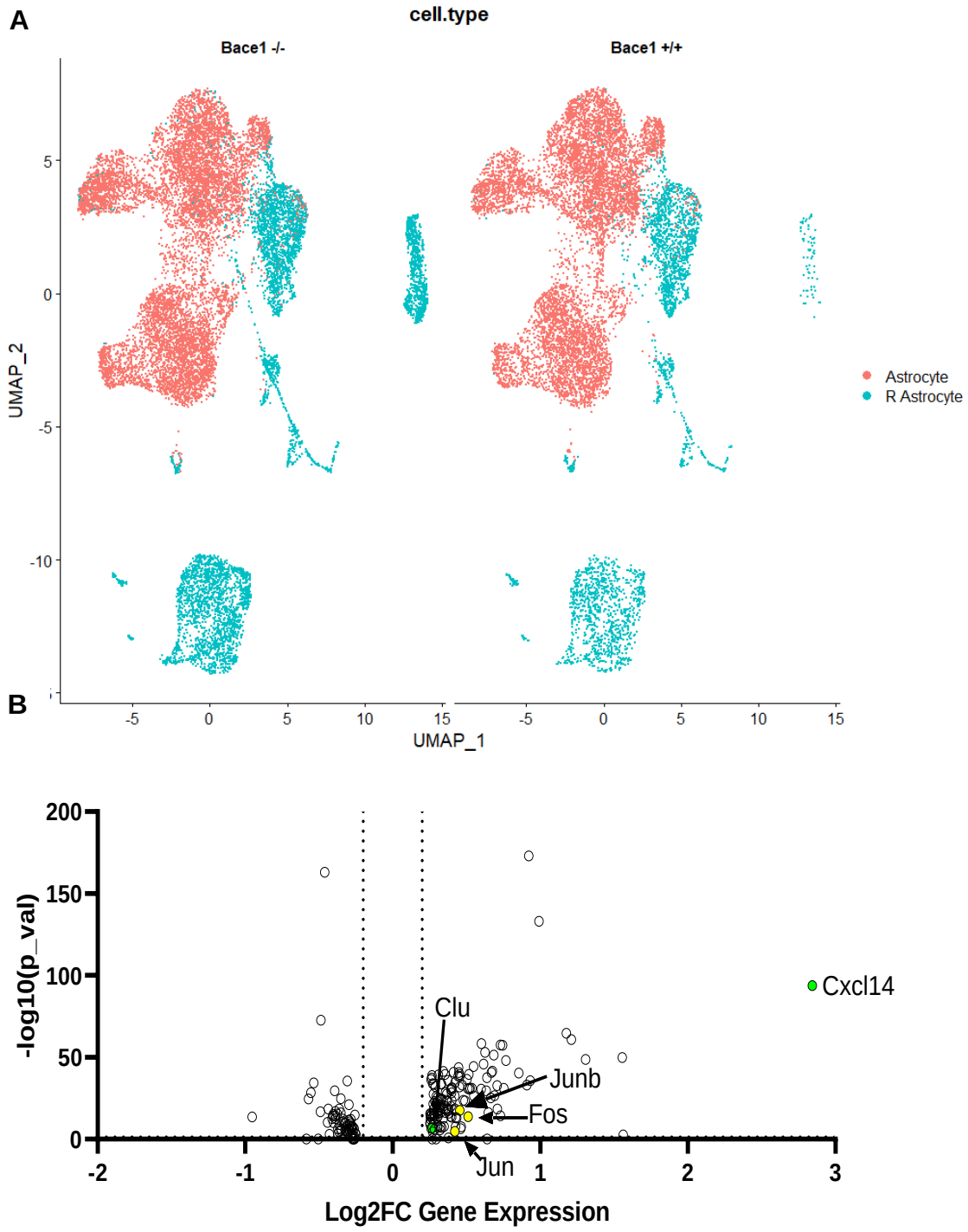
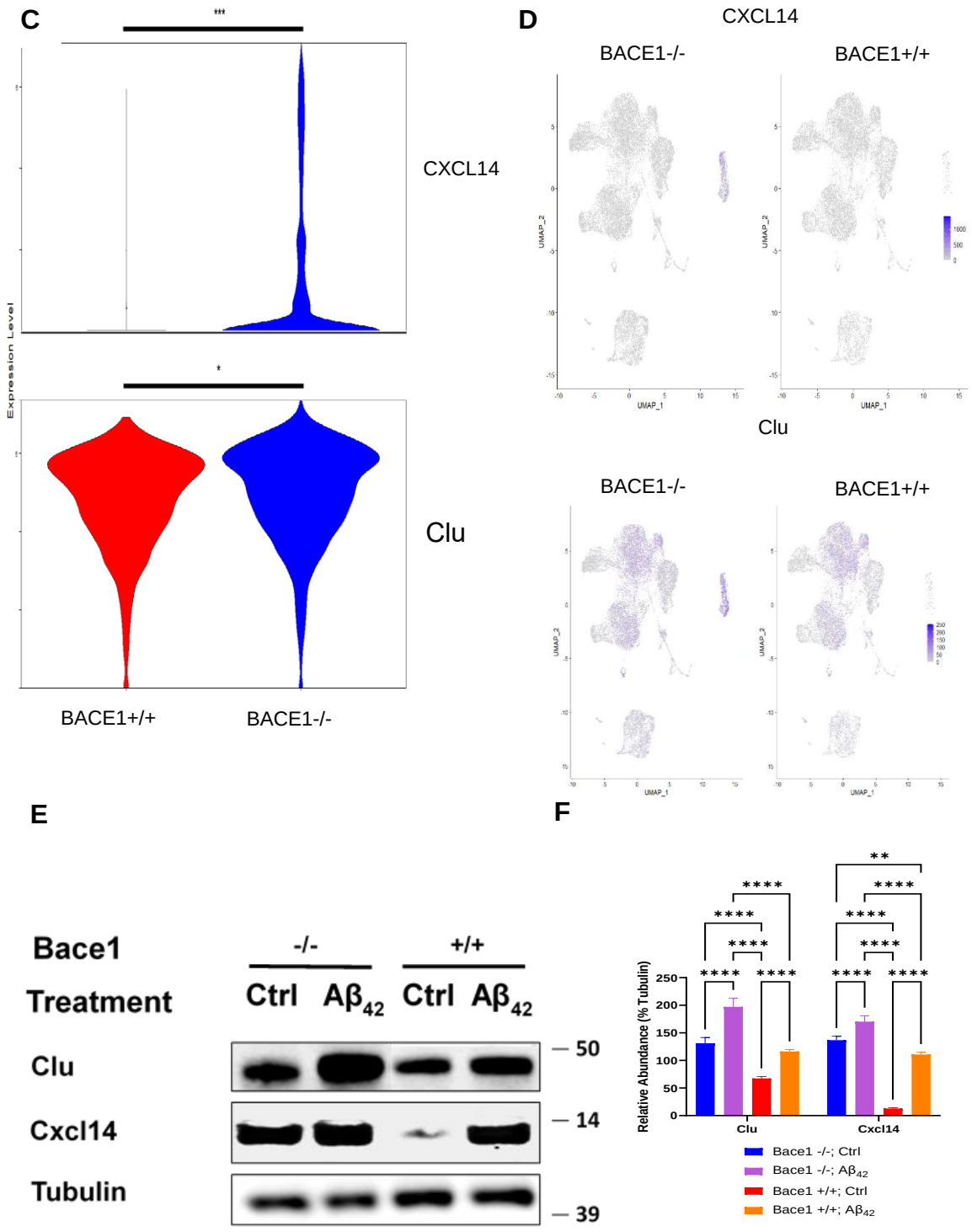
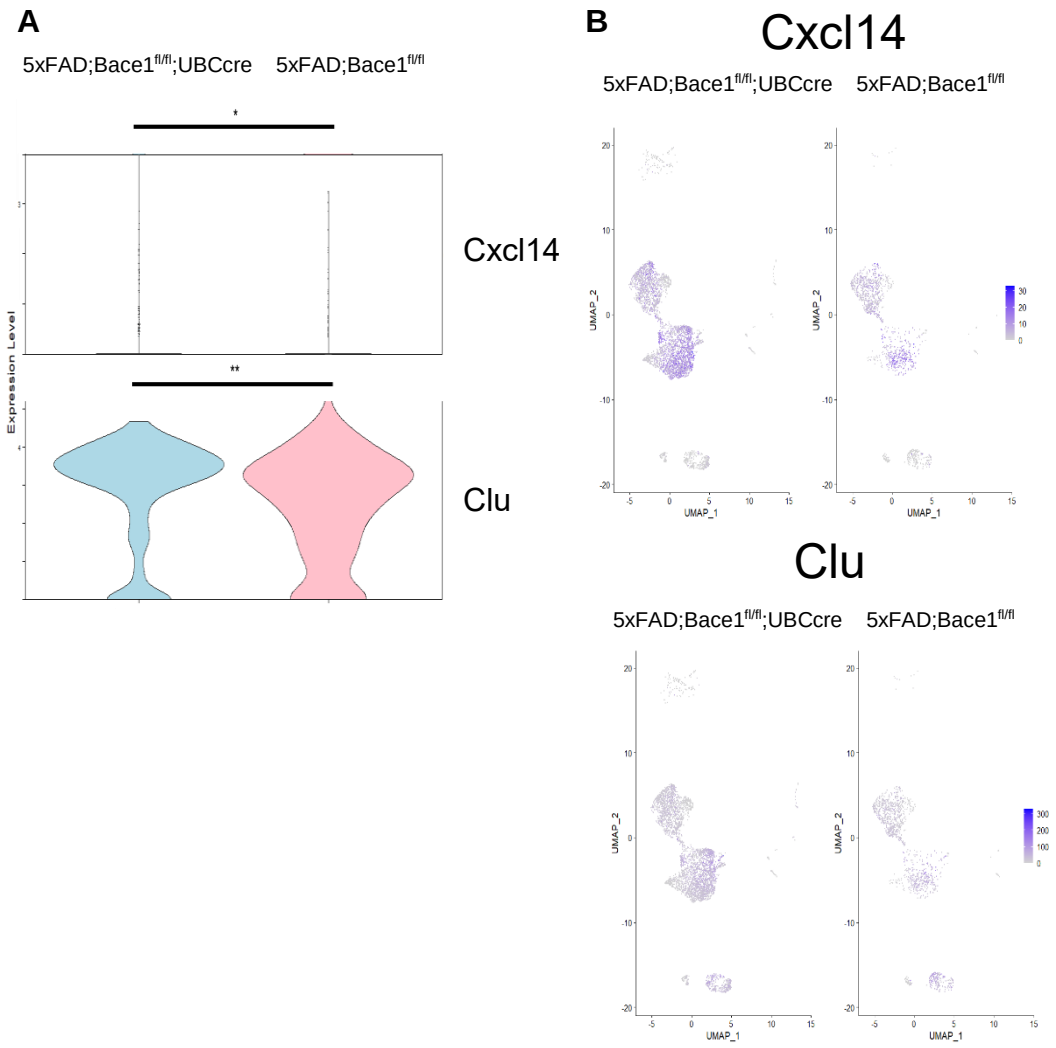


Figure 2.2 Continued



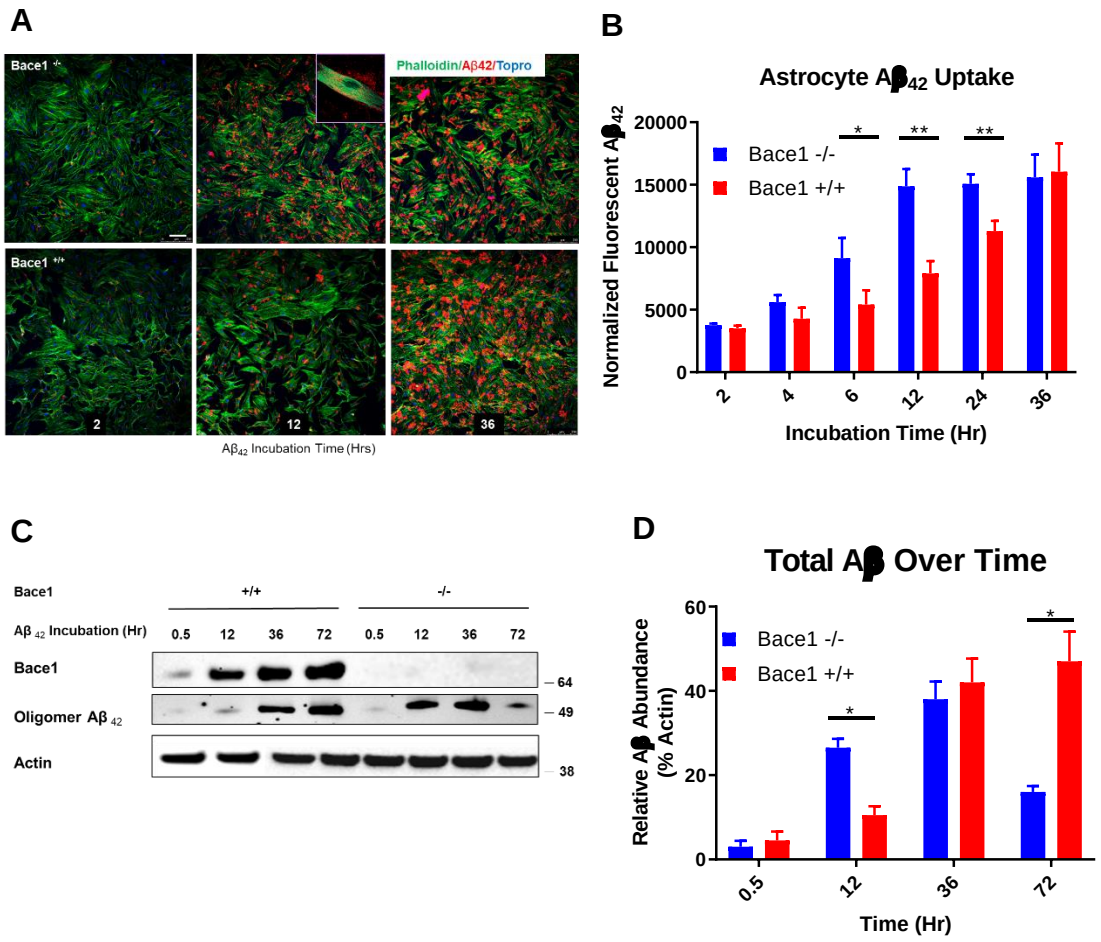
(A) Top differentially expressed genes comparing BACE1-null to WT reactive astrocyte transcriptomes expressed as a volcano plot of log2 fold change value gene expression and –log10(p-value), Wilcoxon ranked sum test was used to calculate p-value. Dotted lines indicate|log2(fold change)| > 0.2 and -log10(p-value) of 1.3, corresponding to p-value < 0.05,) . (B) Violin plot of genes of interest, Clu, and Cxcl14, expression comparing BACE1-null and WT reactive astrocyte clusters. * indicates p-value < 0.05, *** indicates p-value < 0.001, Wilcoxon ranked sum test was used to calculate p-value. (C) Distribution of cells highly expression Clu, and Cxcl14 with indicated log2fold change scales. (D) Western blotting of genes of interest – Clu andCxcl14 - in primary astrocyte culture lysate with or without 2 uM of aggregated Aβ42 treatment. (E) Quantification of western blot band intensity normalized to actin compared to BACE1 +/+ without Aβ42 treatment. N= 4, * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001. One-way ANOVA with Bonferonni post-test.

Figure 2.3 Adult BACE1 deficiency increases known amyloid clearance genes in reactive astrocytes



(A) Violin plot of genes of interest, Clu and Cxcl14, log2 fold change expression comparing 5xFAD;Bace1fl/fl;UBC-Cre BACE1fl/fl; UBC-cre;5xFAD and 5xFAD;Bace1fl/fl BACE1fl/fl; 5xFAD reactive astrocyte clusters. (C) Distribution of cells highly expressing Clu and Cxcl14 with indicated log2 fold change scaling.

Figure 2.4 BACE1 deficiency enhances astrocytic clearance of Aβ in vitro



(A) Confocal imaging of astrocyte primary cultures from BACE1-null and WT perinatal mice pups and treated with 2 uM of Aβ4242 tagged with fluorescent Hilyte-555 for indicated incubation times. Stained with phalloidin (green) to mark f-actin and ToPro3 (blue) to mark nuclei. Scale bars represents 10 uM. Inset shows magnified image of Aβ4242 within phalloidin marked astrocyte boundaries. (B) Quantification of Aβ4242 integrated fluorescence within phalloidin marked astrocyte boundaries and normalized to number of nuclei and astrocyte area. N= 6, * indicates p-value < 0.05, ** indicates p-value < 0.01, One way ANOVA, with Tukey post testing comparing between timepoints. (C) Western blot of astrocyte primary culture lysates from indicated genotypes and treated with 2 uM of aggregated Aβ4242 for the indicated incubation times. Images indicate major bands for BACE1, Aβ4242 (human, oligomeric), and actin. (D) Quantification of western blot Aβ4242 band intensity normalized to actin. N= 4, * indicates p-value < 0.05, One-way ANOVA, with Tukey post testing comparing between samples.

