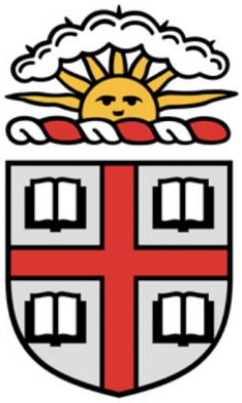
The Neural Effect of Chitinase-3-like Protein 1 (CHI3L1/YKL-40) in Neuroinflammation & Alzheimer's Disease



Kevin Connolly

B.S. Eastern Connecticut State University

M.H.S. Quinnipiac University

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Division of Biology of Medicine at Brown University: Molecular Biology, Cell Biology, and Biochemistry

Providence, Rhode Island

October 2025

Signature Page

This dissertation by Kevin Connolly is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date _____ _____

Yu-Wen Alvin Huang, M.D., Ph.D., Advisor

Recommended to the Graduate Council

Date _____ _____

John Sedivy, Ph.D., Chair/Reader

Date _____ _____

Ashley Webb, Ph.D., Reader

Date _____ _____

Chun Geun Lee, Ph.D., Reader

Date _____ _____

Erik Musiek, M.D., Ph.D., External Reader

Approved by the Graduate Council

Date _____ _____

Thomas A. Lewis Ph.D., Dean of the Graduate School

Curriculum Vitae

KEVIN CONNOLLY

• 70 Ship Street, Providence, RI 02903 •

• kevin_connolly@brown.edu •

EDUCATION

Doctor of Philosophy, Molecular Biology, **Cell Biology**, **& Biochemistry**, Brown University **2019 – 2025**
Cumulative GPA: 4.00
Relevant Coursework: Foundations for Advanced Study in the Life Sciences, Quantitative Approaches to Biology, Biology of Reproduction, Scientific Communication, Molecular Genetics

Master of Health Sciences, Biomedical Sciences, Quinnipiac University **2017 - 2019**
Cumulative GPA: 3.96
Relevant Coursework: Research Methods, Pathophysiology, Immunology, Pharmacology, Leukemia, Histology, Neuropharmacology, Toxicology

Bachelor of Science, Biology, Eastern Connecticut State University **2013 – 2017**
Minor: Spanish
Cumulative GPA: 3.89
Honors: University Honors Program Scholar, *Beta Beta Beta*, *Omicron Delta Kappa*
Relevant Coursework: Cell Structure and Function, Genetics, Microbiology, Developmental Biology, Human Anatomy, Human Physiology, General Chemistry, Organic Chemistry, Physics, Calculus

PROFESSIONAL EXPERIENCE

Brown University – Providence, RI **2019 – 2025**
PhD Research with Dr. Alvin Huang
- Researched "*The Neural Effect of CHI3L1 in Neuroinflammation & Alzheimer's Disease*".
- Utilized human stem cell technology to study the molecular and cellular fundamentals underlying neurodegeneration and brain aging, specifically in the context of Alzheimer's Disease.
- Awarded an NIH Parent F31 and the Carney Institute Graduate Award in Brian Science.

Teaching Assistant
- Taught BIOL0470: Genetics.
- Aided 40 + students in the completion of online, bioinformatic-based laboratory work.
- Graded and provided feedback on laboratory assignments, exams, and oral presentations.

Quinnipiac University – Hamden, CT **2017 – 2019**
Master's Thesis Research with Dr. Martine Mirrione
- Researched "*Analysis of Microglia Expression, Morphology, and Polarization State in the Cornu Ammonis and Dentate Gyrus of the Hippocampus in Female Maternally Separated Rats*".
- Utilized cryostat sectioning of whole rat brain tissue, immunofluorescence, and confocal microscopy to examine hippocampal microglia.
- Managed established research with the execution of novel ideas and work.

Graduate Research Assistant for the School of Health Sciences
- Instructed BMS 502: Research Methods and BMS 472: Biotechnology.
- Assisted students with a variety of biological applications in a laboratory setting.
- Developed lesson plans and communicated essential material through classroom style lectures.
- Troubleshot course laboratory experiments to best enhance student learning experiences.

Graduate Assistant of Student Organizations

- Oversaw student organization scheduling and event approvals.
- Managed New Student Organization Information Sessions.
- Enabled the development and allocation of resources for the creation of new organizations on campus.
- Supervised team of 10 + student workers.

Eastern Connecticut State University – Willimantic, CT **2015 – 2017**

Undergraduate Thesis Research with Dr. Barbara Murdoch

- Researched "*Enhancement of Collagen Production in Dermal Cells Via Transforming Growth Factor-β (TGF-β) Signaling and Other Communication Pathways*".
    - Awarded the Phillip F. Elliot Exceptional Thesis Award and the Yaw Barwuah Award for Outstanding Biology Research.
    - Presented at the Northeast Regional Honors Conference (NRHC), Council of Public Liberal Arts Colleges Conference (COPLAC), and the Eastern Colleges Science Conference (ECSC).
- Cultured and treated cells to isolate collagen protein and quantify via Western Blotting.
- Earned Freeman Scholarship and the Honors Thesis Research Enhancement Fellowship.

Small World Initiative Exhibitor Assistant.

- Educated students throughout Connecticut about antibiotic resistance.
- Communicated findings, applications, and information to target audiences.
- Contributed to community engagement efforts.
- Presented "*Engagement in Science Education and Research for the Eradication of Antibiotic Resistance*" at CT Space Grant Consortium Expo.

Teaching Assistant

- Taught BIO 230: Genetics Laboratory and BIO 220: Cell Structure and Function Laboratory.
- Awarded the Michael F. Gable Biology Student Assistantships Endowed Fund for outstanding performance.
- Educated students about biological applications and information.

ACCOLADES & PROFESSIONAL DEVELOPMENT

Publications

Jiang, W., Zhu, F., Xu, H., Xu, L., Li, H., Yang, X., Afridi, S. K., Lai, S., Qui, X., Liu, C., Li, H., Long, Y., Wang, Y., **Connolly, K.**, Elias, J. A., Lee, C. G., Cui, Y., Huang, Y. A., Qui, W., Tang, C. (2023). CHI3L1 signaling impairs hippocampal neurogenesis and cognitive function in autoimmune-mediated neuroinflammation. *Sci Adv, 9*(39).

Connolly, K., Lehoux, M., Assetta, B., Huang, Y. A. (2023). Modeling cellular cross-talk of neuroinflammation axis by tri-cultures of iPSC-derived human microglia, astrocytes, and neurons. *Methods Mol. Biol.* Vol. 2683.

Lehoux, M., **Connolly, K.**, Assetta, B., Huang, Y. A. (2023). The generation and functional characterization of human microglia-like cells derived from iPS and embryonic stem cells. *Methods Mol. Biol.* Vol. 2683.

Connolly, K., Lehoux, M., O'Rourke, R., Assetta, B., Erdemir, G. A., Elias, J. A., Lee, C. G., Huang, Y. A. (2022). Potential role of chitinase-3-like protein 1 (CHI3L1/YKL-40) in neurodegeneration and Alzheimer's disease. *Alzheimers Dement*.

Assetta, B., Tang, C., Bian, J., O'Rourke, R., **Connolly, K.**, Brickler, T., Chetty, S., Huang, Y. A. (2020). Generation of Human Neurons and Oligodendrocytes from Pluripotent Stem Cells for Modeling Neuron-Oligodendrocyte Interactions. *Journal of visualized experiments: JoVE*.

Awards

NIH Parent F31 Ruth L. Kirschstein Predoctoral Individual National Research Service Award 2023 - 2025
Carney Institute Graduate Award in Brain Science 2022 - 2023
Phillip F. Elliot Exceptional Thesis Award 2017
Yaw Barwuah Award for Outstanding Biology Research 2017
Connecticut State University System Foundation Henry Barnard Distinguished Student Award 2017
Connecticut Space Grant Consortium Travel Grant 2016
Connecticut Space Grant Consortium Undergraduate Scholarship 2016
Freeman Scholarship 2016
Honors Thesis Research Enhancement Fellowship 2016
Michael F. Gable Biology Student Assistantships Endowed Fund - for outstanding performance 2015

Presentations

Keystone Symposium - Neuroimmune Interactions: From Basic Mechanisms to Novel Therapeutic Directions
- *The Neural Effect of CHI3L1 in Neuroinflammation & Alzheimer's Disease* 2023

Northeastern Glenn Symposium on the Biology of Aging
- *The Neuronal Effect of CHI3L1 in Neuroinflammation & Alzheimer's Disease* 2022

Providence Area Aging Research Forum (PAARF)
- *The Neuronal Effect of CHI3L1 in Neuroinflammation & Alzheimer's Disease* 2022

StemCONN: Connecticut's Stem Cell and Regenerative Medicine Symposium
- Highschool Outreach Virtual Presentation: *Stem Cell Applications in Research* 2021

Northeast Under/graduate Research Organization for Neuroscience (NEURON) Conference
- *Analysis of Neuronal Activity and Optimization of Microglial Expression, Morphology, and Polarization State in the Hippocampus in Rat Models of Depressive-Like Behavior* 2019

Northeast Regional Honors Conference
- *Enhancement of Collagen Production in Skin Cells Via Cell Communication Signals* 2017
- *Postwar Reconstruction: Medical Advances Throughout Wartime* 2015

Eastern Colleges Science Conference
- *Enhancement of Collagen Production in Dermal Cells Via Transforming Growth Factor-β (TGF-β) Signaling* 2017

Council of Public Liberal Arts Colleges (COPLAC) Conference
- *Enhancement of Collagen Production in Skin Cells Via Cell Communication Signals* 2016

Connecticut Space Consortium Grants Expo
- *Engagement in Science Education and Research for the Eradication of Antibiotic Resistance* 2016

Connecticut Learn and Works Conference
- *College and University Honors/Scholars Program* 2016

Workshops

Sheridan Course Design Seminar - teaching certificate program 2022
Sheridan Teaching Seminar - teaching certificate program 2021

Leadership & Involvement

Committee Member, MCBGP Retreat Committee 2023, 2024
Peer Mentor, MCBGP Peer Mentorship Program 2021 - 2024
Committee Member, Trainee Advisory Committee for the Center on the Biology of Aging 2022
President of Honors Club 2015 - 2017
Vice President of *Omicron Delta Kappa*, National Leadership Honors Society 2016 - 2017
Secretary of *Beta Beta Beta*, Biology Honors Society 2017
Chair of World Antibiotic Awareness Week Organizing Committee 2016
Public Relations Officer of UNICEF Club 2015 - 2016

Acknowledgements

I thank Dr. Alvin Huang for his support, encouragement, and dedication throughout this entire research process. Alvin readily imparted his wisdom and continuously pushed me toward success in completing this project. I am grateful for all of the knowledge and experience I have gained in working in the Huang Lab. I would also like to extend appreciation to Dr. John Sedivy, Dr. Ashley Webb, Dr. Chun Guen Lee, as well as Dr. Erik Musiek for serving on my thesis committee and for providing helpful comments and important insights contributing to the overall success of the project. Additionally, I would also like to extend my appreciation to my lab members, Dr. Benedetta Assetta, Dr. Xin Yang, Dr. Ryan O'Rourke, Jules Duckhorn, Ellie Doo Kyung Kim, Joshua Wu, Mika Lehoux Marmolejos, and Zachary Pearson for assistance with aspects of the project and for providing support throughout this process. I would also like to acknowledge and thank my funding sources including my NIH Parent F31 Ruth L. Kirschstein Predoctoral Individual National Research Service Award, Carney Institute Graduate Award in Brain Science, and NIH Pre-Doctoral T32 MCB Training Grant. Gratitude is also extended to the Molecular Biology, Cell Biology, & Biochemistry Graduate Program and Brown University for the opportunities that they have afforded me. Lastly, I would like to thank my family and friends for all of their love and support throughout this entire process.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

This chapter was adapted from publication:

Connolly, K., Lehoux, M., O'Rourke, R., Assetta, B., Erdemir, G. A., Elias, J. A., Lee, C. G., Huang, Y. A. (2022). Potential role of chitinase-3-like protein 1 (CHI3L1/YKL-40) in neurodegeneration and Alzheimer's disease. *Alzheimers Dement*.

https://doi.org/10.1002/alz.12612

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is an age-related, neurological disorder with symptomology of progressive cognitive impairment. AD is currently the sixth leading cause of death in the United States[1]. As we age the brain is susceptible to neurodegeneration, resulting in the loss of brain structure and function. Neurodegeneration and AD pathogenesis are dictated by genetics and cellular interactions. Pathologically, AD is characterized by the accumulation of intracellular neurofibrillary tangles comprised of phospho-tau, and extracellular amyloid plaques consisting of amyloid beta (Aβ), derived from amyloid precursor protein (APP). Tau prevents the transport of essential nutrients and molecules inside neurons, while Aβ and APP interfere with neuronal communication[1]. In addition to tau and Aβ accumulation, neuroinflammation plays an important role in neuron dysfunction and degeneration, especially in the context of AD[2-4] (**Figure 1**). Neuroinflammation is a process mainly involving microglia and astrocytes and is necessary for healthy brain function. However, when this process goes awry, it can lead to synapse loss and neuronal death[5-7]. Together, these pathologies are thought to lead to neurodegeneration and AD progression. Despite the identification of some key players, the molecular pathways that lead to neuronal dysfunction and degeneration are still not well understood.

The attention given to hallmark pathologies as causative agents for Alzheimer's Disease (AD), namely extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFTs), comprised of amyloid beta (Aβ) and phospho-tau, respectively, has framed the inflammatory response to these molecules as secondary. However, recent evidence suggests otherwise, implicating neuroinflammation as an early factor in AD progression. Neuroinflammation is a process mainly involving microglia and astrocytes and is necessary for healthy brain function. However, in many neurological disorders such as AD there is an uncontrolled immune imbalance

leading to abnormalities including increased microglial and astroglial activation, enhanced inflammatory cytokines and oxidative stress, vascular impairment and compromised integrity of blood-brain barrier (BBB), decreased neurogenesis, loss of synapses and eventually neuronal death; these abnormalities have been implicated in AD because they have detrimental effects on neuroprotection and cause neuroinflammation [8-11]. Despite the prevalence of these events in AD, the questions still remain: what causes this "uncontrolled immune imbalance", is neuroinflammation a direct causative factor in AD pathogenesis, and, if so, what is the precise mechanism underlying neuroinflammation and neurodegeneration in AD?

The inflammatory molecule Chitinase-3-like protein 1 (CHI3L1) has emerged to be a recent focus in the field of AD. CHI3L1, also called YKL-40 in humans and BRP-39 in mice, is a well-characterized inflammatory molecule and well known as a powerful biomarker that can be detected at the earliest stage of pathogenesis and help differentiate AD from other forms of dementia [4, 12, 13]. Cerebrospinal fluid (CSF) levels of CHI3L1 predict the severity of clinical symptoms and pathological features and can reliably distinguish AD from other forms of dementia [4, 12, 13]. CHI3L1 is a secreted glycoprotein that belongs to the diverse glycoside hydrolase family 18 [14]. While it preserves their chitin binding capacity, CHI3L1 lacks chitinase activity owing to loss-of-function mutations of its chitinase domain [15, 16].

CHI3L1 is indeed most studied in the field of pulmonary inflammation, injury repair and tissue remodeling, e.g. asthma and lung fibrosis, and its multifaceted signaling functions through a specific receptor system have been pioneered by us and documented by others in both innate and adaptive immunity [17-20]. It is now clear that CHI3L1 is a master regulator for a wide range of injury and repair responses, including the innate immunity pathway that resembles the neuroinflammation process initiated by microglia and coordinated by astrocytes (**Figure 1**) [15,

16, 18-22]. Nevertheless, how CHI3L1 operates in neuroinflammation and AD pathogenesis remains to be elucidated. Based on the known function of CHI3L1 in peripheral tissues and recent findings in the central nervous system (CNS), we propose here the potential role of CHI3L1 in neuroinflammation and neurodegeneration, going beyond merely a biomarker. It is our hypothesis that CHI3L1, secreted by astrocytes upon microglia reactivation, functions as an inflammatory signaling molecule to mediate inflammatory responses most likely in a cell type-specific fashion, and that such signaling function can derail to instigate the neurodegeneration process.

1.2. CHI3L1 as a Powerful Biomarker in Neurodegenerative Disease

Chitins and chitinase family members have demonstrated utility as biomarkers for variety of inflammation-prone brain diseases, including AD. Not only are chitin levels elevated in the CNS, CSF, and plasma in AD patients, but chitin has also been implicated in influencing Aβ accumulation and AD pathogenesis [23]. Similarly, the levels of chitinase family members, including chitotriosidase, acidic mammalian chitinase, chitinase 3-like protein 2, chitinase domain–containing 1, and CHI3L1, are increased in AD brains versus healthy controls [24]. In AD, these chitinases were increased at both the protein and RNA levels [24]. While increases in the levels of chitins and several chitinase family members have been documented, CHI3L1 has been the most frequently studied one for its reliability and robustness in serving as an AD biomarker. Scores of groups have long found elevated CHI3L1 levels in the CSF of AD patients (**Table 1**, a summary of most recent results). CHI3L1 is a secreted chitinase-dead, catalytically inactive glycoprotein expressed and secreted mainly by astrocytes in the brain (**Figure 2**) [14, 20, 25]. Longitudinal studies suggest a correlation between higher levels of CHI3L1 and the increased

rate of cognitive decline as well as the increased risk of AD development [13]. CHI3L1's promise as an AD biomarker relates to its increasing levels in the CSF as a function of AD progression.

Increased CHI3L1 levels has been implicated in the reduction of cortical volume in AD, a function of neurodegeneration. High CSF levels of CHI3L1 correlate with cortical thinning in the precuneus and superior parietal cortex, cortical areas involved in episodic and working memory, as well as thinning of the middle and inferior temporal areas [13, 26]. The latter finding is noteworthy, as thinning in these areas occurs first in the spatial distribution of neurodegeneration in AD [27]. Additionally, a longitudinal study showed an association between CHI3L1 expression levels and hippocampal atrophy, an early event in AD progression [28]. Hippocampal CHI3L1 levels were significantly higher in AD brains compared to healthy controls [24]. In addition to the hippocampus, other brain regions vulnerable to AD and aging, including the entorhinal cortex, primary visual cortex, posterior cingulate cortex, superior frontal gyrus, and the middle temporal gyrus, all had significantly increased levels of CHI3L1 in AD compared to age-matched controls [24].

While CHI3L1 levels are increased in a variety of brain regions in AD, CHI3L1-expressing astrocyte number is significantly increased in the cortical layers and white matter in AD [29]. In the frontal cortex, CHI3L1 protein levels showed a positive trend along with disease progression, in line with the enhanced reactivation of astrocytes that express CHI3L1 [29]. Despite this trend, there was a significant increase in CHI3L1-expressing astrocytes in severe AD only [29]. CHI3L1, however, can exist in its secreted form, which is present early in disease state. It may take time for CHI3L1 to accumulate intracellularly, which may explain why this study shows that increased CHI3L1-expressing astrocytes are not apparent until severe stages. The increased CHI3L1-expressing cells in the white matter were also strongly correlated with Iba1-expressing microglia

numbers [29]. Both CHI3L1-postive astrocytes and Iba1-positive microglia numbers negatively correlate with perceptual speed and episodic memory in early AD, suggesting an association between white matter degeneration and early memory deficits in AD [29]. Intriguingly, in the same study, researchers also found a significant correlation between CHI3L1 and neuronal pentraxin II (NPTX2) levels. NPTX2 is a member of the pentraxin family and has been well-characterized to govern glutamatergic synaptic formation and plasticity. Recently, NPTX2 has been implicated in neuroinflammation regulation as well as AD pathogenesis [29, 30]. CHI3L1 and NPTX2 expression are not only correlated, but were the only consistently significant biomarkers that best predicted AD neuropathology over 24 months, as determined via memory performance, medial temporal lobe volume, and relation to inflammation [30]. The levels of NPTX2 are correlated with cognitive performance and hippocampal volume, implicating NPTX2 in neurodegeneration [31]. Conversely to CHI3L1, there is a reduction of NPTX2 in AD brains and CSF [30, 31]. This reduction of NPTX2 in AD was witnessed at both protein and RNA levels. Reduced NPTX2 has been further implicated in AD because NPTX2 knock-out mouse models with amyloidosis have disrupted hippocampal rhythmicity and excitatory synapse function on parvalbumin interneurons, which disrupts hippocampal homeostasis resulting in brain activity that is reportedly increased in mild cognitive impairment and AD [31].

CHI3L1 has been studied in the CSF in relation to other hallmark AD biomarkers as well. CHI3L1 CSF levels correlate with early neurodegeneration in individuals with low CSF levels of Aβ, suggesting that CHI3L1 precedes amyloid pathology [13, 26]. While there is a stronger correlation between tau and CHI3L1 in Aβ-positive individuals, Aβ-negative individuals also display a positive correlation between CHI3L1 CSF levels and CSF t-tau [13]. This further suggests that CHI3L1 is elevated in conditions of neuronal loss prior to Aβ deposition, placing

CHI3L1, and by extension neuroinflammation, to precede Aβ pathology. Additionally, carriers of the *APOE*-ε4 allele, the most significant genetic risk allele for late-onset AD, show a correlation between age and CHI3L1 and Aβ42 CSF levels [26]. Taken together, this shows that CHI3L1 CSF levels increase in proportion to AD progression, from neurodegeneration to cognitive decline, making CHI3L1 a powerful AD biomarker.

Beyond expression and CSF levels, CHI3L1 polymorphisms have also been implicated in AD development. In a study of 166 AD patients in the Chinese Han population, two single-nucleotide polymorphisms (SNPs) in the *Chi3l1* gene were found to modulate the risk of AD development [32]. Notably, the G allele of rs4950928 C>G appears to be protective of AD pathology while the T allele of rs10399931 C>T in the *Chi3l1* gene confers risk. In addition, the protective and risk allele also varied in CHI3L1 plasma concentration, with patients carrying the risk allele showing greater levels of CHI3L1 in the CSF [32]. These recent findings highlight the importance of elucidating the role CHI3L1 and its polymorphisms play in the pathogenesis of AD.

In addition to AD, CHI3L1 expression is associated with other neurodegenerative disorders. Elevated CHI3L1 CSF levels have been found in Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), Parkinson's Disease (PD), and Multiple Sclerosis (MS) in a progression-dependent manner [33-36]. In the early stages of MS, levels of CHI3L1 correlate with worse performance of executive functions [34, 37]. Similarly, in PD, the increase in CHI3L1 CSF levels correlates with faster cognitive decline [33]. In ALS, fold increases in CHI3L1-positive cells, mainly GFAP expressing astrocytes, were observed in motor cortex white matter, with expression being negatively correlated with survival time [35, 38]. CHI3L1 was also detected in the cerebellum and frontal cortex of patients with sporadic Creutzfeldt-Jakob Disease, a neurodegenerative disorder that exhibits a very robust inflammatory response [39]. It is evident

that CHI3L1 has utility as a biomarker in neurodegenerative disease, has an emerging role in AD pathogenesis, and is related to inflammation; however, how CHI3L1 functions and dysfunctions in the brain remains to be elucidated.

1.3. Biological and Pathological Function of CHI3L1

1.3.1 CHI3L1 Expression & Regulation

While largely uncharacterized in the brain, CHI3L1 expression, regulation, and function have been well defined in the periphery. Beyond CNS expression in astrocytes (**Figure 2**), CHI3L1 is expressed in a variety of peripheral cells including macrophages, dendritic cells, neutrophils, CD4 and CD8 T cells, chondrocytes, synovial cells, fibroblasts, osteocytes, epithelial cells, endothelial cells, smooth muscle cells, hepatic stellate cells, Kupffer cells, and tumor cells [18, 40-46]. CHI3L1 expression and secretion by these specific cell types is regulated by a variety of factors, of which cytokines comprise the largest group. Through its expression in a variety of cells, particularly immune cells, as well as its regulation by cytokines and other inflammatory factors, it is clear that CHI3L1 plays a role in immune response and inflammation.

