

Engineering a 3D cortical culture model to investigate spatiotemporal dynamics of microglia-neuron interactions in Alzheimer's disease

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B.S. Bioengineering, 2016, University of California San Diego

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Providence, Rhode Island
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This dissertation by Sophie C. Brown is accepted in its present form by the School of Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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To Jensen

For making each day a good day.

Curriculum Vitae

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EDUCATION

Brown University, Providence, RI – **Master of Science, May 2020**, Biomedical Engineering

University of California San Diego, La Jolla, CA – **Bachelor of Science, March 2016**, Bioengineering

RESEARCH EXPERIENCE

PhD Candidate—Biomedical Engineering—Borton Neural Engineering Lab

May 2020-Current

Brown University, Providence, RI

- Developed a 3D primary culture system to investigate glia-neuron interactions under neuroinflammatory disease conditions. Designed and carried out functional imaging experiments to probe microglia-dependent modulation of the synaptic connectivity.
- Led an independent research project on the development and validation of a three-dimensional xenograft cell culture model to study human iPSC-derived glial cells and decipher glia-neuron interactions in health and Alzheimer's disease.
- Conducted in-depth studies to decipher human iPSC-derived microglia function and neuroinflammatory mechanisms in a 3D microtissue culture system, utilizing advanced techniques in cell biology and molecular analysis.
- Designed and carried out interdisciplinary experimentation including animal handling, 3D primary rodent cell culture, molecular biology assay development, iPSC differentiation and

engraftment, confocal microscopy, and advanced computational analysis methods for analysis of morphofunctional datasets.

- Fostered collaboration between interdisciplinary teams of the Borton Neuroengineering Lab and the Huang Laboratory in Translational Neuroscience which resulted in enhanced experimental design, data analysis, and interpretation of results.
- Presented findings at several conferences, one first-author methods paper, with two additional first-author papers in preparation, contributing to the development of novel tools, understanding of neuroinflammatory processes, and discovery potential therapeutic targets in neurodegenerative diseases.

PhD Student—Biomedical Engineering—Borton Neural Engineering Lab

Sept 2020-May 2022

Brown University, Providence, RI

- Member of the In vitro Brain-Machine Interface (IV-BMI) project which aims to study innate neuroinflammatory mechanisms at the device-tissue interface in an *in vitro* three-dimensional primary cortical culture system.
- Aided in characterization of structural and functional neural dynamics around implanted microwires via live fluorescent probes and investigation of a library of potential therapies to mitigate neuroinflammation at the device-tissue interface.
- Responsible for design and implementation of *in vitro* assays for investigation of novel microelectrode coatings and fabricated devices.
- Responsible for leading experimental design, training undergraduate and masters students on research techniques and fostering collaboration between the Borton Neuroengineering Lab and the Capadona Lab for Bio-Inspired Materials and Neural Interfacing at Case Western University.

Graduate Student—Biomedical Engineering—Borton Neural Engineering Lab

Sept 2018-May 2020

Brown University, Providence, RI

- Successful development of live cell imaging platform for real-time visualization of three-dimensional primary neural microtissues. Optimized methods of simultaneous labeling of three target neural cell types using adeno-associated viruses.
- Real-time monitoring of key cell types involved in neuroinflammation and foreign body reaction to implanted electrodes. Facilitated construction of a microscopy pipeline for live cell imaging including spectral deconvolution and longitudinal analysis on multi-labeled samples.

Research Associate II—Molecular Biology—Tanabe Research Laboratory

Jan 2016-Jun 2018

San Diego, CA

- Integral member of protein development and analytics group. Responsible for the production of all in-house protein and antibody expression systems.
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- Coordinated with multiple project team leads and senior scientists to ensure efficient and rapid large-scale protein production for all investigative projects.

Project Team Leader—Bidirectional Neural Interface—UCSD Dept of Bioengineering

Sept 2014-Jun 2015

University of California San Diego, San Diego, CA

- Executed design of a bidirectional interface capable of establishing and maintaining high levels of synchrony in a hybrid closed-loop system of two neural networks.
- Responsible for design and implementation of the biological neural network parameters, including the manipulation and use of a microelectrode array and primary neuron cultures.
- Coordinated weekly team meetings and effectively communicated between team members, faculty advisors, and laboratory personnel regarding all aspects of the project.

- Paper accepted for presentation at the IEEE Engineering in Medicine and Biology Society Annual International Conference in 2015.

Research Assistant—Dr. Massoud Khraiche Group—UCSD Jacobs Retina Center

Nov 2014-Mar 2015

University of California San Diego, San Diego, CA

- Assisted with technical laboratory procedures and analysis within a time-sensitive project aimed at developing a high-resolution retinal prosthesis.
- Contributing team member supporting research and optimization of primary cortical neuron growth on microelectrode arrays, maintaining cell lines, and animal surgeries.

Research Assistant—Bioscience Division—Los Alamos National Labs

Summer 2012

Los Alamos, NM

- Collaborated on development and design of immobilized bead assay utilized in current plant enzyme and protein analysis research.
- Worked under a multidisciplinary team of world-class researchers, implemented basic biology lab skills including data entry, plasmid preps, and PCR.

CONFERENCE PRESENTATIONS AND PUBLICATIONS

- **Brown, S.**, Dawson-Elli, A., PhD, Drexler, A., Steele, K., Huang, Y.W., MD, PhD, & Borton, D. A., PhD. A 3D cortical microtissue model uncovers microglia-driven synaptic remodeling and disruption in Alzheimer's disease (submitted 2025)
- **Brown, S.**, Atherton, E. & Borton, D. A. A Three-Dimensional Primary Cortical Culture System Compatible with Transgenic Disease Models, Virally Mediated Fluorescence, and Live Microscopy. *Methods Mol Biol* 2683, 153-167. (2023)
- Atherton, E., Hu, Y., **Brown, S.**, Papiez, E., Ling, V., Colvin, V., Borton, D.A., A 3D in vitro model of the device-tissue interface: functional and structural symptoms of innate neuroinflammation are mitigated by ceria nanoparticles. *Journal of Neural Eng.* (2022) 19, 036004.

- Atherton, E., **Brown, S.**, Papiez, E., Restrepo, M.I., Borton, D.A., PhD; Lipopolysaccharide-induced neuroinflammation disrupts functional connectivity and community structure in primary cortical microtissues. *Scientific Reports* (2021) 11, 22303
- **Brown, S.**, Connolly, K., Drexler, A., Conover, I., Peningroth, M., Yang, X., PhD, Huang, Y.A., PhD, Borton, D. A., PhD (2024) "Three-dimensional primary cortical microtissues for studying glia-mediated modulation of the synaptic environment in health and disease." Presented at Glia in Health and Disease Meeting at Cold Spring Harbor, Cold Spring Harbor, NY. (*Poster Presentation*)
- **Brown, S.**, Connolly, K., Drexler, A., Huang, Y.A., PhD, Borton, D. A., PhD (2023) "A three-dimensional xenograft culture system to study human iPSC-derived microglia in health and Alzheimer's disease." Presented at Society for Neuroscience, Washington, D.C. (*Poster Presentation*)
- **Brown, S.**, Connolly, K., Drexler, A., Huang, Y.A., PhD, Borton, D. A., PhD (2023) "Real-time capture of human iPSC-derived microglia dynamics in a three-dimensional xenograft culture system: towards deciphering microglial roles in Alzheimer's disease pathogenesis". Presented at New York Stem Cell Foundation Conference, New York, NY(*Poster Presentation*)
- **Brown, S.**, Assetta, B., Lehoux, M., Drexler, A., Yang, X., PhD, Huang, Y.A., PhD, Borton, D. A., PhD (2022) "A hybrid three-dimensional xenoculture system to study human iPSC-derived microglia." Presented at Development & 3D Modeling of the Human Brain Meeting at Cold Spring Harbor, Cold Spring Harbor, NY. (*Poster Presentation*)
- **Brown, S.**, Assetta, B., Lehoux, M., Omar, J., Huang, Y.A., PhD, Borton, D. A., PhD (2022) "A hybrid three-dimensional xenoculture system to study human iPSC-derived microglia: toward deciphering the role of microglia in Alzheimer's disease." Presented at the Annual Society for Neuroscience Meeting, Chicago, IL. (*Poster Presentation*)
- **Brown, S.**, Atherton, E., A., Borton, D. A., PhD; (2022) "An engineered 3D in vitro model of glial-mediated neuroinflammation: Toward understanding multicellular mechanisms underlying neurodegenerative diseases." Presented at the Annual Winter Conference on Brain Research, Snowmass, CO (*Poster Presentation*)
- **Brown, S.**, Atherton, E., A., Borton, D. A., PhD; (2022) "Toward Understanding Glial-Mediated Neuroinflammatory Responses to Implanted Devices." Presented at the National VA Research Week, Providence, RI (*Poster Presentation*)

- **Brown, S.**, Atherton, E., Allawala, A., Borton, D. A., PhD (2019) “Live cell imaging of 3D cortical microtissue via AAV-induced fluorescence.” Presented at the Annual Society for Neuroscience Meeting, Chicago, IL. (*Poster Presentation*)
- Chou Z., Lim J., **Brown, S.**, Keller, M., Bugbee, J., Broccard, F., PhD, Khraiche, ML, MD, PhD, Silva, GA, PhD, Cauwenberghs, G., PhD (2015). “Bidirectional neural interface: Closed-loop feedback control for hybrid neural systems.” Presented at the Annual Engineering in Medicine and Biology Society 37th Annual International Conference (*Paper Presentation*)

TECHNICAL SKILLS

Computer: Matlab, Python, R, Jupyter, Fusion360, Femap, LabView, ImageJ, Prism, JMP, Adobe Illustrator, and Microsoft Word/Excel/PowerPoint