Figure 2.5 Clusterin underlies astrocyte endocytosis of Aβ in vitro

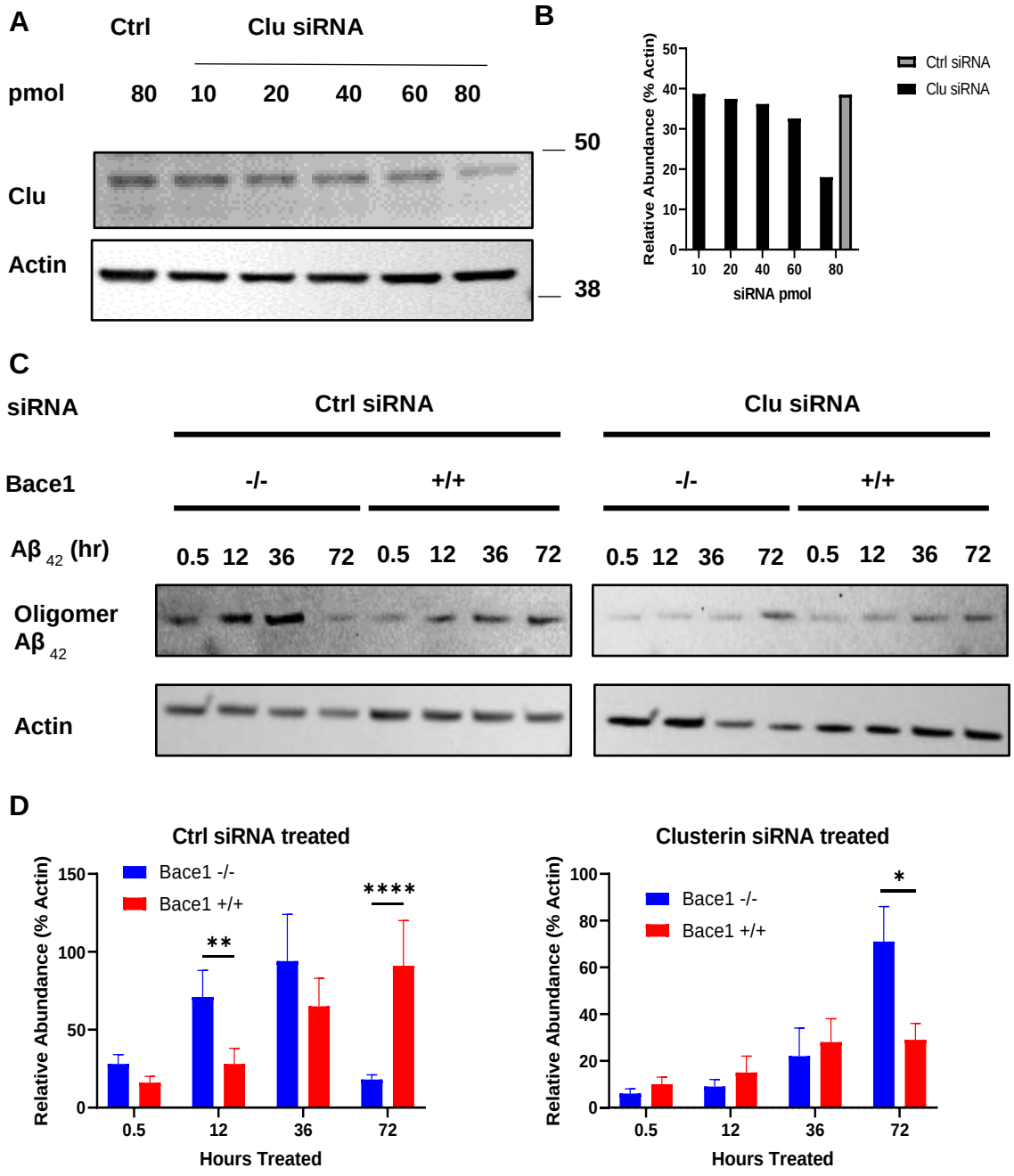
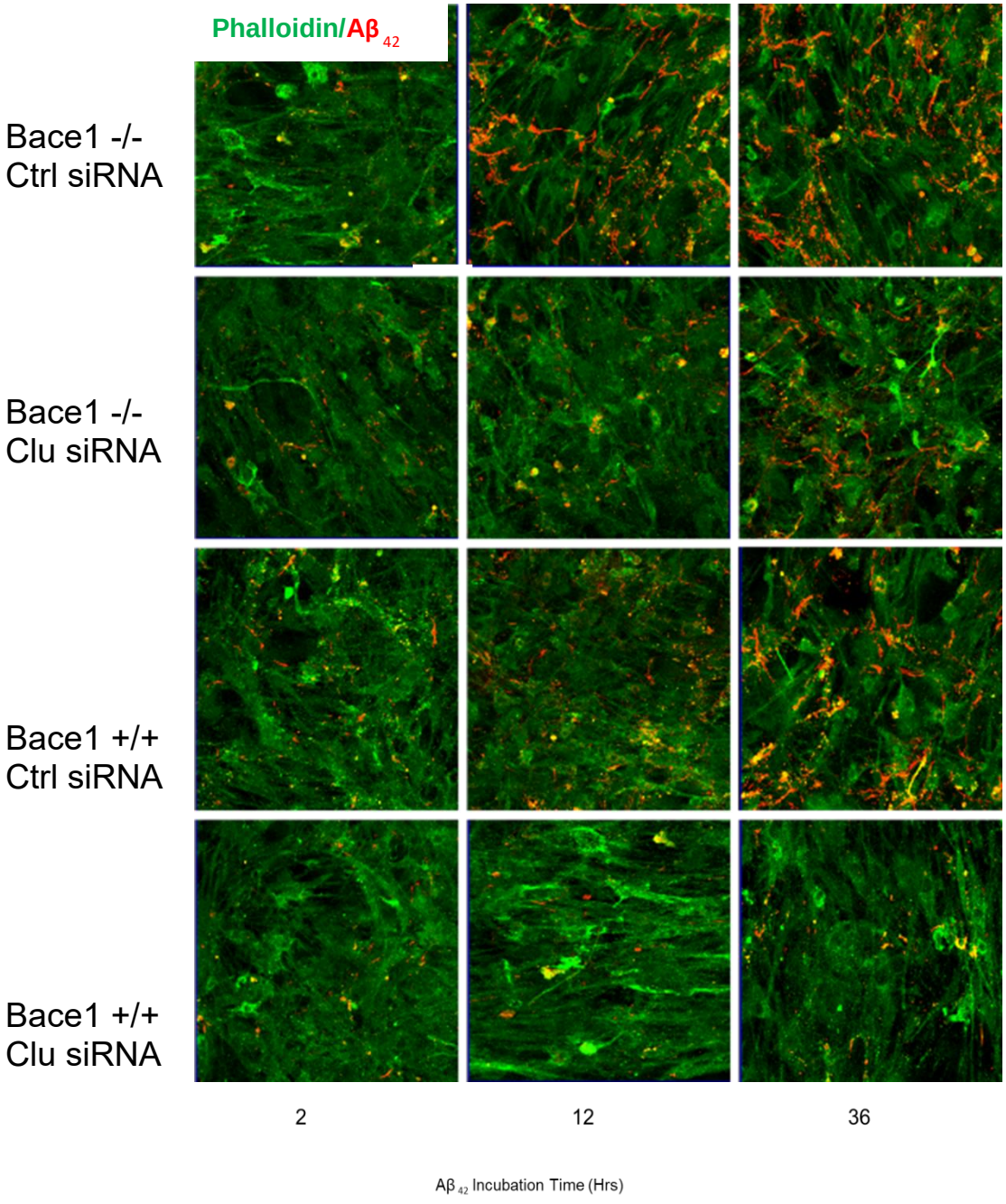


Figure 2.5 Continued

E



(A) Western blot of WT-primary astrocytes treated with either 80, 40, 20, 10 pmol of Clu siRNA or 80 pmol of control scrambled siRNA. Images indicate major bands for Clu and actin. (B) Quantification of Clu band intensity normalized to actin. 80 pmol of Clu siRNA results in an approximately 50% decrease in Clu levels compared to control siRNA. N=1. (C) Western blot of BACE1-null and WT-primary astrocytes pretreated with either 80 pmol of Clu siRNA or 80 pmol of control scrambled siRNA and incubated with 2 uM of Aβ4242 for indicated times. Images indicate major bands for Aβ4242 (human, oligomeric) and actin. (D) Quantification of western blot Aβ4242 band intensity normalized to actin. N= 4, * indicates p-value < 0.05, ** indicates p-value <0.01, **** indicates p-value <0.001, One-way ANOVA, with Tukey post testing comparing between samples.

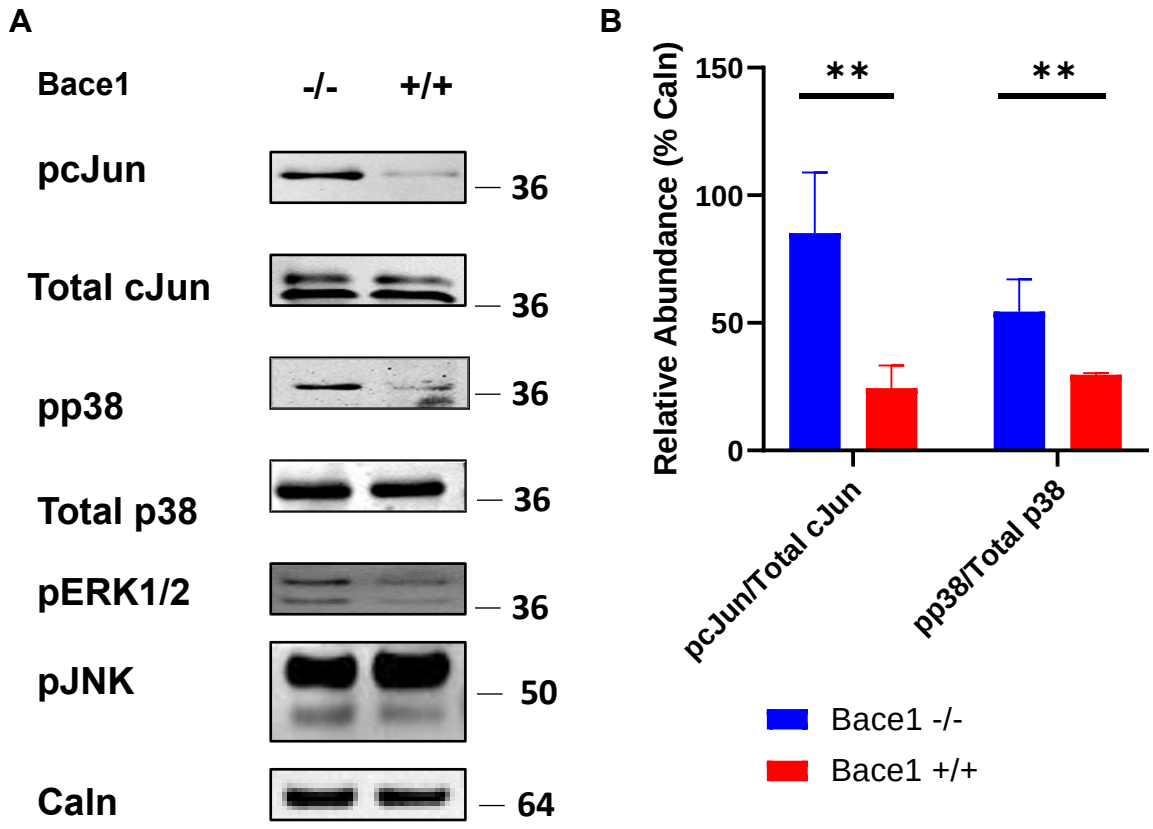
(E) Confocal imaging of astrocyte primary cultures from BACE1-null and WT and pretreated with either Clu or control scrambled siRNA then treated with 2 uM of Aβ4242 tagged with fluorescent Hilyte-555 for indicated incubation times. Stained with phalloidin (green) to mark f-actin and ToPro3 (blue) to mark nuclei. Scale bars represents 10 uM.

Table 2.1 Possible transcription factors underlying DEG transcription.

Transcription factor	Regulated genes	Database	Transcription factor family	
Jun	*ApoE, Ttr, Clu, CXCL14, GluL*	tred	Ap1	
KLF4	Aldoc, Tubb2b, Mfg8, *GluL*	ensembl	Zinc finger	
Creb1	Ntsr2, Scg3, Tubb2b	tred	Leucine zipper	
Ctcf	*GluL, Gpr7l1, ApoE*	ensembl	Zinc finger	
Esrrb	*Acsbg1, ApoE*, Eno1	ensembl	Orphan nuclear	
Tbp	*Clu*, Atp1b2, Sepp1	ucsc	Tata box binding	
Bach1	*Clu,* Prnp	ucsc	Leucine zipper	
Bach2	*Clu, CXCL14*	ucsc	Leucine zipper	
Fos	*ApoE, Clu, CXCL14*	ucsc	Ap1	
Fosb	*ApoE, Clu, CXCL14*	ucsc	Ap1	
Fosl1	*ApoE, Clu, CXCL14*	ucsc	Ap1	
Junb	*ApoE, Clu, CXCL14*	ucsc	Ap1	
Jund	*ApoE, Clu, CXCL14*	ucsc	Ap1	
Pparg	*Acsbg1, ApoE*	kegg	PPAR receptor	

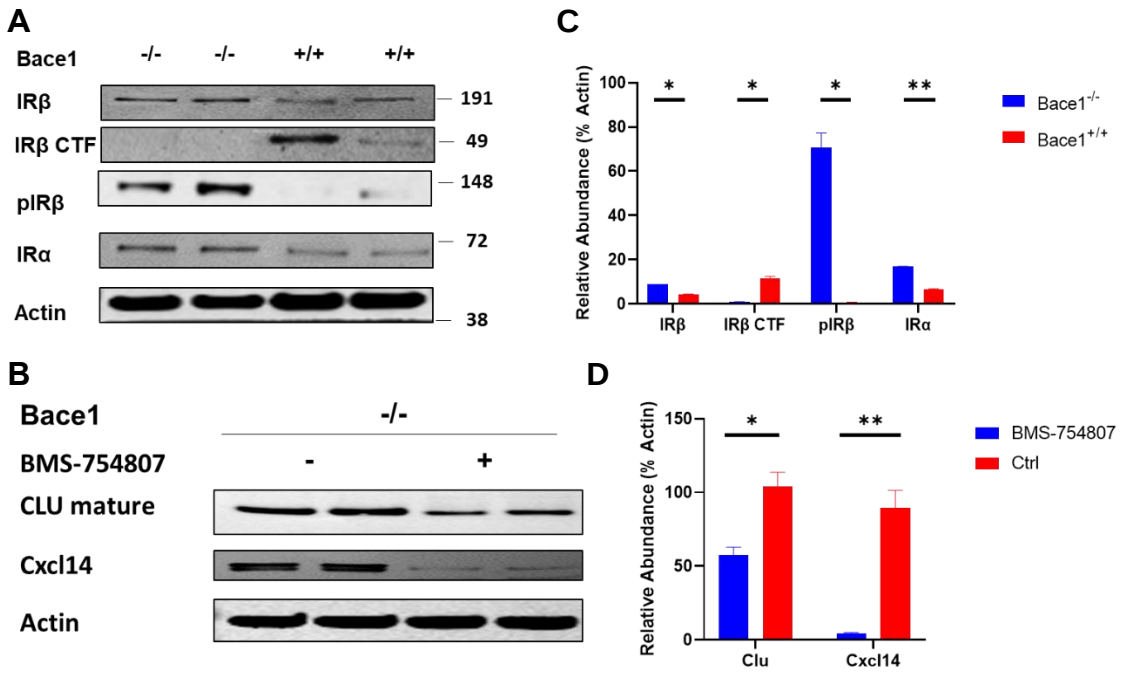
Possible transcription components and families of top 25 DEG from BACE1-null or WT reactive astrocytes using BioMart accessed gene information databases.

Figure 2.6 BACE1 deficiency enhances P38, ERK1/2, and cJun phosphorylation



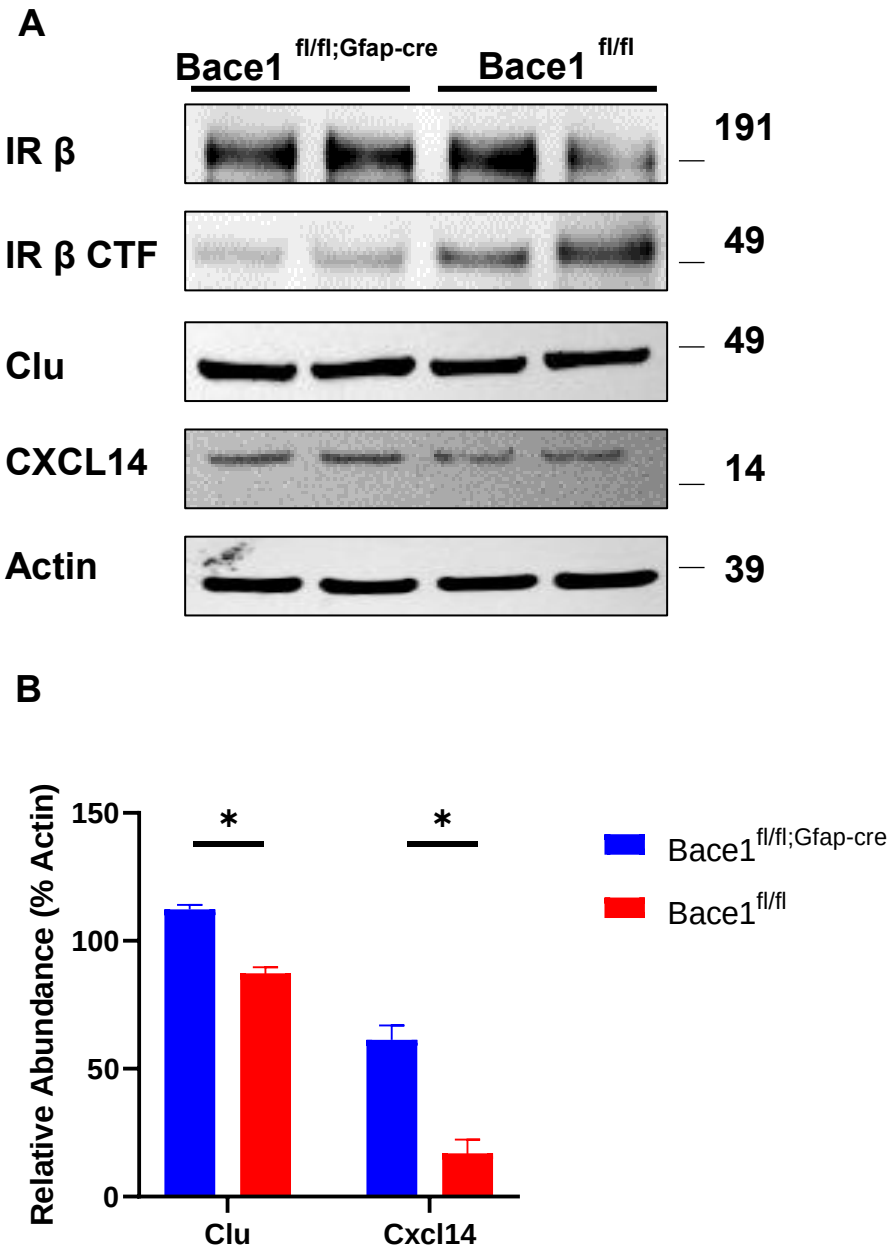
(A) Western blot of BACE1-null and WT primary astrocytes lysates. Images indicate major bands for pcJun, total cJun, pp38, total p38, pErk1/2, pJNK, and Caln. (B) Quantification of pcJun and pp38 band intensity normalized to actin total cJun and p38 respectively as well as Caln. N= 2, * indicates p-value < 0.05, One-way ANOVA, with Tukey post testing comparing between samples.