1.3.2 Identified CHI3L1 Receptors

Currently, there have been 6 identified CHI3L1 receptors: interleukin-13 receptor subunit alpha-2 (IL-13Rα2), transmembrane protein 219 (TMEM219), galectin-3 (Gal-3), CD44, chemo-attractant receptor-homologous 2 (CRTH2), and receptor for advanced glycation end product (RAGE) (**Figure 1A**).

CHI3L1 binds to IL-13Rα2 and, along with IL-13, forms a multimeric complex. There is no competition in binding between CHI3L1 and IL-13 nor is there an abrogative effect on each

other's signaling because they bind different locations on the receptor [15]. Upon binding to IL-13Rα2, CHI3L1 was shown to activate MAPK/ERK, AKT/PKB, and Wnt/β-catenin signaling pathways [15]. The activation of these signaling pathways via CHI3L1-IL-13Rα2 binding resulted in the regulation of oxidant injury, apoptosis, pyroptosis, inflammasome activation, antibacterial responses, melanoma metastasis, and TGF- β1 production [15].

TMEM219 physically interacts with IL-13Rα2-CHI3L1 complex and plays a critical role in subsequent signaling. The complexing of TMEM219 with IL-13Rα2 and CHI3L1 was shown to augment IL-13 binding to IL-13Rα2 [47]. Experimentally silencing TMEM219 decreased CHI3L1-induced MAPK/ERK and AKT/PKB activation, but had no effect on Wnt/β-catenin signaling indicating that TMEM219 interaction with IL-13Rα2 plays a critical role in subsequent MAPK/ERK and AKT/PKB activation but not Wnt/β-catenin signaling [47]. Due to this interaction and activation of similar pathways, TMEM219 also plays a role in the regulation of apoptosis, oxidant injury, melanoma metastasis, and TGF- β1 induction.

The CHI3L1 receptor Gal-3 has also demonstrated to physically interact with IL-13Rα2, where it competes with TMEM219 for IL-13Rα2 binding [46]. Unlike TMEM219 which has an antiapoptotic role, Gal-3 promotes apoptosis when accumulated extracellularly; however, when expressed intracellularly, Gal-3 has been shown to influence macrophage differentiation and fibroproliferative repair [46]. Additionally, it has been demonstrated that CHI3L1 binding to the Gal-3- IL-13Rα2 complex activates Wnt/β-catenin signaling [46].

Another identified CHI3L1 receptor, CD44, also interacts and forms a complex with IL-13Rα2. The physical interaction between CD44 and IL-13Rα2 activates CHI3L1-induced MAPK/ERK, AKT/PKB, and Wnt/β-catenin signaling [48]. CD44 is a cell-surface transmembrane

glycoprotein known to play a role in cell growth, survival, and differentiation, which aligns with the aforementioned processes affected by activation of the three signaling cascades [46, 48].

CRTH2 is a CHI3L1 receptor that was identified in Hermansky-Pudlak mutant cells, which have decreased membrane expression of IL-13Rα2 [46]. CHI3L1 binding to CRTH2 enhances collagen accumulation and promotes fibrotic responses [46].

While many of the identified CHI3L1 receptors interact and complex with IL-13Rα2, RAGE does not interact with IL-13Rα2 in CHI3L1 signaling responses. CHI3L1 binds surface RAGE and activates MAPK/ERK, Wnt/β-catenin, NF-κB, and STAT3 signaling which promote cell proliferation, migration, and survival [45, 49, 50]. While not directly attributed to RAGE binding, it has been shown that CHI3L1 is able to increase the production of IL-8 in a NF-κB-dependent manner, whereby CHI3L1 induces IκBα phosphorylation and degradation, which releases NF-κB from complex and allows it to activate the IL-8 gene [51]. IL-8 then stimulates cell proliferation and migration. CHI3L1-induced stimulation of the STAT3 pathway was also identified to be NF-κB-dependent [50, 52]. RAGE is currently the only known receptor to activate NF-κB, which is critical in mediating continued CHI3L1 production, suggesting a positive feedback loop where CH3L1 is able to increase its expression by activating NF-κB via RAGE binding.

1.3.3 Proliferation & Cell Survival

One of the consequences of CHI3L1 receptor binding, particularly to IL-13Rα2, TMEM219, CD44, and RAGE, is increased proliferation and cell survival (**Figure 1B**). CHI3L1 treatment or overexpression in multiple cell types, either *in vitro* or *in vivo*, has been shown to increase proliferation [43, 53-56]. The increased number of cells witnessed in these cases were in

fact due to increased proliferation and not only decreased apoptosis because DNA synthesis was increased by CHI3L1 [53, 56]. The CHI3L1-induced activation of MAPK/ERK and AKT/PKB signaling are mainly responsible for the increases in proliferation. Both pathways are associated with mitogenesis control and inhibition of MAPK/ERK and AKT/PKB signaling significantly reduced proliferation [15, 43, 56]. In addition to increasing proliferation, CHI3L1 also increases cell survival by inhibiting apoptosis (**Figure 1B**). *In vivo* studies using CHI3L1 null mice have demonstrated that knocking out CHI3L1 enhances levels of the apoptotic marker annexin V, particularly in macrophages, T cells, and eosinophils [57]. Loss of CHI3L1 increased the expression of the death receptor Fas on these cells [57]. The antiapoptotic function of CHI3L1 is driven by the activation of the AKT/PKB signaling pathways as well as by increased expression of Fas apoptosis-inhibiting molecule (Faim) 3 [15, 57].

1.3.4 Tissue Remodeling & Angiogenesis

In addition to increased connective tissue cell proliferation, CHI3L1 is also implicated in tissue remodeling as it is expressed in tissues with high levels of remodeling activity and has the ability to interact with and bind numerous extracellular matrix (ECM) components including collagen types I through IV, hyaluronic acid, and fibronectin [42, 58]. CHI3L1 has also been shown to stimulate fibrosis and ECM gene expression (**Figure 1B**) [55, 59]. The expression of CHI3L1 by chondrocytes, osteocytes, synoviocytes, and fibroblasts can stimulate the synthesis of proteoglycans and increases collagen accumulation, both of which make up a large portion of the ECM [22, 43, 59].

Epithelial-mesenchymal transition (EMT), a biological process that also enhances ECM components, is regulated by CHI3L1 (**Figure 1B**). CHI3L1 overexpression up-regulates the

mesenchymal markers N-cadherin and Vimentin as well as the EMT activators Twist, Snail, and Slug, while the epithelial marker E-cadherin was down-regulated [48, 58]. EMT progression is induced by CHI3L1 and appears to be CD44-dependent because the use of a neutralizing antibody against the CD44 receptor blocked EMT [48].

CHI3L1 promotes migration of human microvascular endothelial cells and increases vascular endothelial growth factor (VEGF), suggesting a role in angiogenesis (**Figure 1B**). Treatment of cells with CHI3L1 enhanced endothelial cell migration and tube formation, while CHI3L1 knockdown reduced VEGF and suppressed angiogenesis [49, 54, 55, 60]. In addition to enhanced migration of human microvascular endothelial cells to form vasculature, CHI3L1 also enhances the migration of macrophages through the ECM and increases macrophage production of matrix metalloprotinase-9 (MMP9), C-chemokine ligand 2 (CCL2), and CX motif ligand 2 (CXCL2), which are all proinflammatory and pro-invasive factors [49, 54, 61].

1.3.5 Macrophage Maturation & Activation

CHI3L1 is expressed in macrophages throughout differentiation, maturation, and activation, but is absent in its precursor, monocytes [14, 41, 61-63]. Similarly, CHI3L1 expression is upregulated during dendritic cell maturation and activation as well [41, 57]. While CHI3L1 aids in cell survival via increasing proliferation and decreasing apoptosis, CHI3L1 also further promotes macrophage survival by inhibiting macrophage pyroptosis. Macrophage pyroptosis is caspase-1 dependent and is therefore prevented by CHI3L1 via inhibition of caspase-1 [15, 64]. This anti-pyroptosis function was witnessed after CHI3L1-IL-13Rα2 binding, which also results in the inhibition of Nlrp3 inflammasome activation, a complex that plays a role in inflammation, immune response, and apoptosis [15, 64].

Macrophages can be activated into different polarization states, characterized as either classically activated, M1 macrophages, or alternatively activated, M2 macrophages. Treatment with CHI3L1 either does not augment or reduces the expression of markers associated with classic M1 activation, such as iNOS; however, CHI3L1 expression has still been witnessed in M1 macrophages [22, 62]. Conversely, CHI3L1 causes a significant increase in arginase-1, macrophage mannose receptor, MHC class II, YM-1, CD206, and FIZZ-1, all of which are molecules associated with alternative M2 activation (**Figure 1B**) [22, 57]. The CHI3L1-induced activation of both macrophages and dendritic cells suggests that CHI3L1 plays an important role in the innate immune response.

1.3.6 Th2 Polarization

CHI3L1 not only functions in regulation of innate immunity, but its role also extends into and affects the adaptive immune response, specifically by exerting effects on T cells. T cells express CHI3L1, which has been shown to increase T cell proliferation and activation [18, 57]. Specifically, CHI3L1 is highly expressed and activates the T-helper 2-type (Th2) state in $CD4^+$ T cells (**Figure 1B**) [18, 52, 57, 65]. Similar to macrophages, $CD4^+$ T cells activate into differing polarization phenotypes depending on the signals received. Th2 cells produce cytokines including IL-4 and IL-13, which evoke antibody responses, particularly IgE, and are typically involved in allergies and helminth infection [16, 66]. CHI3L1 levels are positively correlated with IgE levels and CHI3L1 was shown to play a role in antigen and allergen sensitization [51, 57]. IL-4 and IL-13, produced by CHI3L1-induced Th2 cells, are able to activate macrophages to an M2 phenotype, while macrophages are also able to activate $CD4^+$ T cells via antigen presentation. The ability of these cells to be activated by CHI3L1 and each other indicates that CHI3L1 may both directly and

indirectly activate these cells, thus bridging the innate and adaptive immune response. CHI3L1 not only activates the Th2 phenotype, but also inhibits Th1 polarization because null mutations of CHI3L1 resulted in Th1 differentiation [18, 57]. CHI3L1 is a negative regulator of Th1 cells and drives Th2 differentiation, making CHI3L1 an important factor in the adaptive immune response. Due to its association in both innate and adaptive immunity, CHI3L1 is thought to play a role in a variety of inflammatory diseases, affecting pathology and progression. While numerous studies support the implication of CHI3L1 in the pathogenesis of various peripheral diseases, there could be a bi-phasic effector function (protective vs provocative) of CHI3L1 depending on the levels of expression, activation status of CHI3L1, the presence of certain receptors, and disease context.

CHI3L1 has clear involvement in peripheral innate and adaptive immunity, where it has been determined that CHI3L1 is an important player in injury and repair responses. This innate immunity in the periphery resembles the neuroinflammatory process initiated by microglia and coordinated by astrocytes in the CNS. Considering the similarities between immunities, the information regarding biological and pathological functions of CHI3L1 in the periphery may be important in understanding the inflammatory mechanism in the CNS, especially in the context of AD.

1.4. CHI3L1 in Neuroinflammation

1.4.1 Glial Role in Neuroinflammation

Neuroinflammation was once regarded as merely a secondary response in the neurodegeneration process. Now, after myriad preclinical studies and a number of large genomic data analyses from AD patients and risk carriers, neuroinflammation sits at the center of AD pathogenesis [2, 3]. Neuroinflammation is mediated by microglia and astrocytes, and involves

rapid and elaborate intercellular signaling, which is necessary for healthy brain function. Additionally, these inflammatory cells are responsible for synaptic pruning of neurons and neuroprotective support, thereby further maintaining normal brain function [4]. However, the neuroinflammatory process can become imbalanced and derail normal brain functions to cause neurodegeneration. Specifically, neuroinflammation is mainly driven by activated microglia which stimulate astrocytes to trigger the inflammatory cascades eventually leading to synapse loss and neuronal death [5, 6].

Both microglia and astrocyte reactivity have been classified into differing activation polarization states named M1 and M2 and A1 and A2, respectively, in analogy to the M1/M2 macrophage convention nomenclature. M1/A1 are thought to be more pro-inflammatory while M2/A2 are thought to be anti-inflammatory [5, 6, 11, 67]. Despite the persistent use of this nomenclature by some researchers, others believe this dichotomy to be an oversimplification and that it is likely intermediate activation states exist [68]. Nonetheless, while microglia have become a major focus in AD research, these A1 reactive astrocytes are also believed to underline neurodegenerative disorders, including AD, and are reported to be enriched in aged mouse brains (**Figure 2**) [5, 69]. A1 astrocytes have been shown to be destructive to synapses, hence believed to be neurotoxic, while A2 astrocytes seem to be protective [70-72]. Recent data shows that A1 astrocytes are induced by activated microglia, namely via the secretion of three cytokines, IL-1α, TNF-α and C1q [6, 73]. It has been shown that these A1 reactive astrocytes rapidly kill neurons and oligodendrocytes as they release neurotoxic factors and lose neuroprotective abilities, including the promotion of neuronal survival, outgrowth, synaptogenesis, and phagocytosis [6, 69, 73]. Conversely, blocking A1 astrocyte conversion by microglia is shown to be neuroprotective in models of Parkinson's disease [74]. The rapid and dynamic interactions between microglia and

astrocytes, such as the induction of neurotoxic A1 reactive astrocytes by activated microglia, are among the major drivers for pathogenesis of AD and age-related neurodegeneration, but these mechanisms are still not well understood [6, 69, 75]. Remarkably, CHI3L1 expression is robustly induced in reactivated astrocytes *in viv*o and *in vitro* by independent inflammatory stimuli, and considered to be part of the neurotoxic gene signature [5, 73].

1.4.2 Astrocyte Secretion of CHI3L1

In the brain, CHI3L1 is most abundantly and almost exclusively expressed in astrocytes throughout neural development, and its expression peaks in the last stage of lifespan, coinciding with the upsurge of neurotoxic A1 astrocytes and unchecked inflammatory responses in the aged brains [69, 76, 77]. Of particular relevance to AD, CHI3L1 expression is highly enriched in astrocytes and significantly elevated in the diseased human brain cortical tissues as compared to healthy controls [29, 78]. Such an expression pattern supports an effector role in the neuroinflammatory axis of microglia–astrocyte–neuron. It has been experimentally shown *in vitro* that CHI3L1 expression in astrocytes is increased by IL-1, IL-6, and TNF-α, as well as M1 macrophage conditioned media, while M2 macrophage conditioned media inhibited CHI3L1 transcription [79, 80]. These factors, particularly IL-1 and TNF-α, have been shown to induce A1 astrocyte reactivity, which suggests that CHI3L1 is expressed and secreted by A1 astrocytes. Additionally, the combination of A1 astrocyte induction by cytokines secreted mainly by M1 microglia and the increase in CHI3L1 expression in astrocytes by M1 macrophage conditioned media further ties A1 reactivity and CHI3L1 expression together. If CHI3L1 expression and secretion is increased in A1 astrocytes, perhaps CHI3L1 is neurotoxic because A1 astrocytes have been shown to induce neuronal death. In future AD research, it will be important to uncover a

regulatory mechanism governing the microglia-induced conversion of A1 reactive astrocytes in neuroinflammation and the sequelae of impaired neuronal integrity, with a specific focus on CHI3L1 and its involvement in this process.

1.4.3 CHI3L1 Effect on CNS Cells

Beyond its expression by astrocytes, not much is known about CHI3L1 in the CNS. While CHI3L1 has been well-characterized in the periphery, there have been limited studies on the biological functions of CHI3L1 in the CNS or the effect of CHI3L1 on brain cells. Recent studies have emerged that attempt to define the effects of CHI3L1 on microglia, neurons, and oligodendrocytes.

While microglia are thought to be the initiators in the neuroinflammatory axis of microglia–astrocyte–neuron, microglia can be affected by inflammatory factors, including CHI3L1. It has been demonstrated that CHI3L1 is able to modulate the microglial secretome and polarization activation state *in vitro* and *in vivo*. One group studied the effects of a CHI3L1 inhibitor they generated and found that CHI3L1 inhibition decreased lipopolysaccharide (LPS)-induced expression of the inflammatory proteins COX-2, iNOS, and Iba-1 in microglial BV-2 cells [81]. Furthermore, in the brain, the same CHI3L1 inhibitor decreased the M1 markers TNF, IL-1β, IL-6, and CD86, while CHI3L1 overexpression increased the expression of these M1 markers [82]. Conversely, M2 markers such as Arg1, Mrc1, TGF-β, and IL-10 were unaffected by the inhibition of CHI3L1 [82]. This indicates that CHI3L1 may drive the activation of microglia to an M1, pro-inflammatory phenotype. However, other research using microglia isolated from middle cerebral artery occlusion-injured CHI3L1 knockout mice describe opposite results where CHI3L1 knockout increased the M1 markers iNOS, CD86, IL-1β, and IL-6, and decreased the M2 markers

Arg1, Mrc1, IL-10, and IL-4Rα [83]. These results suggest that CHI3L1 activates microglia toward an M2, anti-inflammatory phenotype. Further study is needed to clarify the effect of CHI3L1 on microglia and the subsequent effects of these microglia on astrocytes and neurons.

There has been very limited research on the direct effect of CHI3L1 on neurons. In one *in vitro* study, researchers added recombinant CHI3L1 to primary mouse cortical neuron cultures and assessed neuronal survival and function [84]. It was found that CHI3L1 was cytotoxic to the neurons as it shortened total neurite length, induced neurite length retraction, and increased cell death after 48 hours of exposure [84]. The researchers wanted to determine if CHI3L1 was cytotoxic to glia, so they treated independently cultured microglia and astrocytes with CHI3L1 and witnessed no effect on cell death, demonstrating CHI3L1 cytotoxicity is neuron-specific [84].

Many neurodegenerative diseases are associated with the loss of myelin sheath, the cellular structure formed by oligodendrocytes that insulates the axons of CNS neurons to promote rapid and efficient neuronal signal conduction, amongst other metabolic support functions [85, 86]. While demyelination has been well characterized in diseases such as Multiple Sclerosis, this process has become increasingly observed in other common neurodegenerative diseases like AD [87]. In fact, a recent publication claims that myelin damage can precede clinical AD symptoms by 20 years [87]. It has been recently reported that CHI3L1 can stimulate the proliferation of oligodendrocyte progenitor cells (OPCs) as an indirect method for myelin formation [85]. One such publication shows that downregulation of CHI3L1 protein expression in OPCs results in reduced proliferation and that this reduced proliferation phenotype was able to be rescued upon exogenous CHI3L1 introduction [85]. Another group found that increased CHI3L1 secretion can activate an EGFR-dependent MAPK signaling cascade in neural stem cells to induce differentiation into OPCs, and subsequently into myelin-forming oligodendrocytes *in vitro* and *in*

vivo, further supporting a role of CHI3L1 in oligodendrogenesis [88]. However, agreement on the effect of CHI3L1 and its mechanism of action pertaining to oligodendrogenesis is not without contention. It has been shown using human iPSC models of Alexander Disease (AxD) that astrocytes harboring disease-causing GFAP mutations have increased CHI3L1 expression and can reduce the proliferative capacity of OPCs [89]. Additionally, proliferation was decreased in OPCs cultured in AxD astrocyte conditioned media and this effect was rescued upon treatment with a CHI3L1 neutralizing antibody [89]. This demonstrates that astrocytes can secrete factors which inhibit OPC proliferation, and that CHI3L1 is a confirmed mediator of this process. Further investigation into the role of CHI3L1 on oligodendrogenesis is needed to gain a more complete understanding of this protein's function in AD and other demyelinating diseases.

Blood brain barrier dysfunction is commonly observed in the early stage of neurodegenerative disorders affected by neuroinflammation, particularly in AD. In AD, BBB homeostasis is disrupted leading to hypoperfusion that prevents appropriate amounts of oxygen, glucose, and other nutrients from reaching the brain [90]. There has been recent evidence that CHI3L1 may physically associate with the BBB and act upon the endothelial cells, possibly contributing to the compromised BBB integrity witnessed in AD. White matter damage has been tied to BBB dysfunction, and as previously discussed, CHI3L1 has been implicated in white matter degeneration in AD brains [29]. Additionally, CHI3L1-expressing astrocytes have been shown to associate with blood vessels, further implicating CHI3L1 in BBB disruption [29]. It has been shown that the increased CSF levels of CHI3L1 in AD patients significantly correlate with increased albumin ratio, a marker for BBB breakdown [91]. This suggests that the increases in CHI3L1 expression in AD lead to decreased stability and increased permeability of BBB. The direct effect of CHI3L1 on BBB integrity is supported by its reported role in angiogenesis and

vascular tissue remodeling in the periphery. While CHI3L1 has been shown to promote angiogenesis in the periphery via increasing VEGF, there has also been evidence that VEGF can downregulate the tight junction protein zona occludens-1 which can lead to BBB damage [49, 54, 92]. It has also been detailed in the periphery that CHI3L1 increases macrophage production of MMP9 and CCL2 [49, 54, 61]. MMPs, such as MMP9, have been shown to cause BBB breakdown by disrupting the basal lamina proteins and by degrading tight junction complexes, while CCL2 can recruit peripheral immune cells which further promotes inflammation that can damage the BBB [92]. Along with peripheral immune cells, inflammation produced by glial cells can also negatively affect expression of tight-junction proteins and result in BBB damage [92]. As detailed above, CHI3L1 can modulate the expression and secretion of pro-inflammatory cytokines by glial cells, further implicating CHI3L1 in BBB dysfunction [81, 82]. While CHI3L1 has been implicated in BBB disruption and remodeling of blood vasculature in AD, the precise mechanism is still unknown and further study is required to determine the precise role of CHI3L1 on BBB dysfunction in AD.

1.4.4 Known Effects of CHI3L1 on Neuroinflammation & AD

In addition to the cell-specific effect of CHI3L1 in the CNS, there are a few studies that have tried to examine the role of CHI3L1 in the context of general neuroinflammation and CNS disease, including AD. In one study, the role of CHI3L1 in AD was assessed via the use of a CHI3L1 inhibitor. The researchers used an $A\beta_{1-42}$-induced AD mouse model to determine the effect of CHI3L1 inhibition on amyloid burden and memory impairment. The CHI3L1 inhibitor decreased the expression levels of the $A\beta_{1-42}$-induced inflammatory proteins iNOS, COX-2, GFAP, and Iba-1 through inactivation of NFκB signaling by reducing translocation of p65, p50, and p-

IκBα into the nucleus [81]. The CHI3L1 inhibitor was also shown to decrease the mRNA levels of TNF-α, IL-1β, and IL-6 in the $A\beta_{1-42}$-induced AD mice brains [81]. In addition to cytokine expression levels, the researchers assessed components involved in β-secretase activity and Aβ generation, namely BACE1 and APP. The CHI3L1 inhibitor decreased the expression of BACE1 and APP in LPS-treated BV-2 cells and cultured astrocytes [81]. Additionally, the CHI3L1 inhibitor decreased the β-secretase activity that was increased in the brains of the $A\beta_{1-42}$-induced AD mice [81]. The $A\beta_{1-42}$-induced AD mice had significantly higher Aβ1-42 levels compared to controls and these levels were reduced by the CHI3L1 inhibitor [81]. Overall, this study determined that inhibition of CHI3L1 can reduce amyloidogenesis resulting in a memory recovery effect in this AD mouse model.