Equipment: microscopy (Confocal, bright field), multielectrode array recording system, circuit design and analysis, oscilloscope use, custom 3D printing and design, all basic molecular biology laboratory equipment

Biological: adeno-associated viral transductions, immunohistochemistry, rodent handling, PCR, western blot, ELISA, mammalian cell transfections, bacterial transformations, expression vector design, gibson assemblies, and DNA plasmid preparation

AWARDS, LEADERSHIP AND COMMUNITY SERVICE

- Carney Institute Graduate Award in Brain Science (2023-2024)
- Lead instructor for Introduction to Eng. as part of high school outreach program with local girls school (2024)
- Mentored four Master’s students, visiting undergraduate student, and Brown University Undergraduates through UTRA program (2021, 2022, 2023, 2024)

- Advisory Board Member, Design and Eng. Career and Technical Edu. Program, Lincoln High School, RI (2021-2023)
- Taught summer-long courses to participating high-school and middle-school students: Biomedical Eng. and Device Design; Introduction to Eng. and Design; and Eng. Biomedical Systems (2019)
- Executive Board Member, Chi Omega Sorority, University of San Diego Chapter (2012-2015)
- Volunteer at Make A Wish Foundation: Annually fundraised over \$10,000 for San Diego Chapter as well as rewarding interaction with Make A Wish Children (2011-2015)
- Los Alamos National Laboratory Foundation Silver Scholarship Recipient (2011)
- Senior Class Salutatorian (2011)

Preface

If there is one thing that my PhD experience has solidified, it is that resilience is forged in the fire of curiosity and challenge. This dissertation represents not only my research but also the perseverance, independence, and passion that have carried me through the highs and lows of scientific discovery. My fascination with the brain—a profound universe of its own filled with constellations of cells and molecular gravitational currents that are carefully crafted in time and space to define our thoughts, emotions, movements, and memories—has been the driving force behind my scientific journey.

Joining a lab with an important history of engineering technologies to better understand the nervous system and improve human lives, I found myself among brilliant researchers with diverse backgrounds. Yet for many years, I have independently pursued my fascination with the brain's cellular landscape, particularly the ever-enigmatic glial cells. I became intent on understanding how these cells shaped neural function and pioneering new ways to study their behavior in neurodegenerative diseases.

My journey began at Brown University as a master's student, eager to engage in cutting-edge neuroengineering research. The intricate mechanisms of the nervous system drew me to the academic frontier where basic neuroscience and engineered innovation intersect. My work began with the development of novel *in vitro* models to visualize cellular behaviors during neuroinflammatory responses, particularly those arising from implanted electrodes used in an array of neurotechnology. This work demonstrated the feasibility of using genetically encoded fluorescent reporters in a microtissue model system—a tool widely utilized across labs at Brown but, until my efforts, never before explored for live fluorescent imaging of multicellular microtissues

via viral expression of cell-specific reporters. It was uncharted territory, and I embraced it wholeheartedly.

The progress and sense of fulfillment found during my master's research encouraged me to continue my academic journey in pursuit of a doctoral degree. Fueled by the curiosities cultivated in my prior research, I was intent on investigating glial cells and their roles in neuroinflammatory mechanisms of disease. As I learned how these shape-shifting cells can sense their surrounding environment and respond with cascades of functional behaviors to sculpt the neural microenvironment, I became painfully aware of our inability to study such processes in the brain in real time. Motivated by these limitations, I centered my doctoral work on the development of *in vitro* tools that enable us to visualize these dynamic processes on a laboratory benchtop. This thesis offers a narrative on my desire and efforts to innovate and details the surprising discoveries made along the way.

Yet, the process of discovery also felt much like opening Pandora's box: each breakthrough revealed new challenges that demanded my independent and scientific fortitude to continually adapt, grow, and see experiments through. There were long nights alone in the lab, troubleshooting, problem-solving, and pushing experiments forward when no roadmap existed. It was thrilling, frustrating, and deeply personal. As enthusiasm and interest in my work grew, as well as an expansion to include broader scientific input and disciplines, the long hours in the lab felt deeply rewarding. This body of work emerged largely from my own determination and belief in the expansive potential of my process. It is a testament to my relentless drive to decipher the mysteries of glia and the dynamic roles they play in shaping the brain's microenvironment.

As I reflect on this journey, I am distinctly proud of the work I have completed. I am also proud of the person I have become. The years working towards my PhD have proven to be more than an academic pursuit. It has been a transformative experience that has instilled in me a sense

of confidence, resilience, fulfillment, and hope. The path ahead is still full of unknowns, but if my years at Brown have taught me anything, it is that with curiosity, persistence, and a belief in the importance of the questions we ask, there is no limit to our potential for discovery.

Acknowledgements

When I began this chapter of my life, I don't think I fully realized how much these PhD years would change me. I think every person who sets out to pursue a doctoral degree is warned of the toll it can take—and yet we pursue it anyways, determined to forge our own path, set on being unflinching against the inevitable challenges ahead. Despite this optimism, I can say with certainty that I would not have survived my graduate studies without the support of friends, family, and those that are both. Every misstep, every triumph, every frustration, and every success became a shared experience.

Growing up in a large family I found that my life has felt so wonderfully “full” because I was able to share it with those around me. Having people in your corner—to be witness to your life, whether it's sharing what happened to you at work that day or meeting up for a celebratory beer after you submitted that paper—instills a sense of connection and purpose that gives joy and meaning to life. At least, it does for me. That connection and purpose were found and nurtured in many ways, with many people, along this journey.

I have been fortunate to work with an incredible team of mentors: professors, my thesis committee, fellow lab members, my advisor Dr. David Borton, and Dr. Alvin Huang. These relationships, founded in respect and collaboration, helped guide my path and taught me to find the joy in science. Dr. Borton and Dr. Huang have been instrumental to my growth as a scientist and as a person. They have challenged, encouraged, consoled, and pushed me to become the strong and resilient person I am today. I am proud of the work we have accomplished and how we have learned alongside one another over these years to develop an interesting and expansive research collaboration. I will forever be appreciative of their patience and belief in me. In my time at Brown, I have been especially grateful to be surrounded by strong female voices—voices like Dr. Frost, Dr. Hoffman-Kim, Dr. Mayoral, Dr. Provenza, Dr. Allawala, and Dr.

Atherton—that taught me to believe our work is important, our challenges are real, and that it's okay to take up space in this world.

I have been inspired by the enthusiasm and creativity of my first mentor, Elaina, who taught me not only how to make a microtissue but how to reframe the challenges in science to find new and exciting paths forward. I learned so much from Dr. Calvert, Dr. Parker, Dr. Darie, Dr. Black, Dr. Celinskis, Dr. Powell, and Dr. Xing, whose kindness and patience supported me as I fought to keep up as they developed new and exciting neurotechnology over the years. My time spent mentoring many undergraduate and master's students in the lab helped me grow as a mentor myself, and I will always be grateful to them for helping me realize my own aptitude for teaching.

Beyond the friendships forged on campus, I have been lucky to have people like my dear friend Aaron in my corner. He has become my family here in Providence, and he has been there every step of the way. Whether meeting me for a coffee on campus to debrief my latest colossal experiment mess-up or taking me to explore little beach towns of Rhode Island, he has been such a good friend. I have also been lucky enough to know—and be fueled by—the magic of strong female friendships. I owe so much of my growth over the past few years to my two dear friends Avanthi and Griffin. They have been my shoulders to cry on, the voices that helped to keep my head above water when I was feeling overwhelmed, alone, or lost in this experience. Their honesty, their ability to help me find the humor in life, and their unwavering belief in me will forever continue to change my life for the better.

Above all, I know that I would not be who I am today, or where I am today, without my family. I know not everyone is lucky enough to find friendship in their siblings, but I am deeply grateful to know that my five best friends are the ones who have been by my side for the last 31 years. Hallie, Olin, Jensen, Soren, and Greta—you define the very best parts of me and I share this accomplishment with you. You have been my current, steadily guiding my boat along this journey and I thank you from the bottom of my heart for simply being the incredible people that

you are. And finally, to my parents, Ann and Merritt—you have been my gravity throughout this experience. Your belief in me has anchored and guided me through every thrilling and challenging chapter of this journey. You have taught me so much—kindness, humility, resilience, compassion—and most importantly, that a steadfast belief in ourselves can take us anywhere we hope to go. For every lesson, every sacrifice, and every moment of encouragement, I am endlessly grateful. This achievement is as much mine as it is yours.

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Chapter 1

1.1 Introduction

1.1.1 The role of microglia in the healthy and diseased brain

Like any organ system or tissue in the human body, the central nervous system (CNS) exists as a complex milieu of cells surrounded by a supportive extracellular matrix and dense network of vasculature. However, unlike many other organ systems, the function and behavior of cells within the CNS ultimately control the function and behavior of nearly every other organ system in the body. Without the diverse array of neurons and glia resident to the CNS, the brain could not operate as the central processing unit that regulates function of cardiovascular, endocrine, respiratory, reproductive, immune, gastrointestinal, and lymphatic systems. The very field of neuroscience was formed with the discovery of the neuron, and for decades our conceptualization of how the CNS exerts control of the human body has progressed from a neuron-centric approach. Neurons, although magnificently complex in their subtype specialization and vital to most every aspect of bodily function, are now widely known to interact with surrounding glial cells in spatially and temporally defined patterns to regulate function of the CNS. Disruption of these mechanisms can lead to devastating neurological disorders and have dramatic impact on human health. In fact, a recent report found that neurological disorders affected nearly 3 billion people, or 43% of the worldwide population, in 2021 (GBD 2021 Nervous System Disorders Collaborators 2024) where 37 conditions affecting the nervous system were identified as having the greatest impact on global disease burden. Improving our understanding of how age, injury, genetics, and the environment can alter the cellular landscape of the CNS to cause disease is paramount to this public health crisis.