Figure 2.7 BACE1 deficiency preserves astrocytic IR bioavailability



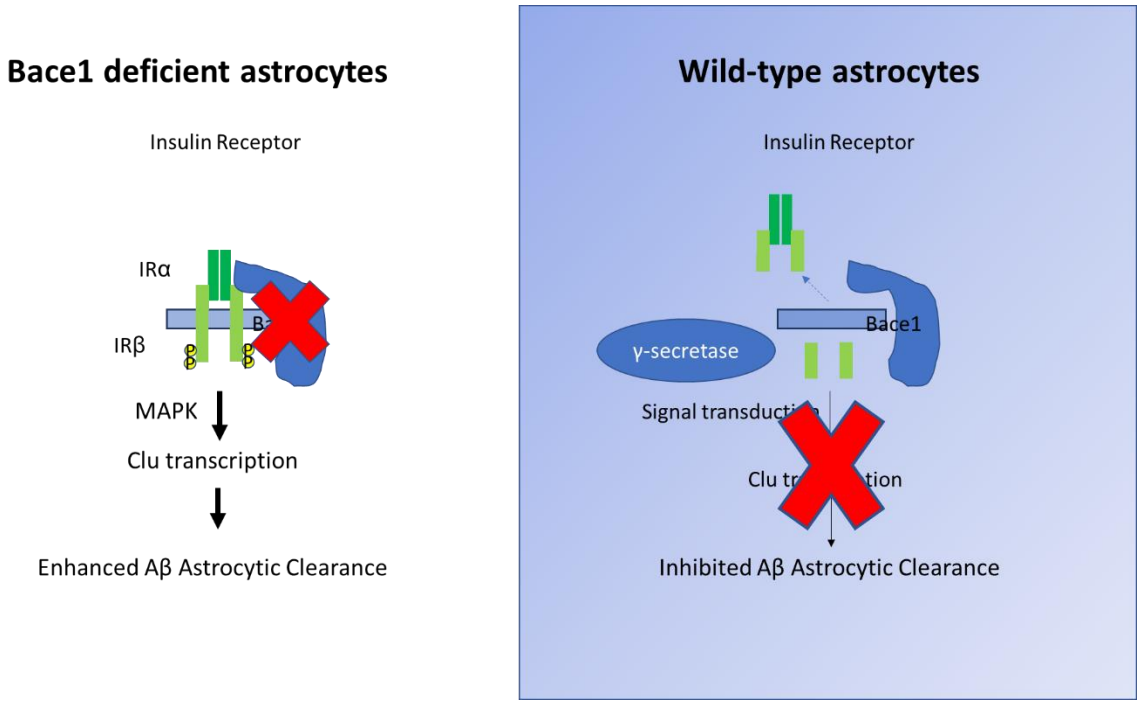
(A) Western blot of BACE1-null and WT primary astrocytes culture lysates. Images indicate major bands for IRβ, IRβ CTF, pIRβ, IRα, and actin. (B) Quantification of IRβ, IRβ CTF, pIRβ, and IRα band intensity normalized to actin. N= 4, * indicates p-value < 0.05, ** indicate p-value <0.01, One-way ANOVA, with Tukey post testing comparing between samples. (C) Western blot of BACE1-null primary astrocytes culture lysates treated with 1 uM of BMS-754807 or control. Images indicate major bands for Clu, Cxcl14, and actin. (D) Quantification of IRβ, IRβ CTF, pIRβ, and IRα band intensity normalized to actin. N= 3, * indicates p-value < 0.05, One-way ANOVA, with Tukey post testing comparing between samples.

Figure 2.8 Targeted astrocytic deletion of BACE1 enhances IR



(A) Western blot of ACSA2+ astrocyte enriched lysates from BACE1fl/fl;GfapFAP-Cre and BACE1fl/fl mice. Images indicate major bands for IRβ, IRβ CTF, ApoE, Clu, Cxcl14, and actin. (B) Quantification of IRβ, IRβ CTF, ApoE, Clu, Cxcl14 band intensity normalized to actin. N= 34, * indicates p-value < 0.05, One-way ANOVA, with Tukey post testing comparing between samples.

Figure 2.9 Model of BACE1 deficiency effect on reactive astrocyte.



Schematic diagram of gain of IR function as a result of BACE1 deficiency and downstream increases in p38, ERK1/2 pathways. This increases AP1 transcription of Clu levels, which then contributes to enhances amyloid endocytosis by BACE1-null reactive astrocytes. Wild type levels of BACE1 contributes to IR cleavage thereby reducing these signaling pathways.

Chapter 3: **Novel role of CXCL14 in AD**

1. Introduction

The complex interplay between the immune system and AD is complex. Various pro and anti-inflammatory cytokines, chemokines, and immunomodulatory make up the AD inflammatory milieu. As in many diseases associated with inflammation, in AD, pro inflammatory cytokines play an important role in activating glia cells for Aβ clearance in the acute phase. However, as AD pathology persist, recurrent or chronic exposure to pro-inflammatory cytokines can inhibit Aβ clearance and perpetuate neuronal death.(Kinney et al., 2018) Initial epidemiological studies suggested that AD patients taking general non-steroidal anti-inflammatory drugs (NSAID) were associated with improved cognition.(Paul S. Aisen, 2002) More recently, long-term comprehensive analysis has shown that the majority of NSAID were not associated with cognitive improvement, although diclofenac was found to be beneficial in reducing AD incidence and improving cognition.(Rivers-Auty et al., 2020) Recent trends in the AD dug pipeline have continued to develop specific immunomodulation rather than broad anti-inflammatories for the treatment of AD. Of the 64 disease modulating therapies in Phase II clinical trials for AD, 19% of these therapies are related to inflammation, infection, or the immune system. (Cummings et al., 2021) Further, inspection reveals that most of these new immunomodulatory drug targeting specific cytokines or immune cell compartments. Therefore, it is critical to understand how these individual cytokines affect AD pathogenesis.

Previous research by our lab (see previous chapter) discovered that astrocytic expression of the orphan chemokine, CXCL14, was positively associated with increased glial Aβ clearance. As an orphan chemokine, CXCL14 has no obligate receptor, although research has primarily focused on CXCL14 positive interaction with Cxcr4 and other homeostatic systems.(Kouzeli et al., 2020) CXCL14 has well known roles in immune surveillance, cancer progression, and metabolism, however its function in the CNS is relatively unknown.(Lu, Chatterjee, Schmid, Beck, & Gawaz, 2016) CXCL14 is expressed in developing neurons, reactive astrocytes, and microglia. (Banisadr et al., 2011; Lee et al., 2017; Liu et al., 2020). In an MCAO stroke model, CXCL14 supplementation almost completely ameliorated the infarct volume, by promoting an anti-inflammatory environment and the recruitment of T-regulatory cells. (Lee et al., 2017) The role of CXCL14 in AD pathogenesis is currently unknown, although as mentioned before CXCL14 was associated with increased glial Aβ clearance and 2 single nucleotide polymorphisms were associated with increased CSF tau. (Chung et al., 2018). Therefore, we aim to understand the role of CXCL14 on AD pathogenesis. We hypothesize that CXCL14 supplementation may act in a beneficial manner for AD pathogenesis, by enhancing microglial clearance of Aβ and promoting neuronal survival.

To understand the role of CXCL14 on microglia Aβ clearance, we studied the role of CXCL14 on microglia chemotaxis and Aβ uptake. We found that primary microglia were attracted to CXCL14 gradients in a dose-dependent manner in an in vitro, trans-well system. Furthermore, we found that lentiviral intracerebral

injection of with a CXCL14 overexpression construct resulted in an intense localization of microglia. We also found that CXCL14 supplementation enhanced microglial uptake of Aβ in both acute and recurrent Aβ attack conditions in a Cxcr4-dependent manner. This suggests that CXCL14 can directly promote microglial Aβ clearance.

To understand the role of CXCL14 on neuronal survival, we isolated primary neurons and treated them with combinations of Aβ, CXCL14, and inhibiting CXCR4 antibody and measured media lactate dehydrogenase-based cytotoxicity assay. We found that CXCL14 protected neurons from recurrent Aβ attack in a Cxcr4 dependent manner. As metabolic distress is associated with Aβ induced neurotoxicity, we investigated role of CXCL14 on mitochondrial dynamics and anti-oxidant defense systems. We found that CXCL14 enhanced expression of both mitochondrial dynamic and anti-oxidant system markers that are depleted by recurrent Aβ attack treatment. This suggests that CXCL14 can act directly to protects neurons from chronic Aβ exposure by activating neuronal metabolism.

Altogether, we found that CXCL14 supplementation may be a novel avenue for AD therapy by activating microglial Aβ clearance and protecting against neurotoxicity.

2. Materials and Methods

Mouse strains and breeding strategy

C57/Bl6 and Tg (APPSwFlLon,PSEN1*M146L*L286V) mice (5xFAD; Jackson Laboratory) were used for this study. All lines were routinely backcrossed with C57BL/6J mice for at least five generations to ensure consistent genetic background for phenotypic analyses.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Lerner Research Institute and University of Connecticut Health Center in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory.Animals.

Preparation of aggregated $A\beta_{42}$

Non-tagged $A\beta_{42}$ peptide (ThermoFisher) and fluorescent Hilyte 555 tagged-$A\beta_{42}$ (Aβ-555) (Anaspec) were prepared according to previous reports (Stine et al., 2011). Briefly, peptides were solubilized in 0.1% NH4OH containing 0.01% (w/v) NaN3, and stored at -20°C. Just prior to use, peptides were oligomerized by incubation with constant rotation for 24 hours at 4°C in DMEMF12 or fibrillized by incubation with constant rotation for 24 hours at 37°C in PBS. Formation of beta-sheets was detected by Thioflavin-S fluorescence indicating the formation of aggregated $A\beta_{42}$ (data not shown). Cells were treated with 2 uM of oligomeric or fibrillar Aβ (oAβ or fAβ) unless otherwise indicated. Furthermore, unless specifically indicated “Aβ” refers to oligomerized Aβ.

Primary perinatal astrocyte culture and isolation

Primary astrocytes cultures were prepared from C57/Bl6 perinatal mouse pups. Primary mixed glia were prepared from P1-3 as described previously (Chen et al., 2010; Schildge et al., 2013). Briefly, brain tissue was isolated from skulls, bisected, and then incubated with 0.25% TriZol at 37°C for 15 minutes, with occasional gently swirling. Afterwards, tissue solution was gently triturated with fire-polished glass pipettes and passed through a 70 micron filter to form a single-cell suspension. The mixed glia culture was cultured in a T75 flask in DMEM-F12 containing 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, and 1% penicillin/streptomycin (Life Technologies) for 12-14 days. Mixed glia cultures were split twice with 4-5 day intervals to achieve a pure mature astrocyte culture.

Astrocytes were seeded 1x10^6 for 6 well plates for Western blot and 1x10^4 for 8 well chamber sides and media was switched to DMEM-F12 medium containing 1% G5 astrocyte media supplement (Gibco) for 12-24 hours before treatment. Cells were maintained and grown in a humidified atmosphere of 5% CO2 at 37°C. Astrocytes were treated for 36 hours for 2 uM of oAβ or fAβ before lysate was collected for Western blot.

Primary microglial isolation and culture

Primary microglial glia were prepared from C57/Bl6 perinatal mouse pups.(Tamashiro, Dalgard, & Byrnes, 2012) Briefly, brain tissue was isolated from skulls, bisected, and then incubated with 0.25% TriZol at 37°C for 15 minutes, with occasional gently swirling. Afterwards, tissue solution was gently triturated with fire-polished glass pipettes and passed through a 70 micron filter to form a single-

cell suspension. The mixed glia culture was cultured in a T175 flask in DMEM-F12 containing 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, and 1% penicillin/streptomycin (P/S) (Life Technologies) for 14-21 days. Mixed glia cultures were oscillated at 100 rps at 37℃ for 1 hours. Media containing microglia was collected and replated at 1x10^6 for 6 well plates for Western blot, 1x10^4 for 8 well chamber sides and 12 well transwell inserts. Media was switched to RPMI medium containing 10% FBS, 1% P/S for 24 hours before treatment. Cells were maintained and grown in a humidified atmosphere of 5% CO2 at 37°C.

Primary neuron isolation and culture

Primary neurons were prepared from C57/Bl6 neonatal mouse pups according to previously published methods.(Subramanian, Michel, Benoit, & Nedivi, 2019) Hippocampi and cortex were carefully isolated from meninges and the rest of the brain. Single cell suspension was prepared by incubated brain tissue with papain and DNase, then gently triturated with fire-polished glasses pipettes, and filtered through a 22 micron filter. 2x10^6 cell solutions were plated on 0.1% coated poly-d lysine coated 6-well plates and Neurobasal (Gibco) with L-glutamine and 10% FBS (Gibco). After 24 hours, media was switched to Neurobasal media with B27 neuronal supplement (Gibco) and Glutamax (Gibco) and maintained for 14 days until treatment. Cells were maintained and grown in a humidified atmosphere of 5% CO2 at 37°C.

In vitro chemotaxis assay

1x10^5 microglia were placed on the apical chamber of 12 well, 8 uM pore inserts (ThermoFisher). RPMI media containing the 0, 10, 100 ng/mL of human,

recombinant CXCL14 protein (R&D) in PBS was placed in the basal portion of the transwell system. After 24 hours, transwell membranes were fixed with 4% PFA for 10 minutes and stained with toluidine blue. Basal side of the transwell membranes were imaged with Keyence microscope, 2x objective. Quantification of microglial was performed manually.

Lentivirus CXCL14 overexpression

pLenti-C-mGFP- Puro- ORF-mCXCL14 (NM_019568) or pLenti-C-mGFP-Puro-ORF was purchased from Origene for the purpose of inducing ubiquitous CXCL14 over expression with GFP as a labeling tag. The promoter for this CXCL14/GFP or GFP construct was CMV. We first tested lentiviral effectiveness in primary astrocytes by incubating lentiviruses at different multiplicity of infections (MOI) for 24 hours. We collected these lysates and probed for GFP by Western blot.

Based on effective MOI, we stereotaxically injected hippocampi of 1.5 month old C57/Bl6 mice with 7.8*10^10 lentiviral particles.(Neurostar) Mice were anesthetized by ketamine/xylazine and anesthesia was maintained by isoflurane inhalation. Post-operative buprenorphine was given as an analgesic. After 2.5 months, mice brains were extracted were extracted for immunohistochemistry staining and imaging.

All handling of replication-deficient lentiviral protocols were performed in BSL2 or ABSL2 containment systems. All experimental protocols were approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee of the University of Connecticut Health Center in compliance with the

guidelines established by the Public Health Service Guide for the Care and Use of Laboratory.Animals.

Microglial Aβ uptake assay

Primary microglial were plated onto 8-well chamber and were pretreated with either 0.1% NH4OH (Ctrl) or 2 uM of oAβ for either acute or recurrent/chronic Aβ conditions according to previous literature.(Baik et al., 2019) Pretreatment occurred for 24 hours, before media refresh for two days afterwards. Microglial were then treated with either oAβ-555 alone, or oAβ-555 in conjunction with CXCL14 (100 ng/mL), or oAβ-555 in conjunction with CXCL14 (100 ng/mL) and a functional inhibitory antibody for CXCR4 (αCXCR4) (2 ug/mL) (ebioscience) (Feldbauer et al., 2016)for 24 hours before immunohistochemical staining.

Immunohistochemistry imaging

For tissue staining, mice were anesthetized with ketamine/xylazine and then perfused with 4% PFA. Brains were carefully extracted, post fixed in 4% PFA for at least 24 hours, then incubated with 30% sucrose for at least 24 hours. Brains were embedded and frozen in optical cutting temperature (OCT) and sections were cut at 14 uM with a cryostat (Leica). Sections were permeabilized in 0.3% Triton-X100 (Sigma Aldritch) for 30 minutes and underwent citric acid antigen retrieval. Next sections were blocked in 5% goat serum and then incubated with primary antibody at 4℃ overnight. Sections were incubated with corresponding host secondary antibody for 2 hours at 37℃. For 5xFAD brains, section were stained with 0.05% thioflavin-S for 10 minutes to mark Aβ plaques.

For cell staining, cells were plated onto 8 well chamber slides. After treatment, cells were fixed in 4% PFA for 10 minutes before blocking.

Images were taken by confocal microscopy (Zeiss). For microglial Aβ uptake assay image quantification was performed by measure raw image density within microglia boundaries marked by Iba1 and normalized to Iba1 area size. Quantification was performed in ImageJ. See antibody list for antibodies.

Cytotoxicity assay

For cytotoxicity assay and WB analysis of metabolic dysfunction markers, we plated microglia and neurons in a 6-well plate. Cells were pretreated in a similar manner as microglia Aβ uptake assay with either 0.1% NH4OH (Ctrl)of 2 uM oAβ for 24 hours before media refresh. Afterwards cells were treated with either Aβ (2 uM), CXCL14 (100 ng/nL), αCXCR4 (2 ug/mL), or different permutation of each. After 24 hours, media was taken and relative cytotoxicity was measured using Cyquant Cytotoxicity Assay (Promega), an LDH-based assay. Absorbance was measured at 490 (signal) and 680 (background). Lysate was collected for WB analysis of metabolic dysfunction markers.

Western blotting

Cell cultures were washed twice with ice-cold PBS and lysed on in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml aprotinin, for 10-30 minutes at 4°C with rotation. The lysate was collected and further bath sonicated on ice for 30 s on and off cycles at 60 hz for 5 minutes and then centrifuged at 15 000 × g for 30 minutes at 4°C. Protein

concentrations were determined using a BCA assay kit (Pierce). Equal amounts of protein from each sample were loaded and electrophoretically resolved on 4%–12% SDS-PAGE (NuPAGE system, Life Technologies) gels. After electrophoresis, proteins were transferred to nitrocellulose membranes at 100V for 2 h. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hr at room temperature. The membranes were probed with primary antibody (see table for antibody list and dilution), followed by incubation with appropriate secondary HRP-conjugated antibody (1:2,000). The antibody-bound proteins were detected by an iBright 1500 imaging system (Invitrogen). To ensure equal loading, the blots were reprobed with monoclonal anti-actin (1:10,000). For quantification purposes, band intensities of immunoblots were analyzed using image J software.

Antibody list used for the study

Antibody Name	Catalog no.	RRID:	Manufacturer
Actin (Clone AC-15)	A5441	AB_476744	Sigma
Catalase	14097	AB_2798391	Cell Signaling
CXCL14	ab137541		Abcam
GFAP	G-3893	AB_477010	Sigma
GFP	8362-1		Clontech
Iba1	019-19741	AB_839504	Wako
Mff	17090-1-AP	AB_2142463	ProteinTech
Mfn2	12186-1-AP	AB_2266320	ProteinTech
Sod1	37385		Cell Signaling
Sod2	13141	AB_2636921	Cell Signaling

Statistical analysis

Bar graph results are expressed as mean±SD. The statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego). Student's t-tests were used to compare between 2 groups. Two-way ANOVAs were used to compare multiple groups, post-test of Sidak or Bonferonni was used to compare between different groups. Differences with * indicating $p < 0.05$, ** indicating $p < 0.01$, and *** indicating $p < 0.001$ were considered significant.

3. Results

CXCL14 expression in AD reactive astrocytes

Previous scRNA-seq revealed that CXCL14 gene expression was increased in BACE1-null reactive astrocytes with enhanced Aβ.(Figure 2.2 B,C, and D) We further confirmed that CXCL14 is expressed in wild-type astrocytes and expression is increased in reactive astrocytes responding to acute Aβ treatment. (Figure 2.2E and F) To confirm expression of CXCL14 in vivo, we subjected 4 month old 5xFAD brains to immunohistochemistry and found concentration of CXCL14 surrounding Aβ plaques and reactive astrocytes stained with GFAP. (Figure 3.1A)

In order to understand overall CXCL14 expression levels as disease progresses, we performed immunoblot of whole brain lysate from 5xFAD or WT littermate controls (Figure 3.1B) At P90, levels of CXCL14 expression are similar between 5xFAD or WT irrespective of sex. However at P266, levels of CXCL14 levels were lower compared to age-matched WT controls, suggesting that continued AD progression results in a downregulation of CXCL14 expression.

This suggests that CXCL14 expression is stimulated by Aβ in a local or acute manner in reactive astrocytes. However, as AD progression increases, either chronic Aβ exposure or inflammatory factors may downregulate total CXCL14 levels. If CXCL14 proves to be protective, then downregulation of CXCL14 in later AD stages may be indicative of failure of homeostatic system at this disease time point. Therefore, supplementation of CXCL14 may help to restore homeostatic conditions and ameliorate AD pathology.