A follow-up study by this same group used the same CHI3L1 inhibitor to investigate its effect on neuroinflammation and memory loss in another AD model, Tg2576 transgenic mice. The CHI3L1 inhibitor reduced the number of iNOS, COX-2, GFAP, and Iba-1 positive cells, indicating an inhibition of microglial and astrocytic activation, and also decreased the levels of TNF-α, IL-1β, and IL-6 in the brains of the Tg2576 transgenic mice [82]. These results are consistent with the group's previous results in the $A\beta_{1-42}$-induced AD model. Reduced NFκB signaling by CHI3L1 inhibition was also supported in the Tg2576 transgenic mouse model of AD as it was shown that the CHI3L1 inhibitor reduced the levels of p-IκBα in the brain [82]. However, in this Tg2576 transgenic model, decreased levels of p-ERK1/2 were also witnessed in the CHI3L1 inhibitor-treated brains, thus implicating the ERK signal transduction pathway in the inhibitory effect on neuroinflammation by the CHI3L1 inhibitor [82]. In addition to reduced neuroinflammation and NFκB and ERK signaling, the CHI3L1 inhibitor reduced the levels of APP and BACE1, β-secretase activity, and Aβ plaque accumulation in the Tg2576 transgenic mice [82]. In accordance

with what was observed in the $A\beta_{1-42}$-induced AD mice, the CHI3L1 inhibitor also alleviated memory impairment and mitigated impaired cognitive function in the Tg2576 AD model [82].

While the previous studies utilized a CHI3L1 inhibitor to assess the role of CHI3L1 in neuroinflammation and AD, a different group aimed to determine this role via CHI3L1 deletion. Our collaborative study used an APP/PS1 model of AD crossed with the *Chi3l1* knockout line we generated to analyze the effect of CHI3L1 deletion on AD progression. The deletion of CHI3L1 decreased amyloid plaque burden in the APP/PS1 mice [78]. In the APP/PS1 mouse, CHI3L1 deletion also increased the microglial lysosomal marker CD68, which suggested to the researchers that the decreased amyloid plaque accumulation may be a result of increased phagocytosis by glia [78]. The researchers tested phagocytic capabilities of glial cells *in vitro* and found that CHI3L1 knockdown increased phagocytosis of zymosan-coated pHrodo-labeled beads as well as Aβ42 peptide in both microglia and astrocytes, but with a more pronounced effect in microglia [78]. In addition to its effect on microglial phagocytosis, CHI3L1 deletion modestly increased microglia activation and LPS-induced inflammatory cytokine expression in astrocytes and microglia [78]. These results disagree with the previous studies that utilize the CHI3L1 inhibitors which witnessed reduced neuroinflammation when CHI3L1 was inhibited. Overall, this study of CHI3L1 deletion in APP/PS1 AD mice demonstrated that CHI3L1 can contribute to AD progression by altering glial function and amyloid burden.

Together these three studies suggest that therapeutic intervention targeting CHI3L1 may perhaps be able to reduce memory impairment by decreasing neuroinflammation and amyloidogenesis alone or in conjunction with each other. However, there have been other studies that have found CHI3L1 to be anti-inflammatory where loss of CHI3L1 can modestly potentiate pro-inflammatory cytokine expression or even exacerbate neuroinflammation [78, 83]. Further

study regarding the effect of CHI3L1 on neuroinflammation is needed to reconcile the discrepancies in this limited field of CHI3L1 CNS function.

Based on this evidence from the periphery and new, recent evidence in the brain, our central hypothesis is that CHI3L1 functions as a neuroinflammation signaling molecule to trigger the receptor system on brain cells and regulate inflammatory responses in a cell type-specific manner. I propose that CHI3L1 plays an essential role in the intricate cross-talks between microglia and astrocytes in the highly dynamic process of neuroinflammation (**Figure 4**). Furthermore, on top of the well-characterized regulation of ECM by CHI3L1, the signaling function in endothelial cells and perhaps pericytes may contribute to the vascular impairment and compromised BBB integrity as observed at the early stage of AD pathology preceding cognitive decline. The effects of CHI3L1 on the self-renewal of oligodendrocyte lineage cells and myelination homeostasis have been documented as mentioned above [89], and such oligodendroglial failures can aggravate the dystrophy and degeneration of neurons. Finally, the CHI3L1 effect on neurons can be potentially linked to the loss of synapses as well as the accumulation of misfolded proteins and the appearance of well-defined neurodegenerative features before the overt formation of plaques and tangles. We believe studying the overall underappreciated CHI3L1 function in the brain cells will help identify the pathogenic determinants of neuroinflammation that contribute to AD and other neurodegenerative disorders.

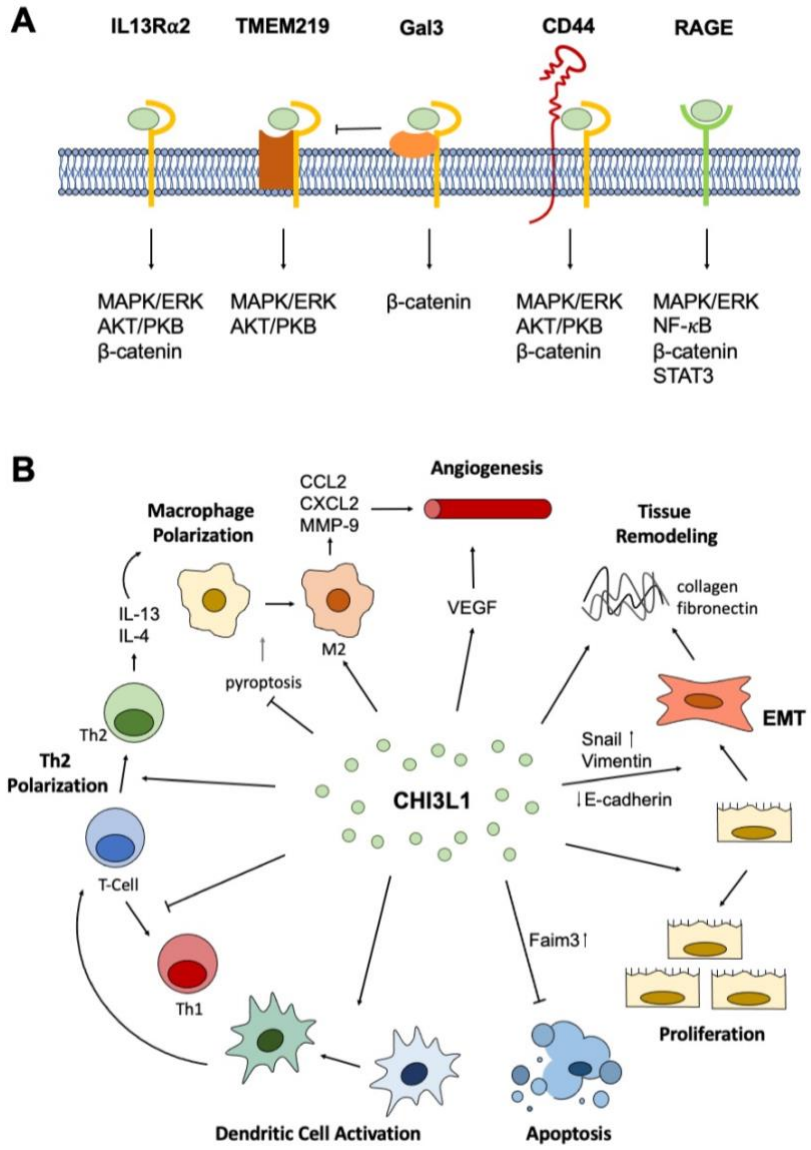


Figure 1. Chitinase-3-like protein 1 (CHI3L1) regulation and function in the periphery. **A.** The known CHI3L1 receptors and the signaling pathways activated by CHI3L1-receptor binding. **B.** The main functions of CHI3L1 identified in the periphery include increased proliferation, cell survival, dendritic cell activation, Th2 polarization, M2 macrophage polarization, angiogenesis, tissue remodeling, and EMT. AKT/PKB, protein kinase B; CCL2, C-chemokine ligand 2; CXCL2, CX motif ligand 2; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloprotinase-9; Th2, T-helper 2-type; VEGF, vascular endothelial growth factor [93].

Table 1. A summary of recent studies (not included in previous review articles) consistently showing elevated levels of CHI3L1 in CSF samples from AD patients [93].

Study	Healthy Controls (HC)	Alzheimer's Disease (AD)	HC (*n*)	AD (*n*)
Baldacci et al., 2017	98 ng/mL	146 ng/mL	21	35
Gispert et al., 2017	271.36 ± 104.49 ng/mL (e4-); 252.65 ± 32.67 ng/mL (e4+)	327.79 ± 133.27 ng/mL (e4-); 339.96 ± 107.99 ng/mL (e4+)	44 (e4-); 5 (e4+)	8 (e4-); 7 (e4+)
Llorens et al., 2017 (Cohort 1)	250 ± 77 pg/mL	386 ± 221 pg/mL	62	84
Llorens et al., 2017 (Cohort 2)	226 ± 82 pg/mL	311 ± 113 pg/mL	35	28
Muszynski et al., 2017	291 ng/mL	387 ng/mL	23	45
Dai & Gong, 2018	87.710 ± 0.928 ng/mL	382.700 ± 6.267 ng/mL	184	166
Paterson et al., 2018	111 ng/mL	163 ng/mL	29	114
Sutphen et al., 2018	384.1 ± 20.08 ng/mL (Ab-), 399.6 ± 19.4 ng/mL (Ab+)	471.9 ± 41.86 ng/mL	35 (Ab-); 21 (Ab+)	16
Zhang et al., 2018	397.2 ± 25.7 ng/mL	471.9 ± 39.5 ng/mL	32	18
Antonell et al., 2019	217.23 ng/mL	305.12 ng/mL	50	108
Bos et al., 2019	127 ± 45.4 ng/mL (Ab-); 175.1 ± 63.6 ng/mL (Ab+)	184.2 ± 64.6 ng/mL (Ab-); 193.6 ± 68.7 ng/mL (Ab+)	95 (Ab-); 45 (Ab+)	23 (Ab-); 157 (Ab+)
Morenas Rodriguez et al., 2019	238.8 ± 49.2 ng/mL	295.3 ± 54.1 ng/mL	44	50
Nordengen et al., 2019	145 ± 46 ng/mL	221 ± 70 ng/mL	36	27
Villar-Pique et al., 2019	84 ± 84 ng/mL	133 ± 110 ng/mL	70	50
Abu-Rumeileh et al., 2020	145 ng/mL	240 ng/mL	40	40
Wang et al., 2020	335.0 ng/mL	467.1 ng/mL	35	11
Woollacott et al., 2020	108 ± 30 ng/mL	163 ± 67 ng/mL	18	15

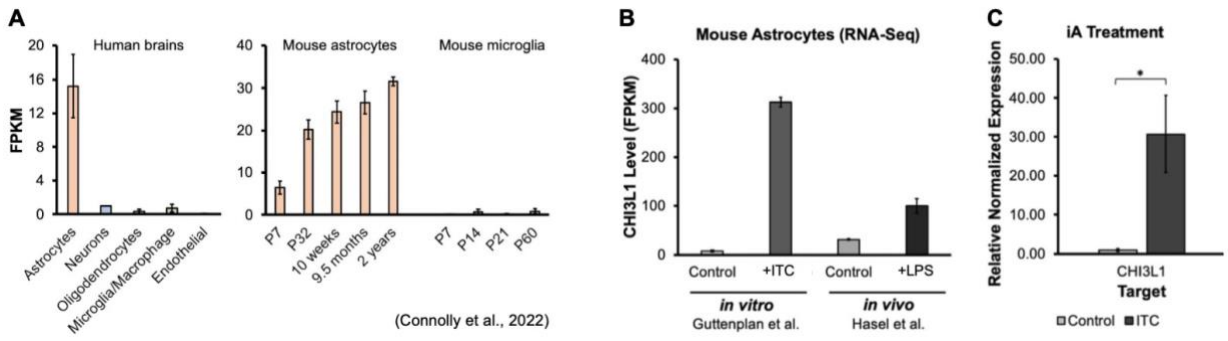


Figure 2. CHI3L1 expression *in vivo* and *in vitro* is highly enriched in astrocytes and is induced by defined stimuli of neurotoxic reactivation. **A**. CHI3L1 expression in living tissues acutely prepared from human brains (left) [77] and in mouse astrocytes [69] and microglia [76] *in vivo* at different ages (right) [93]. RNA-Seq datasets from Dr. Ben Barres group, (https://www.brainrnaseq.org/) **B**. Transcriptomic analyses of wildtype mouse astrocytes in primary cultures treated with IL1α, TNF and C1q (ITC for 24h; '*in vitro*') and acutely purified from mice stimulated by i.p. injection of liposaccharide (LPS after 24h; '*in vivo*'). Bulk RNA-Seq datasets downloaded from Guttenplan et al. and Hasel et al [94, 95]. **C.** iA cells were treated with ITC for 24 hours and CHI3L1 expression was assessed via qPCR. The mean and standard error of the mean (Mean±SEM) are shown. (t-test, * = $p<0.05$).

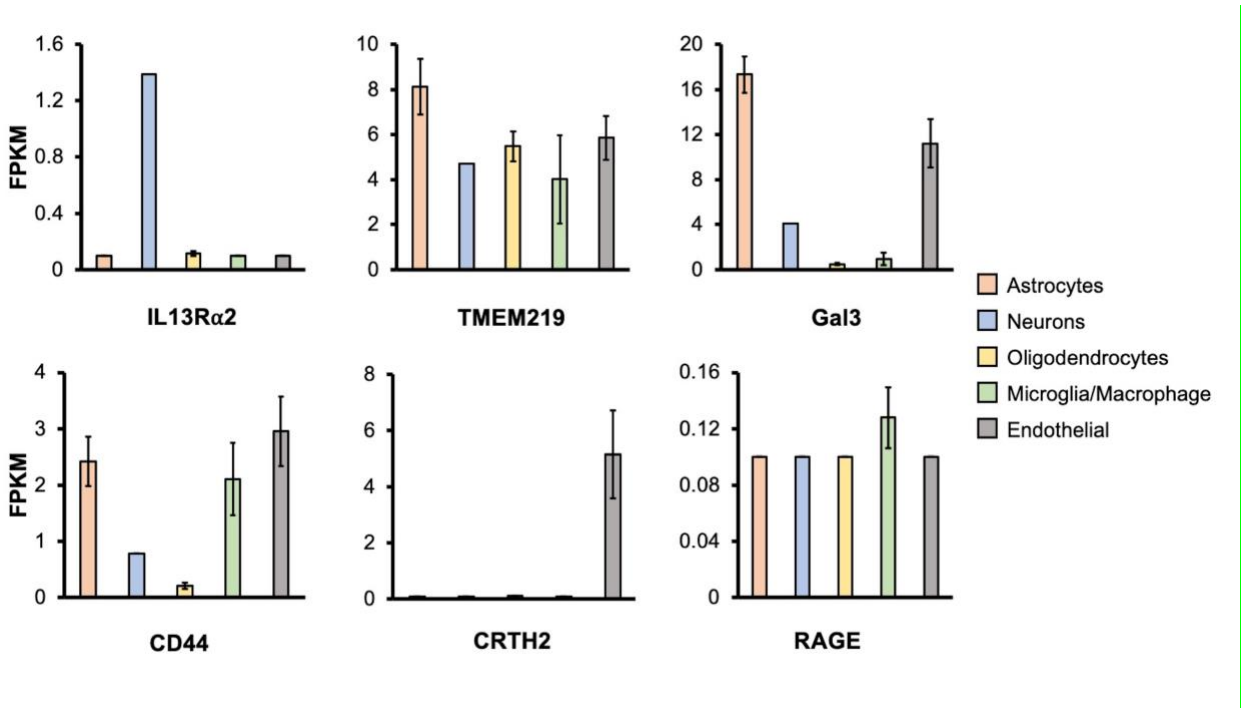


Figure 3. Chitinase-3-like protein 1 (CHI3L1) receptor expression in central nervous system (CNS) cells [93]. RNA-Sequencing transcriptome of the known CHI3L1 receptors in cell types isolated from human brain was profiled [77]. The mean and standard error of the mean (mean ± SEM) are shown. RNA-Seq datasets from Dr. Ben Barres group (https://www.brainrnaseq.org/).

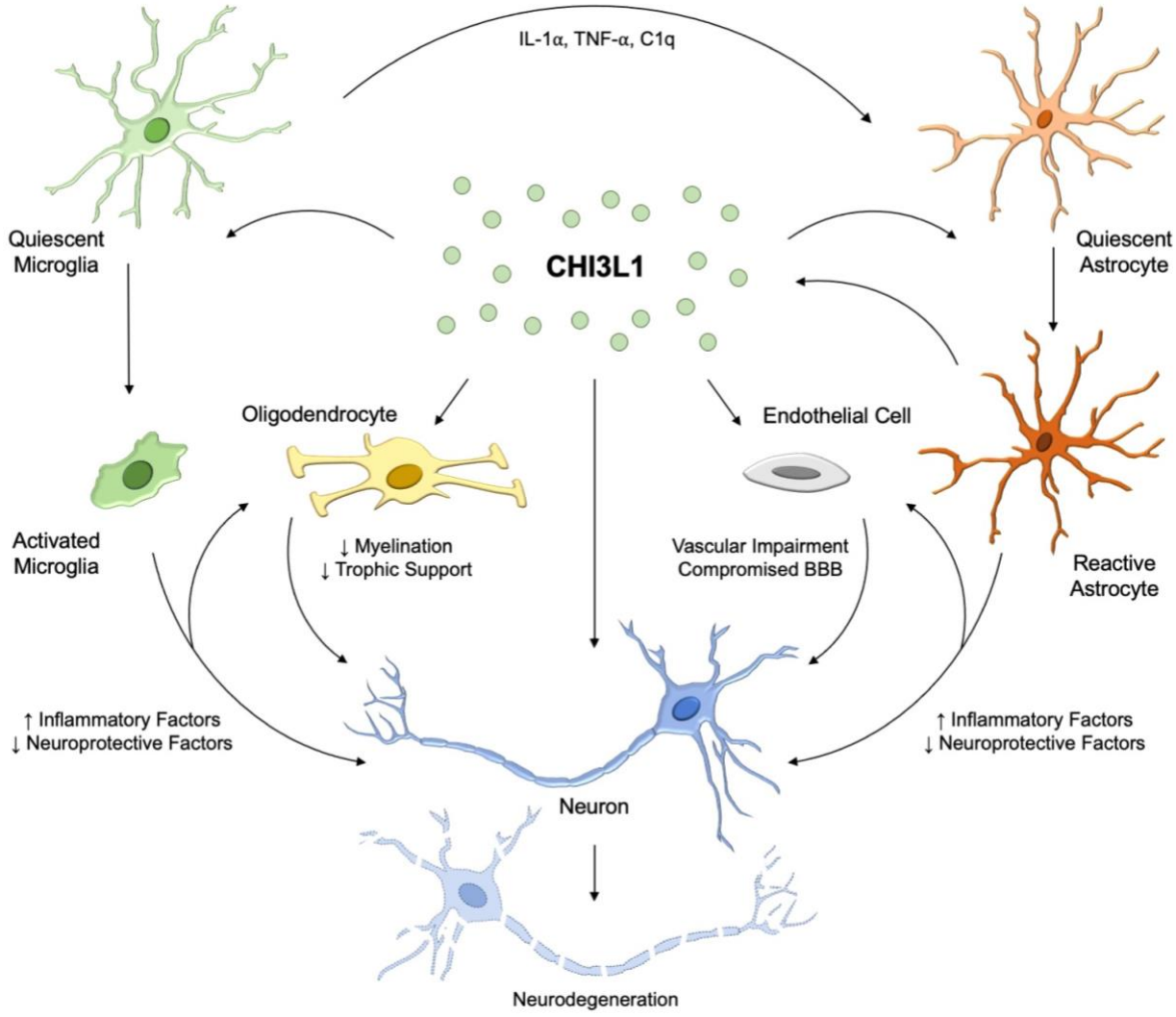


Figure 4. Potential role of chitinase-3-like protein 1 (CHI3L1) in neurodegeneration and Alzheimer's disease (AD). Supported by reported data in the field, our working hypothesis is that microglia-induced CHI3L1 secretion from astrocytes augments inflammation and promotes neural damages. Microglia secrete interleukin (IL)-1α, tumor necrosis factor alpha (TNF-α), and C1q, to activate astrocytes to a reactive phenotype which induces increased CHI3L1 secretion. CHI3L1 can then act upon the glial cells and alter glial activation as well as the expression and secretion of inflammatory or neuroprotective factors. The increased inflammatory factors or direct CHI3L1 action may affect oligodendrocytes and endothelial cells leading to demyelination and reduced trophic support as well as vascular impairment and compromised blood-brain barrier (BBB) integrity, respectively. Similarly, CHI3L1 may act directly on the neurons or in combination with the increased inflammation and reduced neuroprotective factors to lead to neural damages. The increased neuroinflammation and resulting neurotoxicity thus contribute to pathology seen in AD [93].

Chapter 2: CHI3L1 Induces AD and Neurotoxic Features in Neurons

2.1 Introduction

Despite the emerging role of CHI3L1 in AD pathogenesis and its relation to inflammation, how it functions and dysfunctions in the brain remains to be elucidated. To address this question and attempt to further uncover molecular pathways that underlie neuronal dysfunction and degeneration in AD, the Huang laboratory has established expertise in the use of stem cell technology to study the neurobiology of AD. We use human pluripotent stem cells to induce neurons (iNs) in a pure culture without the need of glia, fibroblasts, or feeder cells [96, 97]. This system excludes confounding influences of biological processes or secreted factors by these other cell types on neurons. Using this pure, model iN system, I propose that CHI3L1 is not just an AD biomarker, but a causal agent. Overall, I hypothesize that CHI3L1 engages a neuronal receptor and triggers a signaling cascade in neurons that contributes to the inflammatory neurotoxicity and leads to neurodegeneration and relevant AD features.

2.2 Methodology

2.2.1 iNeuron Induction

H1 human embryonic stem cells (WiCell Research Institute, WA01) were cultured in mTeSR+ (STEMCELL Technologies, 100-0276) on matrigel (Corning, 354234) or geltrex (ThermoFisher Scientific, 1413301) coated plates. To induce stem cells into neurons, cells were lifted at ~80% confluency with accutase (STEMCELL Technologies, 07920), spun down at 300xg for 5min, and resuspended and replated onto coated plates with 10μM ROCK inhibitor (STEMCELL Technologies, Y-27632). The following day, stem cells were infected with an inducible lentivirus system encoding rtTA (Addgene, 19780) and expressing neurogenin-2 (Ngn2) (Addgene, 52047). The next day 2μg/mL doxycycline was added to the cells in fresh media to

activate Ngn2 expression. Puromycin plus doxycycline (2µg/mL) was then added to the cells to select for the transduced cells on the subsequent day. On the final day, the induced neurons (iNs) were detached and replated on extracellular matrix solution coated plates in Neurobasal-A medium (ThermoFisher Scientific, 10888022) with 1X B27 (ThermoFisher Scientific, 17504044) for maturation and experimentation [96, 97] (**Figure 5**).

2.2.2 Cell Treatments

iNs were treated with 1µg/ml of recombinant CHI3L1 protein (R&D Systems; #2599-CH-050). In experiments where Aβ was used, human beta-amyloid peptide 1-42 (Abcam, ab120301) was added to culturing medium at a final concentration of 1µM and incubated for 24 hours while shaking at 37^{o}C. This Aβ solution was added to cultures 1 day prior to CHI3L1 treatment. Depending on the respective experiment, the iNs were treated with CHI3L1 for either 72 hours, a time course within 2 hours, or, in the case of the ELISA, chronically every other day for 10 days.