Astrocytes, microglia, and oligodendrocytes are glial cells in the CNS, each with unique morphological and functional states that play essential roles in brain function. However, unlike astrocytes and oligodendrocytes that originate from the neuroectoderm, microglia are mesoderm-derived immune cells that migrate into the CNS during embryonic development (Schuurmans, Mordelt, and de Witte 2025; Mendes and Majewska 2021; Bennett et al. 2018). Like other immune cells such as circulating monocytes or peripheral macrophages, microglia are the brain-resident macrophage cells responsible for conducting immune surveillance, maintaining tissue homeostasis, and responding to injury (Li and Barres 2018). However, unlike other tissue-resident macrophages that exist in the periphery, microglia are specialized for sensing and responding to changes unique to the neural microenvironment. Such processes include pruning synapses and potentiating synaptic plasticity, maintaining integrity of the blood-brain barrier, phagocytosing neuronal debris and protein aggregates, modulating perineuronal net densities, regulating myelin loss, and orchestrating an innate neuroinflammatory response that is separate from systemic immune mechanisms outside the CNS (Prinz, Jung, and Priller 2019; Paolicelli et al. 2022; Y. Wu et al. 2015; Vilalta and Brown 2018). Although microglia are dynamically involved in several mechanisms critical to healthy brain function, under pathological conditions such as neurodegenerative diseases, they can adopt pro-inflammatory phenotypes that contribute to neuronal damage. For these reasons, microglia have emerged as an important target for understanding and modulating CNS disorders (Hickman et al. 2018; Valiukas et al. 2025; Subhramanyam et al. 2019).

Neuroinflammation in the brain can be initiated in response to a variety of endogenous and exogenous stimuli including invading pathogens or infection, injury, and even abnormal protein aggregation. It is characterized by glial cell activation, the release of cytokines and inflammatory molecules, increased blood-brain barrier permeability, and recruitment of peripheral immune cells into the brain. As the tissue-resident immune cells of the brain, microglia are central

to these processes (Heneka, Kummer, and Latz 2014). With their motile processes, microglia survey the parenchyma and can respond to perturbations via their pattern recognition receptors (PRRs). Microglia activation is initiated via ligand-receptor binding of the PRRs to pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs) that can arise in the surrounding neural environment (Kigerl et al. 2014). PRRs represent a large group of receptors, each associated with PAMP/DAMP detection in various compartments of the intracellular and extracellular space. Toll-like receptors (TLRs) are a family of PRRs that reside on the cell surface to detect extracellular PAMPs whereas other PRRs such as the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), can detect cytoplasmic intracellular PAMPs (Takeda and Akira 2005; Inohara et al. 2005; Akira and Takeda 2004).

Historically, microglia were thought to acquire one of two phenotypes during activation: a proinflammatory M1 or an anti-inflammatory M2. However, the current state of the field now suggests that there is a wide range of microglial activation states or phenotypes (Sousa et al. 2018; Keren-Shaul et al. 2017; Dyne et al. 2022; Islam et al. 2022; L. Wang et al. 2021; Sun et al. 2023; Paolicelli et al. 2022). As they acquire different phenotypes depending on the surrounding environmental cues or PAMP/DAMP activation, microglia demonstrate dynamic functional plasticity that correlates with complex transcriptional programming. Beyond the diverse functional capabilities, upon activation microglia will also exhibit a morphological transformation from highly ramified with small soma and numerous branches to a less ramified more amoeboid shape with a larger soma and fewer branches (Dyne et al. 2022; Jung et al. 2023; Fernández-Arjona et al. 2017; Madore et al. 2013). These distinct morphological changes are widely utilized as a biomarker to quantify microglial activation across experimental platforms as both cell shape and motility represent a fundamental aspect of the cell identity and can be highly predictive of function. The morphofunctional state is a combined representation of an acquired morphological

response that corresponds to function. Capturing and quantifying changes in microglia morphology is essential to deciphering their functional response in the environment.

1.1.2 Microglia in neural circuit development and maturation

The nearly 86 billion neurons in the adult human brain are interconnected by approximately 100 trillion synapses that together form the dense circuitry with which the human brain is wired (Azevedo et al. 2009; Pakkenberg et al 2003). It is from these intricate electrical pathways that the very identity of who we are as people—the way we learn, feel, adapt, and remember—is defined. Remarkably, the very structure of these synaptic connections and their varying strengths are significantly shaped by glial cells in a dynamic and spatiotemporally regulated manner. In early brain development, neural circuits form and refine through a process involving synaptogenesis (formation of new synapses), synaptic pruning (elimination of unnecessary or excess synapses), and myelination (insulation of axons). These processes are widely shaped by neuronal activity elicited by receiving and processing information from our surrounding environment. In the 1970s the work of Peter Richard Huttenlocher showed that large spikes in synaptogenesis occurred postnatally and interestingly was followed by substantial synaptic loss during early childhood (Huttenlocher 1979). The dynamic shift in the number of synapses in the brain correlated with onsets of specific developmental milestones such as learning to walk and speak. This seminal work showed that neural circuitry undergoes significant remodeling during early development. However, it wasn't until the early 2000's that it became clear that glial cells—specifically microglia and astrocytes—were responsible for carrying out this structural remodeling by pruning away synapses during periods of learning (Stevens et al. 2007; Bernardinelli, Muller, and Nikonenko 2014; Chung et al. 2013; D. P. Schafer, Lehrman, and Stevens 2013; Paolicelli et al. 2011).

The molecular drivers regulating synaptic removal by astrocytes and microglia constitute a field still in its infancy. However, several key studies have identified components of the complement system—part of the innate immune system involved in the clearance of pathogens and damaged cells—namely C1q, C3, and CR3, as well as the (fractalkine) Cx3CL1-Cx3Cr1 axis, as possible regulatory pathways (Paolicelli et al. 2011; Stephan, Barres, and Stevens 2012; Shi et al. 2017; Gunner et al. 2019). The complement pathway involves the tagging of synapses for removal via increased expression of neuronal C1q followed by increased expression of C3, which localizes to the synapse and subsequently binds to microglial CR3, triggering microglia engulfment of the synapse. In particular, evidence of complement-mediated removal of synapses by microglia was shown to be essential for the normal formation of circuitry in the developing visual thalamus of mice with C3, C1q or CR3-deficiency leading to sustained synaptic-pruning deficits (D. P. Schafer et al. 2012). The CX3CL1-CX3CR1 axis is dependent upon neuronal upregulation of the transmembrane glycoprotein Cx3CL1 which, in its soluble state, acts as a chemotactic cytokine inducing microglia migration via binding to the microglial CX3CR1 receptor. Precise mechanisms of how CX3CL1-CX3CR1 signaling then leads to the removal of synapses by microglia is still incompletely understood. While some studies have shown that binding of CX3CL1 to microglia CX3CR1 is required to maintain microglia quiescence (Cardona et al. 2006; Biber et al. 2007; Bolós et al. 2017), little has been discussed as to how quiescence or dampening of inflammatory mechanisms in microglia would induce synaptic engulfment in developing neural circuitry. Additionally, evidence of microglia-engulfment of synapses in thalamocortical circuits following manipulation of sensory input was shown to be dependent on CX3CL1-CX3CR1 signaling (Gunner et al. 2019) but whether or not this molecular pathway is involved in normal circuit development and across other brain regions remains unclear.

Although *in vivo* models enable the study of microglia-neuron interactions in a physiologically environment, they are severely limited by a lack of precise control of experimental conditions and incompatibility of high-resolution imaging techniques. In particular, imaging

submicron synaptic morphologies with high spatial resolution in an intact brain environment of a living animal is inhibited by diffraction limits of classical optical microscopy techniques (~200nm; (Conchello, Kim, and Hansen 1994) and minimal imaging depths due to light scattering in brain tissue (Favre-Bulle et al. 2015). Additionally, capturing the dynamic nature of microglia-mediated synaptic pruning in real time requires intensive control of genetic reporters for fluorescent live imaging and is often performed at lower spatial resolution due to slow imaging speeds of nanoscale super-resolution microscopy such as stimulated emission depletion (STED) (Urban et al. 2011; Lakadamyali et al. 2012; Tønnesen and Nägerl 2013). Alternatively, *in vitro* models allow for more controlled experimental manipulation and are widely compatible with high resolution microscopy platforms. However, *in vitro* systems, and in particular 2D *in vitro* models lack a 3D microenvironment and the endogenous microglia-neuron or microglia-synapse signaling and physical interactions that exist in the brain. To overcome this, several techniques have been developed to establish 3D cultures of the neural microenvironment (Irons et al. 2008; Cullen et al. 2007; Murphy et al. 2017; Dingle et al. 2015; Zhuang et al. 2018). Despite these advances, several challenges remain in fully replicating the complex spatiotemporal cues that regulate microglia-neuron interactions in the brain.

1.1.3 Three-dimensional neural culture models

Improving our understanding of how glial cells influence the neural microenvironment requires development of advanced cellular models that accurately reflect the heterogeneous nature of the CNS from both a structural and functional perspective. Replicating cortical cell diversity and recapitulating dynamic glial-neuron interactions *in vitro* has proven a major challenge to the field. Perhaps the most pronounced efforts to replicate the cellular architecture of the human brain *in vitro* are exemplified by a growing transition from 2D to 3D culture systems. Recent advances in 3D neural culture systems—including generation of blood-brain barrier (BBB) or

region-specific brain organoids from human induced pluripotent stem cells (iPSCs) and primary or iPSC-derived co-cultures—have demonstrated physiological cell-cell signaling, morphological maturation, proliferation, and gene expression patterns that more closely resemble physiological conditions compared to 2D culture (Campisi et al. 2021; Liu et al. 2022; Watson et al. 2017; Papamichail et al. 2025; Chiaradia and Lancaster 2020; Sloan et al. 2018). These advances in 3D neural culturing have enabled a new generation of research in brain development and disease and opened doors to major therapeutic discoveries.