CXCL14 chemotactic effect on microglia

Previous research has largely focused on the effect of CXCL14 on peripheral immune cell migration but the effect on microglia migration, the main phagocytic cell in the CNS, has not been investigated. To understand if CXCL14 can induce microglia migration, we used a transwell migratory assay and found a significant increase in microglia migration with increasing doses of CXCL14. (Figure 3.2A and B) As an additional negative control we also tested microglial chemotaxis with 10% FBS and found no appreciable migration, thereby suggesting that CXCL14 specifically induced chemotaxis and not a non-specific protein. (Data not shown)

Next, we tested whether CXCL14 overexpression can induce microglia chemotaxis in vivo. To induce CXCL14 overexpression, we first designed a lentiviral particle containing an open reading frame for CXCL14 fused with a GFP tag. (LV-CXCL14/GFP) (Origene) We then treated primary astrocytes cultures with LV-CXCL14/GFP or LV-GFP with different multiplicities of infections – i.e. the ratio between viral particles to cells. (Figure 3.2C) We probed for GFP, which expectedly showed GFP bands at approximately 30 kD molecular weight for LV-GFP treated samples, as well as CXCL14/GFP fusion protein at 44 kD for LV-CXCL14/GFP. We found that a 1 MOI was sufficient to induce CXCL14/GFP expression comparable to 10 MOI. Based on this effective dosage of 1 MOI of CXCL14/GFP, we stereotaxically injected 1.5 month old C57/Bl6 mice with 7.8*10^10 LV- CXCL14/GFP or LV-GFP particles into the right cortex.(Figure 3.2D) Afterwards, mice brains section were subjected to IHC for Iba1 and

CXCL14. We found that LV-CXCL14/GFP injection resulted in intense local concentration of Iba1 positive microglia, whereas LV-GFP did not induce microglia migration. This indicates a specific attraction by microglia to CXCL14 rather than attraction to injection sites.

Altogether this suggests a model where initial Aβ stimulation from reactive astrocytes results in CXCL14 expression. CXCL14 then specifically can enhance microglia migration to Aβ plaque sites. Once at the Aβ plaque site microglia can participate in Aβ clearance.

CXCL14 enhance microglial Aβ uptake

Next, we wanted to know if CXCL14 played a directed role in microglial Aβ uptake within the context of acute and recurrent Aβ treatment. To do this we pretreated primary microglia with either 0,1% NH4OH (Ctrl) or 2uM of Aβ for 24 hours to mimic acute and recurrent conditions.(Baik et al., 2019) After two days of media refresh, primary cultures were treated either Aβ- 555 (Anaspec) with a red fluorescent tag alone, or Aβ- 555 in conjunction with 100 ng/mL of CXCL14 and/or 0,2 ug/mL of an functional inhibitory antibody that targets the CXCR4 receptor for internalization. (αCXCR4) (ebioscience) (Feldbauer et al., 2016) We found that CXCL14 treatment significantly increased uptake of fluorescent Aβ in both control and Aβ pretreatment groups. (Figure 3.3 A and B) In the acute case (Ctrl pretreatment), CXCL14 enhanced Aβ uptake by almost 50% compared to control. Co-treatment with CXCL14 and αCXCR4 did not enhance Aβ uptake, suggesting that CXCL14 activation of microglial Aβ uptake is CXCR4 dependent. A similar pattern emerges in the recurrent conditions (Aβ pretreatment), with

CXCL14 enhance microglial Aβ uptake in a CXCR4-dependent manner. However, overall levels of uptake were reduced by approximately 50% compared to the acute cases. This confirms previous treatment paradigms that showed reduced phagocytic capacity by microglial and monocytes after recurrent or chronic Aβ exposure. (Baik et al., 2019)

Together, this data suggests that CXCL14 enhances microglial Aβ uptake in both acute and chronic Aβ conditions and via the CXCR4 axis. To extend the previously described model for CXCL14 role in microglial Aβ clearance, CXCL14 not only promotes microglial attraction to Aβ plaques, but once microglia arrives CXCL14 also facilitates the Aβ uptake. Afterwards, microglia can directly degrade Aβ by shuttling to lysosome and endosomes containing Aβ degrading enzymes.

CXCL14 protects against chronic Aβ toxicity

Previous reports have shown that chronic Aβ exposure can result in neuronal cell death in vitro via metabolic dysfunction and that restoration with anti-inflammatory or homeostatic system can correct (Giordano, Terlecky, Bollig-Fischer, Walton, & Terlecky, 2014; Kadowaki et al., 2005) To understand if CXCL14 might also be able to protect against Aβ toxicity, we treated both microglia and neuron (3.4A and B respectively) with various permutations of Aβ, CXCL14, and αCXCR4.in both acute (control pretreatment) and recurrent (Aβ pretreatment) conditions. Media was collected and cytotoxicity was measured by Cyquant Cytotoxicity assay (Promega) an LDH-based assay. We found that in acute setting both neurons and microglia were resistant to Aβ induced

cytotoxicity. However, in recurrent Aβ pretreatment groups, a second Aβ insult did result in increased cytotoxicity for both microglia and neurons, although neuronal cell death was more sensitive to Aβ. Furthermore, we found that in neurons, the protective effect of CXCL14 was CXCR dependent, whereas in microglia it was not.

Our results suggest that in addition to promoting microglial clearance of Aβ, CXCL14 may act to protect cells, especially neurons, against chronic Aβ exposure. In neurons, CXCL14 may act through the CXCR4 receptor, although the protective effect of CXCL14 on microglia may act through other potential receptors, such as CXCR7.

CXCL14 activates neuronal metabolic dynamics after chronic Aβ

As mentioned previously, chronic Aβ exposure results in neuronal dysfunction via metabolic dysfunction by disruption of mitochondrial fission/fusion and decreased protection against reactive oxygen species (ROS). (Giordano et al., 2014; Kadowaki et al., 2005) In order to understand how CXCL14 might protect against metabolic dysfunction, we performed Western Blots with neuronal lysates from the cytotoxicity assay described in the previous section.(Figure 3.5)

We first examined mitochondrial dynamics by probing for Mitofusin protein 2 (Mfn2) and Mitochondrial fission factor (Mff).(Figure 3.5A) In the acute settings, treatments did not appear to significantly effect Mfn2 or Mff levels, indicating little changes in mitochondrial dynamics. However, in the chronic setting, pretreatment with Aβ resulted in slight downregulation of Mfn2, but significant reduction in Mff, even without secondary Aβ treatment. CXCL14 treatment

appears to significantly increase both Mf2 and Mff levels, indicating robust reactivation of mitochondrial fission/fusion dynamics. CXCL14 treatment also appears to slightly elevate Mf2 and Mff even in the presence of secondary Aβ treatment. This protection of mitochondrial dynamics via CXCL14 appears to by CXCR4 dependent, as treatment with αCXCR4 prevent Mf2 and Mff elevation.

Next we examined key members of the antioxidant defense system Superoxide dismutase 1 (Sod1), Superoxide dismutase 2 (Sod2), and Catalase.(Figure 3.5B) Interestingly, we see a similar pattern with these markers of the antioxidant defense system and mitochondrial dynamics. Acute exposure to these treatment have no effect on Sod1, Sod2, or Catalase. Chronic Aβ insult however results in downregulation of these markers, but is rescued by CXCL14 treatment even with secondary Aβ treatment.

Because of similar patterns between CXCL14 protective effects on cytotoxicity, mitochondrial dynamics, and antioxidant defense system, it appears that CXCL14 does appear to have a protective effect on neuronal cell death via reactivation of cellular metabolism in chronic Aβ conditions. In neurons, these CXCL14 protective effects appears to be mediated by CXCR4, while in microglial, CXCL14 did not appear to be CXCR4 dependent. This suggests that CXCL14 may act in complex cell and receptor-specific manners that remain to be uncovered.

4. Discussion

CXCL14 is a homeostatic chemokine with known roles in the immune system, metabolism, and cancer.(Lu et al., 2016) As an orphan chemokine, CXCL14 has potential to synergistically bind to several homeostatic receptors, but CXCL14 interaction with CXCR4 has been most well studied.(Kouzeli et al., 2020) Although CXCL14 has been detected in developing neurons and astrocytes, relatively little is understood about the role of CXCL14 in the central nervous system diseases, let alone Alzheimer's disease.(Banisadr et al., 2011; Lee et al., 2017) Although one report indicates that CXCL14 acts in a beneficial manner to protect against hypoxia induced by MCAO model by recruitment of anti-inflammatory T-regulatory cells and promoting a homeostatic environment. (Lee et al., 2017) In chapter 2 of this thesis, I described previous research by our lab, which found a high expression of CXCL14 expression in BACE1-null reactive astrocytes with enhanced Aβ clearance function.(John Zhou, 2022) Furthermore, 2 SNPS were found on the anti-sense strand for CXCL14 were associated with enhanced tau and p-tau, markers for AD.(Chung et al., 2018). Based on this previous research and literature in the field, we decided to study the potential role of CXCL14 in the context of AD. Because AD-associated pro-inflammatory environment is considered deleterious and CXCL14 is regarded as an anti-inflammatory chemokine, we hypothesized that CXCL14 may act in a beneficial manner for AD.(Baik et al., 2019; Kouzeli et al., 2020; Lu et al., 2016; Rangaraju et al., 2018) To explore this aim, we explored CXCL14 expression and found CXCL14 upregulation in reactive astrocytes both in vitro when treated

with Aβ and in vivo surrounding Aβ plaques. Next, we found that CXCL14 could enhance microglial Aβ uptake by enhancing microglia chemotaxis and Aβ uptake. Furthermore, we found CXCL14 to be protective of chronic Aβ neuronal cell death and metabolic dysfunction.

Under normal physiological conditions, CXCL14 expression was found in neurons and astrocytes.(Banisadr et al., 2011; Lee et al., 2017; Liu et al., 2020) We discovered that astrocytes treated with Aβ significantly upregulated expression of CXCL14 in vitro.(Figure 2.2D and E) We also found localization of CXCL14 surrounding Aβ plaques (Figure 3.1B), possibly acting as a signaling agent to enhance chemotaxis of glia or immune cells for Aβ clearance. This is analogous to the action of CCL2, which potently encourages migration of astrocytes and microglia to plaque sites.(Tony Wyss-Coray et al., 2003) Interestingly, we also found that as disease progresses that total brain levels of CXCL14 actually decreased in 5xFAD mouse model as disease progresses.(Figure 3.1B) We also found that CXCL14 RNA expression is also reduced in in AD human patients scRNA-seq databases. (Grubman et al., 2019) Most likely, chronic and systemic pro-inflammatory AD environment leads to upstream changes that downregulate homeostatic mediators like CXCL14, thereby worsening the disease. Interestingly, Hypoxia Inducible Factor 1-α (HIF1A), a known compensatory mechanism against hypoxia and known transcription factor for CXCL14 appears to be decreased in AD patients.(Ashok, Ajith, & Sivanesan, 2017; Lu et al., 2016; Ogunshola & Antoniou, 2009) Altogether, it does appear that CXCL14 is expressed in reactive astrocytes in

the context of AD, at least at the local level. But further exploration of how CXCL14 is regulated in the context of chronic AD should also occur.

Microglial Aβ clearance is a multi-step process including migration to plaque sites and then uptake. Interestingly, in the acute phase pro-inflammatory cytokines may enhance microglial and monocyte derived macrophage Aβ clearance, however long term exposure can actually result in downregulation of Aβ uptake function.(Paul S. Aisen, 2002; Baik et al., 2019; Bonneh-Barkay et al., 2010; Holmes et al., 2009) Therefore, we first examined the direct role CXCL14 might have on Aβ clearance by examining microglia migration. CXCL14 is a known chemoattractant for multiple immune cell types, but it is unknown whether it acts in a similar fashion towards microglia, the main, resident immune cell of the brain.(Lu et al., 2016) We found that CXCL14 does indeed induce potent microglial chemotaxis in a transwell system, as well as in vivo. (Figure 3.2) Next, we examined the direct role CXCL14 might have on microglial Aβ uptake. Previous literature suggests that CXCL14 may play a role in enhancing monocyte phagocytosis of *E. coli* particles.(Witte, Chatterjee, Lang, & Gawaz, 2017) Our results, show that CXCL14 appears to enhance microglial Aβ uptake in both acute and chronic conditions. (Figure 3.3) Furthermore, co-treatment of CXCL14 with αCXCR4 appears to reverse this effect, suggesting CXCL14 mediation of Aβ uptake is CXCR4 dependent. Overall, this suggests that CXCL14 does directly act on microglial to enhance their Aβ clearance activity. Still, further research needs to be done to see if CXCL14 enhances other

components of microglial Aβ clearance, including lysosomal degradation and secretion of extracellular Aβ degradation enzymes.

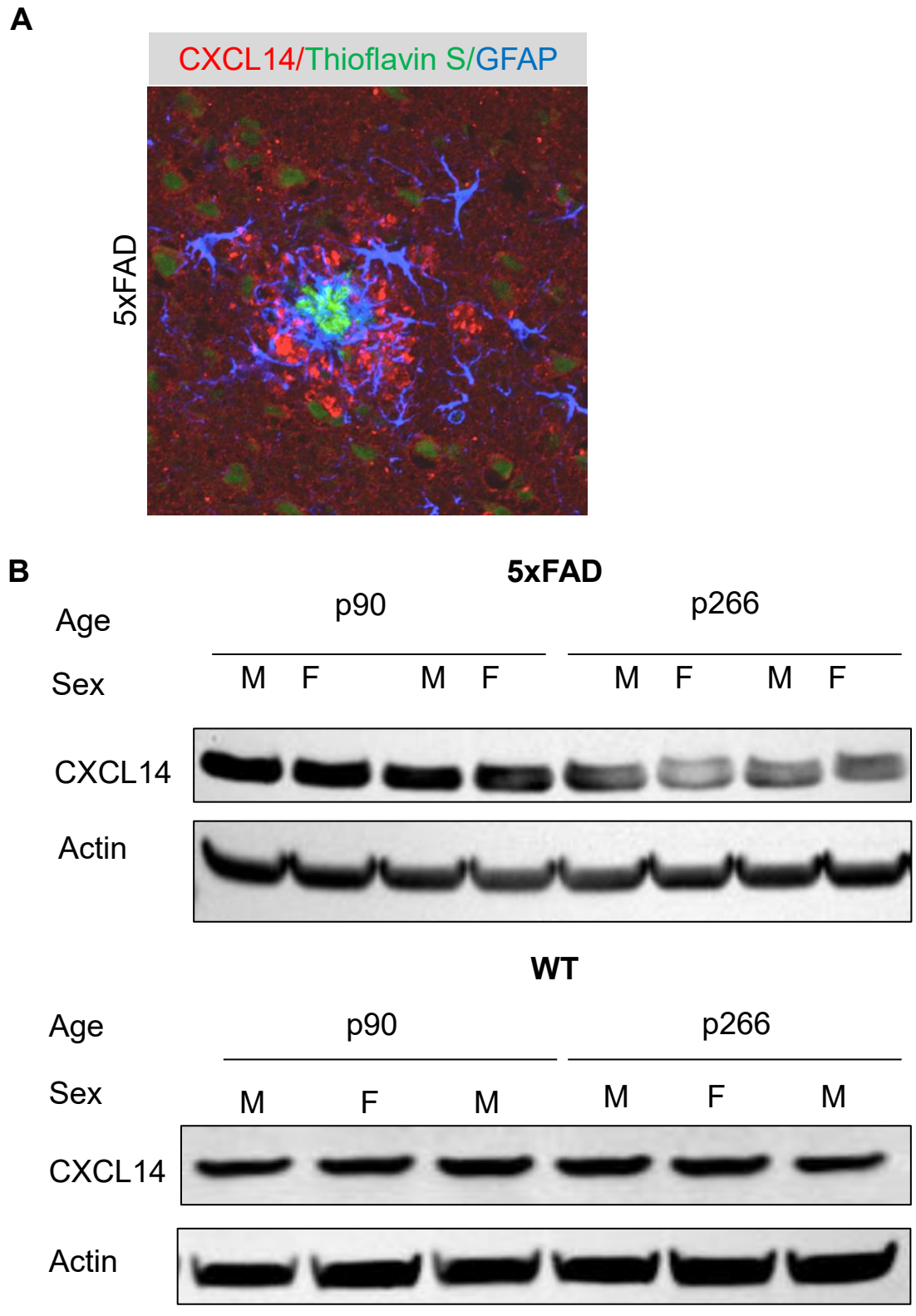
Atrophy and cell death as a result of prolonged Aβ or pro-inflammatory environment is another hall mark of AD, with disease induced metabolic dysfunction a key pathway for cell death.(Holmes et al., 2009; Janelidze et al., 2018; Kinney et al., 2018; Zhou et al., 2021) Therefore, anti-inflammatory agents have been hypothesized to alleviate AD atrophy.(Cummings et al., 2021; Kinney et al., 2018; Zhong & Chen, 2019) We tested to see if CXCL14 may also act to protect cells from both acute and recurrent Aβ insult. (Figure 3.4) Recurrent Aβ insult appeared to be much more effective in inducing both neuronal and microglial cytotoxicity, although the microglial appear to be much more resistant to Aβ toxicity. Furthermore, CXCL14 appears to protect neurons in a CXCR4 dependent manner, but not microglia. We also tested if CXCL14 protected against cellular metabolic dysfunction, specifically mitochondrial fusion/fission dynamics and reactive oxygen species defense.(Figure 3.5) CXCL14 treatment markedly enhances markers of mitochondrial fusion/fission dynamics and the reactive oxygen species defense under recurrent Aβ conditions, suggesting a reactivation of neuronal metabolism. CXCL14 metabolic reactivation of neurons also appears to be CXCR4 dependent. Because of the strikingly similar patterns between these observations, it is likely that CXCL14 has the potential to protect neurons from Aβ insult by activating neuronal metabolism. Future explorations might be to more closely examine

neuronal cytotoxicity and metabolism by more advanced assays, such as FACS or SeaHorse respectively.

In summary, we provide evidence where CXCL14 treatment may be beneficial for AD patients, by enhancing microglial Aβ clearance and protecting against neuronal cell death.(Figure 3.6) Further mechanistic studies need to be performed in order to understand CXCL14 expression in AD, as well as CXCL14 effect on signaling pathways and systems in different cell types. As AD disease progresses, declining level of CXCL14 and other homeostatic systems might foster the acceleration of the disease. Therefore, enhancing CXCL14 might be a key part in restoring balance to the AD inflammatory milieu and slow the progression of AD.

5. Figures and figure legends

Figure 3.1 CXCL14 expression in AD



(A) Immunohistochemistry of 5xFAD mouse cortical tissue showing representing image of CXCL14 (red) expression surrounding amyloid plaques stained by Thioflain-S (green) and surrounding reactive astrocytes stained by GFAP (blue). (B) Western blot of CXCL14 expression in 5xFAD and WT litter mates total brain lysate. Male and females samples and different age points are represented. CXCL14 expression appears to be reduced in more aged 5xFAD mice.