2.2.3 RNA Sequencing (RNASeq)

RNA was harvested via an RNeasy Mini Kit (Qiagen, 74104) and genomic DNA was degraded. RNA was sent off for bulk RNA sequencing at GeneWiz/Azenta Life Sciences. RNASeq library preparation was conducted by Genewiz via mRNA enrichment, mRNA fragmentation using RNAase III, and random priming followed by first and second strand cDNA synthesis, end repair, 5' phosphorylation, and dA-tailing, then adaptor ligation, PCR enrichment, and sequencing. The Illumina HiSeq platform was used with a 2x150bp configuration with 20 to 30 million read pairs per sample. Bioinformatic analysis was conducted by Genewiz in which they evaluate sequence quality, trim reads, map reads to genome, generate hit counts for genes, compare the

gene hit counts, and analyze gene ontology. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Homo sapiens GRCh38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using FeatureCounts from the Subread package v.1.5.2. The hit counts were summarized and reported using the gene_id feature in the annotation file. Only unique reads that fell within exon regions were counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the customer-defined groups of samples was performed. The Wald test was used to generate p-values and log2 fold changes. Genes with an adjusted p-value < 0.05 and absolute log2 fold change > 1 were called as differentially expressed genes for each comparison. A gene ontology analysis was performed on the statistically significant set of genes by implementing the software GeneSCF v.1.1-p2. The mgi GO list was used to cluster the set of genes based on their biological processes and determine their statistical significance (Azenta). KEGG analysis was independently conducted.

2.2.4 Quantitative PCR (qPCR)

RNA was harvested via an RNeasy Mini Kit (Qiagen, 74104). RNA concentration was determined via nanodrop. The genomic DNA was degraded and cDNA was subsequently synthesized via the iScript gDNA Clear cDNA synthesis kit (Bio-Rad, 1725034). Using SsoAdvanced Universal Probes Supermix (Bio-Rad, 172-5281), respective probes (**Table 2**) were

mixed with the supermix and water, then added on top of cDNA in a 96-well qPCR plate. The plates were run on a Bio-Rad CFX96 Real-Time System.

2.2.5 Western Blotting

Media was removed and cells were lysed in radioimmunoprecipitation, RIPA, buffer. The lysate was centrifuged at 14,000 rpm for 10 minutes at 4°C to remove debris. Criterion TGX Gels, 4-20% (Bio-Rad, 5671094) were loaded with protein and the gels were run at 100 volts for 10 minutes, followed by 180 volts for 30 minutes. Gels were transferred onto Midi Format, 0.2 µm nitrocellulose membranes (Bio-Rad, 1704159) using a semi-dry transfer via a Trans-Blot Turbo transfer system. Membranes were blocked in 1X TBS 1% Caesin Blocker (Bio-Rad, 1610782EDU) for 1 hour, rocking. The membranes were then incubated overnight with respective primary antibodies (**Table 3**) suspended in 1X TBS 1% Caesin Blocker at 4°C. Membranes were washed with TBS-T 4 times for 5 minutes each. Primary antibodies were detected with species-specific horseradish-peroxidase-labeled secondary antibody (1:2000) incubated for 1 hour, rocking, protected from light. Membranes were again washed with TBS-T 4 times for 5 minutes each. Clarity Max Western ECL Substrate (Bio-Rad, 1705062) was used to obtain chemiluminescent reaction indicative of protein quantity. Membranes were rocked in the substrate for 5 minutes and then visualized using an iBright 1500. Protein bands of interest were quantified using FIJI software and were normalized with β-actin protein as a loading control for each sample.

2.2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

A Human Amyloid beta 42 Ultrasensitive ELISA Kit (Invitrogen, KHB554) was used to determine the amount of Aβ42 secreted by iNs. Neurons were treated chronically with CHI3L1

every other day for 10 days after which the iN medium/supernatant was collected. The provided Aβ42 standards were serial diluted and added to respective wells. After standards and samples were added to their respective wells, Hu Aβ42 US Detection Antibody solution was added to each well and incubated for 3 hours. Wells were subsequently aspirated and washed. An anti-Rabbit IgG HRP solution was added to each well and incubated for 30 minutes. Wells were again aspirated and washed. Stabilized Chromogen was added to each well and incubated for 30 minutes in the dark. Stop Solution was added to each well and then the plate absorbance was read at 450 nm. A standard curve was generated and Aβ42 concentration was calculated.

2.2.7 Caspase 3/7 Kit

To test for CHI3L1-induced apoptosis in neurons, CellEvent Caspase-3/7 Detection Reagents (Invitrogen, C10423) were used. Four hours prior to fixation, cells were treated with 0.5 μM staurosporine as a positive control for apoptosis. Media was aspirated off the cells and were subsequently washed with plain DMEM. CellEvent green was diluted in plain DMEM to 5 μM, added to each well, and incubated for 1 hour. Cells were then fixed with 4% paraformaldehyde. Cells were washed with PBS and then PBS with 1% triton was added and incubated for 15 minutes. Subsequently, a blocking buffer composed of 10% goat serum was added and incubated for 1 hour. Primary antibody solutions composed of 2% serum in PBS with anti-β-tubulin antibody (1:1000; Santa Cruz Biotechnology) was added and incubated overnight at 4°C. The following day, cells were washed with PBS 3 times for 10 minutes. A secondary antibody solution with PBS and goat anti-mouse 647 florescent antibody (1:2000, Invitrogen) was added and incubated covered for 2 hours at room temperature. Cells were again washed with PBS 3 times for 10 minutes. DAPI (1:1000) was added and incubated covered for 10 minutes followed by another PBS wash 3 times

for 10 minutes. Cells were visualized in the 405, 488, and 647 channels via a Lionheart FX automated microscope from BioTek. For analysis, images were taken from 3 different wells per condition with 5 pictures from each well. This imaging strategy was be replicated 3 times and all images were analyzed blind to condition.

2.2.8 Short Hairpin RNA (shRNA) Mediated Knockdowns

Predesigned, commercially available lentiviral shRNAs were used to knockdown specific genes of interest. Neurons were treated with a RAGE lentiviral shRNA (Sigma-Aldrich, SHCLNV TRCN0000371281). To confirm adequate knockdown, a titer was conducted to identify the amount of viral particles needed. A knockdown of 75% or greater was considered an efficient knockdown. One, 2.5, 5, 7.5, and 10 viral particles were tested and it was determined that 7.5 viral particles of the RAGE shRNA lentivirus successfully knocked down roughly 80% of RAGE expression (**Figure 6**).

2.2.9 Statistical Analysis

For statistical and bioinformatic analyses, in brief, the normality of the data distribution was routinely determined by a Shapiro-Wilk normality test ($p<0.05$ indicating a non-normal distribution). For the data confirmed to be normally distributed data, Student's t-test for pair-wise comparisons and ANOVA (followed by post-hoc analysis) for 3 groups or more were used. For data that is not normally distributed, non-parametric alternatives, such as Mann-Whitney or Kruskal-Wallis were tested. $p<0.05$ is considered to be statistically significant.

2.3 Results

2.3.1 CHI3L1 Treatment Alters the iN Transcriptome

To investigate the potential CHI3L1 function in neurons, I performed RNA Sequencing to determine the effect of CHI3L1 on the neuronal transcriptome. Principal component analysis (PCA) shows that iNs treated with CHI3L1 separate from the control iNs and group together within treatment, indicating a CHI3L1 effect on iN gene expression (**Figure 7A**). A volcano plot further validates this change in gene expression as significant differentially expressed genes are present, those that are downregulated appearing in blue and those that are upregulated in red (**Figure 7B**). Furthermore, CHI3L1 treatment significantly altering gene expression and gene ontology terms in iNs, supports an effector function of CHI3L1 on neurons.

From this altered iN gene profile, gene ontology (GO) analysis was conducted to determine further information or function of these significant differentially expressed genes. The top 20 most significant GO terms are shown (**Figure 7C**). Of note, "signal transduction", "apoptotic process", and "inflammatory response" were present in the top 20 most significant terms. These terms align with CHI3L1 processes and functions that have been defined in the periphery [93] and may further corroborate that CHI3L1 retains said functions in the central nervous system. To further investigate the effect of CHI3L1 on neuronal signaling, apoptosis, and inflammation, I continued to probe the significant GO terms relating to these processes (**Figure 8**). Other GO terms relevant to signaling include pathways that CHI3L1 has been known to activate in the periphery, including NFκB and MAPK/ERK [93]. Similarly, further probing the GO terms relevant to inflammation and apoptosis also reveal a CHI3L1 role in neurons similar to that which have been seen in the periphery [93]. In order to determine if these gene ontology terms were significantly changed by CHI3L1 inducing or reducing gene expression, the specific altered genes within these ontology terms were investigated. Overall, CHI3L1 largely increased the expression of genes relevant to signal

transduction, inflammatory response, and apoptosis indicating a pro-signaling, pro-inflammatory, and pro-apoptotic function of CHI3L1 in neurons (**Figure 9**). Additionally, several of these upregulated genes have been associated with neurodegeneration and AD pathogenesis (**Figure 9**), suggesting an AD-promoting effect on neurons from CHI3L1. Interestingly, some of the top upregulated genes comprise a group of cytokines and chemokines, which have long been known to be expressed in neurons with poorly defined function. This finding indicates that some reported mechanisms of CHI3L1 in regulating cytokine release in peripheral immune cells might operate similarly in neurons.

In addition to GO analysis, KEGG pathway analysis was performed to provide further insight into active pathways. Similarly to the GO, KEGG pathway analysis on the iNs also aligned with CHI3L1 processes that have been identified in the periphery, including receptor interactions and signaling via MAPK, NFκB, AKT, Wnt, STAT, and RAGE signaling [93] (**Table 4**). Of note, additional significant KEGG pathways include "Alzheimer's Disease" and "Pathways of Neurodegeneration", providing CNS-specific implications of CHI3L1 and further support that CHI3L1 plays a role in AD (**Figure 10**).

2.3.2 CHI3L1 Increases AD and Neurotoxic Features in iNs

In order to validate the RNASeq findings and to further tie CHI3L1 to Alzheimer's Disease and neurodegeneration, I treated iNs with CHI3L1 and assessed the effect on AD-relevant features. CHI3L1 treatment of iNs increases Amyloid precursor protein (APP) at both the RNA and protein levels as confirmed by qPCR and immunoblotting (**Figure 11A-B**). While APP in it of itself is not directly pathogenic in AD, APP is sequentially cleaved by a β- followed by γ-secretase toward an amyloidogenic processing and secretion of Aβ, which is indeed a hallmark pathology of AD. As

such, I characterized the CHI3L1 effect on amyloidogenic Aβ42 secretion by iNs via an enzyme-linked immunosorbent assay (ELISA). Chronic, but not acute, treatment of CHI3L1 significantly increases the amount of Aβ42 secreted by iNs (**Figure 11C**). In addition to Aβ, another hallmark AD pathology is hyperphosphorylated tau tangles. Therefore, I also probed the levels of phosphorylated tau in iNs and found that CHI3L1 significantly increases phospho-tau (**Figure 11B**). Taken together, these results show that CHI3L1 is able to induce AD-relevant features in neurons.

While I witnessed an increase in AD features in iNs, it was still unclear if CHI3L1 played a direct role in neuronal viability or neurodegeneration. As such, I aimed to confirm that CHI3L1 also had a neurodegenerative effect, namely to determine if CHI3L1 is neurotoxic via apoptosis assays. CHI3L1-treated iNs were probed for caspase-3, a protein specifically involved in an apoptotic pathway. Immunoblotting of caspase-3 shows an increase in caspase-3 and its cleavage products, indicating CHI3L1 induces apoptosis in neurons (**Figure 12A**). The apoptotic effect of CHI3L1 on iNs was confirmed via a caspase3/7 imaging kit, which shows CHI3L1 significantly increases caspase levels in neurons, similar to that of reactive astrocyte supernatant and staurosporine, a positive control for apoptosis. This pro-apoptotic finding further supports the RNASeq data implicating CHI3L1 in apoptosis, and overall confirms a CHI3L1 neurotoxic effect.

2.3.3 CHI3L1 Treatment Modulates Known Receptor Expression, Implicating RAGE

Amongst the widespread transcriptomic alterations, I examined and confirmed the expression of the receptors documented in peripheral immunity on iNs (**Figure 13A**). The presence of these known CHI3L1 receptors on iNs was then further validated by qPCR (**Figure 13B**). Consistent with the prior *in vivo* transcriptomic results[77], control iNs do express all the known

CHI3L1 receptors; upon treatment with recombinant CHI3L1 proteins, along with Aβ, the expression of known receptors is modulated, particularly RAGE (**Fig. 13B**). These results thus suggest the potential engagement of a receptor to mediate the neuronal responses to CHI3L1 secreted by activated astrocytes in neuroinflammation. While RAGE expression was significantly altered, signaling pathways known to be activated by CHI3L1, RAGE binding were probed and found to be largely unchanged over time (**Figure 13C**).

Determining the receptor CHI3L1 binds to on neurons is critical to elucidate the mechanism by which CHI3L1 modulates AD and neurotoxic outputs. RAGE is one of the only defined CHI3L1 receptors that does not require complexing with the IL-13Rα2 receptor in order to elicit CHI3L1 binding. This individual accessibility, along with it being one of the more highly expressed receptors on iNs (**Figure 13A**) that is modulated by CHI3L1/Aβ stimuli (**Figure 13B**), positions RAGE as a potential main neuronal CHI3L1 receptor. I treated iNs with a RAGE short hairpin RNA (shRNA) lentivirus to silence the receptor expression in order to determine the role RAGE plays in these CHI3L1 induced AD and neurotoxic features. RAGE knockdowns showed reduced levels of APP, at both the protein and RNA levels, (**Figures 14 & 15**) as well as a reduction of APH1a (**Figure 15**), a subunit of the γ-secretase responsible for cleaving APP toward an amyloidogenic route. While RAGE may have an effect on Aβ-relevant pathologies in iNs, other hallmark AD features, namely phosphorylated tau levels, were unchanged with RAGE knockdown. Additionally, while there was a reduction in the amount of pro-caspase in response to RAGE knockdown, the cleaved caspase remains unchanged (**Figure 14**), as such the role RAGE plays in regulated CHI3L1-induced apoptosis remains unclear. RAGE knockdown also significantly reduced the amount of CHI3L1-induced NFκB at the protein and transcript level, however phosphorylated NFκB remains unchanged (**Figures 14 & 15**). Overall, knocking down

RAGE reduced some features relevant to AD and neurotoxicity, further implicating RAGE as a main neuronal CHI3L1 receptor; however, other receptors should be probed to determine their potential impact on these defined outputs.

2.4 Discussion

Here, I hypothesized that upon the reactivation of astrocytes in neuroinflammation, the secreted Chitinase-3-like protein 1 engages a neuronal receptor and triggers a signal transduction cascade in neurons leading to an increase in Aβ production and neurotoxicity. In order to test this hypothesis, I treated neurons induced from pluripotent stem cells with recombinant CHI3L1 and assessed functional outputs via RNASeq, immunoblotting, and quantitative PCR, among other assays. I determined that CHI3L1 induces widespread transcriptomic alterations in human neurons via RNASeq. Specifically, CHI3L1 modulates the expression of certain genes implicated in inflammation, apoptosis, signaling, and AD. Via a caspase imaging assay and western blotting, I confirmed my RNASeq results and found that CHI3L1 induces apoptosis in neurons. Additionally, I confirmed via western blotting that CHI3L1 significantly increases levels of AD-associated features, including APP and phospho-tau, in neurons. Via an amyloid beta ELISA, I also found that chronic CHI3L1 treatment significantly increases Aβ42 levels in the induced neurons, further supporting that CHI3L1 increases AD-relevant features.

These established effects in neurons and their reliable, significant readouts can be used to assess the effects of candidate receptor knockdowns in the neurons. Therefore, I began to determine if RAGE is the neuronal CHI3L1 receptor by using RNAi on iNs treated with recombinant CHI3L1 versus untreated controls. With the RAGE knockdown, I saw a reduction in genes and proteins implicated in CHI3L1 signaling and AD features, specifically NFκB, caspase,

and APP. Specifically, RAGE knockdowns showed reduced levels of APP, at both the protein and RNA levels, as well as a reduction of APH1a, a subunit of the γ-secretase responsible for cleaving APP toward an amyloidogenic route. While RAGE may have an effect on Aβ-relevant pathologies in iNs, other hallmark AD features, namely phosphorylated tau levels, were unchanged with RAGE knockdown. Additionally, RAGE knockdown significantly reduced the amount of CHI3L1-induced NFκB at the protein and transcript level. Overall, knocking down RAGE reduced some features relevant to AD and neurotoxicity, further implicating RAGE as a main neuronal CHI3L1 receptor; however, other receptors should be probed to determine other potential CHI3L1 neuronal mechanisms and their potential impact on these defined outputs.

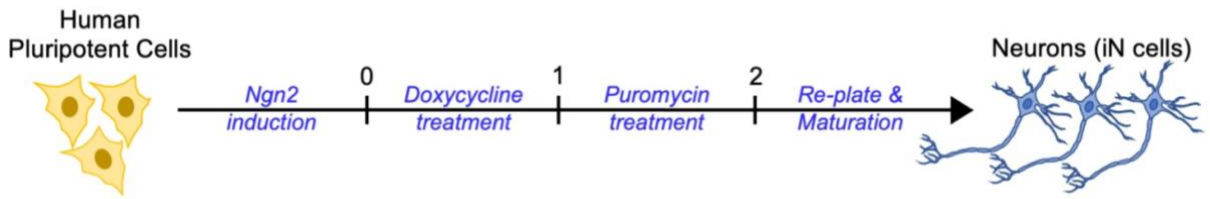


Figure 5. Induced neuron cell induction from human pluripotent stem cells, embryonic stem (ES) and iPS cells. On day -1, hPSCs are transfected with a lentivirus expressing Ngn2. On day 0, doxycycline is added to induces expression of Ngn2. On Day 1, puromycin is added to select for transfected cells. On day 2, iNs are replated and allowed to mature.

Table 2. Probes used for qPCR analysis.

Probe	Function	Reference #	Manufacturer
CHI3L1	Chitinase-3-like protein 1 Immune Modulator/Protein of interest	Hs01072228_m1	ThermoFisher Scientific
RAGE/AGER	CHI3L1 Receptor	qHsaCED0001302	BioRad
APP	Amyloid Precursor Protein/ AD feature	Hs.PT.56a.38877427 exon5-6	Integrated DNA Technologies
IL13Ra2	CHI3L1 Receptor	qHsaCID0017264	BioRad
TMEM219	CHI3L1 Receptor	qHsaCID0015277	BioRad
Gal3	CHI3L1 Receptor	qHsaCED0044416	BioRad
CD44	CHI3L1 Receptor	qHsaCID0013679	BioRad
CRTH2	CHI3L1 Receptor	qHsaCID0012826	BioRad
APH1a	Gamma Secretase Subunit	qHsaCEP0025677	BioRad
NFkB1	Pro-inflammatory Marker	Hs.PT.58.20344216	Integrated DNA Technologies
NFkB2	Pro-inflammatory Marker	Hs.PT.58.46592905	Integrated DNA Technologies
SYN1	Pre-synaptic Marker	Hs.PT.58.4027324	Integrated DNA Technologies
BDNF	Anti-inflammatory Marker	Hs.PT.56a.24829701	Integrated DNA Technologies
C3	Pro-inflammatory Marker	Hs.PT.56a.2840009	Integrated DNA Technologies
IL1B	Pro-inflammatory Marker	Hs.PT.58.1518186	Integrated DNA Technologies
TNF	Pro-inflammatory Marker	Hs.PT.58.45380900	Integrated DNA Technologies
DLG4	Post-synaptic Marker	Hs.PT.58.20575145	Integrated DNA Technologies
IBA1/AIF1	Microglia Marker	qHsaCEP0040047	BioRad
C1qa	Pro-inflammatory Marker	qHsaCED0038525	BioRad
IL6	Pro-inflammatory Marker	Hs.PT.58.40226675	Integrated DNA Technologies

Table 3. Primary antibodies used for immunoblotting analysis.

Function	1º Antibody	Dilution Factor	Manufacturer
Astrocyte Marker	anti-GFAP made in mouse	1:1000	UC Davis
Microglia Marker	anti-IBA1 made in rabbit	1:1000	Wako
Apoptotic Marker	anti-Caspase3 made in mouse	1:500	ProteinTech
Amyloid Precursor Protein/ AD feature	anti-APP C99 made in mouse	1:1000	Millipore Sigma
Total tau/ AD feature	anti-HT7 tau made in mouse	1:1000	ThermoFisher Scientific
Phospho-tau/ AD feature	Anti-AT8 tau	1:1000	Invitrogen
Chitinase-3-like protein 1 Immune Modulator/Protein of interest	anti-CHI3L1 made in rabbit	1:1000	Abcam
Signaling Protein	anti-β-catenin (D10A8) made in rabbit	1:1000	Cell Signaling Technology
Phospho-ERK/ Signaling Protein	anti-phospho-p44/42 MAPK (Erk1/2) made in rabbit	1:1000	Cell Signaling Technology
Total ERK/ Signaling Protein	anti-p44/42 MAPK (Erk1/2) made in mouse	1:1000	Cell Signaling Technology
Phospho-NFκB/ Signaling Protein	anti-phospho-NFκB p65 (Ser536) made in rabbit	1:1000	Cell Signaling Technology
Total NFκB/ Signaling Protein	anti-NFκB p65 made in rabbit	1:1000	Cell Signaling Technology
Loading control	ant-β-actin made in mouse	1:4000	Sigma-Aldrich
Loading control	anti-β-tubulin made in mouse	1:4000	Santa Cruz Biotechnology

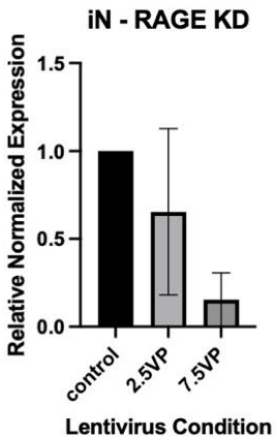


Figure 6. Lentiviral shRNA mediated knockdown of desired genes. Predesigned, commercially available lentiviral shRNAs were used to knockdown RAGE in neurons. A titer was performed for each lentivirus to determine the appropriate amount of viral particles needed for adequate gene knockdown. Reduced gene expression was confirmed via qPCR. A knockdown of 75% or greater was considered an efficient knockdown (n = 3).