Despite such progress, several technical barriers remain in the 3D culture systems. In the case of investigating dynamic glial-neuron interactions, most iPSC-derived organoid protocols are insufficient as they often lack cells of non-neuronal or non-ectoderm origin, such as mesodermal-derived vascular and microglia cells. Although there are some studies that describe the incorporation of microglia into iPSC-derived neural organoids (Zhang et al. 2023; Ormel et al. 2018; Popova et al. 2021) these protocols often have high variability, limited microglia integration and proliferation, require complex and delicate timing of separate microglia differentiation in 2D culture prior to integration into organoids, and are not highly reproducible. Additionally, most 3D cell culture platforms require an exogenous matrix to mimic extracellular matrix (ECM) support, and in the case of stem cell organoid models, take 250-300 days to reach postnatal stages thus making it difficult to study cellular behaviors and mechanisms at varying temporal scales (Gordon et al. 2021).

Another significant challenge in modeling physiological glial-neuron interactions *in vitro* arises in the context of studying complex disease states, particularly in neurodegenerative disorders like Alzheimer's disease. While 3D *in vitro* models, such as iPSC-derived cortical organoids and tri-culture systems, provide a more physiologically relevant environment than traditional 2D cultures, they often struggle to fully recapitulate key aspects of Alzheimer's disease pathology. A major limitation is the incomplete representation of amyloid-beta (A β) plaque

deposition and tau pathology, which are hallmarks of the disease. Although incorporation of genetic mutations associated with familial AD to induce A β aggregation has improved some iPSC-modeling (Raja et al. 2016; Gonzalez et al. 2018; Chiaradia and Lancaster 2020; Park et al. 2018; Karmirian et al. 2023), the plaques often fail to form in a manner that mimics *in vivo* progression. Similarly, tau hyperphosphorylation and neurofibrillary tangle formation remain inconsistent, limiting their utility in studying tauopathies (Hossain, Kim, and Chae 2024). But beyond the canonical amyloid and tau pathologies, microglial dysfunction in AD—now widely characterized as a critical component of disease progression—is difficult to model accurately due to the immature state of microglia or entire lack thereof in most organoid systems. Overcoming these limitations will require new approaches that not only support microglia maturation and inflammatory signaling but promote temporal regulation of pathological processes such as amyloid and tau accumulation.

1.1.4 Alzheimer's disease pathology and microglia-mediated synaptic dysfunction

Alzheimer's disease is a progressive neurodegenerative disease that accounts for 60-80% of the 55 million dementia cases worldwide. In 2023, Alzheimer's disease was ranked as the seventh leading cause of death in the United States and has been estimated that nearly one in three senior citizens dies with Alzheimer's disease or a related dementia. Despite many decades of research dedicated to improving our understanding of this deleterious disease, the underlying pathogenesis and ultimate etiology of Alzheimer's disease remains unknown. Currently, there are seven FDA-approved therapies to treat symptoms associated with Alzheimer's disease that are differentially prescribed depending on diagnosis of disease severity. Six out of the seven symptom-treating therapies are cholinesterase or glutamate inhibitors which combat the breakdown of the neurotransmitter acetylcholine and the excessive binding of glutamate to N-methyl-D-aspartate (NMDA) receptors respectively. Additionally, there is 1 FDA-approved anti-

psychosis therapy prescribed to ameliorate agitation in AD patients by modulating neurotransmitter activity through partial agonism at dopamine D2 receptors, antagonism at serotonin 5-hydroxytryptamine receptor 2A (5-HT2A) receptors, and partial agonism at serotonin 5-HT1A receptors. Beyond these symptom-treating medications the FDA has also approved the use of 2 disease modifying immunotherapies, which target abnormal amyloid protein aggregation. The two monoclonal antibodies, Donanemab and Lecanemab target two forms of amyloid-beta protein that are thought to be more neurotoxic: pyroglutamate-modified amyloid beta (N3pG) and soluble A β protofibrils.

These disease-modifying therapies target only one aspect of AD pathology—the abnormal buildup of amyloid-beta protein—however, AD is characterized by other pathologies besides A β aggregation including the formation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, neuroinflammation, reactive gliosis, neurovascular dysfunction, as well as neuronal and synaptic loss. The spatiotemporal scales at which each of these pathological features arise and possibly interact to spur disease onset and catalyze disease progression is still not fully understood. One of the common theories regarding AD pathogenesis and progression is that of the amyloid cascade (Karran, Mercken, and De Strooper 2011; Hardy and Selkoe 2002). This hypothesis suggests amyloid protein aggregation in the brain parenchyma results from the improper cleavage of amyloid precursor protein (APP) and subsequently initiates a cascade of downstream neurotoxic events. Amyloid beta plaque accumulation can trigger microglial activation that can lead to chronic neuroinflammation and oxidative stress (Wang et al. 2023; Hansen, Hanson, and Sheng 2018; Gallagher et al. 2012; Lian et al. 2016; Keren-Shaul et al. 2017). Although, there have been conflicting findings on the neuroprotective versus neurotoxic roles of microglia in the amyloid cascade (Merlo et al. 2020; Tsay et al. 2013; Richter et al. 2020; Mancuso et al. 2024; Mathys et al. 2019; Salter and Stevens 2017). Within the complex research landscape that has emerged in the effort to probe the roles of microglia in Alzheimer's disease, there are recent reports which describe a biphasic response of microglia in the amyloid cascade

(Baligács et al. 2024; He et al. 2025). Here, researchers show that microglia operate both upstream and downstream of amyloid plaque formation, with homeostatic microglia playing a deleterious role in initiating plaque formation and compaction before shifting to engage in plaque clearance. Building upon this, researchers used mouse models of prodromal AD to ascertain that in early disease states, microglia exhibit a protective phenotype by phagocytosing A β aggregates, clearing cellular debris, and releasing anti-inflammatory cytokines to maintain homeostasis (Zhao et al. 2017; Fruhwürth, Zetterberg, and Paludan 2024). However, as the disease progresses, chronic exposure to A β plaques and tau pathology drives microglia into a prolonged activated state, leading to excessive secretion of pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6 (De Strooper and Karran 2016). This sustained inflammatory response contributes to neuronal toxicity, synaptic loss, and blood-brain barrier dysfunction (Ransohoff 2016; Vilalta and Brown 2018; Hickman et al. 2018; Sun et al. 2023). Additionally, genetic studies have identified risk-associated microglial genes, such as *APOE*, *TREM2* and *CD33*, which modulate microglial function and are implicated in AD susceptibility (Villegas-Llerena et al. 2016). Understanding how microglia transition from a protective to a neurotoxic state, and how those states interact with other disease pathologies is crucial for developing therapeutic strategies aimed at modulating their response in AD.

Synaptic dysfunction is an early and central feature of AD and is reported to be the strongest correlate of cognitive decline in AD (Dejanovic et al. 2018; Tzioras, McGeachan, et al. 2023; Tzioras, Daniels, et al. 2023; Taddei et al. 2023; Deture and Dickson 2019; Mathys et al. 2023; M. Wu et al. 2021). However, exactly how and why synapses become vulnerable and eventually lost is still unclear. Evidence of abnormal overexpression of complement mediators known to regulate synapse elimination in both rodent models of AD (Shi et al. 2017; Hong et al. 2016; Reichwald et al. 2009; Fonseca et al. 2004) and human AD brain tissue (Yasojima et al. 1999; T. Wu et al. 2019) and has been linked to excessive synapse pruning by microglia. Although

microglia-mediated synaptic pruning has been shown to be essential for neural circuit refinement during development (Paolicelli et al. 2011; Konishi et al. 2019; Sancho et al. 2021; Prinz and Priller 2019), aberrant reactivation of such pathways in AD could underlie key aspects of disease progression and cognitive failure. Understanding the spatiotemporal dynamics of microglia-synapse interactions and neuroinflammatory signaling, both before and after the onset of amyloidosis, is crucial for identifying strategies to preserve synaptic integrity and cognitive function in AD patients. Deciphering the cellular and molecular dysfunction that drives such microglia-mediated synaptic control in AD will require use of advanced *in vitro* or *in vivo* systems that enable researchers to simultaneously track high-resolution structural changes and real-time functional readouts of microglia-driven synaptic remodeling over extended periods.

1.1.5 Human iPSC-derived microglia: a new frontier in disease modeling

As microglia have been well established as an integral player in complex AD mechanisms, the development of human microglia from induced pluripotent stem cells (iMG) has been critical to the field of Alzheimer's research. Importantly, the development of human iPSCs-based research tools has dramatically shifted the landscape of translation neuroscience as they can offer profound advantages over other cell-based systems and *in vivo* rodent models. Given the poor representation of many disease-associated gene orthologs in commonly used rodent models (Marsh et al. 2022; Hasselmann et al. 2019; Thrupp et al. 2020; Geirsdottir et al. 2020; Mancuso et al. 2019; Zerbino et al. 2018; Miller, Horvath, and Geschwind 2010; Friedman et al. 2018; Galatro et al. 2017), perhaps the most significant advantage to using human iPSCs is that they have a human genetic background and are highly compatible with gene editing technologies. Moreover, the emergence of iPSC culture systems and the numerous experimental protocols that outline methods for establishing human iPSC-derived neural cells offer great promise as a rigorous platform to study human neurodegenerative diseases at the single cell and molecular scale (McQuade et al. 2018; Haenseler et al. 2017; Tano et al. 2014; Takahashi et al. 2007).