Figure 3.2 CXCL14 induces microglia chemotaxis

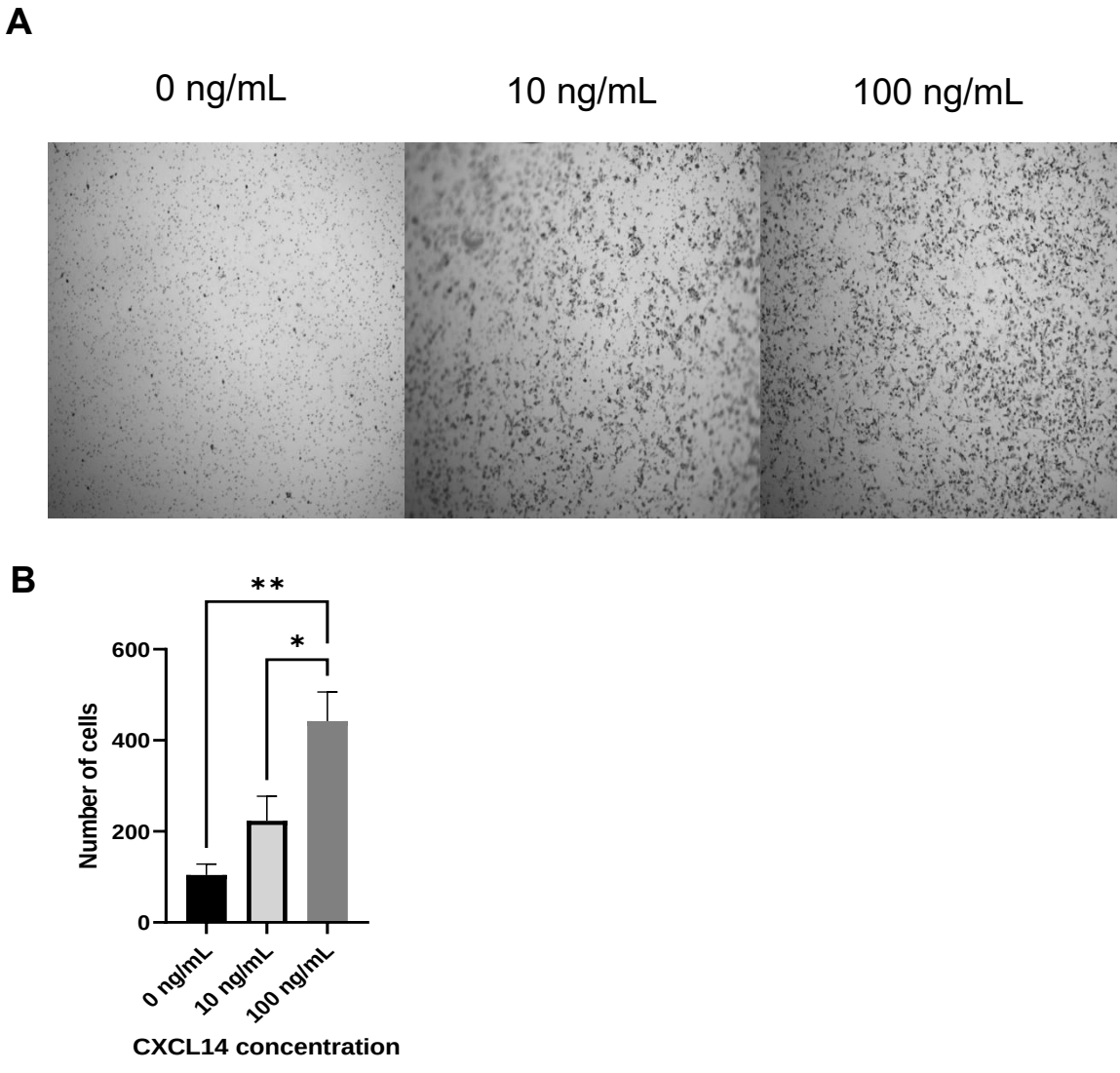
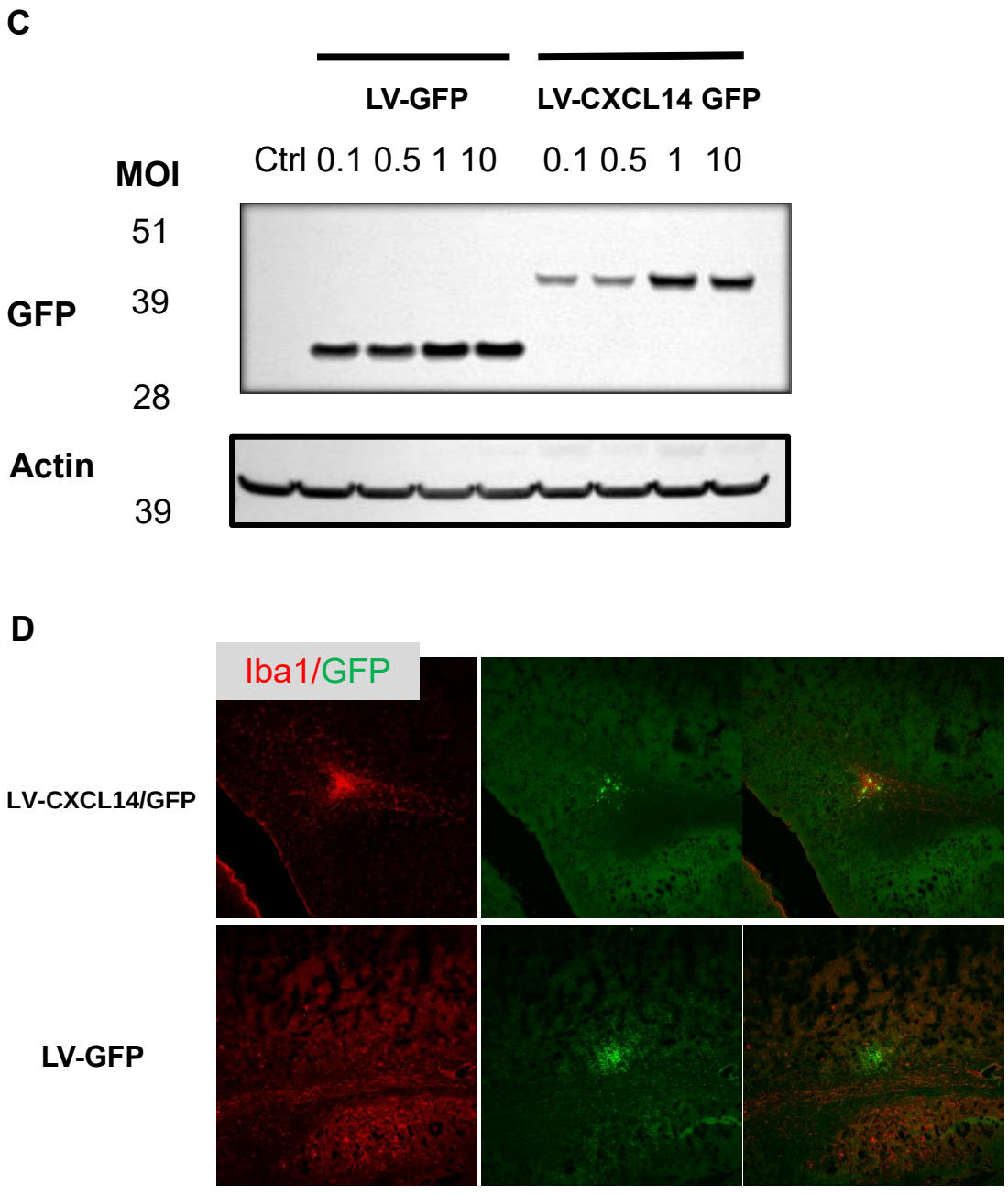
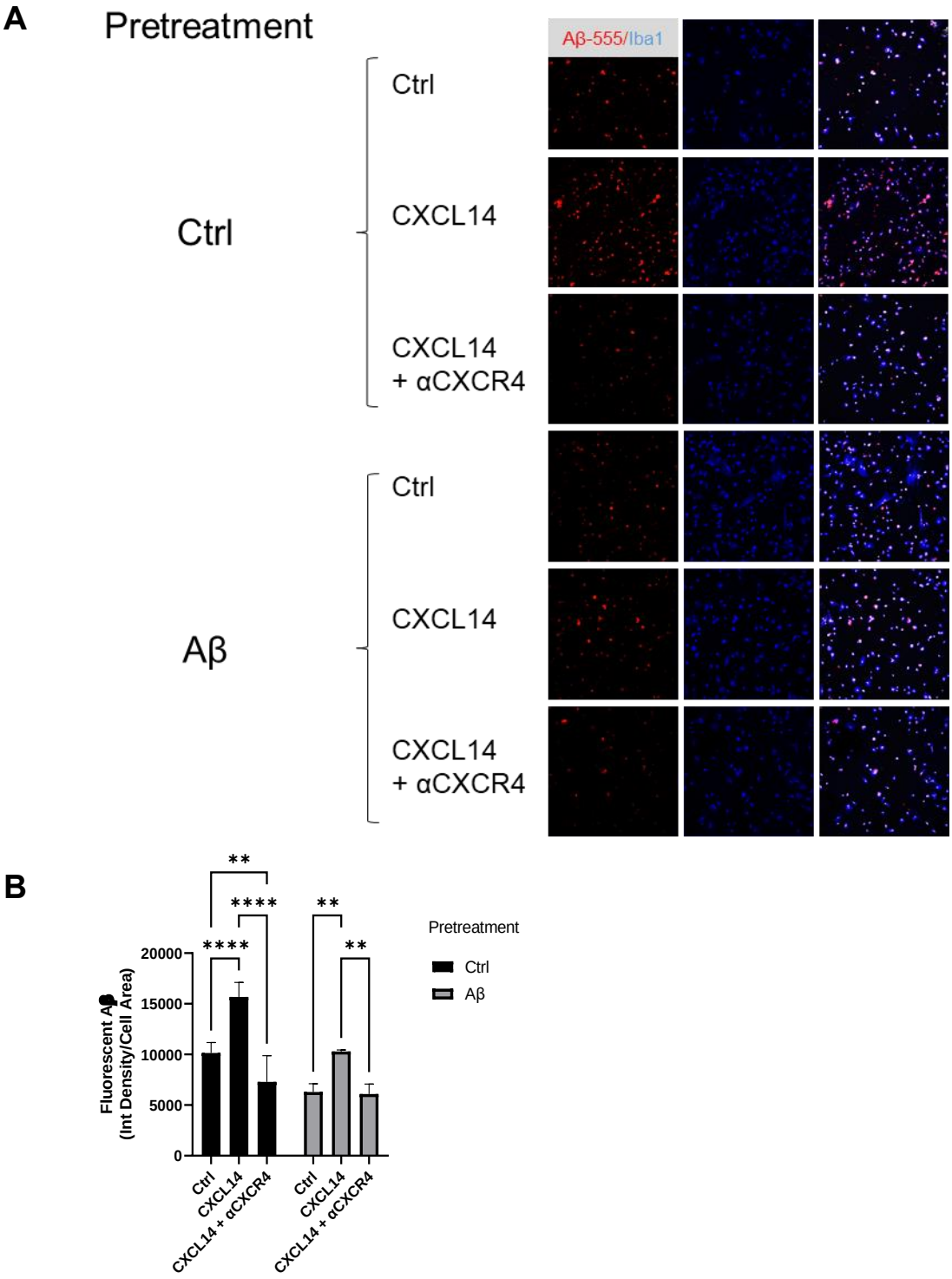


Figure 3.2 cont.



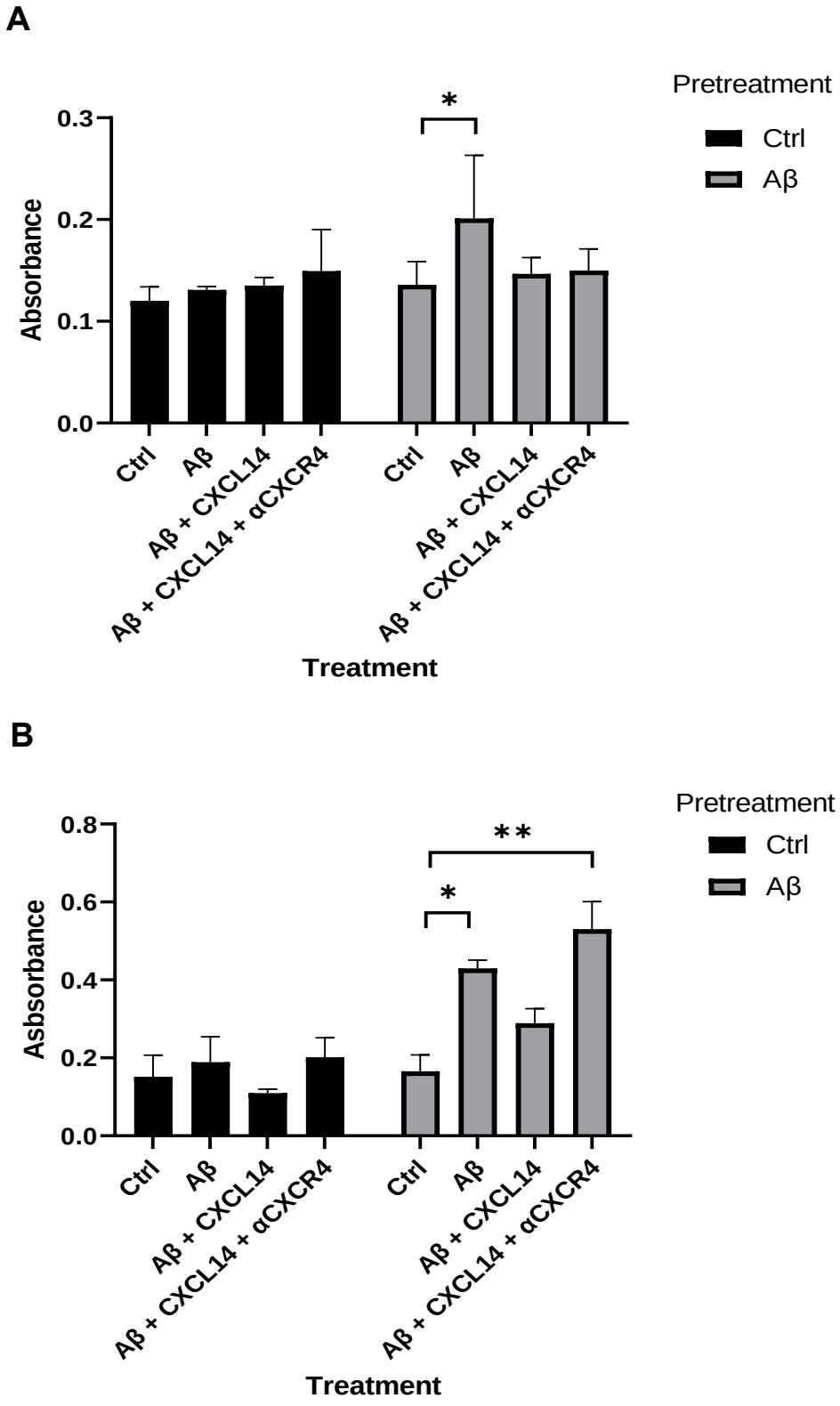
(A) Representative images of toluidine-stained microglia on basal transwell membrane subjected to indicated CXCL14 concentrations. (B) Quantification of average number of microglia that have crossed transwell membrane. Microglia were manually counted across entire membranes. N=3, * indicates p-value < 0.05, ** indicates p-value < 0.01, one-way ANOVA with Bonferonni post-test. (C) GFP Western blot of astrocytes treated with LV-CXCL14/GFP and LV-GFP at indicated MOI. (D) Immunohistochemistry of Iba1 (red) and GFP (green) of WT brains injected with LV-CXCL14/GFP and LV-GFP in the cortex.

Figure 3.3 CXCL14 enhances Aβ uptake under acute and recurrent conditions



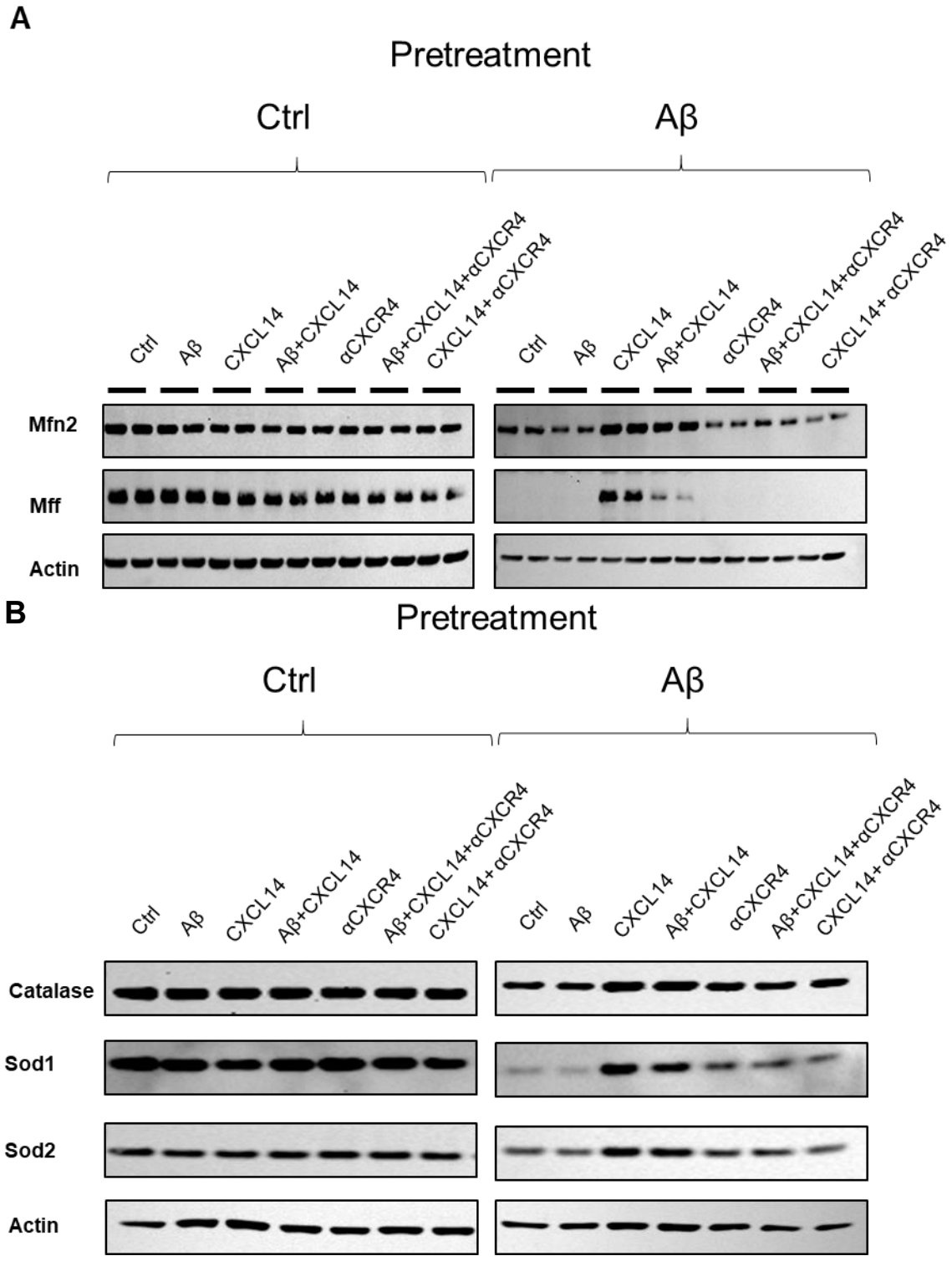
(A) Representative immunocytochemistry of microglia cultures stained with Iba1 (blue). Cultures were either pretreated with or without Aβ before being treated with Aβ-555 (red), as well as CXCL14 and/or αCXCR4 inhibiting antibody. (B) Quantification of average Aβ-555 fluorescent density within the bound of Iba1 stained microglia area, normalized to microglia area. N=3, ** indicates p-value <- 0.01, *** indicates p-value < 0.001, *** indicates p-value <- 0.0001, one way ANOVA with Tukey post-test.