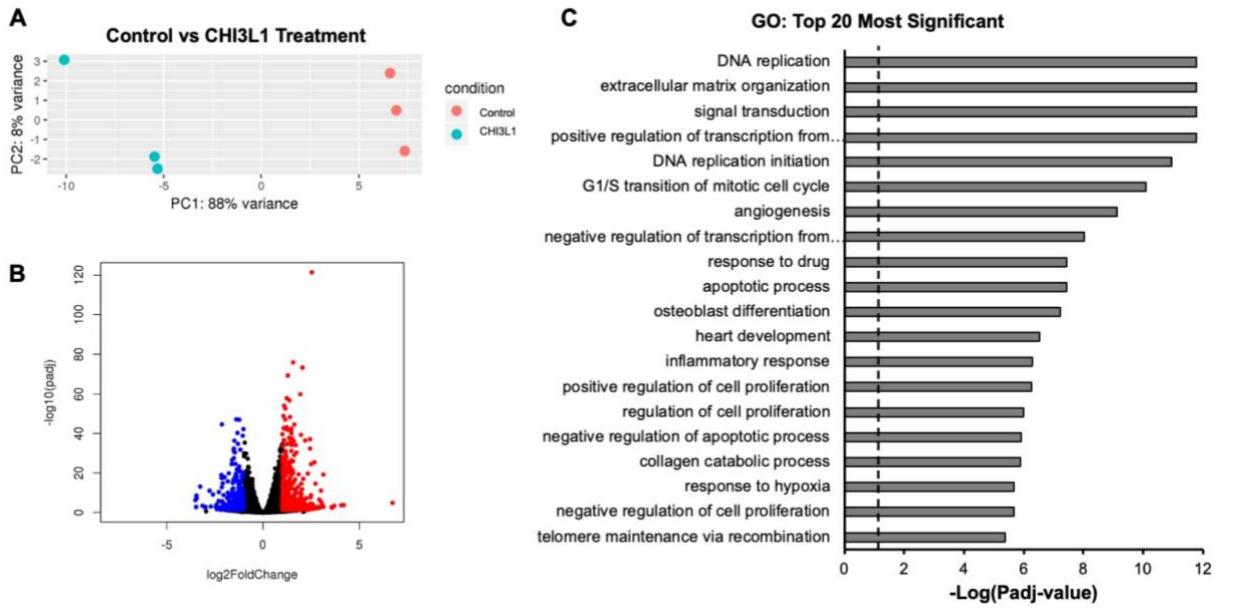


Figure 7. CHI3L1 treatment induces widespread transcriptional alterations in human neurons. Transcriptomic analysis of human induced neurons derived from iPSCs. RNA-Seq of iNs reveals (**A**) differential clustering of control vs. CHI3L1 treated neurons and (**B**) significantly up/downregulated genes and (**C**) relevant gene ontology terms. The dotted line in C demarks the significance threshold.

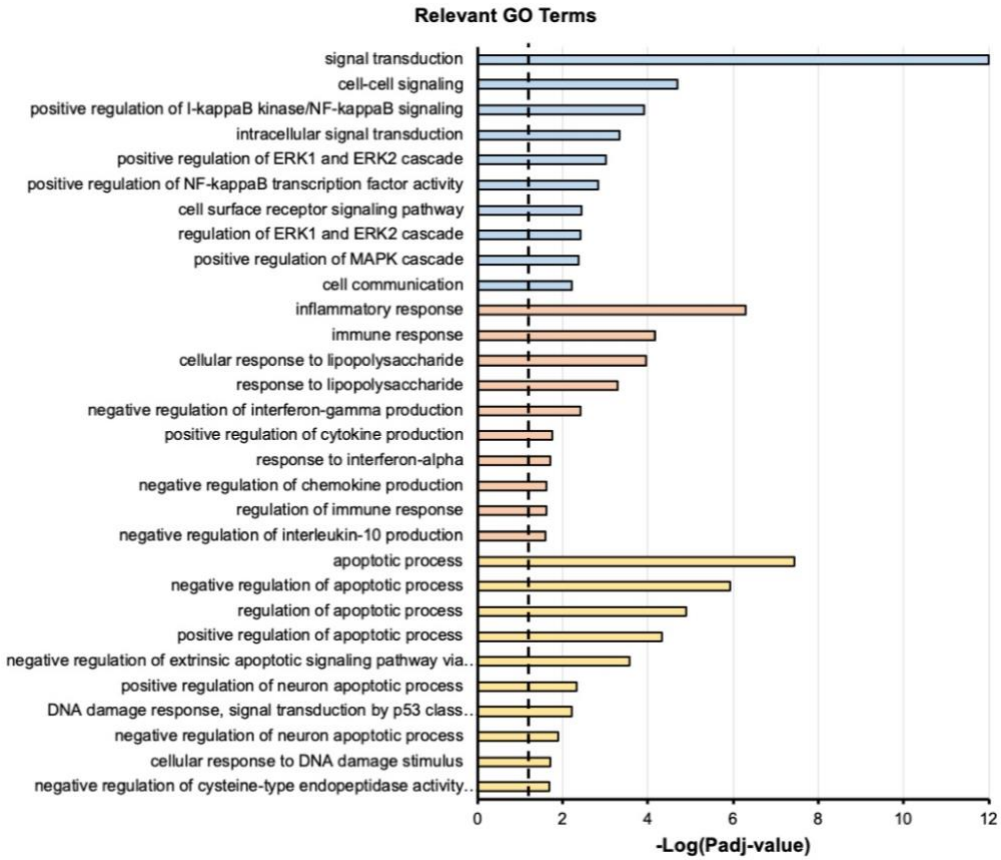


Figure 8. Transcriptomic analysis further implicates CHI3L1 in signaling, inflammatory, and apoptotic processes. Gene ontology terms were further investigated and the top 10 significant, additional terms specific to signaling, inflammation, and apoptosis were included. The dotted line demarks the significance threshold.

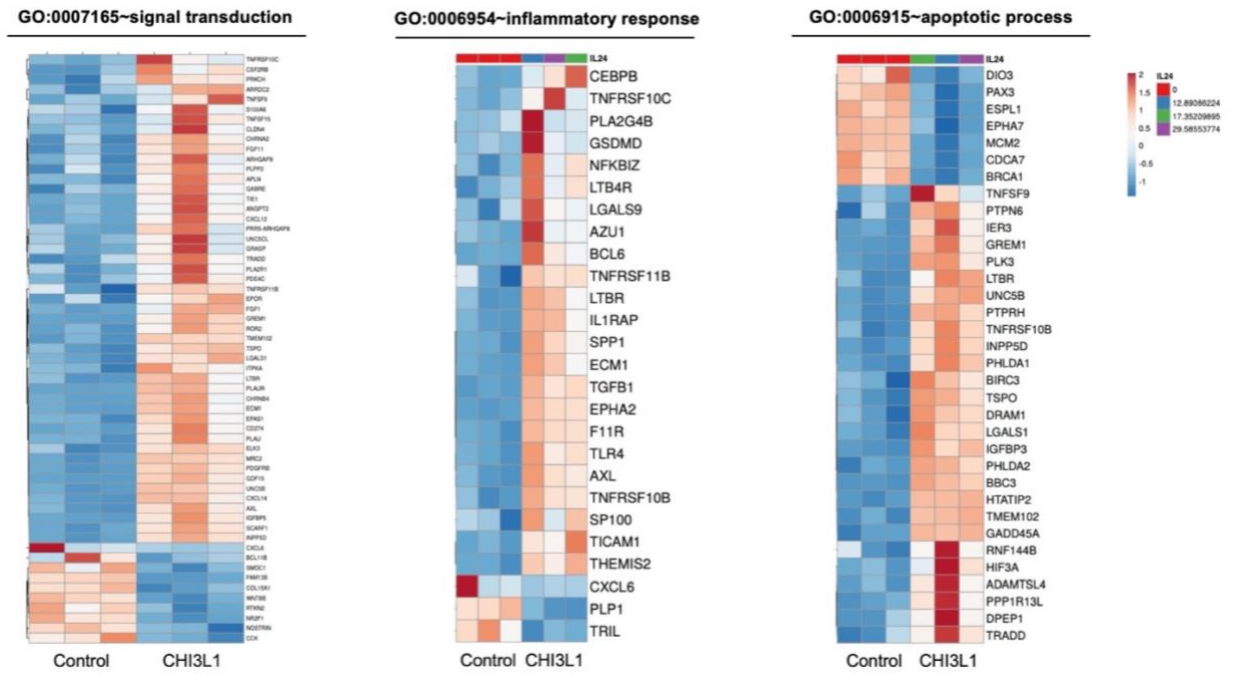


Figure 9. CHI3L1 stimulation induces in neurons the expression of AD-associated and inflammatory genes. Transcriptomic changes caused by treatment of induced neurons (iNs) with recombinant CHI3L1 protein, assayed by RNA-seq. CHI3L1 treatment of iNs significantly increases the expression of genes from defined GO terms in signaling, inflammation, and apoptosis.

Table 4. Relevant KEGG pathways significantly affected by CHI3L1 treatment of iNs.

Identifier	Pathway
hsa04010	MAPK signaling pathway
hsa04060	cytokine-cytokine receptor interaction
hsa04064	NFκB signaling pathway
hsa04151	PI3K-AKT signaling pathway
hsa04210	Apoptosis
hsa04217	Necroptosis
hsa04310	Wnt signaling pathway
hsa04630	JAK-STAT signaling pathway
hsa04933	AGE-RAGE signaling pathway
hsa05010	Alzheimer's Disease
hsa05022	Pathways of Neurodegeneration

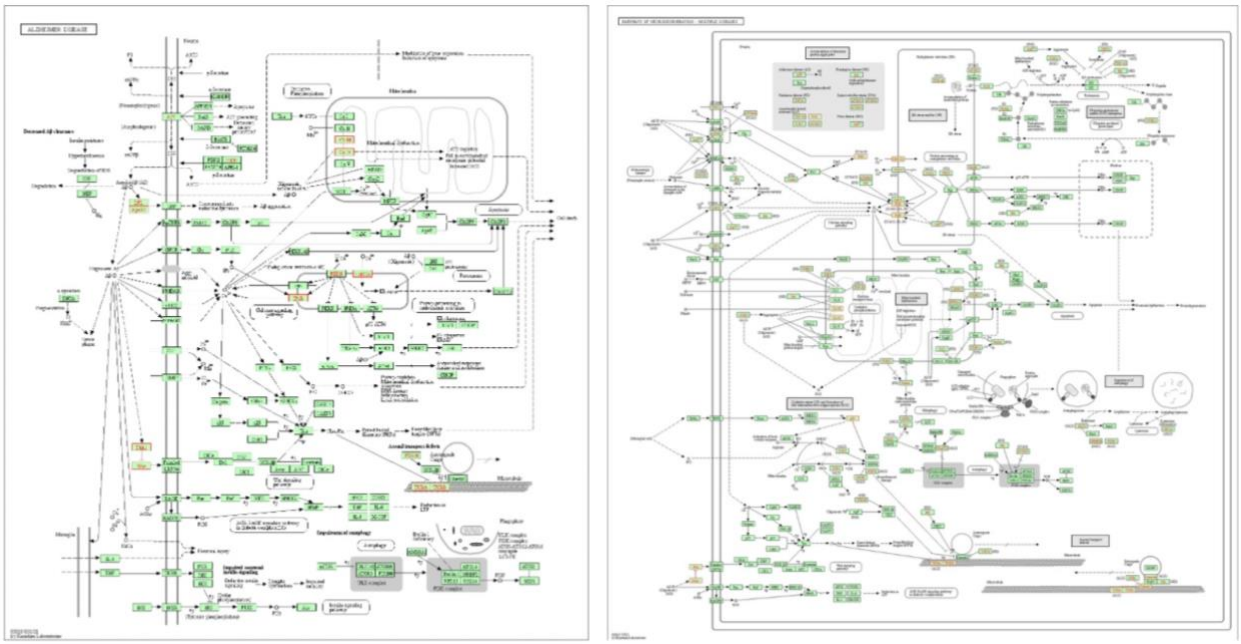


Figure 10. CHI3L1 alters genes implicated in Alzheimer's Disease and neurodegeneration pathways. Differentially expressed genes were input into the KEGG search tool which identified significantly effected pathways. Found KEGG objects (including genes) are marked in red.

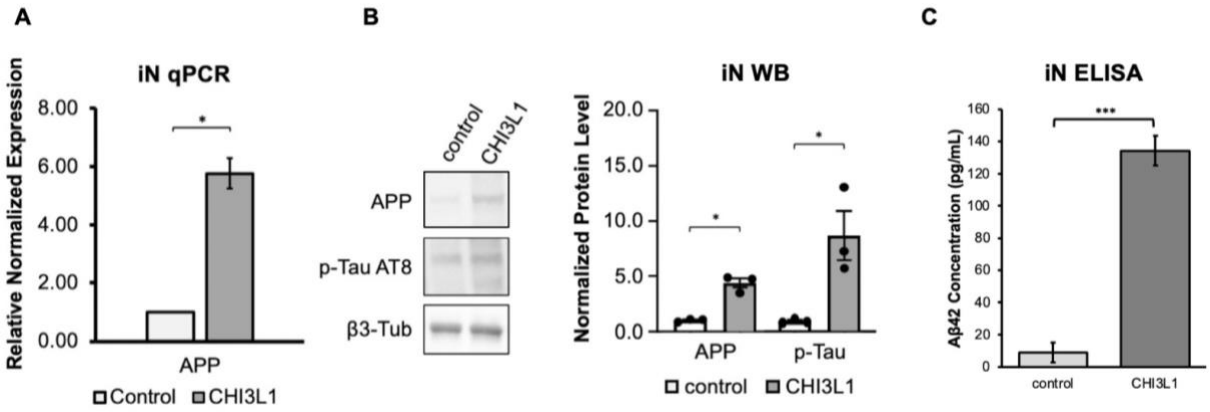


Figure 11. CHI3L1 increases AD-associated features in neurons. **A.** Upregulation of APP was validated by individual qPCR (n = 5). **B.** Increased APP and p-Tau were witnessed via immunoblotting (n = 3). **C.** Aβ secretion was measured via ELISA (n = 4). The mean and standard error of the mean are shown (mean ± SEM). Student's t-test. ** = $p<0.01$, * = $p<0.05$.

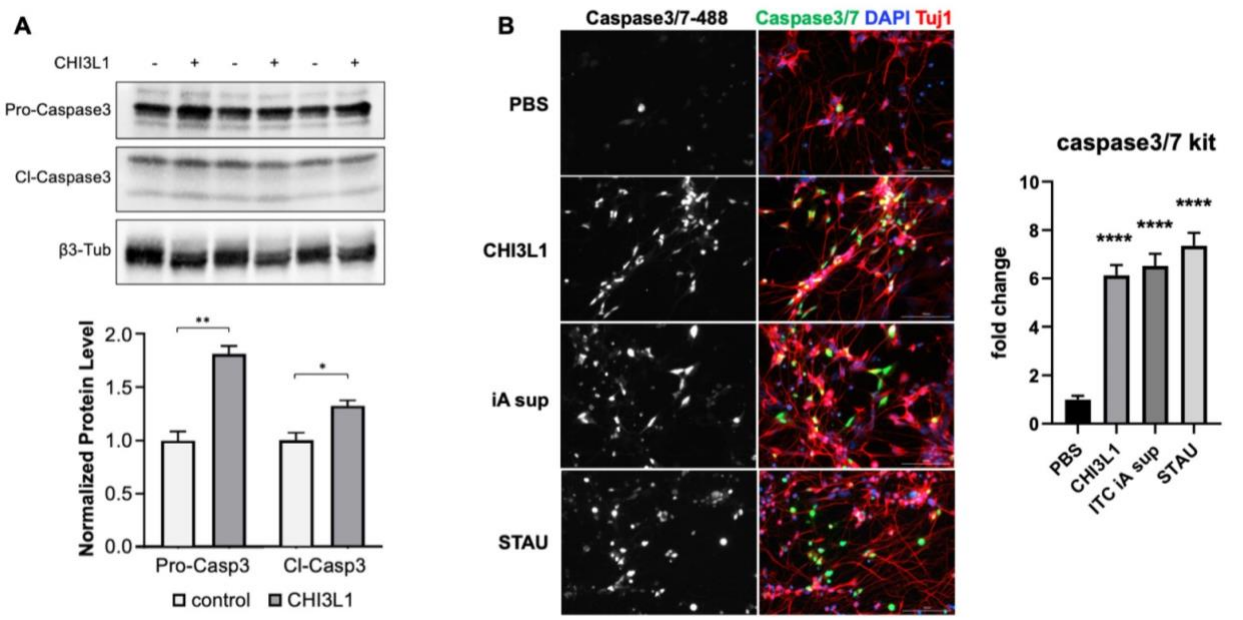


Figure 12. CHI3L1 increases neurotoxic features in neurons. CHI3L1 treatment of iNs significantly increases the levels of caspase 3, supporting the induction of apoptosis by CHI3L1. Shown via immunoblotting (A) and validated with a Caspase3/7 kit. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Student's t-test and ordinary one-way ANOVA with Bonferroni multiple comparisons test. **** = $p<0.0001$, ** = $p<0.01$, * = $p<0.05$.

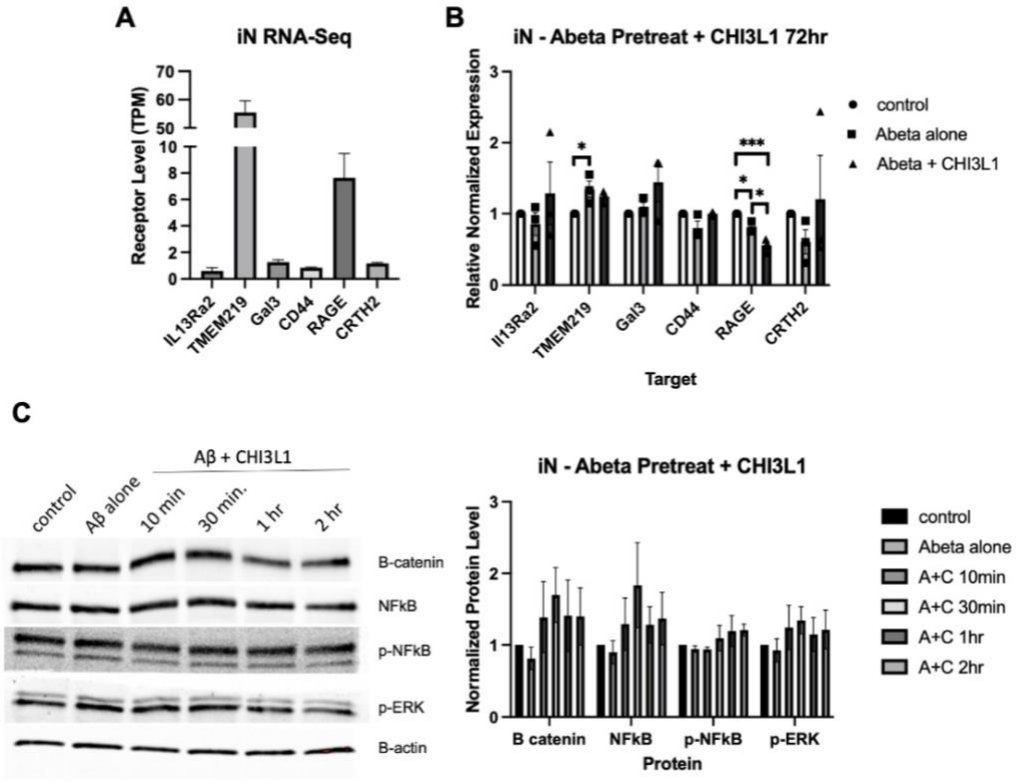


Figure 13. Human neurons express known CHI3L1 receptors in vitro. Transcriptomic analysis shows presence of CHI3L1 receptors on iNs (**A**), confirmed via qPCR (**B**). Of the known receptors, CHI3L1 treatment significantly alters the expression of only RAGE in iNs. **C.** iNs were treated with CHI3L1 at various timepoints and protein levels were assessed via immunoblotting. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary one-way ANOVA with Bonferroni multiple comparisons test. *** = $p<0.001$, * = $p<0.05$.

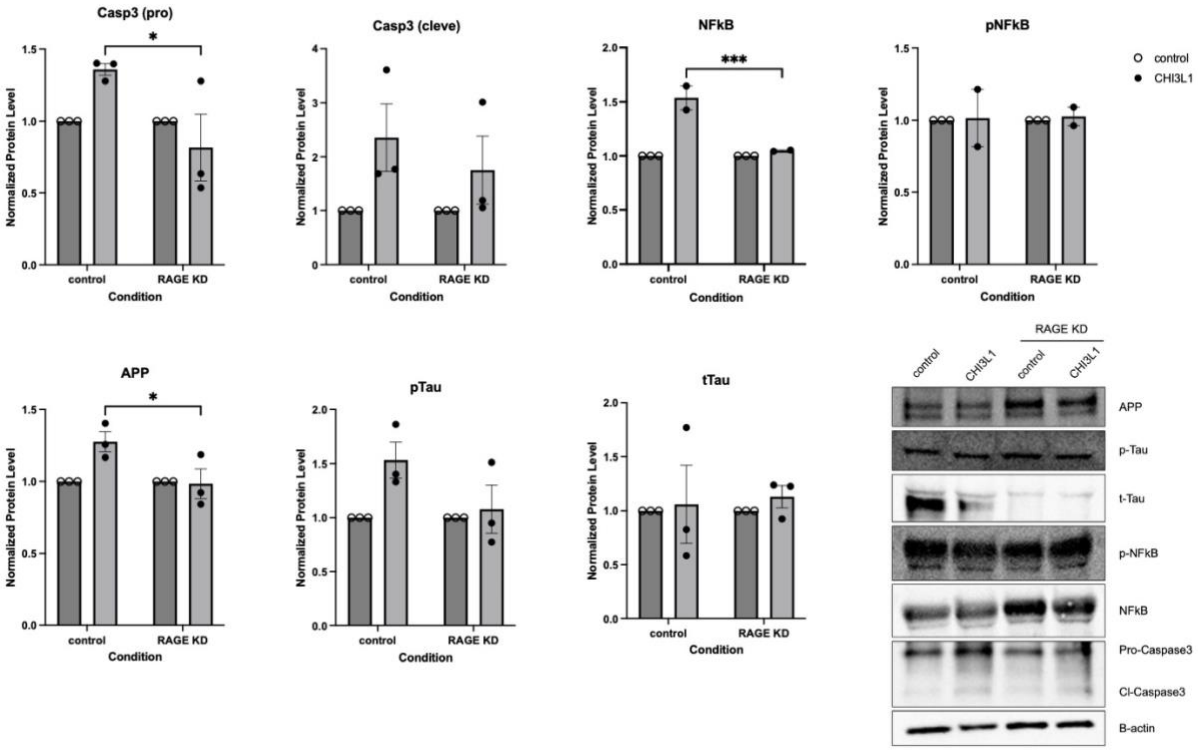


Figure 14. RAGE KD reduces CHI3L1-induced expression of certain AD-relevant and neurotoxic proteins in iNs. RAGE was knocked down in iNs via a lentiviral shRNA and protein levels of desired targets were compared to those of control (WT) iNs via immunoblotting. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary two-way ANOVA with Bonferroni multiple comparisons test. *** = $p<0.001$, * = $p<0.05$.

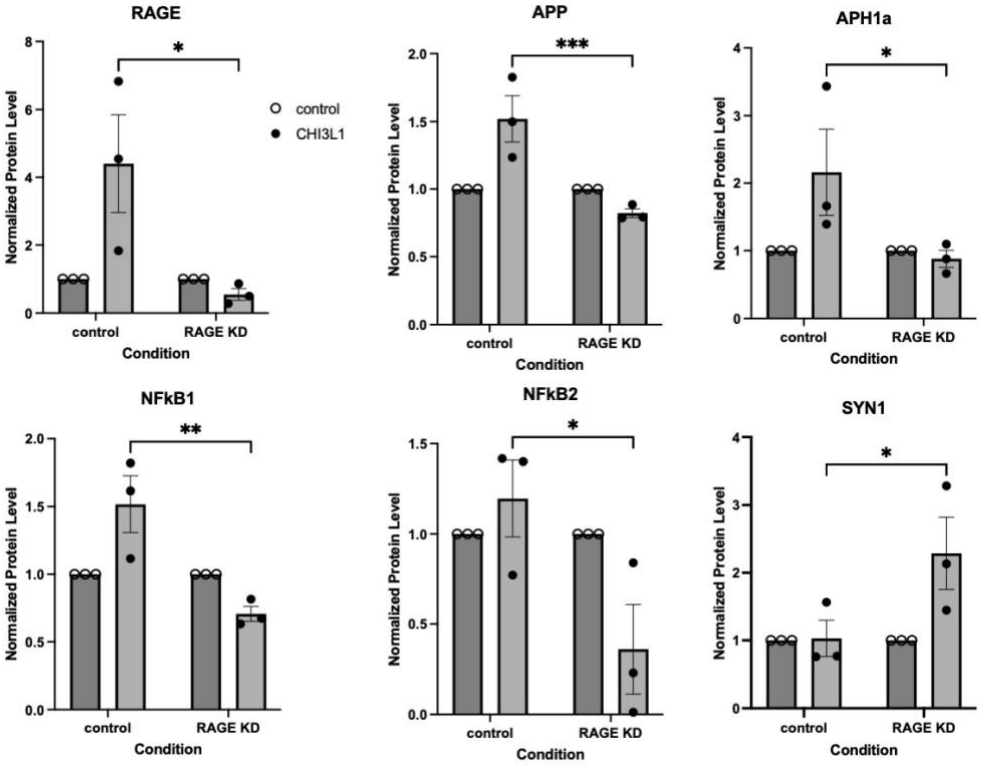


Figure 15. RAGE KD reduces CHI3L1-induced expression of certain signaling and AD-relevant transcripts in iNs. RAGE was knocked down in iNs via a lentiviral shRNA and mRNA levels of desired targets were compared to those of control (WT) iNs via qPCR. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary two-way ANOVA with Bonferroni multiple comparisons test. *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.