Although there are many studies describing the successful differentiation of iPSCs into several key neural cell types including neurons, astrocytes, and oligodendrocytes (Mertens et al. 2018; Santos et al. 2017; Mostafavi Abdolmaleky et al. 2024; Ehrlich et al. 2017; Zeldich and Rajkumar 2024), there are a limited number of studies that demonstrate successful and robust differentiation of human microglia. Amongst them are two prominent protocols that describe differentiation of human microglia from iPSCs based on 1) initial differentiation toward a mesodermal, hematopoietic lineage using commercially available defined medias (Abud et al. 2017) and 2) by forced expression of defined transcription factors SPI1 and CEBPA (Chen et al. 2021). Single cell RNAseq and subsequent principal component analysis has demonstrated that generation of iMG under these conditions acquire genome-wide gene expression patterns similar to that of human fetal and adult microglia (Abud et al. 2017; McQuade et al. 2018; Chen et al. 2021). Despite these advances, several groups have shown that iMG cultured *in vitro* adopt a homeostatic signature that can overlap with peripheral macrophages (Popova et al. 2021) suggesting that the *in vitro* differentiation process lacks some key brain-specific signaling cues (Popova et al. 2021; Dolan et al. 2023) required to induce brain-like homeostatic signature including expression of *P2RY12*, *SALL1*, and *TMEM119* (S. T. Schafer et al. 2023).

The artifactual gene expression signature of iMG cultured *in vitro* may be attributed to an inherent characteristic of microglia to sense and respond to the surrounding microenvironment (Gosselin et al. 2017; Bohlen et al. 2017). Thus a highly engineered culture environment required to develop iPSCs may prohibit maturation of iMG that fully resemble microglia found in the brain, especially when that environment lacks CNS-specific cues from physiological cell-cell interactions and cell-cell signaling of surrounding neurons, astrocytes, or oligodendrocytes (Dolan et al. 2023). To this end, attempts have been made to incorporate iMG into 3D culture environments including scaffolded co- and tri-culture systems and iPSC-derived organoids (Guttikonda et al. 2021; Dumas, Borst, and Prinz 2021; Popova et al. 2021; S. T. Schafer et al. 2023; Zhang et al. 2023; Takata et al. 2017). However, significant challenges have been observed including limited

microglia integration and proliferation in a 3D environment, complex and delicate timing of separate microglia differentiation in 2D culture prior to integration into organoids, and low reproducibility. Beyond these *in vitro* approaches to incorporate iMG into a 3D brain-like environment, several groups have begun to show the utility of a xenograft model. Here, they demonstrate that the diverse array of transcriptomic profiles characteristic of human microglia can be restored when iMG are placed into the physiological neural microenvironment of a rodent brain (Svoboda et al. 2019; Mancuso et al. 2019; Fattorelli et al. 2021; Hasselmann et al. 2019).

These findings describe use of xenograft models to promote maturation of iMG towards a primary-like signature with expression of several homeostatic genes including *P2RY12*, *SALL1*, *IBA1*, *CX3CR1*, and *TMEM119* in a ramified morphological state (S. T. Schafer et al. 2023; Xu et al. 2020; Hasselmann et al. 2019). To achieve this, iMG hematopoietic progenitor cells were transplanted into the brains of immunodeficient mice and followed by in-depth transcriptomic analysis. Their findings revealed robust maturation of an iMG population that resembles primary human microglia (Svoboda et al. 2019; Fattorelli et al. 2021; Hasselmann et al. 2019) including brain-like homeostatic gene marker expression. Together this collective of data grounds a hypothesis where interaction from the neural microenvironment and surrounding neural cell types is essential in establishing iMG phenotypes that most-accurately resemble primary human microglia. Despite this groundbreaking work, the lack of high-throughput experimentation hinders these types of xeno-engraftment model systems for large scale studies of iMG. As such, further improving high-throughput *in vitro* iMG culture models that better represent the CNS microenvironment remains an important but understudied area.

1.1.6 Current approaches and limitations of existing *in vitro* and *in vivo* models of microglia function in Alzheimer's disease

Although our understanding of microglial function in health and disease has significantly improved in the last two decades of research both *in vitro* and *in vivo*, existing models still exhibit critical limitations that hinder a comprehensive exploration of microglia influence on the neural microenvironment. Importantly, our understanding of the deleterious roles that microglia play during AD pathogenesis and progression remain incomplete as many models fail to fully recapitulate the human-specific features of disease pathology while maintaining a controlled experimental environment. Significant advancements of *in vitro* and *in vivo* approaches including use of iPSC-based technologies, three-dimensional culture systems, and transgenic rodent models of disease have strengthened our understanding of microglia-associated dysfunction in AD. However, these models remain limited by an inability to fully capture the cellular complexity of the human brain microenvironment under physiological and pathological conditions in an experimentally controlled setting.

Current *in vitro* approaches include 2D culture of microglia that can subsequently be co-cultured with neurons and iPSC-based cultures, in either 2D or organoid (3D) models. 2D neural cultures have long served as a fundamental tool for studying neuronal and glial interactions. However, these cultures lack the three-dimensional cytoarchitecture and extracellular matrix composition, both essential features of cell-cell signaling, that define the *in vivo* neural environment. Importantly, microglia cultured in 2D typically exhibit amoeboid morphology and fail to adopt the dynamic, ramified state observed in healthy brain tissue, leading to an inaccurate representation of microglial surveillance. Although human iMG have dramatically improved our understanding of species-specific features of disease and are highly compatible with gene editing technologies, these cells frequently exhibit an immature phenotype, lacking the transcriptional and morphological features characteristic of mature, brain-resident microglia (Popova et al. 2021; Dolan et al. 2023; S. T. Schafer et al. 2023).

Furthermore, human iMG often cultured in isolation or co-cultured with neurons in a non-physiological 2D format, limited the ability to fully recapitulate cell-cell signaling cues required for proper microglial maturation and surveillance (Centeno, Cimarosti, and Bithell 2018).

While transgenic rodent models of AD have advanced *in vivo* systems and provided significant insights into microglia-mediated synaptic remodeling in amyloidogenic environments (Ding et al. 2021; Shi et al. 2017; Hong et al. 2016), they present challenges in species-specific differences and experimental control including easy visual access of such tightly regulated and dynamic cell-cell interactions (Marsh et al. 2022; Thrupp et al. 2020; Geirsdottir et al. 2020; Hasselmann et al. 2019; Galatro et al. 2017; Miller, Horvath, and Geschwind 2010; Friedman et al. 2018). Additionally, *in vivo* models are often labor-intensive, costly, and constrained by slow disease progression, limiting their utility for high-throughput mechanistic studies.

Overcoming several of these limitations, our 3D primary cortical microtissue model provides a powerful, cellularly diverse framework with endogenous ECM support and physiologically relevant cell-cell interactions for studying microglial function in both health and disease. Specifically, this unique model system offers 1) enhanced cellular diversity and an ability to easily model postnatal developmental trajectories compared to canonical iPSC organoid systems, 2) compatibility advanced imaging modalities to capture dynamic cellular behaviors in real time, 3) flexibility of tissue sourcing from transgenic mice or rat models, and 4) translational compatibility with high throughput screening. Furthermore, by improving microglial maturation, supporting complex neuronal circuitry, and enabling real-time imaging of microglia-neuron interactions, this model is well suited for investigations into the spatiotemporal dynamics of microglia-neuron interactions under physiological and pathological conditions such as AD. As we continue to refine and expand its applications, this platform holds great promise for uncovering novel mechanisms of microglia-mediated synaptic dysfunction and guiding the development of targeted therapies for neurodegenerative diseases.

1.2 Motivation from Prior Work

Since the start of my research career, I have always had an interest in studying neurodegenerative diseases and developing tools to better understand the brain. This is evidenced in my prior research experiences that span neuroscience and engineering disciplines; each providing me with technical and analytical skills that have propelled my doctoral research. The work completed during my master's studies and in the first year of my doctoral studies laid a strong foundation for the research presented in this thesis.

Over the course of the two-year master's in biomedical engineering graduate program, I became immersed in a research project that deepened my interests in neuroinflammation and accelerated my abilities at the bench. My master's thesis work—entitled “*NeuroBorealis: Live cell imaging of 3D cortical microtissues via AAV-induced fluorescence*”—included development of an *in vitro* model for real-time imaging of neurons, astrocytes, and microglia in a 3D cortical microtissue. This was motivated in large part by a project—led by fellow PhD student in the lab at the time—aimed at characterizing cortical tissue response to implanted electrode materials *in vitro* with a particular focus on neuroinflammatory mechanisms of the foreign body response. Together, the combined goal of our projects was to develop an *in vitro* platform to visualize the tissue response during neuroinflammatory responses to electrodes implanted into three-dimensional cortical cultures. My contributions resulted in some of the first functional imaging assays developed in our lab and helped lead to two publications showcasing the use of genetically encoded calcium indicators to characterize neural activity in 3D microtissues during response to implanted electrodes (Atherton et al. 2022) and in response to systemic neuroinflammatory stimuli (Atherton et al. 2021).