Figure 3.4 CXCL14 protects against recurrent Aβ cytotoxicity



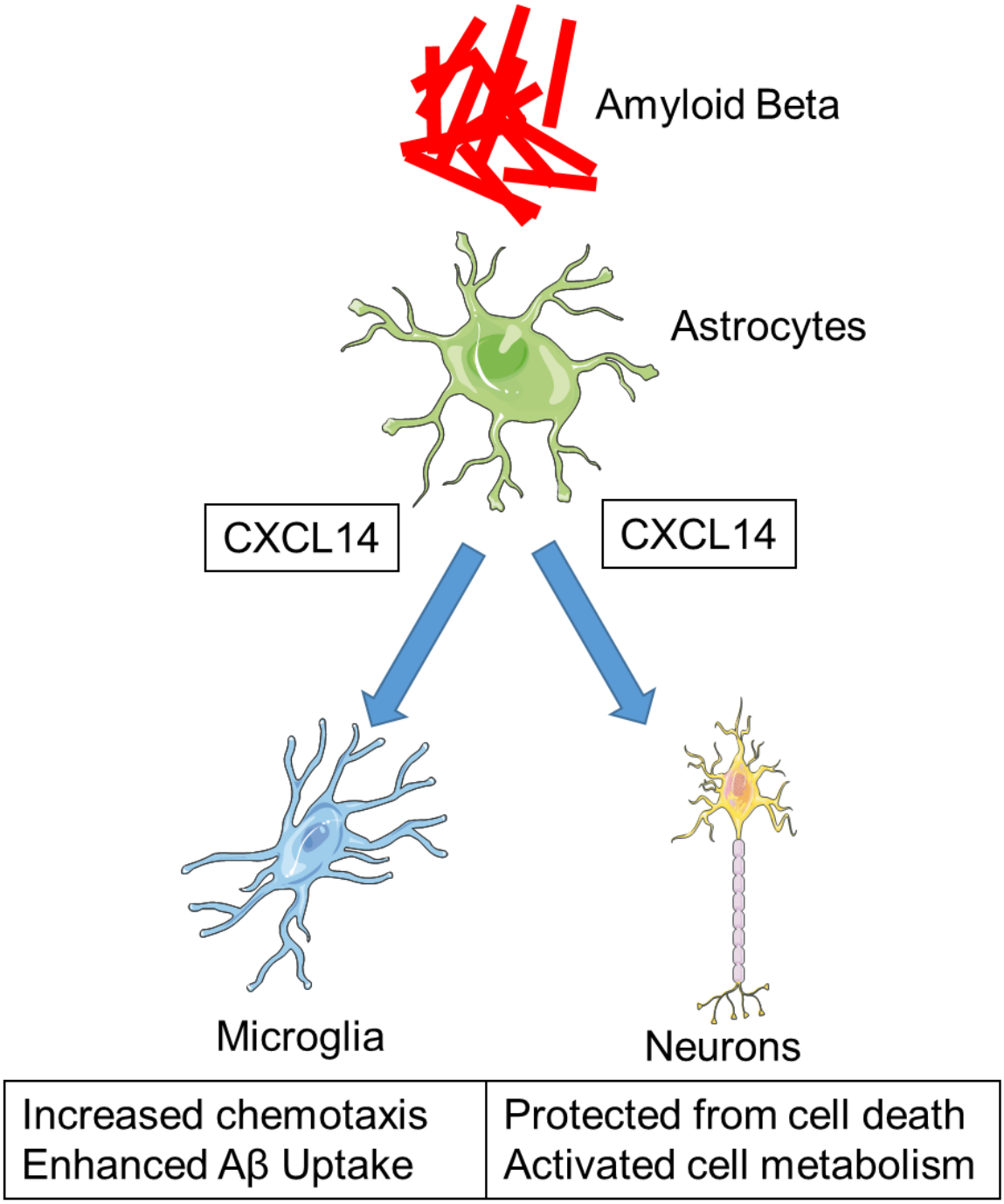
(A) Quantification of relative absorbance (490 nM – 680 nM) from LDH cytotoxicity assay from microglia cultures pretreated with either control of Aβ and then treated again with various permutations of Aβ, CXCL14, and αCXCR4. N=4 * indicates p-value < 0.05, one-way ANOVA with Tukey post-test. (B) (A) Quantification of relative absorbance from LDH cytotoxicity assay from neurons cultures pretreated with either control of Aβ and then treated again with various permutations of Aβ, CXCL14, and αCXCR4. N=4 * indicates p-value < 0.05, ** indicates p-value < 0.01, one-way ANOVA with Tukey post-test.

Figure 3.5 CXCL14 protects from recurrent Aβ induced cellular metabolism dysfunction



(A) Western blot of neuron lysate pretreated with either control of Aβ and then treated again with various permutations of Aβ, CXCL14, and αCXCR4. Probed by antibodies for Mfn2 and Mff, markers of mitochondria fusion/fission. (B) Western blot of neuron lysate pretreated with either control of Aβ and then treated again with various permutations of Aβ, CXCL14, and αCXCR4. Probed by antibodies for Sod1, Sod2, and Catalase, members of the reactive oxygen species defense system.

Figure 3.6 Model of CXCL14 expression and effect on various cell types



Schematic diagram of CXCL14 expression by reactive astrocytes acutely stimulated by Aβ and effect of CXCL14 on microglia and neuron function in the context of AD

Chapter 4: **Discussion**

Introduction

The studies presented in this dissertation focused on using reactive astrocytes for the purposes of treating AD. In the first set of studies, I described a population of reactive astrocytes in BACE1-null mice with enhanced Aβ clearance function and genes. In particular, I focused on function and signaling pathways CLU, a known enhancer of astrocytic Aβ clearance, which involved BACE1 mediated cleavage of IR. In this context, specific inhibition of astrocytic BACE1 or downstream pathways may prove to be beneficial for AD by enhancing Aβ clearance, while avoiding mostly neuronal side effects from global BACE1 deletion. In the second set of studies, I evaluated the potential role for a new target for AD, CXCL14. CXCL14 was found to be expressed in Aβ treated reactive astrocytes. We next found that CXCL14 enhanced microglia Aβ clearance, while protecting neurons from recurrent Aβ insult. In both studies, we found that it was possible to modify reactive astrocyte behavior to either directly or with other cell types to reduce AD pathology. In this section, I will discuss future implications of the works, as well as outstanding questions raised by each study.

BACE1 inhibition for the treatment of AD

BACE1 inhibition has been a major therapeutic goal for AD therapies, but it is clear that global inhibition of BACE1 has consequences. (P. S. Aisen et al., 2018; Egan et al., 2018; Hawkes, 2017; Yan, 2016) Ongoing research has learned from these failures and it has become increasingly evident that dose, timing, or cell-type specific inhibition of BACE1 may be beneficial for AD. (X. Hu

et al., 2018; John Zhou, 2022; B. D. Neeraj Singh, John Zhou, Xiangyou Hu and Riqiang Yan 2022; X. H. Neeraj Singh, Marc Benoit, Brati Das, John Zhou, Jose Davila, Li-Huei Tsai, Manolis Kellis, Riqiang Yan 2022) This is in line with the prevailing trend for medical research and therapies to be more personalized and specific.(Hampel et al., 2016) The complexities of reactive astrocytes, BACE1 inhibition, and AD have to be untangled before proper understanding and treatment of AD can occur.

Reactive astrocytes for direct Aβ clearance

While astrogliosis surrounding Aβ plaques was discovered in AD brains very early on, whether not these reactive astrocytes are beneficial, harmful, or bystanders in AD remains an ongoing debate. The work presented in chapter 2 shows that reactive astrocytes can by utilized to enhance Aβ clearance by inhibition of BACE1, activating IR, and increasing Clu.(John Zhou, 2022) Together with previous research in adult BACE1 inhibition and microglial BACE1 inhibition, it is clear that specific BACE1 inhibition has the potential to reduce AD pathology, while avoiding neuronal side effects. (X. Hu et al., 2018; B. D. Neeraj Singh, John Zhou, Xiangyou Hu and Riqiang Yan 2022; X. H. Neeraj Singh, Marc Benoit, Brati Das, John Zhou, Jose Davila, Li-Huei Tsai, Manolis Kellis, Riqiang Yan 2022) Although we've observed the benefits of BACE1 inhibition in reactive astrocytes, still several questions remain to be explored in the next sections and by future work.

Does BACE1 inhibition in astrocytes enhance Aβ clearance in a cell autonomous manner?

The work present in chapter 2 has presented a model where BACE1 inhibition acts specifically in astrocytes to increase Clu and directly enhance astrocyte ability to clear Aβ. (John Zhou, 2022) Still, how astrocytic BACE1 inhibition might affect other cell types in the AD context remains to be explored. Astrocytes are a major secretory cell of the brain and secreted Clu can facilitate clearance in not just astrocyte, but microglia and endothelial cells. (Bettens et al., 2015; Evangeline M. Foster et al., 2019; Nelson et al., 2017; Nuutinen et al., 2007; Aleksandra M. Wojtas et al., 2020) As can be seen in Figures 1.7 and 1.8, total adult BACE1 ablation is more effecting in reducing Aβ plaque load than microglial BACE1 deletion alone. This could be due to reduced neuronal Aβ production as well, but more likely BACE1 mediated astrocyte contributions to Aβ clearance are not accounted for in these studies. This suggests that BACE1 inhibition in both microglia and astrocytes may play a synergistic role in enhancing Aβ clearance and reducing AD pathology.

Additionally, ApoE, a lipoprotein and AD GWAS risk factor like Clu, has been well explored for its role as a signaling molecule for TREM2 and this interaction directly leads to DAM1/2 microglia signaling.(Nguyen et al., 2020; Parhizkar et al., 2019) BACE1 inhibition in microglia alone was found to increase DAM1 signaling, (Figure 1.8B) however whether or not extracellular Clu signaling can influence microglia or other cell types in AD is unknown.

To answer these queries, we are actively working on examining the effects of astrocyte-specific BACE1 inhibition on AD pathology and other cell types in an in vivo model. In this model, it may be also possible to examine the role of astrocyte-specific BACE1 inhibition on synaptic physiology and behavior.

Is it feasible to target astrocytic BACE1 directly in human AD patients?

As mentioned previously, precision medicine and research has resulted in a trend of researching cell-specific changes in diseases. The growing abundance of scRNAseq capabilities and usage has only facilitated this trend. Although, we have found that BACE1 inhibition enhances microglia and astrocytes Aβ clearance, so far BACE1 inhibitor have been global, with the deleterious effect on neuronal function outweighing reduction in plaque load. Therefore, we propose that specific inhibition of BACE1 in glia cells can bypass neuronal side effects, while still reducing Aβ loads. Despite this promising ideas, it remains under question how this might be feasible in a clinical setting. Although genetic ablation in specific cell-types is relatively simple task in animals and in vitro, human gene therapy is dangerous, unpredictable, and not feasible. Nanomedicine platforms have been proposed to deliver cell-specific drugs to the CNS, however these are still under development and targeting specific subclusters of reactive astrocytes may be especially difficult.(F. Zhang, Lin, Kannan, & Kannan, 2016) Therefore, it was important for us to fully explore downstream mechanism of BACE1 cleavage in order to identify potential targets where intervention is more feasible.

For example, enhancing astrocytic insulin receptor or preventing BACE1 cleavage of this receptor could be a major therapeutic option for the future of AD. Nasal insulin sprays have been under consideration for the treatment of AD for its benefits in maintaining neuronal metabolism and synaptic physiology.(Craft et al., 2020; Mustapic, Tran, Craft, & Kapogiannis, 2019; Steen et al., 2005) Our research is the first to imply that enhancing insulin signaling might promote astrocytic Aβ. Furthermore, we found specific MAPK signaling pathway downstream of insulin that could be targeted by small molecule inhibitors. Even still; there are concerns that providing insulin alone can result in insulin insensitivity and alteration or IR availability. We found that BACE1 does not appear to change IR availability or signaling in total brain lysate, (data not shown) this suggests that BACE1 cleavage of IR is an astrocyte specific phenomenon. Therefore, understanding how BACE1 specifically mediates astrocytic IR cleavage might be the key to bypassing possible insulin insensitivity brought on by nasal insulin implementation.

Anti-inflammatory chemokines and AD

As mentioned previously, specific targeting of anti-inflammatory systems is an increasingly important target for AD therapies. The work focusing on CXCL14 in chapter 3 of this dissertation reveals a potential new homeostatic target for study and therapy. We found that CXCL14 appears to have dual beneficial effects by enhancing microglial Aβ clearance and neuronal survival. As there is limited knowledge about CXCL14 in the case of neurological diseases, let alone AD, this research has great potential for providing additional insight into AD

associated inflammation. These promising results though raise further questions and avenues for exploration discussed in the next two sections and with ongoing research.

How does CXCL14 affect microglia and neuronal signaling?

Previous literature on CXCL14 has extensively explored potential signaling mechanisms of CXCL14 and CXCR4. For example CXCL14/CXCR4 axis has been implicated in endothelial cell angiogenesis and monocyte phagocytosis via vascular endothelial cell growth factor (VEGF). (Witte et al., 2017) This may form the basis for further exploration of CXCL14 on microglial Aβ uptake, which we've found to be to be CXCR4 dependent. CXCL14 effect on other aspects of microglial Aβ clearance, such as lysosomal degradation or DAM1/2 signaling, also needs to be further explored. We also found that CXCL14 protects neurons from recurrent Aβ toxicity via activating cellular metabolism. This is in line with numerous reports showing the positive effects of other homeostatic chemokines on neuronal metabolism.(Baik et al., 2019; Namioka et al., 2017; Nordengen et al., 2019; Parbo et al., 2017; Park, Han, & Mook-Jung, 2020; Zulfiqar et al., 2019) CXCL14 effect on specific mechanisms and functions of cell death and metabolism need to be examined with other assays. Furthermore, CXCL14 was found to have a potential inhibitory effect on GABA-ergic neurons of the dentate gyrus via CXCR4 signaling.(Banisadr et al., 2011) Whether or not the potential for CXCL14 regulation of neuron synaptic function might be relevant in the context of AD, remains to be seen. Also, the role of CXCL12, the primary chemokine for CXCR4, in AD remains to be

explored. One paper did indicate that CXCL12 appears to upregulate Aβ clearance in AD mice, although this was in the context of intranasal delivery of human nerve growth factor.(Capsoni et al., 2017)

As an orphan chemokine CXCL14, has been shown to bind to a variety of receptors and whether these interactions are negative or positive is context dependent.(Kouzeli et al., 2020; Lu et al., 2016) CCR7 is another potential receptor for CXCL14 and could explain additional phenomena not through the CXCR4, such as CXCL14 protection of microglia. Whether or not these non-CXCL14/CXCR4 interaction play a role in AD remains to be seen.

Can CXCL14 recruit peripheral immune cells for the benefit of AD?

Although, our work found direct roles on microglia for CXCL14 in AD, previous research has focused on CXCL14 role in the peripheral immune system. The papers that examined CXCL14 role in neurological diseases have also focused primarily at the recruitment of CXCL14 or peripheral immune cells. One paper showed that CXCL14 ameliorates infarct volume by recruiting T-regulatory cells and immature dendritic cells.(Lee et al., 2017) T-reg cells have been well implicated in slowing AD disease progression. (Dansokho et al., 2016) Furthermore, T-reg cells are in early Phase I clinical trials for AD. (Cummings et al., 2021; LTD, 2021) Two recent papers have examined CXCL14 in the context of glioblastoma, as CXCL14 was originally discovered in breast and kidney cell carcinoma, however they provide contradictory findings. The first paper by Fazi et al. found CXCL14 was found to be pro-tumorigenic by enhancing sphere formation of cancer stem cells. (Fazi et al., 2019) Another more recent paper by

Kumar et al. stated that CXCL14 actually has an anti-tumorigenic role by enhancing recruitment of CD8+ cells targeted against cancer stem cells. (Kumar et al., 2022) Whether or not these previous studies focusing on CXCL14 effect on peripheral immune cells and neurological diseases have any relevance in the field of AD remains to be seen. In both stroke and glioblastoma, there is significant disruption of the brain-blood barrier resulting in much easier invasion by the peripheral immune cells. In AD, brain blood barrier disruptions typically occurs later than plaque formation in humans and much later in amyloid mouse models. Although we performed LV-CXCL14/GFP injection in vivo and used an LV/GFP control, whether brain CXCL14 can recruit peripheral immune cells without disruption of the brain blood barrier remain to be seen. It is possible that CXCL14 might signal endothelial cells to upregulate proteins necessary for peripheral immune cell migration into the brain, as endothelial cells express CXCR4 and other potential CXCL14 potential receptors. These questions are actively being researched in our lab through the development of a mouse model of conditional overexpression of CXCL14. This would enable study of CXCL14 effect on Aβ load – by crossing with amyloid mouse models – atrophy – by crossing with tau atrophy mouse models – or peripheral immune cell invasion – adoptive transfer of GFP-bone marrow.

Future AD therapies utilizing reactive astrocytes

The body of work presented in this dissertation demonstrates that reactive astrocytes play a key role in AD pathogenesis. As the most numerous glia cell type and the most secretory cell type, astrocytes may be the key first responder

to Aβ plaque. By first starting with the clue that BACE1 inhibition might enhance Aβ clearance, we found a unique reactive astrocyte population with enhanced Aβ clearance function. We the expanded our search to discover novel ways in which reactive astrocytes can both directly and indirectly ameliorate AD disease.

Our results show that BACE1-null reactive astrocytes were more efficient in direct Aβ uptake and degradation, primarily through increased astrocytic expression of Clu. Next, we found that BACE1 mediated cleavage of the IR and downstream MAPK were responsible for astrocytic expression of Clu. Our second set of experiments examined how astrocytic expression of the anti-inflammatory chemokine CXCL14. We found that CXCL14 works with microglia to enhance their Aβ uptake and with neurons to protect them from cellular toxicity. Based on these results, we find that BACE1 inhibition in reactive astrocytes can affect AD directly by enhancing astrocytic Aβ clearance, or by indirectly synergizing with other cell types. Furthermore, we propose downstream mechanisms of BACE1 in astrocytes that might specifically be targeted to enhance astrocytic Aβ clearance of reactive astrocytes, while bypassing neuronal side effects. Thus, future studies should aim to target therapies to reactive astrocyte systems that can have widespread benefits and prevent AD.