Chapter 3: CHI3L1 Modulates Glial Reactivity in the Presence of Neurons

3.1 Introduction

Overall, the use of pure cultures of individual CNS cells induced from stem cell allows for a reductionist approach that enables the dissection of cell type-specific effects or events that can be difficult to study in the context of the whole brain due to its inherent complexity.

In addition to determining cell-specific effects by using pure cultures of induced cells, the differing induced cell types can be combined and cultured together as co- or tri-cultures in order to determine cellular cross-talk and interactions. While pure cultures are useful tools for studying individual cell types, these models alone do not address the important, intricate hetero-cellular interactions or the cross-talk interactions that may be implicated in health, neuroinflammation, or diseased states. Thus, a co- and/or tri-culture approach allows for further probing into multiple cell types while still maintaining a reductionist approach that is again able to avoid issues posed by the inherent complexity inside the whole brain [96, 98-100]. By combining induced neurons (iNs), induced astrocytes (iAs), and induced microglia (iMGs) together, I attempt to unravel the potential intercellular communications, interactions, underlying mechanisms, and their implication in the CHI3L1-induced neuroinflammatory process.

3.2 Methodology

3.2.1 iNeuron Induction

H1 human embryonic stem cells (WiCell Research Institute, WA01) were cultured in mTeSR+ (STEMCELL Technologies, 100-0276) on matrigel (Corning, 354234) or geltrex (ThermoFisher Scientific, 1413301) coated plates. To induce stem cells into neurons, cells were lifted at ~80% confluency with accutase (STEMCELL Technologies, 07920), spun down at 300xg for 5min, and resuspended and replated onto coated plates with 10μM ROCK inhibitor

(STEMCELL Technologies, Y-27632). The following day, stem cells were infected with an inducible lentivirus system encoding rtTA (Addgene, 19780) and expressing neurogenin-2 (Ngn2) (Addgene, 52047). The next day 2µg/mL doxycycline was added to the cells in fresh media to activate Ngn2 expression. Puromycin plus doxycycline (2µg/mL) was then added to the cells to select for the transduced cells on the subsequent day. On the final day, the induced neurons (iNs) were detached and replated on extracellular matrix solution coated plates in Neurobasal-A medium (ThermoFisher Scientific, 10888022) with 1X B27 (ThermoFisher Scientific, 17504044) for maturation and experimentation [96, 97] (**Figure 16**).

3.2.2 iAstrocyte Induction

H1 human embryonic stem cells (WiCell Research Institute, WA01) were cultured in mTeSR+ (STEMCELL Technologies, 100-0276) on matrigel (Corning, 354234) or geltrex (ThermoFisher Scientific, 1413301) coated plates. To induce stem cells into microglia, cells were lifted at ~80% confluency with accutase (STEMCELL Technologies, 07920), spun down at 300xg for 5min, and resuspended and replated onto coated plates with 10µM ROCK inhibitor (STEMCELL Technologies, Y-27632). The following day, DMSO was added to the stem cells in fresh media, then each day for the following 6 days, fresh neural induction media containing SMAD inhibitors was added to the cells to generate neural progenitor cells (NPCs) via a STEMdiff SMADi Neural Induction Cell Differentiation Kit (STEMCELL Technologies, 08582). The next day, NPCs were detached and replated on extracellular matrix solution coated plates in Astrocyte Medium supplemented with fetal bovine serum (FBS), astrocyte growth supplement (AGS), and penicillin/streptomycin solution (P/S) (ScienCell, 1801). Astrocytes were allowed to mature and fresh astrocyte medium was replaced every 48 hours. The induced astrocytes (iAs) were detached

and replated on extracellular matrix solution coated plates for experimentation [96, 101] (**Figure 16**).

3.2.3 iMicroglia Induction

H1 human embryonic stem cells (WiCell Research Institute, WA01) were cultured in mTeSR+ (STEMCELL Technologies, 100-0276) on matrigel (Corning, 354234) or geltrex (ThermoFisher Scientific, 1413301) coated plates. To induce stem cells into microglia, cells were lifted at ~80% confluency with accutase (STEMCELL Technologies, 07920), spun down at 300xg for 5min, and resuspended and replated onto coated plates with 10μM ROCK inhibitor (STEMCELL Technologies, Y-27632). The following day, stem cells were infected with an inducible lentivirus system encoding rtTA (Addgene, 19780) and expressing both CCAAT/enhancer-binding protein α and SFFV pro-viral integration 1 (CEBPA/SPI1) (Addgene,). The next day 2μg/mL doxycycline along with cytokines and signaling molecules required for microglia development were added to the cells in fresh media to activate gene expression (**Table 5**). Puromycin plus doxycycline, along with cytokines and signaling molecules, was then added to the cells to select for the transduced cells on the subsequent day. Full medium changes were performed D1-4, while half medium changes were conducted D6+ until treatment/harvest [102] (**Figure 16**).

3.2.4 Co-/Tri-cultures

Individual, pure cultures of iNs, iAs, and iMGs were generated and maintained in culture as described above. The final ratio of neurons to astrocytes and neurons to microglia in co-culture is 6:1 respectively, while in tri-culture the ratio of iNs to iAs to iMGs is 6:1:1. On day -2, iNs and

iAs were thawed (or passaged), counted, and plated individually based on the desired ratio/culture set-up. The respective media (NBA/B27 and Astrocyte medium) were changed the following day. On day 0, co- and/or tri-culture medium was prepared at a 3:1 or 3:1:1 ratio, NBA/B27 to respective glial medium or NBA/B27 to Astrocyte Medium to iMG Medium. Once the respective culture medium was prepared, iMGs were thawed, iAs were passaged, and the cells were counted and plated together with the iNs, either as co-cultures or all together as tri-cultures. The respective co-/tri-culture media was changed the next day and subsequently every other day [100] (**Figure 17**).

3.2.5 Cell Treatments

iNs were treated with 1µg/ml of recombinant CHI3L1 protein (R&D Systems; #2599-CH-050). In experiments where Aβ was used, human beta-amyloid peptide 1-42 (Abcam, ab120301) was added to culturing medium at a final concentration of 1µM and incubated for 24 hours while shaking at 37ºC. This Aβ solution was added to cultures 1 day prior to CHI3L1 treatment. Depending on the respective experiment, the iNs were treated with CHI3L1 for either 72 hours, a time course within 2 hours, or, in the case of the ELISA, chronically every other day for 10 days.

3.2.6 Quantitative PCR (qPCR)

RNA was harvested via an RNeasy Mini Kit (Qiagen, 74104). RNA concentration was determined via nanodrop. The genomic DNA was degraded and cDNA was subsequently synthesized via the iScript gDNA Clear cDNA synthesis kit (Bio-Rad, 1725034). Using SsoAdvanced Universal Probes Supermix (Bio-Rad, 172-5281), respective probes (**Table 2**) were

mixed with the supermix and water, then added on top of cDNA in a 96-well qPCR plate. The plates were run on a Bio-Rad CFX96 Real-Time System.

3.2.7 Western Blotting

Media was removed and cells were lysed in radioimmunoprecipitation, RIPA, buffer. The lysate was centrifuged at 14,000 rpm for 10 minutes at 4^{o}C to remove debris. Criterion TGX Gels, 4-20% (Bio-Rad, 5671094) were loaded with protein and the gels were run at 100 volts for 10 minutes, followed by 180 volts for 30 minutes. Gels were transferred onto Midi Format, 0.2 µm nitrocellulose membranes (Bio-Rad, 1704159) using a semi-dry transfer via a Trans-Blot Turbo transfer system. Membranes were blocked in 1X TBS 1% Caesin Blocker (Bio-Rad, 1610782EDU) for 1 hour, rocking. The membranes were then incubated overnight with respective primary antibodies (**Table 3**) suspended in 1X TBS 1% Caesin Blocker at 4^{o}C. Membranes were washed with TBS-T 4 times for 5 minutes each. Primary antibodies were detected with species-specific horseradish-peroxidase-labeled secondary antibody (1:2000) incubated for 1 hour, rocking, protected from light. Membranes were again washed with TBS-T 4 times for 5 minutes each. Clarity Max Western ECL Substrate (Bio-Rad, 1705062) was used to obtain chemiluminescent reaction indicative of protein quantity. Membranes were rocked in the substrate for 5 minutes and then visualized using an iBright 1500. Protein bands of interest were quantified using FIJI software and were normalized with β-actin protein as a loading control for each sample.

3.2.8 Short Hairpin RNA (shRNA) Mediated Knockdowns

Predesigned, commercially available lentiviral shRNAs were used to knockdown specific genes of interest. Astrocytes were treated with a CHI3L1 lentiviral shRNA (Sigma-Aldrich,

SHCLNV TRCN0000051954). To confirm adequate knockdown, a titer was conducted to identify the amount of viral particles needed. One, 2.5, 5, 7.5, and 10 viral particles were tested and it was determined that 5 viral particles of the CHI3L1 shRNA lentivirus successfully knocked down roughly all CHI3L1 expression in astrocytes (**Figure 18**). Scramble shRNA or an FUGW lentivirus were used as controls.

3.2.9 Statistical Analysis

For statistical and bioinformatic analyses, in brief, the normality of the data distribution was routinely determined by a Shapiro-Wilk normality test ($p<0.05$ indicating a non-normal distribution). For the data confirmed to be normally distributed data, Student's t-test for pair-wise comparisons and ANOVA (followed by post-hoc analysis) for 3 groups or more were used. For data that is not normally distributed, non-parametric alternatives, such as Mann-Whitney or Kruskal-Wallis were tested. $p<0.05$ is considered to be statistically significant.

3.3 Results

Astrocytes are the main cell responsible for the expression and secretion of CHI3L1 in the CNS[25, 69, 77]. Therefore, in characterizing the CHI3L1 effect on iN/iA co-cultures, I knocked down CHI3L1 in iAs via a lentiviral shRNA prior to culturing with iNs. Overall, the iN/iA co-cultures showed little overall response to CHI3L1 knockdown or CHI3L1 treatment, whether alone or in conjunction with Aβ (**Figures 19 & 20**). CHI3L1 knockdown or treatment had no effect on AD relevant (APP, p-Tau), apoptotic (caspase3), or astrocytic reactivity (GFAP) proteins as shown via immunoblotting (**Figure 19**). APP, along with anti-inflammatory BDNF and pro-inflammatory C3, was also unchanged at the transcript level (**Figure 20**). There were some transcript-level

changes in the iN/iA co-cultures due to the astrocyte-specific CHI3L1 knockdown. CHI3L1 knockdown reduced the levels of pro-inflammatory IL1β and TNF as well as reduced levels of presynaptic marker SYN1 (**Figure 20**). This may indicate that CHI3L1 can potentially modulate astrocyte inflammatory response or synaptic connectivity with neurons; however further experimentation is required for more conclusive results.

In addition to astrocytes, microglia are responsible for immune and inflammatory responses. While microglia do not express or secrete CHI3L1 to the extent of astrocytes, it is important to determine a potential CHI3L1 effect on microglia and their interactions with neurons. I established an iN/iMG co-culture to test the effects of CHI3L1, independently or in conjunction with Aβ, again on AD-relevant and neuroinflammatory outputs (**Figures 21 & 22**). Similar to what was seen in the iN/iA co-cultures, the levels of AD relevant (APP, p-Tau), apoptotic (caspase3), or microglial reactivity (IBA1) proteins were unchanged, as witnessed via immunoblotting (**Figure 21**). Targets of interest were largely unchanged at the transcript level as well in the iN/iMG co cultures; however, there was a trend in increased pro-inflammatory cytokines with CHI3L1 treatment (C3, IL1β, IL6, TNF) though unsignificant (**Figure 22**). Microglial reactivity marker IBA1 was significantly increased at the mRNA level upon CHI3L1 treatment as was pro-inflammatory cytokine C1qa (**Figure 22**). Based on the iN/iMG co-cultures, CHI3L1 was shown to modulate aspects of microglial reactivity.

To then attempt to identify effects of CHI3L1 on all three cell types together and to better model the neuroinflammatory axis, tri-cultures of iNs/iAs/iMGs were developed. Cells were cultured together and treated with a variety of inflammatory stimuli (CHI3L1, Aβ, and LPS) either independently or in some combination together. Again, previously established AD and neurotoxic features were probed and it was found that treatments did not alter levels of APP, p-tau, or caspase3

in tri-cultures (**Figure 23**). The increased complexity of the culture system likely made it more difficult to capture subtle, yet appreciable changes. For example, the proliferative glial cells versus the post-mitotic neurons may have overwhelmed the culture masking some specific CHI3L1-neuron effects. Further optimization of this tri-culture system as well as pure, individual cultures of iAs and iMGs should be used moving forward to better determine a CHI3L1-glial effect.

3.4 Discussion

I hypothesized that upon the reactivation of astrocytes in neuroinflammation, the secreted Chitinase-3-like protein 1 (CHI3L1) engages a neuronal receptor and triggers a signal transduction cascade in neurons leading to an increase in Aβ production and neurotoxicity. Via RNA Sequencing (RNASeq), immunoblotting, and quantitative PCR, among other assays, I previously found that CHI3L1 induces widespread transcriptomic alterations, CHI3L1 induces apoptosis in neurons, and significantly increases levels of AD-associated features.

I aimed to further characterize the neuronal effect of CHI3L1 while in the presence of additional cells to isolate CHI3L1 effect from other cell type communication and determine potential cell-specific interactions. Therefore, I utilized induced astrocytes (iA) and induced microglia (iMGs) in co-cultures with iNs to determine how CHI3L1 modulates glial cells in the presence of neurons. I knocked down CHI3L1 in iAs via a lentiviral shRNA in the iN/iA co-cultures due to astrocytes being the main expressor and secretor of CHI3L1. CHI3L1 had little to no effect on AD-relevant features in the iN/iA co-cultures, but did display an effect on inflammatory cytokine expression as well as presynaptic marker expression. In iN/iMG co-cultures, CHI3L1 appeared to effect aspects of glial reactivity and inflammation. Namely, CHI3L1 induced an increased trend of interleukins and TNF, and a significant increase in C1qa, of which

are pro-inflammatory cytokines known to reactivate astrocytes. These findings provide support to the idea that CHI3L1 can reactivate microglia to express and secrete pro-inflammatory molecules that in turn reactivate astrocytes, which then secrete more CHI3L1, overall increasing neuroinflammation and resultant neurodegeneration.

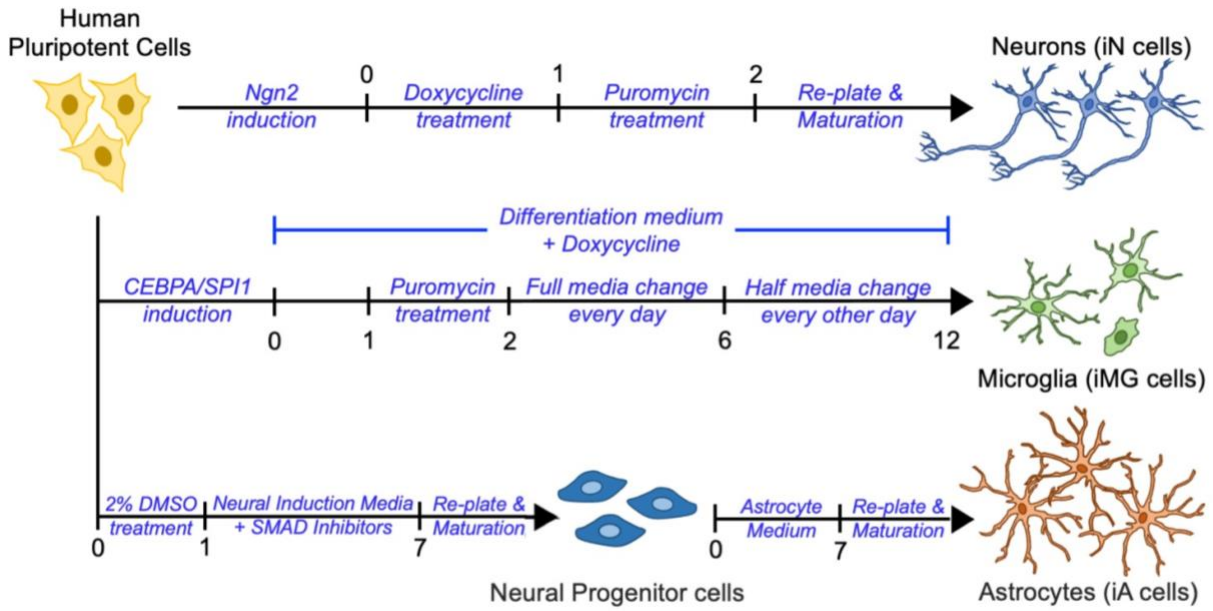


Figure 16. CNS cell induction from human pluripotent stem cells, embryonic stem (ES) and iPS cells. (**Top**) For neurons, on day -1, hPSCs are transfected with a lentivirus expressing Ngn2. On day 0, doxycycline is added to induces expression of Ngn2. On Day 1, puromycin is added to select for transfected cells. On day 2, iNs are replated and allowed to mature. (**Middle**) For microglia, on day -1, hPSCs are transfected with a lentivirus expressing CEBPA/SPI1. On day 0, doxycycline, along with cytokines and signaling molecules, is added to induces expression of CEBPA/SPI1. On Day 1, puromycin is added together with the doxycycline and differentiation medium to select for transfected cells. On day 6 and beyond, half medium changes are performed and iMGs are allowed to mature. (**Bottom**) For astrocytes, on day 0, 2% DMSO is added to PS cells in fresh media. On day 1 through 6, neural induction media with SMAD inhibitors are added each day to generate NPCs. On day 7, NPCs are replated and are subsequently maintained in astrocyte medium to allow for iA maturation.

Table 5. Media composition for iMG induction.

	Reagent	Concentration
Basal Media	NEAA	1X
	N2	1X
D0	βMe	50 μM
	Activin	20 ng/mL
	BMP-4	50 ng/mL
	FGF	50 ng/mL
	Doxycycline	2 μg/mL
D1	βMe	50uM
	VEGF	50 ng/mL
	SCF	50 ng/mL
	FGF	20 ng/mL
	Doxycycline	2 μg/mL
	Puromycin	1 μg/mL
D2	IL34	10 ng/mL
	MCSF	10 ng/mL
	TGFβ	10 ng/mL
	Doxycycline	2 μg/mL
	Puromycin	1 μg/mL
D3	IL34	10 ng/mL
	MCSF	10 ng/mL
	TGFβ	10 ng/mL
	Doxycycline	2 μg/mL
D4/6/8/10/12	IL34	100 ng/mL
	MCSF	20 ng/mL
	TGFβ	20 ng/mL
	GM-CSF	20 ng/mL
	Doxycycline	2 μg/mL

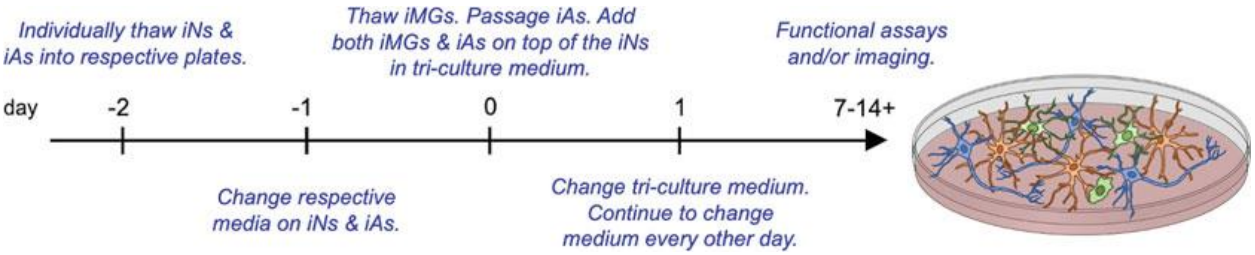


Figure 17. Schematic overview of tri-culture preparation and maintenance. In brief, on day-2, iNs and iAs are thawed and plated individually. The respective media are changed the following day. On day 0, iMGs are thawed, iAs are passaged, and the cells are plated together with the iNs. The media is changed the next day and subsequently every other day [100].

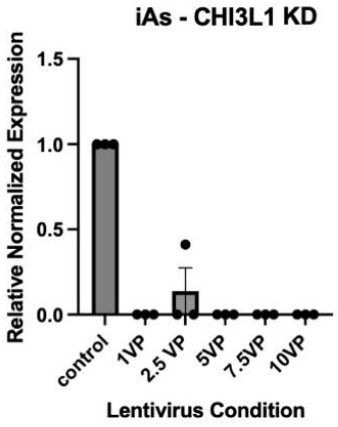


Figure 18. Lentiviral shRNA mediated knockdown of desired genes. Predesigned, commercially available lentiviral shRNAs were used to knockdown CHI3L1 in astrocytes. A titer was performed for each lentivirus to determine the appropriate amount of viral particles needed for adequate gene knockdown. Reduced gene expression was confirmed via qPCR. A knockdown of 75% or greater was considered an efficient knockdown (n = 3).