In my master's thesis I leveraged adeno-associated viral vectors to mediate stable expression of fluorescent reporter proteins to key cell types of interest to establish a real-time imaging platform for longitudinal visualization of dynamic cellular morphology and cell-cell

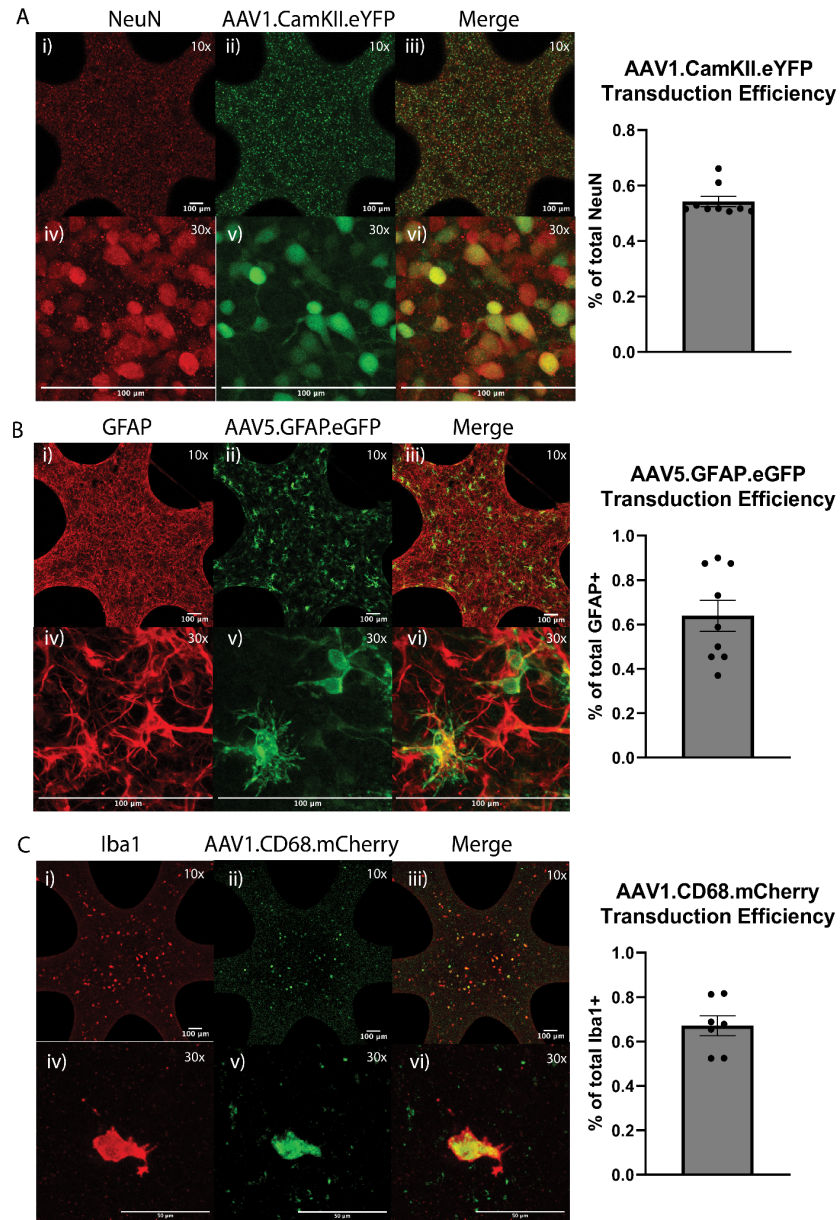


Figure 1.1 | AAV-mediated fluorescent labeling of neurons, astrocytes and microglia in a 3D microtissue model

a, Neuron targeting AAV1.CamKII(1.3).eYFP expression results from transduction at day *in vitro* 1 (TDIV1) and subsequently imaged at 7 days post transduction (DPT7). Representative images from immunohistochemical analysis confirmed colocalization of YFP in NeuN+ neurons at day 7 post transduction. **b**, Astrocyte-targeting AAV5.GFAP.eGFP expression results from transduction at day *in vitro* 1 (TDIV1) and subsequently imaged at 7 days post transduction (DPT7). Representative images from immunohistochemical analysis confirmed colocalization of GFP in GFAP+ astrocytes at day 7 post transduction. **c**, Microglia-targeting AAV1.CD68.hM4D(Gi).mCherry expression results from transduction at day *in vitro* 7 (TDIV7) and subsequently imaged at 14 days post transduction (DPT7). Representative images from immunohistochemical analysis confirmed colocalization of mCherry in CD68+ microglia at day 7 post transduction. *Figure adapted from Sophie C. Brown Thesis for master's degree in biomedical engineering ©2020.*

interactions. I optimized fluorescent expression in neurons, astrocytes, and microglia driven by CamKII, GFAP, and CD68 promoters respectively. Following optimization of viral transduction strategies to efficiently transduce the densely populated 3D microtissues (data not shown) proceeded to characterize neuronal, astrocytic, and microglial expression of three fluorescent reporter proteins. The fluorescent proteins EGFP, EYFP, and mCherry were specifically chosen to enable simultaneous visualization of three cell types. However, because EGFP and EYFP have spectral maxima approximately ~20nm apart (EGFP=510nm and EYFP=530nm emission maxima) I performed spectral deconvolution to resolve the two fluorescent proteins when being expressed in the same microtissue. Cell-specific transduction of the three fluorescent reporter proteins was evaluated separately and confirmed via immunohistochemistry (**Fig. 1.1**).

To achieve simultaneous fluorescent expression in the three cell types of interest within a single microtissue I developed a triple transduction strategy. A unique challenge in aligning the onset of stable fluorescent signals from three cell types that each exhibited unique transduction efficiency that was dependent on the time at which virus was introduced into the microtissue. Stable fluorescence was determined by analyzing signal to noise ratios during a window of fluorescent onset for each fluorophore. Based on varying times of fluorescence onset for each fluorophore the transduction day was selected as follows: DIV12 (TDIV12) for the AAV1.CaMKII(1.3).eYFP mediated fluorescence in neurons, TDIV17 for AAV5.GFAP.eGFP mediated fluorescence in astrocytes, and TDIV7 for the AAV1.CD68.hM4D(Gi).mCherry mediated fluorescence in microglia. Based on this approach of offset transduction days, stable fluorescent onset was observed by DIV24. A schematic of the triple transduction timeline can be seen in **Fig. 1.2**.

A major challenge in designing the microtissue culture system to enable real-time tracking of cells was to bring the microtissues within a working distance of higher-power objectives of a confocal microscope while maintaining the culture conditions in the agarose micro-molded multi-well plates. To achieve this, we designed a custom stainless steel injection mold to make the

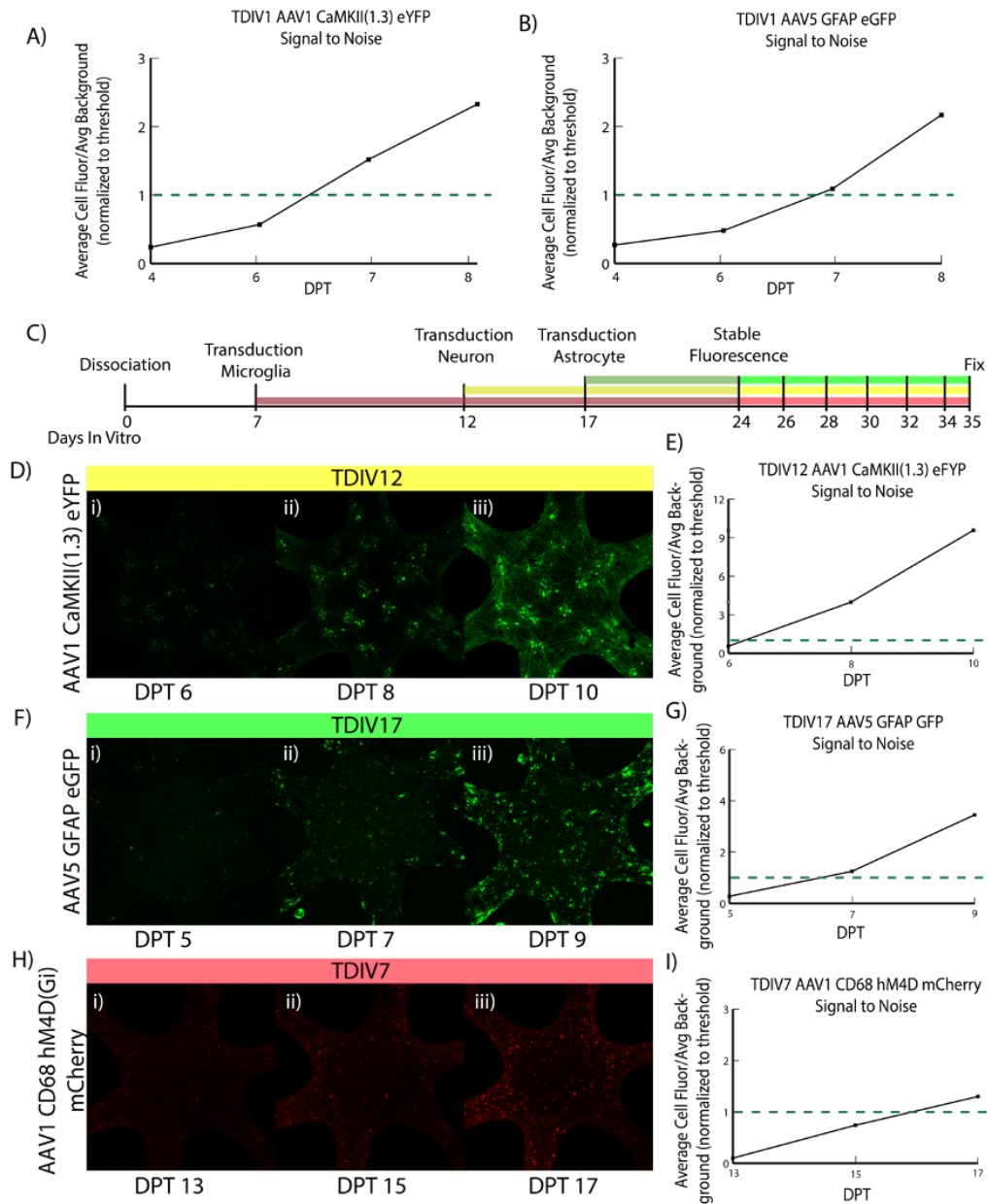


Figure 1.2 | Triple Transduction Timeline

The ratio of signal to noise was measured during fluorescence onset for neuron-targeting AAV1 (a) and for astrocyte-targeting AAV5 (b) following transduction at day *in vitro* 1 (TDIV1). Triple AAV experimental timeline for multiple transductions in a single microtissue (c). Monitoring of fluorescence onset over time of all three AAVs following transductions at days determined by triple AAV experimental timeline (d, f, & h) and the corresponding signal to noise results for assessment of stable fluorescence onset (e, g, & i). Figure adapted from Sophie C. Brown Thesis for master's degree in biomedical engineering ©2020.