Bibliography

2021 Alzheimer's disease facts and figures. (2021). *Alzheimers Dement, 17*(3), 327-406. doi:10.1002/alz.12328

Aisen, P. S. (2002). The potential of anti-inflammatory drugs for the treatment of Alzheimer's disease. *The Lancet Neurology, 1*(5), 279-284. doi:https://doi.org/10.1016/S1474-4422(02)00133-3

Aisen, P. S., Siemers, E., Michelson, D., Salloway, S., Sampaio, C., Carrillo, M. C., . . . Vellas, B. (2018). What Have We Learned from Expedition III and EPOCH Trials? Perspective of the CTAD Task Force. *J Prev Alzheimers Dis, 5*(3), 171-174. doi:10.14283/jpad.2018.23

Ashok, B. S., Ajith, T. A., & Sivanesan, S. (2017). Hypoxia-inducible factors as neuroprotective agent in Alzheimer's disease. *Clin Exp Pharmacol Physiol, 44*(3), 327-334. doi:10.1111/1440-1681.12717

Baik, S. H., Kang, S., Lee, W., Choi, H., Chung, S., Kim, J. I., & Mook-Jung, I. (2019). A Breakdown in Metabolic Reprogramming Causes Microglia Dysfunction in Alzheimer's Disease. *Cell Metab, 30*(3), 493-507.e496. doi:10.1016/j.cmet.2019.06.005

Banisadr, G., Bhattacharyya, B. J., Belmadani, A., Izen, S. C., Ren, D., Tran, P. B., & Miller, R. J. (2011). The chemokine BRAK/CXCL14 regulates synaptic transmission in the adult mouse dentate gyrus stem cell niche. *J Neurochem, 119*(6), 1173-1182. doi:10.1111/j.1471-4159.2011.07509.x

Bettegazzi, B., Mihailovich, M., Di Cesare, A., Consonni, A., Macco, R., Pelizzoni, I., . . . Zacchetti, D. (2011). β-Secretase activity in rat astrocytes: translational block of BACE1 and modulation of BACE2 expression. *33*(2), 236-243. doi:https://doi.org/10.1111/j.1460-9568.2010.07482.x

Bettens, K., Vermeulen, S., Van Cauwenberghe, C., Heeman, B., Asselbergh, B., Robberecht, C., . . . Sleegers, K. (2015). Reduced secreted clusterin as a mechanism for Alzheimer-associated CLU mutations. *Mol Neurodegener, 10*, 30. doi:10.1186/s13024-015-0024-9

Boisvert, M. M., Erikson, G. A., Shokhirev, M. N., & Allen, N. J. (2018). The Aging Astrocyte Transcriptome from Multiple Regions of the Mouse Brain. *Cell Reports, 22*(1), 269-285. doi:10.1016/j.celrep.2017.12.039

Bonneh-Barkay, D., Wang, G., Starkey, A., Hamilton, R. L., & Wiley, C. A. (2010). In vivo CHI3L1 (YKL-40) expression in astrocytes in acute and chronic neurological diseases. *J Neuroinflammation, 7*, 34. doi:10.1186/1742-2094-7-34

Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H., & Del Tredici, K. (2006). Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol, 112*(4), 389-404. doi:10.1007/s00401-006-0127-z

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol, 82*(4), 239-259. doi:10.1007/BF00308809

Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D. R., Price, D. L., & Wong, P. C. (2001). BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nat Neurosci, 4*(3), 233-234. doi:10.1038/85064

Capsoni, S., Malerba, F., Carucci, N. M., Rizzi, C., Criscuolo, C., Origlia, N., . . . Cattaneo, A. (2017). The chemokine CXCL12 mediates the anti-amyloidogenic action of painless human nerve growth factor. *Brain, 140*(1), 201-217. doi:10.1093/brain/aww271

Chacón-Quintero, M. V., Pineda-López, L. G., Villegas-Lanau, C. A., Posada-Duque, R., & Cardona-Gómez, G. P. (2021). Beta-Secretase 1 Underlies Reactive Astrocytes and Endothelial Disruption in Neurodegeneration. *15*(158). doi:10.3389/fncel.2021.656832

Chen, X., Stoeck, A., Lee, S. J., Shih, I., Wang, M. M., & Wang, T. L. (2010). Jagged1 expression regulated by Notch3 and Wnt/beta-catenin signaling pathways in ovarian cancer. *Oncotarget, 1*(3), 210-218.

Chung, J., Wang, X., Maruyama, T., Ma, Y., Zhang, X., Mez, J., . . . Jun, G. R. (2018). Genome-wide association study of Alzheimer's disease endophenotypes at prediagnosis stages. *Alzheimers Dement, 14*(5), 623-633. doi:10.1016/j.jalz.2017.11.006

Clarke, L. E., Liddelow, S. A., Chakraborty, C., Münch, A. E., Heiman, M., & Barres, B. A. (2018). Normal aging induces A1-like astrocyte reactivity. *Proc Natl Acad Sci U S A, 115*(8), E1896-e1905. doi:10.1073/pnas.1800165115

Cline, E. N., Bicca, M. A., Viola, K. L., & Klein, W. L. (2018). The Amyloid-β Oligomer Hypothesis: Beginning of the Third Decade. *J Alzheimers Dis, 64*(s1), S567-s610. doi:10.3233/jad-179941

Corriveau, R. A., Koroshetz, W. J., Gladman, J. T., Jeon, S., Babcock, D., Bennett, D. A., . . . Holtzman, D. M. (2017). Alzheimer's Disease-Related Dementias Summit 2016: National research priorities. *Neurology, 89*(23), 2381-2391.

Craft, S., Raman, R., Chow, T. W., Rafii, M. S., Sun, C.-K., Rissman, R. A., . . . Aisen, P. S. (2020). Safety, Efficacy, and Feasibility of Intranasal Insulin for the Treatment of Mild Cognitive Impairment and Alzheimer Disease Dementia: A Randomized Clinical Trial. *JAMA Neurol, 77*(9), 1099-1109. doi:10.1001/jamaneurol.2020.1840 %J JAMA Neurology

Cummings, J. (2019). The Role of Biomarkers in Alzheimer's Disease Drug Development. *Adv Exp Med Biol, 1118*, 29-61. doi:10.1007/978-3-030-05542-4_2

Cummings, J., Lee, G., Zhong, K., Fonseca, J., & Taghva, K. (2021). Alzheimer's disease drug development pipeline: 2021. *Alzheimers Dement (N Y), 7*(1), e12179. doi:10.1002/trc2.12179

Dansokho, C., Ait Ahmed, D., Aid, S., Toly-Ndour, C., Chaigneau, T., Calle, V., . . . Dorothée, G. (2016). Regulatory T cells delay disease progression in Alzheimer-like pathology. *Brain, 139*(Pt 4), 1237-1251. doi:10.1093/brain/awv408

Das, B., Singh, N., Yao, A. Y., Zhou, J., He, W., Hu, X., & Yan, R. (2021). BACE1 controls synaptic function through modulating release of synaptic vesicles. *Mol Psychiatry*. doi:10.1038/s41380-021-01166-2

Das, B., & Yan, R. (2019). A Close Look at BACE1 Inhibitors for Alzheimer's Disease Treatment. *CNS Drugs, 33*(3), 251-263. doi:10.1007/s40263-019-00613-7

Davis, N., Mota, B. C., Stead, L., Palmer, E. O. C., Lombardero, L., Rodríguez-Puertas, R., . . . Sastre, M. (2021). Pharmacological ablation of astrocytes reduces Aβ degradation and synaptic connectivity in an ex vivo model of Alzheimer's disease. *J Neuroinflammation, 18*(1), 73. doi:10.1186/s12974-021-02117-y

DeTure, M. A., & Dickson, D. W. (2019). The neuropathological diagnosis of Alzheimer's disease. *Mol Neurodegener, 14*(1), 32. doi:10.1186/s13024-019-0333-5

Dislich, B., Wohlrab, F., Bachhuber, T., Muller, S. A., Kuhn, P. H., Hogl, S., . . . Lichtenthaler, S. F. (2015). Label-free Quantitative Proteomics of Mouse Cerebrospinal Fluid Detects beta-Site APP Cleaving Enzyme (BACE1) Protease Substrates In Vivo. *Mol. Cell Proteomics, 14*(10), 2550-2563.

Dorfman, V. B., Pasquini, L., Riudavets, M., López-Costa, J. J., Villegas, A., Troncoso, J. C., . . . Morelli, L. (2010). Differential cerebral deposition of IDE and NEP in sporadic and familial Alzheimer's disease. *Neurobiol Aging, 31*(10), 1743-1757. doi:10.1016/j.neurobiolaging.2008.09.016

Dubois, B., Feldman, H. H., Jacova, C., Hampel, H., Molinuevo, J. L., Blennow, K., . . . Cummings, J. L. (2014). Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria. *Lancet Neurol, 13*(6), 614-629. doi:10.1016/S1474-4422(14)70090-0

Dubois, B., Hampel, H., Feldman, H. H., Scheltens, P., Aisen, P., Andrieu, S., . . . Washington Dc, U. S. A. (2016). Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria. *Alzheimers Dement, 12*(3), 292-323. doi:10.1016/j.jalz.2016.02.002

Egan, M. F., Kost, J., Tariot, P. N., Aisen, P. S., Cummings, J. L., Vellas, B., . . . Michelson, D. (2018). Randomized Trial of Verubecestat for Mild-to-Moderate Alzheimer's Disease. *N Engl J Med, 378*(18), 1691-1703. doi:10.1056/NEJMoa1706441

Egan, M. F., Mukai, Y., Voss, T., Kost, J., Stone, J., Furtek, C., . . . Michelson, D. (2019). Further analyses of the safety of verubecestat in the phase 3 EPOCH trial of mild-to-moderate Alzheimer's disease. *Alzheimers Res Ther, 11*(1), 68. doi:10.1186/s13195-019-0520-1

Ewers, M., Biechele, G., Suárez-Calvet, M., Sacher, C., Blume, T., Morenas-Rodriguez, E., . . . Franzmeier, N. (2020). Higher CSF sTREM2 and microglia activation are associated with slower rates of beta-amyloid accumulation. *12*(9), e12308. doi:https://doi.org/10.15252/emmm.202012308

Fazi, B., Proserpio, C., Galardi, S., Annesi, F., Cola, M., Mangiola, A., . . . Ciafrè, S. A. (2019). The Expression of the Chemokine CXCL14 Correlates with Several Aggressive Aspects of Glioblastoma and Promotes Key

Properties of Glioblastoma Cells. *Int J Mol Sci, 20*(10). doi:10.3390/ijms20102496
Feldbauer, K., Schlegel, J., Weissbecker, J., Sauer, F., Wood, P. G., Bamberg, E., & Terpitz, U. (2016). Optochemokine Tandem for Light-Control of Intracellular Ca2. *PLoS One, 11*(10), e0165344. doi:10.1371/journal.pone.0165344
Finder, V. H., & Glockshuber, R. (2007). Amyloid-beta aggregation. *Neurodegener Dis, 4*(1), 13-27. doi:10.1159/000100355
Foster, E. M., Dangla-Valls, A., Lovestone, S., Ribe, E. M., & Buckley, N. J. (2019). Clusterin in Alzheimer's Disease: Mechanisms, Genetics, and Lessons From Other Pathologies. *Front Neurosci, 13*, 164. doi:10.3389/fnins.2019.00164
Foster, E. M., Dangla-Valls, A., Lovestone, S., Ribe, E. M., & Buckley, N. J. (2019). Clusterin in Alzheimer’s Disease: Mechanisms, Genetics, and Lessons From Other Pathologies. *13*(164). doi:10.3389/fnins.2019.00164
Gauthier, S., Rosa-Neto, P. (2019). Amyloid, tau, neurodegeneration (ATN)—where do we go from here? *Alzheimer's and other dementias*.
Giordano, C. R., Terlecky, L. J., Bollig-Fischer, A., Walton, P. A., & Terlecky, S. R. (2014). Amyloid-beta neuroprotection mediated by a targeted antioxidant. *Sci Rep, 4*(1), 4983. doi:10.1038/srep04983
Grubman, A., Chew, G., Ouyang, J. F., Sun, G., Choo, X. Y., McLean, C., . . . Polo, J. M. (2019). A single-cell atlas of entorhinal cortex from individuals with Alzheimer's disease reveals cell-type-specific gene expression regulation. *Nat Neurosci, 22*(12), 2087-2097. doi:10.1038/s41593-019-0539-4
Guo, T., Zhang, D., Zeng, Y., Huang, T. Y., Xu, H., & Zhao, Y. (2020). Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease. *Mol Neurodegener, 15*(1), 40. doi:10.1186/s13024-020-00391-7
Haass, C., & Selkoe, D. J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol, 8*(2), 101-112. doi:10.1038/nrm2101
Hampel, H., Cummings, J., Blennow, K., Gao, P., Jack, C. R., & Vergallo, A. (2021). Developing the ATX(N) classification for use across the Alzheimer disease continuum. *Nature Reviews Neurology, 17*(9), 580-589. doi:10.1038/s41582-021-00520-w
Hampel, H., O'Bryant, S. E., Castrillo, J. I., Ritchie, C., Rojkova, K., Broich, K., . . . Lista, S. (2016). PRECISION MEDICINE - The Golden Gate for Detection, Treatment and Prevention of Alzheimer's Disease. *J Prev Alzheimers Dis, 3*(4), 243-259. doi:10.14283/jpad.2016.112
Hampel, H., Vassar, R., De Strooper, B., Hardy, J., Willem, M., Singh, N., . . . Vergallo, A. (2020). The β-Secretase BACE1 in Alzheimer's Disease. *Biol Psychiatry*. doi:10.1016/j.biopsych.2020.02.001
Hampel, H., Vassar, R., De Strooper, B., Hardy, J., Willem, M., Singh, N., . . . Vergallo, A. (2021). The β-Secretase BACE1 in Alzheimer's Disease. *Biol Psychiatry, 89*(8), 745-756. doi:10.1016/j.biopsych.2020.02.001

Hawkes, N. (2017). Merck ends trial of potential Alzheimer's drug verubecestat. *BMJ, 356*, j845. doi:10.1136/bmj.j845

Hippius, H., & Neundörfer, G. (2003). The discovery of Alzheimer's disease. *Dialogues Clin Neurosci, 5*(1), 101-108. doi:10.31887/DCNS.2003.5.1/hhippius

Holmes, C., Cunningham, C., Zotova, E., Woolford, J., Dean, C., Kerr, S., . . . Perry, V. H. (2009). Systemic inflammation and disease progression in Alzheimer disease. *Neurology, 73*(10), 768-774. doi:10.1212/WNL.0b013e3181b6bb95

Hu, X., Das, B., Hou, H., He, W., & Yan, R. (2018). BACE1 deletion in the adult mouse reverses preformed amyloid deposition and improves cognitive functions. *J Exp Med, 215*(3), 927-940. doi:10.1084/jem.20171831

Hu, X., Das, B., Hou, H., He, W., & Yan, R. (2018). BACE1 deletion in the adult mouse reverses preformed amyloid deposition and improves cognitive functions. *Journal of Experimental Medicine, 215*(3), 927-940. doi:10.1084/jem.20171831 %J Journal of Experimental Medicine

Hu, X., He, W., Luo, X., Tsubota, K. E., & Yan, R. (2013). BACE1 regulates hippocampal astrogenesis via the Jagged1-Notch pathway. *Cell Rep, 4*(1), 40-49.

Hu, X., He, W., Luo, X., Tsubota, Katherine E., & Yan, R. (2013). BACE1 Regulates Hippocampal Astrogenesis via the Jagged1-Notch Pathway. *Cell Reports, 4*(1), 40-49. doi:https://doi.org/10.1016/j.celrep.2013.06.005

Hu, X., Zhou, X., He, W., Yang, J., Xiong, W., Wong, P., . . . Yan, R. (2010). BACE1 Deficiency Causes Altered Neuronal Activity and Neurodegeneration. *30*(26), 8819-8829. doi:10.1523/JNEUROSCI.1334-10.2010 %J The Journal of Neuroscience

Jack, C. R., Jr., Bennett, D. A., Blennow, K., Carrillo, M. C., Dunn, B., Haeberlein, S. B., . . . Sperling, R. (2018). NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. *Alzheimers Dement, 14*(4), 535-562. doi:10.1016/j.jalz.2018.02.018

Janelidze, S., Hertze, J., Zetterberg, H., Landqvist Waldö, M., Santillo, A., Blennow, K., & Hansson, O. (2016). Cerebrospinal fluid neurogranin and YKL-40 as biomarkers of Alzheimer's disease. *Ann Clin Transl Neurol, 3*(1), 12-20. doi:10.1002/acn3.266

Janelidze, S., Mattsson, N., Stomrud, E., Lindberg, O., Palmqvist, S., Zetterberg, H., . . . Hansson, O. (2018). CSF biomarkers of neuroinflammation and cerebrovascular dysfunction in early Alzheimer disease. *Neurology, 91*(9), e867-e877. doi:10.1212/wnl.0000000000006082

John Zhou, N. S., James Galske, Jacob Hudobenko, Xiangyou Hu and Riqiang Yan (2022). Bace1 regulates expression of Clusterin in astrocytes for enhancing clearance of β-amyloid peptides. *Mol Neurodegener*.

Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., . . . Ichijo, H. (2005). Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death & Differentiation, 12*(1), 19-24. doi:10.1038/sj.cdd.4401528

Katsouri, L., Birch, A. M., Renziehausen, A. W. J., Zach, C., Aman, Y., Steeds, H., . . . Sastre, M. (2020). Ablation of reactive astrocytes exacerbates disease pathology in a model of Alzheimer's disease. *Glia, 68*(5), 1017-1030. doi:https://doi.org/10.1002/glia.23759

Kinney, J. W., Bemiller, S. M., Murtishaw, A. S., Leisgang, A. M., Salazar, A. M., & Lamb, B. T. (2018). Inflammation as a central mechanism in Alzheimer's disease. *Alzheimers Dement (N Y), 4*, 575-590. doi:10.1016/j.trci.2018.06.014

Kouzeli, A., Collins, P. J., Metzemaekers, M., Meyrath, M., Szpakowska, M., Artinger, M., . . . Moser, B. (2020). CXCL14 Preferentially Synergizes With Homeostatic Chemokine Receptor Systems. *11*. doi:10.3389/fimmu.2020.561404

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., . . . Butovsky, O. (2017). The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity, 47*(3), 566-581.e569. doi:10.1016/j.immuni.2017.08.008

Kumar, A., Mohamed, E., Tong, S., Chen, K., Mukherjee, J., Lim, Y., . . . Phillips, J. J. (2022). CXCL14 Promotes a Robust Brain Tumor-Associated Immune Response in Glioma. *Clinical Cancer Research*, OF1-OF13. doi:10.1158/1078-0432.CCR-21-2830 %J Clinical Cancer Research

Kunkle, B. W., Grenier-Boley, B., Sims, R., Bis, J. C., Damotte, V., Naj, A. C., . . . Lieberman, A. P. (2019). Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Aβ, tau, immunity and lipid processing. *Nature Genetics, 51*(3), 414-430. doi:10.1038/s41588-019-0358-2

Lambert, J. C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., . . . Amouyel, P. (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat Genet, 41*(10), 1094-1099. doi:10.1038/ng.439

Lee, H. T., Liu, S. P., Lin, C. H., Lee, S. W., Hsu, C. Y., Sytwu, H. K., . . . Shyu, W. C. (2017). A Crucial Role of CXCL14 for Promoting Regulatory T Cells Activation in Stroke. *Theranostics, 7*(4), 855-875. doi:10.7150/thno.17558

Liddelow, S. A., & Barres, B. A. (2017). Reactive Astrocytes: Production, Function, and Therapeutic Potential. *Immunity, 46*(6), 957-967. doi:10.1016/j.immuni.2017.06.006

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., . . . Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature, 541*(7638), 481-487.