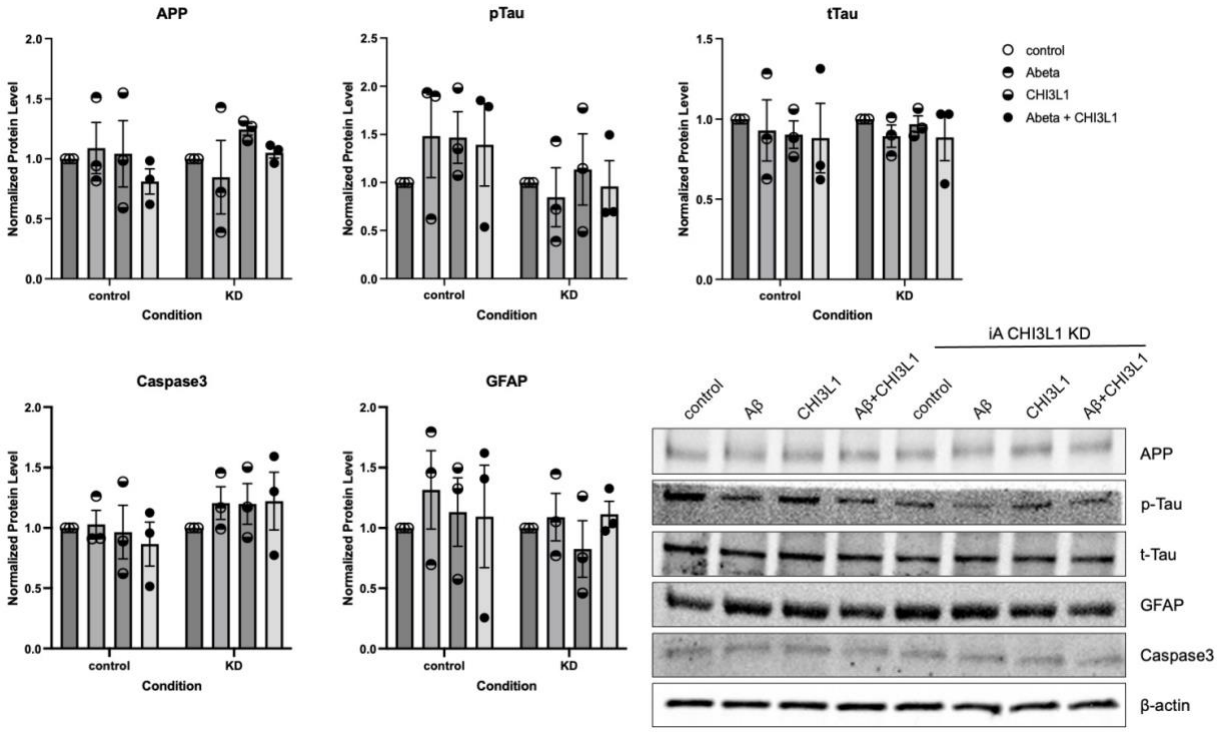


Figure 19. CHI3L1 does not modulate AD, neurotoxic, or glial reactivity at the protein level in iN/iA co-cultures. CHI3L1 was knocked down in iAs via a lentiviral shRNA and protein levels of desired targets were compared to those of control (WT) iAs in co-cultures with iNs via immunoblotting. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary two-way ANOVA with Bonferroni multiple comparisons test.

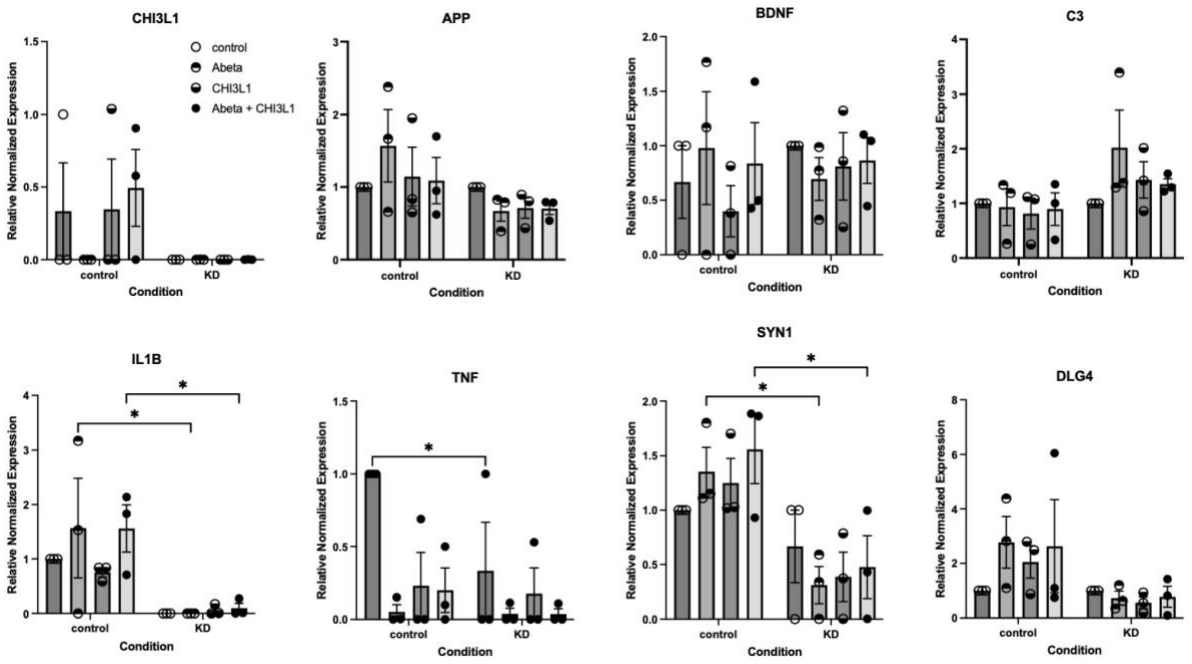


Figure 20. CHI3L1 KD reduces the expression of specific pro-inflammatory and synaptic genes in iN/iA co-cultures. CHI3L1 was knocked down in iAs via a lentiviral shRNA and transcript levels of desired targets were compared to those of control (WT) iAs in co-cultures with iNs via qPCR. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary two-way ANOVA with Bonferroni multiple comparisons test. * = $p < 0.05$.

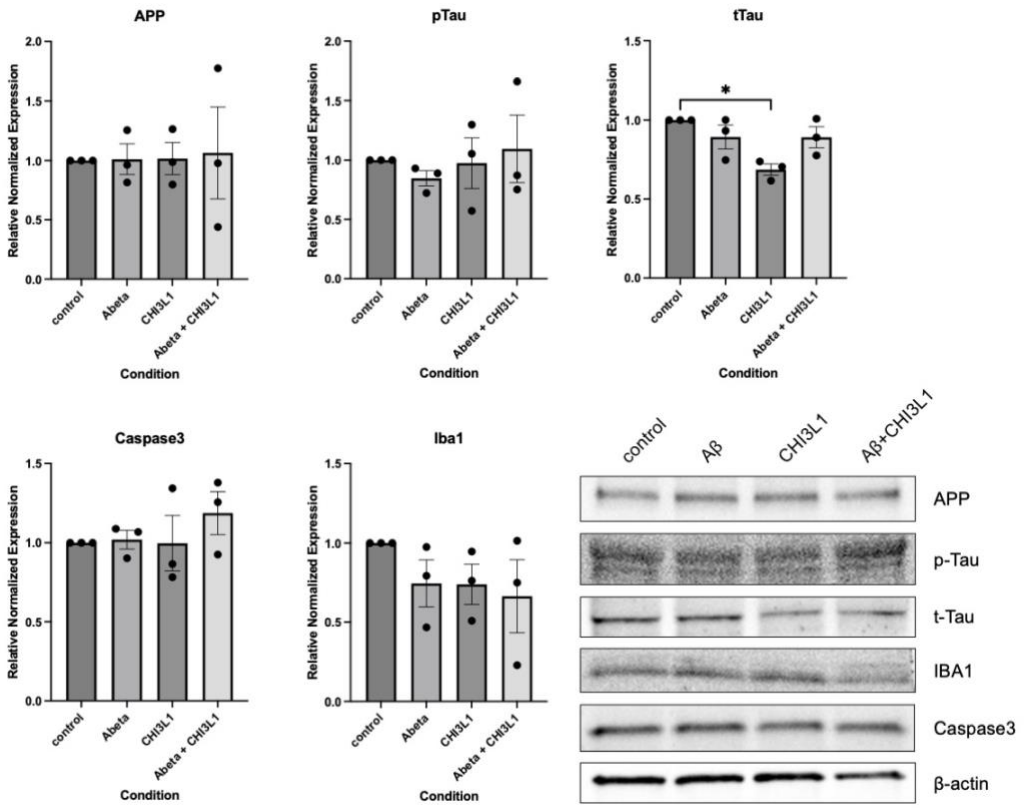


Figure 21. AD-relevant, neurotoxic, and glial reactivity proteins are largely unchanged in iN/iMG co-cultures treated with CHI3L1. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary one-way ANOVA with Bonferroni multiple comparisons test. * = $p < 0.05$.

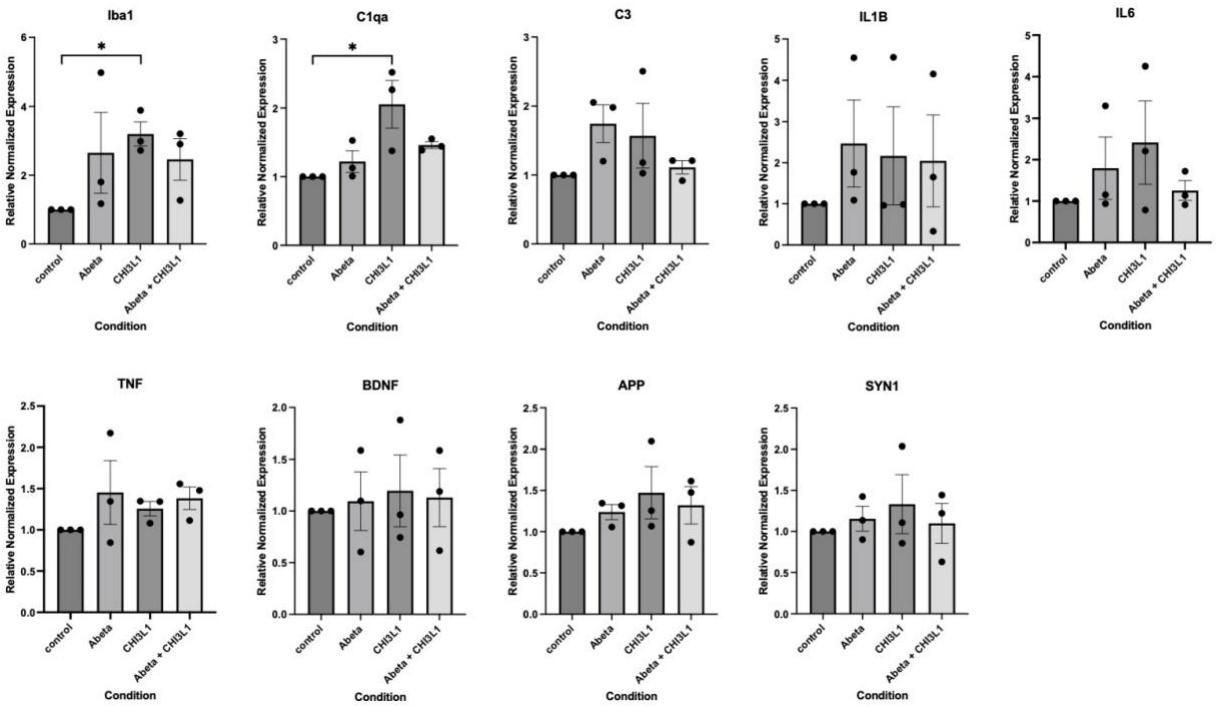


Figure 22. CHI3L1 increases certain pro-inflammatory genes in iN/iMG co-cultures. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary one-way ANOVA with Bonferroni multiple comparisons test. * = $p < 0.05$.

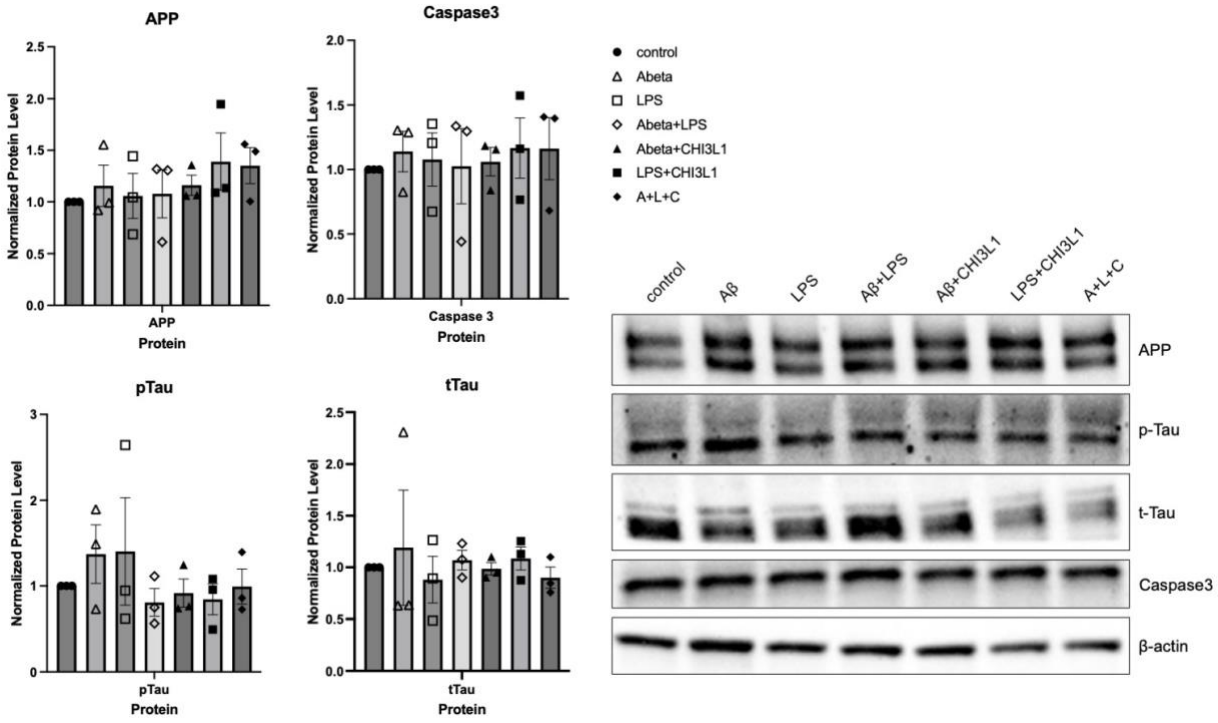


Figure 23. AD and neurotoxic features remain unchanged in iN/iA/iMG tri-cultures upon CHI3L1 or pro-inflammatory stimuli. CHI3L1, whether alone or in combination with Aβ or LPS, does not alter protein levels of APP, tau, or caspase, as shown via immunoblotting. The mean and standard error of the mean are shown (mean ± SEM) (n = 3). Ordinary one-way ANOVA with Bonferroni multiple comparisons test.

Chapter 4: Discussion & Future Directions

This chapter was partially adapted from publication:

Connolly, K., Lehoux, M., O'Rourke, R., Assetta, B., Erdemir, G. A., Elias, J. A., Lee, C. G., Huang, Y. A. (2022). Potential role of chitinase-3-like protein 1 (CHI3L1/YKL-40) in neurodegeneration and Alzheimer's disease. *Alzheimers Dement*.

https://doi.org/10.1002/alz.12612

4.1 Summary

Regarding this project, I hypothesized that upon the reactivation of astrocytes in neuroinflammation, the secreted Chitinase-3-like protein 1 (CHI3L1) engages a neuronal receptor and triggers a signal transduction cascade in neurons that contributes to the inflammatory neurotoxicity and leads to neurodegeneration and relevant AD features.

In order to test this hypothesis, I treated neurons induced (iNs) from pluripotent stem cells with recombinant CHI3L1 and assessed functional outputs via RNA Sequencing (RNASeq), immunoblotting, and quantitative PCR, among other assays. I determined that CHI3L1 induces widespread transcriptomic alterations in human neurons via RNASeq. Specifically, CHI3L1 modulates the expression of certain genes implicated in inflammation, apoptosis, signaling, and AD. Via a caspase imaging assay and western blotting, I confirmed my RNASeq results and found that CHI3L1 induces apoptosis in neurons. Additionally, I confirmed via western blotting that CHI3L1 significantly increases levels of AD-associated features, including amyloid precursor protein (APP) and phospho-tau, in neurons. Via an amyloid beta (Aβ) ELISA, I also found that chronic CHI3L1 treatment significantly increases Aβ42 levels in the induced neurons, further supporting that CHI3L1 increases AD-relevant features. I further characterized these effects in neurons to establish reliable, significant readouts that can be used to assess the effects of candidate receptor knockdowns in the neurons. As such, I knocked down RAGE in iNs via a lentiviral shRNA and saw a reduction in genes and proteins implicated in CHI3L1 signaling and AD features, specifically NFκB, caspase, and APP. This may implicate RAGE as a main CHI3L1 receptor on neurons; however, alternative receptors should be probed in the future to further define other potential CHI3L1 neuronal mechanisms.

Astrocytes are the main cell responsible for the expression and secretion of CHI3L1 in the CNS [25, 69, 77]. The role of the astrocyte-secreted CHI3L1 or the mechanism by which it affects neuroinflammation and neurodegeneration, particularly in the context of AD, is uncharacterized. Therefore, I utilized induced astrocytes (iA) and induced microglia (iMGs) in co-cultures with iNs, or together with iNs in tri-cultures to determine how CHI3L1 modulates glial cells in the presence of neurons. Furthermore, I knocked down CHI3L1 in iAs via a lentiviral shRNA in the iN/iA co-cultures due to astrocytes being the main expressor and secretor of CHI3L1. CHI3L1 had little to no effect on AD-relevant features in the iN/iA co-cultures, but did display an effect on inflammatory cytokine expression as well as presynaptic marker expression. Perhaps, in this context, Aβ alone may not have been sufficient to prime astrocytes and elicit a reactive astrocyte state. Future iN/iA co-cultures should be reactivated with a proinflammatory cytokine cocktail known to elicit astrocyte reactivation as well as increased CHI3L1 expression [6, 73, 93] (**Figure 2B-C**).

In iN/iMG co-cultures, CHI3L1 appeared to effect aspects of glial reactivity and inflammation. Namely, CHI3L1 induced an increased trend of interleukins and TNF, and a significant increase in C1qa, of which are pro-inflammatory cytokines known to reactivate astrocytes [6, 73, 93]. These findings provide support to the idea that CHI3L1 can reactivate microglia to express and secrete pro-inflammatory molecules that in turn reactivate astrocytes, which then secrete more CHI3L1, overall increasing neuroinflammation and resultant neurodegeneration (**Figure 24**). However, when testing the CHI3L1 effect on the microglia-astrocyte-neuron neuroinflammatory axis via tri-cultures, no significant changes were witnessed. This is likely due to the proliferative nature of the iAs, which may have overwhelmed the culture, masking the potential subtle CHI3L1 effects [100]. Further optimization of tri-cultures as well as

individual analysis of pure iA and pure iMG cultures are needed to fully appreciate the effect of CHI3L1 on the neuroinflammatory axis. Overall, these data, taken together from pure neuron and co-/tri-cultures with glial cells, suggest that CHI3L1 is not just an AD biomarker, but may be a causal agent.

4.2 Conclusion

4.2.1 Limitations & Knowledge Gaps

Understanding the mechanisms of CHI3L1 in AD represents a knowledge void to be filled with high priority. A delineation of the key regulatory mechanism for neuroinflammation by CHI3L1 is likely to offer an important scientific framework by which new strategies to anti-neuroinflammation therapy for AD can be developed. Thus, the overall focus in this field should be to i) characterize the signaling mechanism(s) whereby CHI3L1 governs neuroinflammation, both physiologically and pathologically, and ii) to explore the translational potential of CHI3L1 signaling in remedying the pro-inflammatory status in AD brains. In peripheral tissues, it has been well documented that CHI3L1 is a master regulator for a wide range of injury and repair responses, including the innate immunity pathway that resembles the neuroinflammation process initiated by microglia and coordinated by astrocytes [15, 16, 18-22]. Given this similarity, the signaling function of CHI3L1 in microglia and astrocytes may mirror that of the peripheral cells. In fact, all of the known CHI3L1 receptors identified in the periphery are expressed, though some lowly expressed, in major CNS cell types, further supporting the idea that CHI3L1 may signal through similar known pathways in the brain (**Figure 3**) [77]. These receptors expressed in the brain are capable of engaging the ligand of CHI3L1 and may convey neuroinflammatory signals in individual brain cell types.

Based on this evidence from the periphery and new, recent evidence in the brain, our central hypothesis is that CHI3L1 functions as a neuroinflammation signaling molecule to trigger the receptor system on brain cells and regulate inflammatory responses in a cell type-specific manner. We propose that CHI3L1 plays an essential role in the intricate cross-talks between microglia and astrocytes in the highly dynamic process of neuroinflammation (**Figure 24**).

The major limitation of our conclusion here lies in the complexity of intercellular interactions among brain cell types and the potentially diverse composition of CHI3L1 receptors in distinct inflammatory processes and cellular states. This notion has been complicating the interpretation of findings uncovered in some peripheral diseases, in which the biological function of CHI3L1 can be bi-phasic (protective vs provocative) depending on the levels of expression as well as activation status of CHI3L1 along the transition from acute inflammation to chronic repair. For example, the effects of complete loss of CHI3L1 by knockout can be different from the findings based on partial inhibition of CHI3L1 by an inhibitor compound or neutralizing antibody. In this perspective, future studies need to be directed to determine the importance of homeostatic as well as stimulated regulation of CHI3L1 in various *in vivo* and *in vitro* experimental settings as well.

4.4.2 Future Directions

As such, recommended forward action would be to i) identify the functional outputs of CHI3L1 expression in neuroinflammation, ii) determine the effects of CHI3L1 manipulations in pro-inflammatory context relevant to AD, and iii) evaluate the *in vivo* significance of CHI3L1 in neuroinflammation and AD manifestations. First, in single (or mixed cultures) of microglia, astrocytes, and neurons, cell type-specific CHI3L1 manipulations and the known CHI3L1 receptor

manipulations should be conducted. This will allow researchers to dissect out cell type-specific contributions to neuroinflammation and determine if CHI3L1 acts upon the known receptors and signaling pathways in the brain. Alternative receptors should also be probed via an unbiased approach to determine any potential new CHI3L1 receptors outside of the ones already characterized in the periphery. Glial transcriptomics and essential functions altered by the CHI3L1 and receptor modulations should be profiled in order to further characterize the CHI3L1 CNS biology.

Secondly, similar experiments in which CHI3L1 expression is modulated should be conducted in cells with relevant AD mutations, including those of amyloid precursor protein, presenilin, and apolipoprotein E. The effects of inhibition or overexpression of CHI3L1 signaling on AD relevant mutation-associated neuroinflammation should be examined. In both cases, the *in vitro* single or mixed cell cultures can be garnered from animal models, or, alternatively, by cells induced from stem cells. Induced CNS cell models derived from stem cells allow for the use of human cells, which may be able to better recapitulate AD compared to the animal counterparts. Bypassing the limitation of animal models, namely the intrinsic human nature of AD and its associated genetics, data gathered from experiments with induced neurons, astrocytes, and microglia will allow the characterization of the effects of CHI3L1 manipulations in the context of AD [90, 96, 97, 101]. These *in vitro* stem cell models, however, clearly have technical limitations. First, this reductionist approach fails to factor in the functions from diverse cell types and signals present in physiological brain microenvironments, because the iPSC-derived neural cells mostly display features that represent only a particular cell subtype or cellular state. Secondly, the lack of interactions among multiple brain cell types – required to reconstitute a niche for proper neural development – actually retards the maturation and impairs the overall functionality in the cultures.

This is of particular importance in the context of aging and neurodegeneration [90]. To address this, the emerging 3D culture models that simulate a higher level of cell type and tissue diversity and thus better recapitulate the *in vivo* CNS milieu can be considered in conjunction with established animal models to stringently test the role of CHI3L1 in AD.

Third, the *in vivo* significance of CHI3L1 in neuroinflammation and AD manifestations should be examined by the use of Chi3l1-floxed mice for astrocyte- or microglia-specific conditional CHI3L1 knockout via stereotaxic viral injections as well as established mouse models of AD. Using cell-specific CHI3L1 knockouts in established models of AD can allow for the determination of the CHI3L1 necessity in AD pathogenesis. Once CHI3L1 CNS biology and its role in AD pathogenesis is determined, further tests can be conducted in the context of other neurodegenerative diseases to identify if such neuroinflammatory control can underlie neurodegenerative disease. This information can potentially be used in targeted therapeutics to combat AD and its progression or in other medical advances related to this disease. For example, there have been recent studies in the pulmonary system in which an anti-CHI3L1 antibody was used to neutralize secreted CHI3L1 and decreased lung inflammation [103]. Additionally, as discussed above, a CHI3L1 inhibitor has been used in the context of AD and was shown to reduce amyloid burden and improve impaired memory and cognitive function witnessed in the AD mouse models [81, 82]. Perhaps this antibody or inhibitor could be used to neutralize or inhibit CHI3L1, respectively, in the CNS in humans in clinical investigation settings. This anti-CHI3L1 antibody, CHI3L1 inhibitor, and the development of additional therapeutics may be critical in slowing neurodegeneration and AD progression.