bottom thickness of the agarose micro-molds approximately 200µm. By limiting the thickness of agarose in the bottom of the dish, we were able to functionally bring the microtissues with the

working distance of a 30x objective. When limited to use of a 10x objective, we noticed severely low-resolution images of individual cells (**Fig. 1.3, a**). With the fabrication of the specialized injection mold (**Fig. 1.3, b**), the microtissue is placed within the working distance of the 30x objective therefore allowing us to obtain higher-resolution images where cell-specific morphologies and features could be easily distinguished (**Fig. 1.4, c**). Where previous unique morphological features such as the bushy extensions in astrocytes were not clearly visible in the 10x objective (**Fig1.3, a**), the increased objective power allowed for a drastic increase in image resolution where the morphological features were not clear and distinguishable (**Fig1.3, c**).

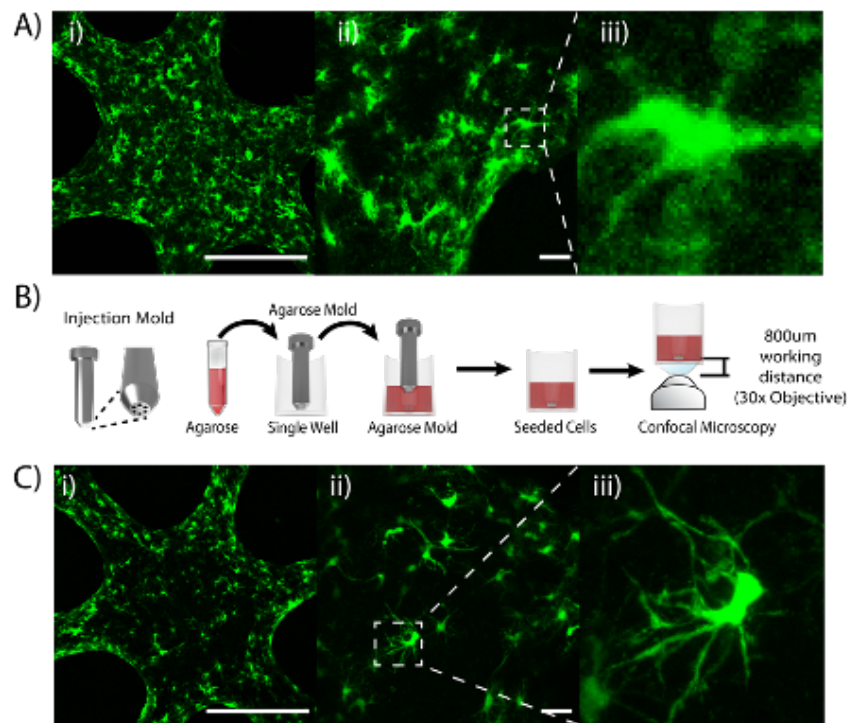


Figure 1.3 | Injection molding enables high resolution imaging of cortical microtissues

Limited spatial resolution of images taken with 10x objective (**a, i**) and 10x objective with 2.5 digital zoom (**a, ii**) prior to the development of specialized injection mold (**b**) designed to bring microtissues within working distance of the 30x objective while remaining in agarose molded cell culture well. 10x (**a, iii**) images compared to 30x with 2.5 digital zoom (**c, iii**) demonstrate the increased resolution with implementation of the injection mold. *Figure adapted from Sophie C. Brown Thesis for master's degree in biomedical engineering ©2020.*

This model allowed for longitudinal analysis of the neural microenvironment in a single 3D culture without the implementation of traditional cross-sectional studies using fixed sample

immunohistochemical staining. I was able to demonstrate, for the first time, AAV-mediated fluorescent targeting of neurons and astrocytes and microglia for continuous visualization in a 3D primary cortical microtissue. Leveraging the success of this work we next sought to quantify cellular dynamics during the neuroinflammatory response to chronically implanted tungsten wires or during systemic inflammatory challenges.

In two separate studies, we used 3D primary cortical microtissues to study the effects of neuroinflammation on neural microcircuits. In the first study we demonstrated the use of a genetically encoded calcium indicator combined with a novel live-imaging platform to record spontaneous calcium transients in microtissues from day 14–34 *in vitro*. Briefly, we transduced microtissues at DIV1 with an AAV particles to induce expression of the genetically encoded calcium sensor GCaMP6s under the human synapsin promoter in the neuronal population. Changes in neuronal calcium concentration (i.e. calcium transients) in large populations of GCaMP6s expressing neurons in the microtissues were captured during 4 minute recording sessions over the course of several weeks (**Fig. 1.4**).

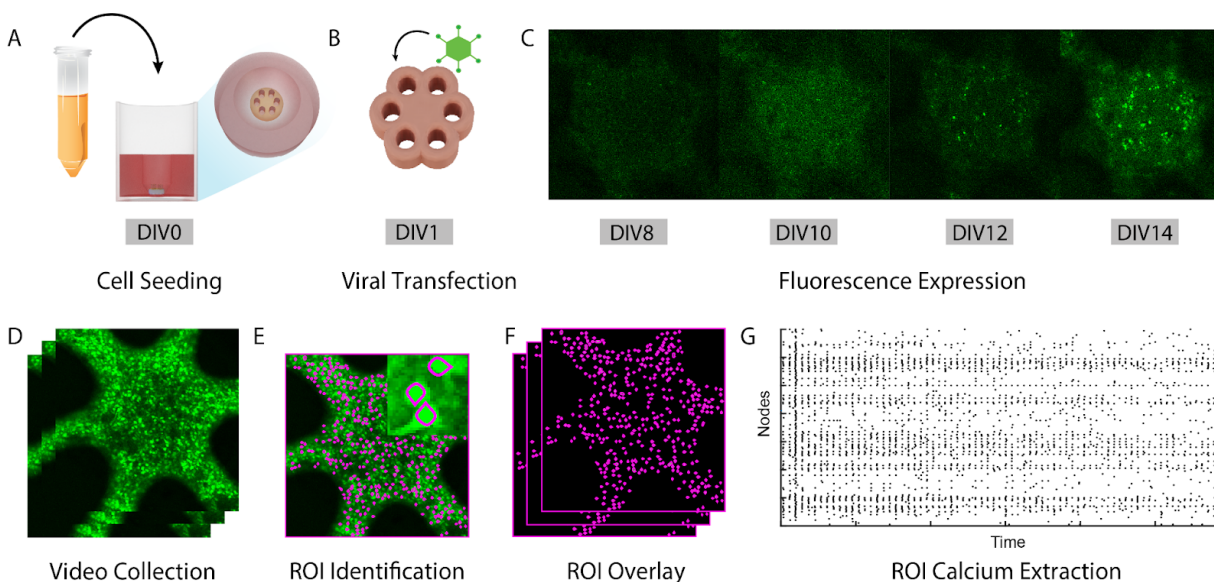


Figure 1.4 | Three-dimensional self-assembled neural cultures express stable GCaMP6s fluorescence and exhibit spontaneous activity by DIV14

a, Dissociated P1 primary rat cells are seeded into a custom injection molded agarose well. **b**, Cells, which form a “microtissue” (tan) in the first 24 hours, were transfected with a commercially available viral vector to encode the GCaMP6s calcium indicator. **c**, Microtissues display progressive expression of baseline calcium fluorescence from DIV8-DIV14. **d**, Time-lapse videos of calcium transients were

collected for 4 min. **e**, Python code was used to perform semi-automated segmentation of cell bodies. **f**, Segmented regions of interest (ROIs) were overlaid onto the 4 minute video for single-cell calcium trace extraction. **g**, A raster plot of calcium events detected from calcium traces was extracted from segmented videos using the MATLAB based software package FluoroSNNAP(Patel et al. 2015). *Figure adapted from Atherton et al. 2021.*

We implemented graph theory analysis of calcium activity to characterize underlying functional connectivity and community structure of microcircuits, which are capable of capturing subtle changes in network dynamics during early disease states. We found that microtissues