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., . . . Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature, 541*(7638), 481-487. doi:10.1038/nature21029

Liu, M., Zhang, S.-B., Luo, Y.-X., Yang, Y.-L., Zhang, X.-Z., Li, B., . . . Li, D. (2020). NFATc2-dependent epigenetic upregulation of CXCL14 is involved

in the development of neuropathic pain induced by paclitaxel. *J Neuroinflammation, 17*(1), 310. doi:10.1186/s12974-020-01992-1
Llorens, F., Thüne, K., Tahir, W., Kanata, E., Diaz-Lucena, D., Xanthopoulos, K., . . . Zerr, I. (2017). YKL-40 in the brain and cerebrospinal fluid of neurodegenerative dementias. *Mol Neurodegener, 12*(1), 83. doi:10.1186/s13024-017-0226-4
Lombardo, S., Chiacchiaretta, M., Tarr, A., Kim, W., Cao, T., Sigal, G., . . . Tesco, G. (2019). BACE1 partial deletion induces synaptic plasticity deficit in adult mice. *Sci Rep, 9*(1), 19877. doi:10.1038/s41598-019-56329-7
LTD, V. C. (2021). A Study of Possibility of Using Regulatory T Cells(VT301) for Treatment of Alzheimer's Disease. Retrieved from https://clinicaltrials.gov/ct2/show/NCT05016427
Lu, J., Chatterjee, M., Schmid, H., Beck, S., & Gawaz, M. (2016). CXCL14 as an emerging immune and inflammatory modulator. *Journal of Inflammation, 13*(1), 1. doi:10.1186/s12950-015-0109-9
Maarouf, C. L., Walker, J. E., Sue, L. I., Dugger, B. N., Beach, T. G., & Serrano, G. E. (2018). Impaired hepatic amyloid-beta degradation in Alzheimer's disease. *PLoS One, 13*(9), e0203659. doi:10.1371/journal.pone.0203659
Mahmoud, S., Gharagozloo, M., Simard, C., & Gris, D. (2019). Astrocytes Maintain Glutamate Homeostasis in the CNS by Controlling the Balance between Glutamate Uptake and Release. *Cells, 8*(2). doi:10.3390/cells8020184
MATSUBARA, E., SOTO, C., GOVERNALE, S., FRANGIONE, B., & GHISO, J. (1996). Apolipoprotein J and Alzheimer's amyloid β solubility. *Biochemical Journal, 316*(2), 671-679. doi:10.1042/bj3160671 %J Biochemical Journal
McDade, E., Llibre-Guerra, J. J., Holtzman, D. M., Morris, J. C., & Bateman, R. J. (2021). The informed road map to prevention of Alzheimer Disease: A call to arms. *Mol Neurodegener, 16*(1), 49. doi:10.1186/s13024-021-00467-y
McDade, E., Voytyuk, I., Aisen, P., Bateman, R. J., Carrillo, M. C., De Strooper, B., . . . Lichtenthaler, S. F. (2021). The case for low-level BACE1 inhibition for the prevention of Alzheimer disease. *Nat Rev Neurol*. doi:10.1038/s41582-021-00545-1
Meakin, P. J., Mezzapesa, A., Benabou, E., Haas, M. E., Bonardo, B., Grino, M., . . . Peiretti, F. (2018). The beta secretase BACE1 regulates the expression of insulin receptor in the liver. *Nat Commun, 9*(1), 1306. doi:10.1038/s41467-018-03755-2
Mustapic, M., Tran, J., Craft, S., & Kapogiannis, D. (2019). Extracellular Vesicle Biomarkers Track Cognitive Changes Following Intranasal Insulin in Alzheimer's Disease. *J Alzheimers Dis, 69*(2), 489-498. doi:10.3233/jad-180578
Namioka, N., Hanyu, H., Hirose, D., Hatanaka, H., Sato, T., & Shimizu, S. (2017). Oxidative stress and inflammation are associated with physical frailty in patients with Alzheimer's disease. *17*(6), 913-918. doi:https://doi.org/10.1111/ggi.12804

Neeraj Singh, B. D., John Zhou, Xiangyou Hu and Riqiang Yan (2022). Targeted BACE-1 inhibition in microglia enhances amyloid clearance and improved cognitive performance. *Science Advances*.

Neeraj Singh, X. H., Marc Benoit, Brati Das, John Zhou, Jose Davila, Li-Huei Tsai, Manolis Kellis, Riqiang Yan (2022). Bace-1 inhibition facilitates the transition from homeostatic microglia to DAM-1. *Science Advances*.

Nelson, A. R., Sagare, A. P., & Zlokovic, B. V. (2017). Role of clusterin in the brain vascular clearance of amyloid-β. *114*(33), 8681-8682. doi:10.1073/pnas.1711357114 %J Proceedings of the National Academy of Sciences

Nguyen, A. T., Wang, K., Hu, G., Wang, X., Miao, Z., Azevedo, J. A., . . . Lee, E. B. (2020). APOE and TREM2 regulate amyloid-responsive microglia in Alzheimer's disease. *Acta Neuropathol, 140*(4), 477-493. doi:10.1007/s00401-020-02200-3

Nordengen, K., Kirsebom, B.-E., Henjum, K., Selnes, P., Gísladóttir, B., Wettergreen, M., . . . Fladby, T. (2019). Glial activation and inflammation along the Alzheimer's disease continuum. *J Neuroinflammation, 16*(1), 46. doi:10.1186/s12974-019-1399-2

Novak, G., Streffer, J. R., Timmers, M., Henley, D., Brashear, H. R., Bogert, J., . . . Engelborghs, S. (2020). Long-term safety and tolerability of atabecestat (JNJ-54861911), an oral BACE1 inhibitor, in early Alzheimer's disease spectrum patients: a randomized, double-blind, placebo-controlled study and a two-period extension study. *Alzheimers Res Ther, 12*(1), 58. doi:10.1186/s13195-020-00614-5

Nuutinen, T., Huuskonen, J., Suuronen, T., Ojala, J., Miettinen, R., & Salminen, A. (2007). Amyloid-β 1–42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes. *Neurochemistry International, 50*(3), 540-547. doi:https://doi.org/10.1016/j.neuint.2006.11.002

Ogunshola, O. O., & Antoniou, X. (2009). Contribution of hypoxia to Alzheimer's disease: is HIF-1alpha a mediator of neurodegeneration? *Cell Mol Life Sci, 66*(22), 3555-3563. doi:10.1007/s00018-009-0141-0

Ou-Yang, M. H., Kurz, J. E., Nomura, T., Popovic, J., Rajapaksha, T. W., Dong, H., . . . Vassar, R. (2018). Axonal organization defects in the hippocampus of adult conditional BACE1 knockout mice. *Sci Transl Med, 10*(459). doi:10.1126/scitranslmed.aao5620

Parbo, P., Ismail, R., Hansen, K. V., Amidi, A., Mårup, F. H., Gottrup, H., . . . Brooks, D. J. (2017). Brain inflammation accompanies amyloid in the majority of mild cognitive impairment cases due to Alzheimer's disease. *Brain: A Journal of Neurology, 140*(7), 2002-2011. doi:10.1093/brain/awx120

Parhizkar, S., Arzberger, T., Brendel, M., Kleinberger, G., Deussing, M., Focke, C., . . . Haass, C. (2019). Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE. *Nature Neuroscience, 22*(2), 191-204. doi:10.1038/s41593-018-0296-9

Park, J. C., Han, S. H., & Mook-Jung, I. (2020). Peripheral inflammatory biomarkers in Alzheimer's disease: a brief review. *BMB Rep, 53*(1), 10-19. doi:10.5483/BMBRep.2020.53.1.309

Perez-Nievas, B. G., & Serrano-Pozo, A. (2018). Deciphering the Astrocyte Reaction in Alzheimer's Disease. *10*(114). doi:10.3389/fnagi.2018.00114

Rangaraju, S., Dammer, E. B., Raza, S. A., Rathakrishnan, P., Xiao, H., Gao, T., . . . Levey, A. I. (2018). Identification and therapeutic modulation of a pro-inflammatory subset of disease-associated-microglia in Alzheimer's disease. *Mol Neurodegener, 13*(1), 24. doi:10.1186/s13024-018-0254-8

Ries, M., & Sastre, M. (2016). Mechanisms of Aβ Clearance and Degradation by Glial Cells. *Front Aging Neurosci, 8*, 160. doi:10.3389/fnagi.2016.00160

Rivers-Auty, J., Mather, A. E., Peters, R., Lawrence, C. B., Brough, D., & Initiative, A. s. D. N. (2020). Anti-inflammatories in Alzheimer's disease—potential therapy or spurious correlate? *Brain Communications, 2*(2). doi:10.1093/braincomms/fcaa109

Schildge, S., Bohrer, C., Beck, K., & Schachtrup, C. (2013). Isolation and culture of mouse cortical astrocytes. *Journal of visualized experiments : JoVE*(71), 50079. doi:10.3791/50079

Schneider, L. (2020). A resurrection of aducanumab for Alzheimer's disease. *Lancet Neurol, 19*(2), 111-112. doi:10.1016/s1474-4422(19)30480-6

Schoemaker, D., Poirier, J., Escobar, S., Gauthier, S., & Pruessner, J. (2016). Selective familiarity deficits in otherwise cognitively intact aging individuals with genetic risk for Alzheimer's disease. *Alzheimers Dement (Amst), 2*, 132-139. doi:10.1016/j.dadm.2015.11.007

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med, 8*(6), 595-608.

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med, 8*(6), 595-608. doi:10.15252/emmm.201606210

Sevigny, J., Chiao, P., Bussière, T., Weinreb, P. H., Williams, L., Maier, M., . . . Sandrock, A. (2016). The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. *Nature, 537*(7618), 50-56. doi:10.1038/nature19323

Söllvander, S., Nikitidou, E., Brolin, R., Söderberg, L., Sehlin, D., Lannfelt, L., & Erlandsson, A. (2016). Accumulation of amyloid-β by astrocytes result in enlarged endosomes and microvesicle-induced apoptosis of neurons. *Mol Neurodegener, 11*(1), 38. doi:10.1186/s13024-016-0098-z

Sompol, P., & Norris, C. M. (2018). Ca(2+), Astrocyte Activation and Calcineurin/NFAT Signaling in Age-Related Neurodegenerative Diseases. *Front Aging Neurosci, 10*, 199. doi:10.3389/fnagi.2018.00199

Soreq, L., Rose, J., Soreq, E., Hardy, J., Trabzuni, D., Cookson, M. R., . . . Ule, J. (2017). Major Shifts in Glial Regional Identity Are a Transcriptional Hallmark of Human Brain Aging. *Cell Reports, 18*(2), 557-570. doi:10.1016/j.celrep.2016.12.011

Steen, E., Terry, B. M., Rivera, E. J., Cannon, J. L., Neely, T. R., Tavares, R., . . . de la Monte, S. M. (2005). Impaired insulin and insulin-like growth factor

expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *J Alzheimers Dis, 7*(1), 63-80. doi:10.3233/jad-2005-7107
Stine, W. B., Jungbauer, L., Yu, C., & LaDu, M. J. (2011). Preparing synthetic Aβ in different aggregation states. *Methods Mol Biol, 670*, 13-32. doi:10.1007/978-1-60761-744-0_2
Subramanian, J., Michel, K., Benoit, M., & Nedivi, E. (2019). CPG15/Neuritin Mimics Experience in Selecting Excitatory Synapses for Stabilization by Facilitating PSD95 Recruitment. *Cell Rep, 28*(6), 1584-1595.e1585. doi:10.1016/j.celrep.2019.07.012
Sur, C., Kost, J., Scott, D., Adamczuk, K., Fox, N. C., Cummings, J. L., . . . Egan, M. F. (2020). BACE inhibition causes rapid, regional, and non-progressive volume reduction in Alzheimer's disease brain. *Brain, 143*(12), 3816-3826. doi:10.1093/brain/awaa332
Tamashiro, T. T., Dalgard, C. L., & Byrnes, K. R. (2012). Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. *Journal of visualized experiments : JoVE*(66), e3814. doi:10.3791/3814
Thakker, D. R., Sankaranarayanan, S., Weatherspoon, M. R., Harrison, J., Pierdomenico, M., Heisel, J. M., . . . Shafer, L. L. (2015). Centrally Delivered BACE1 Inhibitor Activates Microglia, and Reverses Amyloid Pathology and Cognitive Deficit in Aged Tg2576 Mice. *J. Neurosci, 35*(17), 6931-6936.
Thakker, D. R., Sankaranarayanan, S., Weatherspoon, M. R., Harrison, J., Pierdomenico, M., Heisel, J. M., . . . Shafer, L. L. (2015). Centrally Delivered BACE1 Inhibitor Activates Microglia, and Reverses Amyloid Pathology and Cognitive Deficit in Aged Tg2576 Mice. *35*(17), 6931-6936. doi:10.1523/JNEUROSCI.2262-14.2015 %J The Journal of Neuroscience
Thal, D. R., Rub, U., Orantes, M., & Braak, H. (2002). Phases of A beta-deposition in the human brain and its relevance for the development of AD. *Neurology, 58*(12), 1791-1800. doi:10.1212/wnl.58.12.1791
Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., . . . Citron, M. (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science, 286*(5440), 735-741. doi:10.1126/science.286.5440.735
Vekrellis, K., Ye, Z., Qiu, W. Q., Walsh, D., Hartley, D., Chesneau, V., . . . Selkoe, D. J. (2000). Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. *J Neurosci, 20*(5), 1657-1665. doi:10.1523/jneurosci.20-05-01657.2000
Vermunt, L., Sikkes, S. A. M., van den Hout, A., Handels, R., Bos, I., van der Flier, W. M., . . . Visser, P. J. (2019). Duration of preclinical, prodromal, and dementia stages of Alzheimer's disease in relation to age, sex, and APOE genotype. *Alzheimers Dement, 15*(7), 888-898. doi:10.1016/j.jalz.2019.04.001
Villemagne, V. L., Klunk, W. E., Mathis, C. A., Rowe, C. C., Brooks, D. J., Hyman, B. T., . . . Drzezga, A. (2012). Aβ Imaging: feasible, pertinent, and vital to progress in Alzheimer's disease. *European Journal of Nuclear*

Medicine and Molecular Imaging, 39(2), 209-219. doi:10.1007/s00259-011-2045-0

Wang, H., Song, L., Laird, F., Wong, P. C., & Lee, H. K. (2008). BACE1 knock-outs display deficits in activity-dependent potentiation of synaptic transmission at mossy fiber to CA3 synapses in the hippocampus. *J. Neurosci, 28*(35), 8677-8681.

Wang, H., Song, L., Lee, A., Laird, F., Wong, P. C., & Lee, H. K. (2010). Mossy fiber long-term potentiation deficits in BACE1 knock-outs can be rescued by activation of alpha7 nicotinic acetylcholine receptors. *J. Neurosci, 30*(41), 13808-13813.

Waring, S. C., & Rosenberg, R. N. (2008). Genome-wide association studies in Alzheimer disease. *Arch Neurol, 65*(3), 329-334. doi:10.1001/archneur.65.3.329

Witte, A., Chatterjee, M., Lang, F., & Gawaz, M. (2017). Platelets as a Novel Source of Pro-Inflammatory Chemokine CXCL14. *Cell Physiol Biochem, 41*(4), 1684-1696. doi:10.1159/000471821

Wojtas, A. M., Kang, S. S., Olley, B. M., Gatherer, M., Shinohara, M., Lozano, P. A., . . . Fryer, J. D. (2017). Loss of clusterin shifts amyloid deposition to the cerebrovasculature via disruption of perivascular drainage pathways. *Proc Natl Acad Sci U S A, 114*(33), E6962-e6971. doi:10.1073/pnas.1701137114

Wojtas, A. M., Sens, J. P., Kang, S. S., Baker, K. E., Berry, T. J., Kurti, A., . . . Fryer, J. D. (2020). Astrocyte-derived clusterin suppresses amyloid formation in vivo. *Mol Neurodegener, 15*(1), 71. doi:10.1186/s13024-020-00416-1

Wyss-Coray, T., Loike, J. D., Brionne, T. C., Lu, E., Anankov, R., Yan, F., . . . Husemann, J. (2003). Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. *Nat Med, 9*(4), 453-457. doi:10.1038/nm838

Wyss-Coray, T., Loike, J. D., Brionne, T. C., Lu, E., Anankov, R., Yan, F., . . . Husemann, J. (2003). Adult mouse astrocytes degrade amyloid-β in vitro and in situ. *Nat Med, 9*(4), 453-457. doi:10.1038/nm838

Xiao, Q., Yan, P., Ma, X., Liu, H., Perez, R., Zhu, A., . . . Lee, J.-M. (2014). Enhancing Astrocytic Lysosome Biogenesis Facilitates Aβ Clearance and Attenuates Amyloid Plaque Pathogenesis. *34*(29), 9607-9620. doi:10.1523/JNEUROSCI.3788-13.2014 %J The Journal of Neuroscience

Yan, R. (2016). Stepping closer to treating Alzheimer's disease patients with BACE1 inhibitor drugs. *Transl Neurodegener, 5*, 13. doi:10.1186/s40035-016-0061-5

Yan, R. (2017a). Physiological Functions of the beta-Site Amyloid Precursor Protein Cleaving Enzyme 1 and 2. *Front Mol. Neurosci, 10*, 97.

Yan, R. (2017). Physiological Functions of the β-Site Amyloid Precursor Protein Cleaving Enzyme 1 and 2. *10*(97). doi:10.3389/fnmol.2017.00097

Yan, R. (2017b). Physiological Functions of the β-Site Amyloid Precursor Protein Cleaving Enzyme 1 and 2. *Front Mol Neurosci, 10*, 97. doi:10.3389/fnmol.2017.00097

Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., . . . Gurney, M. E. (1999). Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature, 402*(6761), 533-537. doi:10.1038/990107

Yan, R., Fan, Q., Zhou, J., & Vassar, R. (2016). Inhibiting BACE1 to reverse synaptic dysfunctions in Alzheimer's disease. *Neurosci Biobehav Rev, 65*, 326-340. doi:10.1016/j.neubiorev.2016.03.025

Yan, R., & Vassar, R. (2014). Targeting the beta secretase BACE1 for Alzheimer's disease therapy. *Lancet Neurol, 13*(3), 319-329.

Zhang, F., Lin, Y. A., Kannan, S., & Kannan, R. M. (2016). Targeting specific cells in the brain with nanomedicines for CNS therapies. *J Control Release, 240*, 212-226. doi:10.1016/j.jconrel.2015.12.013

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., . . . Wu, J. Q. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci, 34*(36), 11929-11947. doi:10.1523/jneurosci.1860-14.2014

Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., . . . Barres, B. A. (2016). Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron, 89*(1), 37-53. doi:10.1016/j.neuron.2015.11.013

Zhao, J., O'Connor, T., & Vassar, R. (2011). The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. *J Neuroinflammation, 8*, 150. doi:10.1186/1742-2094-8-150

Zhong, L., & Chen, X.-F. (2019). The Emerging Roles and Therapeutic Potential of Soluble TREM2 in Alzheimer’s Disease. *11*(328). doi:10.3389/fnagi.2019.00328

Zhou, J., Benoit, M., & Sharoar, M. G. (2021). Recent advances in pre-clinical diagnosis of Alzheimer's disease. *Metab Brain Dis*. doi:10.1007/s11011-021-00733-4

Zhu, K., Peters, F., Filser, S., & Herms, J. (2018). Consequences of Pharmacological BACE Inhibition on Synaptic Structure and Function. *Biol Psychiatry, 84*(7), 478-487. doi:10.1016/j.biopsych.2018.04.022

Zulfiqar, S., Garg, P., & Nieweg, K. (2019). Contribution of astrocytes to metabolic dysfunction in the Alzheimer's disease brain. *Biol Chem, 400*(9), 1113-1127. doi:10.1515/hsz-2019-0140