Future research will not only greatly advance our knowledge on the CHI3L1 contributions to AD pathogenesis but also present a proof of principle for forthcoming studies focusing on

neuroinflammation. It is our expectation that we can conclude the necessity of CHI3L1 in astrocytes for neuroinflammation and AD development and uncover an *in vivo* CHI3L1 mechanism that can be targeted to lower disease manifestations. Characterizing this signaling mechanism in depth as well as identifying the functional outputs of CHI3L1 expression in neuroinflammation will ultimately provide new opportunities for the development of much-needed intervention and prevention strategies for AD patients and risk carriers and potentially other neurodegenerative conditions.

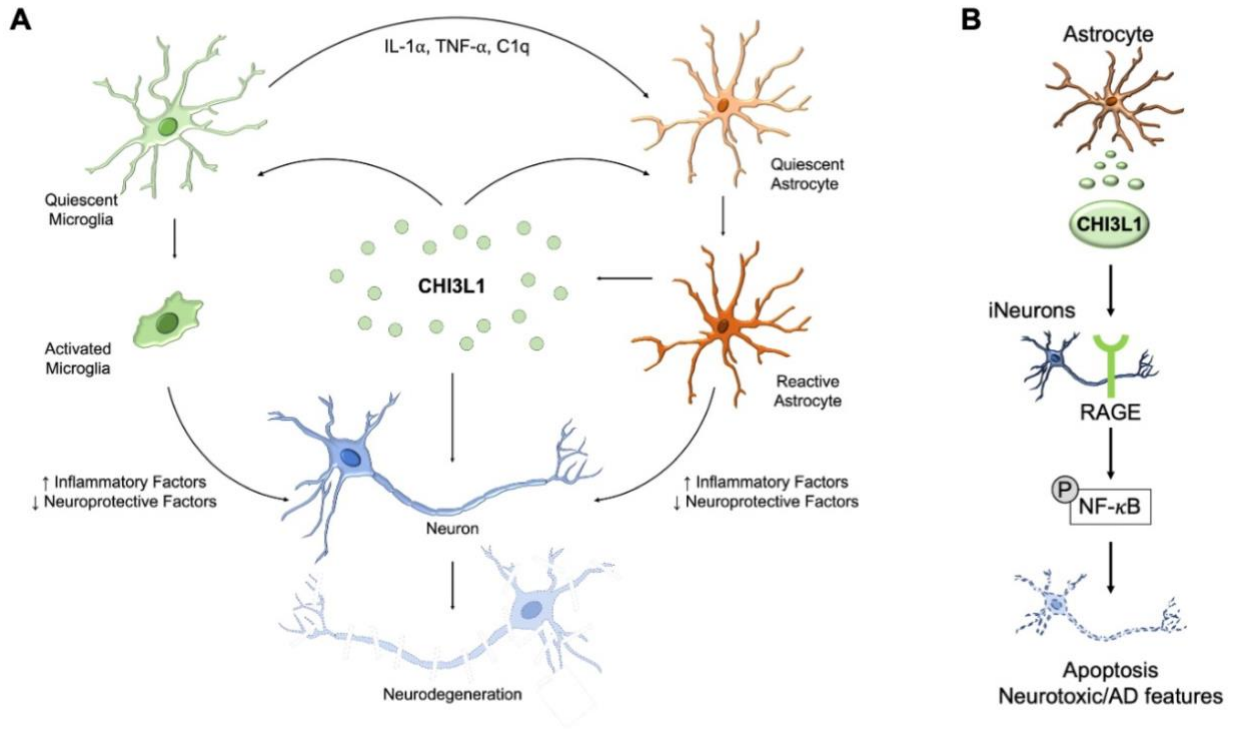


Figure 24. Proposed role and potential mechanism of chitinase-3-like protein 1 (CHI3L1) in neurodegeneration and Alzheimer's disease (AD). Microglia-induced CHI3L1 secretion from astrocytes augments inflammation and promotes neural damages. CHI3L1 can engage the neuronal receptor RAGE, potentially eliciting NFκB signaling, ultimately leading to increased AD and neurotoxic features.

References

1. *2020 Alzheimer's disease facts and figures.* Alzheimers Dement, 2020.
2. Jansen, I.E., et al., *Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk.* Nat Genet, 2019. **51**(3): p. 404-413.
3. Mathys, H., et al., *Single-cell transcriptomic analysis of Alzheimer's disease.* Nature, 2019. **570**(7761): p. 332-337.
4. Muszynski, P., et al., *YKL-40 as a Potential Biomarker and a Possible Target in Therapeutic Strategies of Alzheimer's Disease.* Curr Neuropharmacol, 2017. **15**(6): p. 906-917.
5. Liddelow, S.A. and B.A. Barres, *Reactive Astrocytes: Production, Function, and Therapeutic Potential.* Immunity, 2017. **46**(6): p. 957-967.
6. Liddelow, S.A., et al., *Neurotoxic reactive astrocytes are induced by activated microglia.* Nature, 2017. **541**(7638): p. 481-487.
7. Mattson, M.P. and T.V. Arumugam, *Hallmarks of Brain Aging: Adaptive and Pathological Modification by Metabolic States.* Cell Metab, 2018. **27**(6): p. 1176-1199.
8. Bishop, N.A., T. Lu, and B.A. Yankner, *Neural mechanisms of ageing and cognitive decline.* Nature, 2010. **464**(7288): p. 529-35.
9. Frick, L.R., K. Williams, and C. Pittenger, *Microglial dysregulation in psychiatric disease.* Clin Dev Immunol, 2013. **2013**: p. 608654.
10. Han, X., et al., *Astrocyte Senescence and Alzheimer's Disease: A Review.* Front Aging Neurosci, 2020. **12**: p. 148.
11. Singhal, G. and B.T. Baune, *Microglia: An Interface between the Loss of Neuroplasticity and Depression.* Front Cell Neurosci, 2017. **11**: p. 270.

12. Hampel, H., et al., *Alzheimer's disease biomarker-guided diagnostic workflow using the added value of six combined cerebrospinal fluid candidates: Abeta1-42, total-tau, phosphorylated-tau, NFL, neurogranin, and YKL-40.* Alzheimers Dement, 2018. **14**(4): p. 492-501.

13. Janelidze, S., et al., *CSF biomarkers of neuroinflammation and cerebrovascular dysfunction in early Alzheimer disease.* Neurology, 2018. **91**(9): p. e867-e877.

14. Rehli, M., S.W. Krause, and R. Andreesen, *Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation.* Genomics, 1997. **43**(2): p. 221-5.

15. He, C.H., et al., *Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor alpha2.* Cell Rep, 2013. **4**(4): p. 830-41.

16. Sutherland, T.E., *Chitinase-like proteins as regulators of innate immunity and tissue repair: helpful lessons for asthma?* Biochem Soc Trans, 2018. **46**(1): p. 141-151.

17. He, C.H., et al., *Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor α2.* Cell Rep, 2013. **4**(4): p. 830-41.

18. Kim, D.H., et al., *Regulation of chitinase-3-like-1 in T cell elicits Th1 and cytotoxic responses to inhibit lung metastasis.* Nat Commun, 2018. **9**(1): p. 503.

19. Ober, C., et al., *Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function.* N Engl J Med, 2008. **358**(16): p. 1682-91.

20. Lee, C.G., et al., *Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury.* Annu Rev Physiol, 2011. **73**: p. 479-501.

21. Kwak, E.J., et al., *Chitinase 3-like 1 drives allergic skin inflammation via Th2 immunity and M2 macrophage activation.* Clin Exp Allergy, 2019. **49**(11): p. 1464-1474.

22. Zhou, Y., et al., *Chitinase 3-like 1 suppresses injury and promotes fibroproliferative responses in Mammalian lung fibrosis.* Sci Transl Med, 2014. **6**(240): p. 240ra76.

23. Lomiguen, C., et al., *Possible Role of Chitin-Like Proteins in the Etiology of Alzheimer's Disease.* J Alzheimers Dis, 2018. **66**(2): p. 439-444.

24. Sanfilippo, C., L. Malaguarnera, and M. Di Rosa, *Chitinase expression in Alzheimer's disease and non-demented brains regions.* J Neurol Sci, 2016. **369**: p. 242-249.

25. Wurm, J., et al., *Astrogliosis Releases Pro-Oncogenic Chitinase 3-Like 1 Causing MAPK Signaling in Glioblastoma.* Cancers (Basel), 2019. **11**(10).

26. Alcolea, D., et al., *Relationship between cortical thickness and cerebrospinal fluid YKL-40 in predementia stages of Alzheimer's disease.* Neurobiol Aging, 2015. **36**(6): p. 2018-23.

27. Dickerson, B.C., et al., *The cortical signature of Alzheimer's disease: regionally specific cortical thinning relates to symptom severity in very mild to mild AD dementia and is detectable in asymptomatic amyloid-positive individuals.* Cereb Cortex, 2009. **19**(3): p. 497-510.

28. Zhang, H., et al., *Cerebrospinal fluid phosphorylated tau, visinin-like protein-1, and chitinase-3-like protein 1 in mild cognitive impairment and Alzheimer's disease.* Transl Neurodegener, 2018. **7**: p. 23.

29. Moreno-Rodriguez, M., et al., *Frontal cortex chitinase and pentraxin neuroinflammatory alterations during the progression of Alzheimer's disease.* J Neuroinflammation, 2020. **17**(1): p. 58.

30. Swanson, A., A.A. Willette, and I. Alzheimer's Disease Neuroimaging, *Neuronal Pentraxin 2 predicts medial temporal atrophy and memory decline across the Alzheimer's disease spectrum.* Brain Behav Immun, 2016. **58**: p. 201-208.

31. Xiao, M.F., et al., *NPTX2 and cognitive dysfunction in Alzheimer's Disease.* Elife, 2017. **6**.

32. Dai, Q.H. and D.K. Gong, *Association of the Polymorphisms and Plasma Level of CHI3L1 with Alzheimer's Disease in the Chinese Han Population: A Case-Control Study.* Neuropsychobiology, 2019. **77**(1): p. 29-37.

33. Hall, S., et al., *Longitudinal Measurements of Cerebrospinal Fluid Biomarkers in Parkinson's Disease.* Mov Disord, 2016. **31**(6): p. 898-905.

34. Quintana, E., et al., *Cognitive impairment in early stages of multiple sclerosis is associated with high cerebrospinal fluid levels of chitinase 3-like 1 and neurofilament light chain.* Eur J Neurol, 2018. **25**(9): p. 1189-1191.

35. Vu, L., et al., *Cross-sectional and longitudinal measures of chitinase proteins in amyotrophic lateral sclerosis and expression of CHI3L1 in activated astrocytes.* J Neurol Neurosurg Psychiatry, 2020. **91**(4): p. 350-358.

36. Woollacott, I.O.C., et al., *Cerebrospinal Fluid YKL-40 and Chitotriosidase Levels in Frontotemporal Dementia Vary by Clinical, Genetic and Pathological Subtype.* Dement Geriatr Cogn Disord, 2020. **49**(1): p. 56-76.

37. Hojsgaard Chow, H., et al., *Progressive multiple sclerosis, cognitive function, and quality of life.* Brain Behav, 2018. **8**(2): p. e00875.

38. Sanfilippo, C., et al., *CHI3L1 and CHI3L2 overexpression in motor cortex and spinal cord of sALS patients.* Mol Cell Neurosci, 2017. **85**: p. 162-169.

39. Llorens, F., et al., *YKL-40 in the brain and cerebrospinal fluid of neurodegenerative dementias.* Mol Neurodegener, 2017. **12**(1): p. 83.

40. Nordenbaek, C., et al., *YKL-40, a matrix protein of specific granules in neutrophils, is elevated in serum of patients with community-acquired pneumonia requiring hospitalization.* J Infect Dis, 1999. **180**(5): p. 1722-6.

41. Di Rosa, M., et al., *CHI3L1 nuclear localization in monocyte derived dendritic cells.* Immunobiology, 2016. **221**(2): p. 347-56.

42. Connor, J.R., et al., *Human cartilage glycoprotein 39 (HC gp-39) mRNA expression in adult and fetal chondrocytes, osteoblasts and osteocytes by in-situ hybridization.* Osteoarthritis Cartilage, 2000. **8**(2): p. 87-95.

43. De Ceuninck, F., et al., *YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes.* Biochem Biophys Res Commun, 2001. **285**(4): p. 926-31.

44. Hakala, B.E., C. White, and A.D. Recklies, *Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family.* J Biol Chem, 1993. **268**(34): p. 25803-10.

45. Hamilton, G. and B. Rath, *Circulating tumor cell interactions with macrophages: implications for biology and treatment.* Transl Lung Cancer Res, 2017. **6**(4): p. 418-430.

46. Zhao, T., et al., *Chitinase-3 like-protein-1 function and its role in diseases.* Signal Transduct Target Ther, 2020. **5**(1): p. 201.

47. Lee, C.M., et al., *IL-13Ralpha2 uses TMEM219 in chitinase 3-like-1-induced signalling and effector responses.* Nat Commun, 2016. **7**: p. 12752.

48. Geng, B., et al., *Chitinase 3-like 1-CD44 interaction promotes metastasis and epithelial-to-mesenchymal transition through beta-catenin/Erk/Akt signaling in gastric cancer.* J Exp Clin Cancer Res, 2018. **37**(1): p. 208.

49. Kzhyshkowska, J., et al., *Role of chitinase-like proteins in cancer.* Biol Chem, 2016. **397**(3): p. 231-47.

50. Low, D., et al., *Chitinase 3-like 1 induces survival and proliferation of intestinal epithelial cells during chronic inflammation and colitis-associated cancer by regulating S100A9.* Oncotarget, 2015. **6**(34): p. 36535-50.

51. Tang, H., et al., *YKL-40 induces IL-8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-kappaB pathways, causing bronchial smooth muscle proliferation and migration.* J Immunol, 2013. **190**(1): p. 438-46.

52. Tran, H.T., et al., *Chitinase 3-like 1 synergistically activates IL6-mediated STAT3 phosphorylation in intestinal epithelial cells in murine models of infectious colitis.* Inflamm Bowel Dis, 2014. **20**(5): p. 835-46.

53. Bara, I., et al., *Role of YKL-40 in bronchial smooth muscle remodeling in asthma.* Am J Respir Crit Care Med, 2012. **185**(7): p. 715-22.

54. Kawada, M., et al., *Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer.* Oncogene, 2012. **31**(26): p. 3111-23.

55. Recklies, A.D., et al., *Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes.* J Biol Chem, 2005. **280**(50): p. 41213-21.

56. Recklies, A.D., C. White, and H. Ling, *The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and*

activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem J, 2002. **365**(Pt 1): p. 119-26.

57. Lee, C.G., et al., *Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis.* J Exp Med, 2009. **206**(5): p. 1149-66.

58. Jefri, M., et al., *YKL-40 regulated epithelial-mesenchymal transition and migration/invasion enhancement in non-small cell lung cancer.* BMC Cancer, 2015. **15**: p. 590.

59. Zhou, Y., et al., *Chitinase 3-like-1 and its receptors in Hermansky-Pudlak syndrome-associated lung disease.* J Clin Invest, 2015. **125**(8): p. 3178-92.

60. Eurich, K., et al., *Potential role of chitinase 3-like-1 in inflammation-associated carcinogenic changes of epithelial cells.* World J Gastroenterol, 2009. **15**(42): p. 5249-59.

61. Libreros, S., et al., *Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor metastasis in a breast cancer model.* Int J Cancer, 2012. **131**(2): p. 377-86.

62. Di Rosa, M., et al., *Evaluation of CHI3L-1 and CHIT-1 expression in differentiated and polarized macrophages.* Inflammation, 2013. **36**(2): p. 482-92.

63. Rehli, M., et al., *Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation.* J Biol Chem, 2003. **278**(45): p. 44058-67.

64. Dela Cruz, C.S., et al., *Chitinase 3-like-1 promotes Streptococcus pneumoniae killing and augments host tolerance to lung antibacterial responses.* Cell Host Microbe, 2012. **12**(1): p. 34-46.

65. Kang, M.J., et al., *Role of Chitinase 3-Like-1 in Interleukin-18-Induced Pulmonary Type 1, Type 2, and Type 17 Inflammation; Alveolar Destruction; and Airway Fibrosis in the Murine Lung.* Am J Respir Cell Mol Biol, 2015. **53**(6): p. 863-71.

66. Romagnani, S., *T-cell subsets (Th1 versus Th2).* Ann Allergy Asthma Immunol, 2000. **85**(1): p. 9-18; quiz 18, 21.

67. Tang, Y. and W. Le, *Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases.* Mol Neurobiol, 2016. **53**(2): p. 1181-1194.

68. Escartin, C., et al., *Reactive astrocyte nomenclature, definitions, and future directions.* Nat Neurosci, 2021. **24**(3): p. 312-325.

69. Clarke, L.E., et al., *Normal aging induces A1-like astrocyte reactivity.* Proc Natl Acad Sci U S A, 2018. **115**(8): p. E1896-E1905.

70. Bush, T.G., et al., *Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice.* Neuron, 1999. **23**(2): p. 297-308.

71. Zador, Z., et al., *Role of aquaporin-4 in cerebral edema and stroke.* Handb Exp Pharmacol, 2009(190): p. 159-70.

72. Zamanian, J.L., et al., *Genomic analysis of reactive astrogliosis.* J Neurosci, 2012. **32**(18): p. 6391-410.

73. Barbar, L., et al., *CD49f Is a Novel Marker of Functional and Reactive Human iPSC-Derived Astrocytes.* Neuron, 2020. **107**(3): p. 436-453 e12.

74. Yun, S.P., et al., *Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease.* Nat Med, 2018. **24**(7): p. 931-938.

75. Shi, Y., et al., *ApoE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy.* Nature, 2017. **549**(7673): p. 523-527.

76. Bennett, M.L., et al., *New tools for studying microglia in the mouse and human CNS.* Proc Natl Acad Sci U S A, 2016. **113**(12): p. E1738-46.

77. Zhang, Y., et al., *Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse.* Neuron, 2016. **89**(1): p. 37-53.

78. Lananna, B.V., et al., *Chi3l1/YKL-40 is controlled by the astrocyte circadian clock and regulates neuroinflammation and Alzheimer's disease pathogenesis.* Sci Transl Med, 2020. **12**(574).

79. Bhardwaj, R., et al., *RelB/p50 complexes regulate cytokine-induced YKL-40 expression.* J Immunol, 2015. **194**(6): p. 2862-70.

80. Bonneh-Barkay, D., et al., *Astrocyte and macrophage regulation of YKL-40 expression and cellular response in neuroinflammation.* Brain Pathol, 2012. **22**(4): p. 530-46.

81. Choi, J.Y., et al., *K284-6111 prevents the amyloid beta-induced neuroinflammation and impairment of recognition memory through inhibition of NF-kappaB-mediated CHI3L1 expression.* J Neuroinflammation, 2018. **15**(1): p. 224.

82. Ham, H.J., et al., *K284-6111 alleviates memory impairment and neuroinflammation in Tg2576 mice by inhibition of Chitinase-3-like 1 regulating ERK-dependent PTX3 pathway.* J Neuroinflammation, 2020. **17**(1): p. 350.

83. Im, J.H., et al., *Deletion of Chitinase-3-like 1 accelerates stroke development through enhancement of Neuroinflammation by STAT6-dependent M2 microglial inactivation in Chitinase-3-like 1 knockout mice.* Exp Neurol, 2020. **323**: p. 113082.

84. Matute-Blanch, C., et al., *Chitinase 3-like 1 is neurotoxic in primary cultured neurons.* Sci Rep, 2020. **10**(1): p. 7118.

85. Jiang, L., et al., *Astrocytes induce proliferation of oligodendrocyte progenitor cells via connexin 47-mediated activation of Chi3l1 expression.* Eur Rev Med Pharmacol Sci, 2019. **23**(7): p. 3012-3020.

86. Stadelmann, C., et al., *Myelin in the Central Nervous System: Structure, Function, and Pathology.* Physiol Rev, 2019. **99**(3): p. 1381-1431.

87. Nasrabady, S.E., et al., *White matter changes in Alzheimer's disease: a focus on myelin and oligodendrocytes.* Acta Neuropathol Commun, 2018. **6**(1): p. 22.

88. Starossom, S.C., et al., *Chi3l3 induces oligodendrogenesis in an experimental model of autoimmune neuroinflammation.* Nat Commun, 2019. **10**(1): p. 217.

89. Li, L., et al., *GFAP Mutations in Astrocytes Impair Oligodendrocyte Progenitor Proliferation and Myelination in an hiPSC Model of Alexander Disease.* Cell Stem Cell, 2018. **23**(2): p. 239-251 e6.

90. Essayan-Perez, S., et al., *Modeling Alzheimer's disease with human iPS cells: advancements, lessons, and applications.* Neurobiol Dis, 2019. **130**: p. 104503.

91. Muszynski, P., et al., *The Relationship between Markers of Inflammation and Degeneration in the Central Nervous System and the Blood-Brain Barrier Impairment in Alzheimer's Disease.* J Alzheimers Dis, 2017. **59**(3): p. 903-912.

92. da Fonseca, A.C., et al., *The impact of microglial activation on blood-brain barrier in brain diseases.* Front Cell Neurosci, 2014. **8**: p. 362.

93. Connolly, K., et al., *Potential role of chitinase-3-like protein 1 (CHI3L1/YKL-40) in neurodegeneration and Alzheimer's disease.* Alzheimers Dement, 2022.

94. Guttenplan, K.A., et al., *Knockout of reactive astrocyte activating factors slows disease progression in an ALS mouse model.* Nat Commun, 2020. **11**(1): p. 3753.

95. Hasel, P., et al., *Neuroinflammatory astrocyte subtypes in the mouse brain.* Nat Neurosci, 2021. **24**(10): p. 1475-1487.

96. Assetta, B., et al., *Generation of Human Neurons and Oligodendrocytes from Pluripotent Stem Cells for Modeling Neuron-Oligodendrocyte Interactions.* J Vis Exp, 2020(165).

97. Huang, Y.A., et al., *ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Abeta Secretion.* Cell, 2017. **168**(3): p. 427-441 e21.

98. Goshi, N., et al., *A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation.* J Neuroinflammation, 2020. **17**(1): p. 155.

99. Guttikonda, S.R., et al., *Fully defined human pluripotent stem cell-derived microglia and tri-culture system model C3 production in Alzheimer's disease.* Nat Neurosci, 2021. **24**(3): p. 343-354.

100. Connolly, K., et al., *Modeling Cellular Crosstalk of Neuroinflammation Axis by Tri-cultures of iPSC-Derived Human Microglia, Astrocytes, and Neurons.* Methods Mol Biol, 2023. **2683**: p. 79-87.

101. Tcw, J., et al., *An Efficient Platform for Astrocyte Differentiation from Human Induced Pluripotent Stem Cells.* Stem Cell Reports, 2017. **9**(2): p. 600-614.

102. Chen, S.W., et al., *Efficient conversion of human induced pluripotent stem cells into microglia by defined transcription factors.* Stem Cell Reports, 2021. **16**(5): p. 1363-1380.

103. Kim, M.J., et al., *Chitinase 3-like 1 protein plays a critical role in respiratory syncytial virus-induced airway inflammation.* Allergy, 2019. **74**(4): p. 685-697.