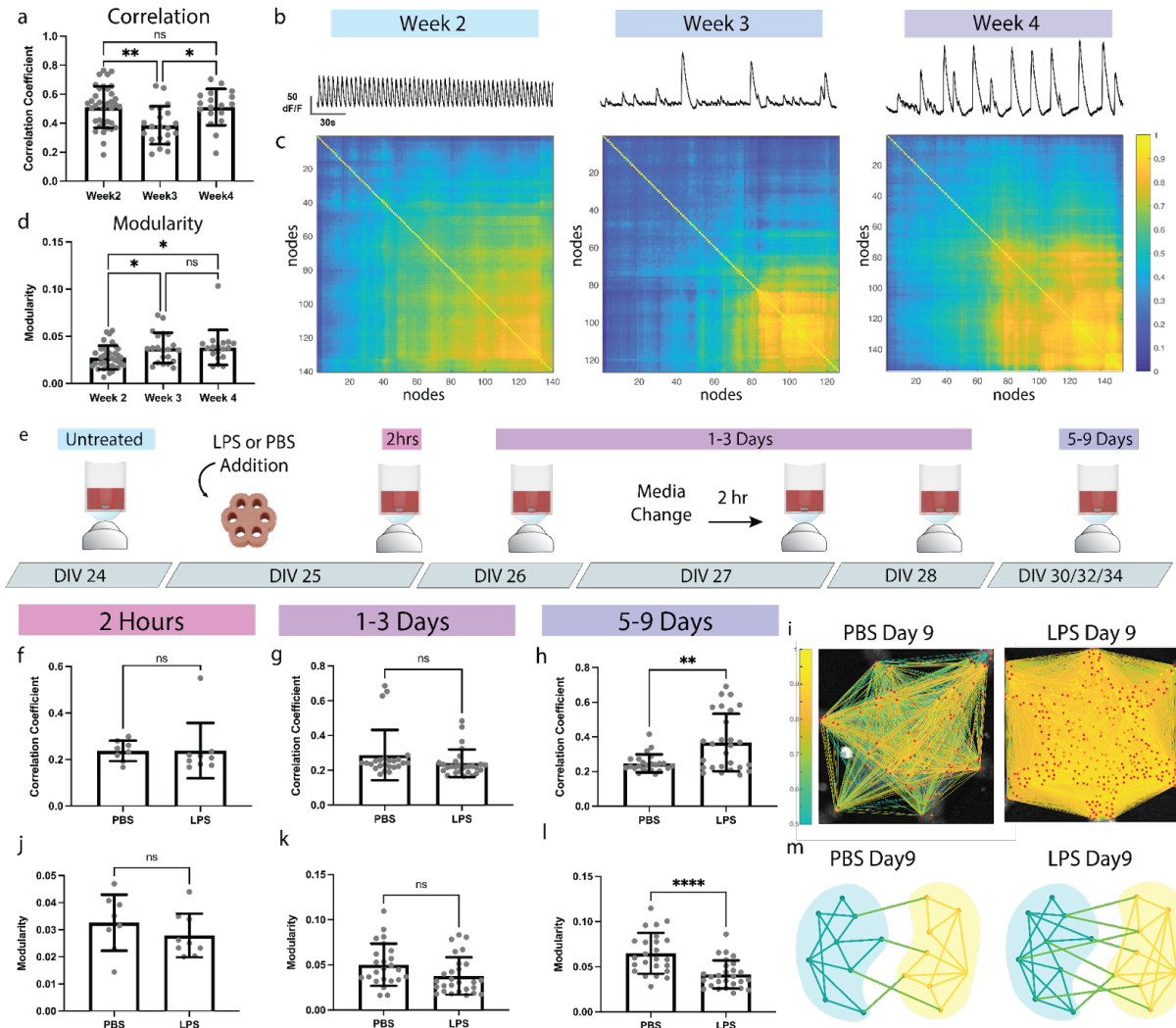


Figure 1.5 | Lipopolysaccharide-induced neuroinflammation disrupts functional connectivity and community structure in primary cortical microtissues

a, average correlation coefficients of microtissues significantly decrease ($p=0.0026$) from week 2 to week 3, followed by a significant increase ($p=0.0151$) from week 3 to week 4. **b**, Example single cell F/F₀ traces from week 2, 3, & 4 along with **(c)** pairwise Pearson correlation matrices of node pairs from example recordings across weeks. **d**, Overall modularity of microtissues significantly increased ($p=0.046$)

($p=0.046$) at week 3 and remained high ($p=0.0475$) at week 4. **e**, Schematic of experimental timeline for addition of LPS or PBS (vehicle control) at DIV25 followed by subsequent imaging at 2hr, 1-3 days, or 5-9 days. **f-h**, whole tissue average correlation coefficient in PBS vs LPS-treated microtissues at **(f)** 2hr, **(g)** 1-3 days, or **(h)** 5-9 days post treatment. LPS-treated samples showed a significant increase in whole tissue correlation ($p=0.0014$) only at 5-9 days post treatment. **i**, representative correlational connectomes from LPS vs PBS treated microtissues at 5-9 days post treatment. **j-l**, Whole tissue modularity in LPS vs PBS-treated microtissues at **(j)** 2hr, **(k)** 1-3 days, **(l)** 5-9 days post treatment. LPS-treated samples showed a significant decrease in modularity ($p=p<0.0001$) at 5-9 days post treatment. **m**, schematic representations of modular connectivity in PBS vs LPS-treated microtissues at 5-9 days post-treatment. *Figure adapted from Atherton et al. 2021.*

cultured for 34 days displayed functional remodeling of microcircuits and that community structure strengthened over time (**Fig. 1.5, a**). Lipopolysaccharide, a neuroinflammatory agent, significantly increased functional connectivity and disrupted community structure 5–9 days after exposure. These microcircuit-level changes have broad implications for the role of neuroinflammation in functional dysregulation of neural networks.

In a second study we characterized the structural and functional neural dynamics in 3D cortical cultures in response to implanted microwires (**Fig. 1.6**). In this study a similar approach

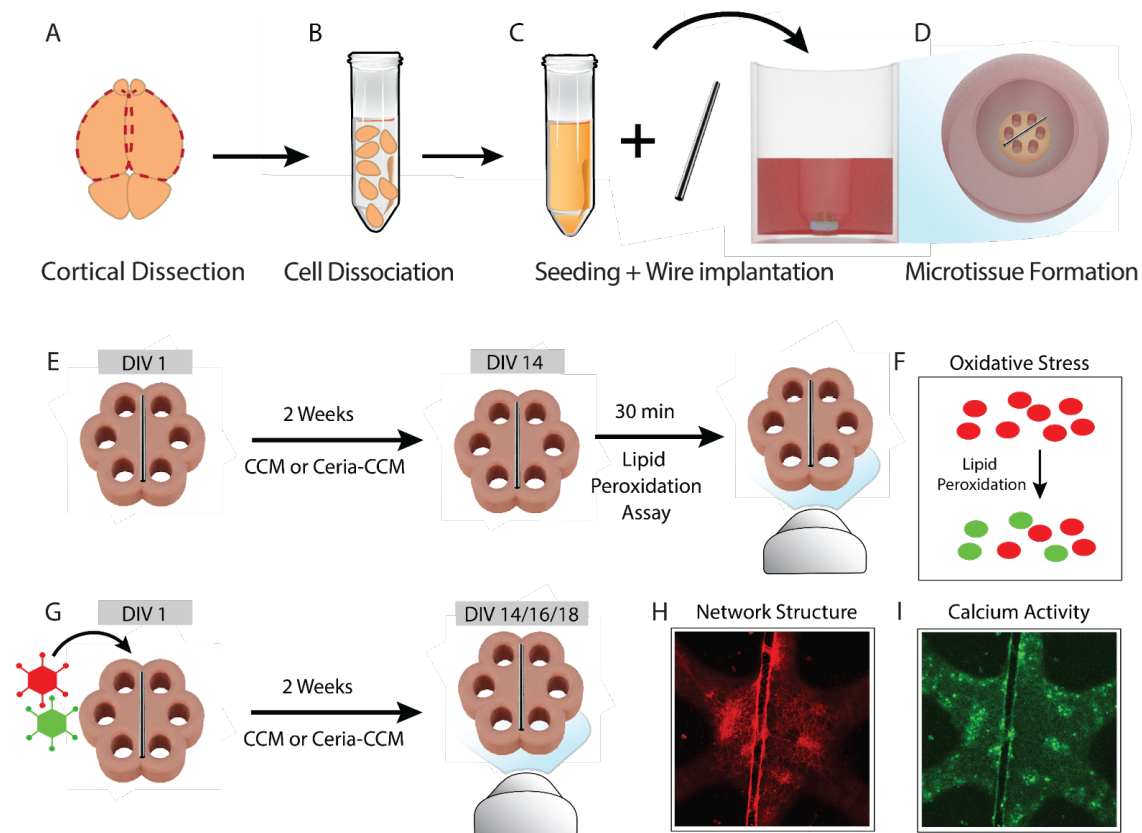


Figure 1.6 | Development of an *in vitro* device-tissue interface for measuring oxidative stress, functional activity, and structural changes around implanted wires

a, Cortices were dissected away from P1 rat brains. **b**, Primary tissue was dissociated into a single cell suspension by papain. **c**, Cells were seeded alongside a tungsten wire into an agarose microwell. **d**, Cells formed a microtissue with an embedded microwire. **e**, Oxidative stress assays were performed after 14 days of culturing. **f**, Image-IT kit shifts live cell fluorescence from red to green in the presence of lipid peroxidation over 30 minutes. **g**, AAV mediated fluorescent protein delivery was performed at day one *in vitro*, and transfected microtissues were imaged on DIV 14, 16 and 18. **h**, Network structure was imaged in the red channel with a 30x oil object for high resolution volumetric data. **i**, Calcium activity was recorded in the green channel with a 10x objective for 4min with a 15Hz sampling rate. *Figure adapted from Atherton et al. 2022.*

to our prior work was used in that we deployed genetically encoded calcium indicators, delivered via AAV, to neuronal populations within a microtissue to capture changes in response to a foreign body (a tungsten microwire) that was implanted into the middle of the tissue (**Fig. 1.6, a-d**). Leveraging the bicistronic vector to express both GCaMP6s and a secondary mRUBY reporter protein under the same human synapsin promoter allowed us to track both structural changes to neurite densities as well as functional changes in neuronal calcium activity over several week *in vitro* (**Fig. 1.6, g-i**). Additionally, we measured oxidative stress via fluorometric lipid peroxidation assay (**Fig. 1.6, e-f**).

Our findings demonstrated that the wire implanted microtissues exhibited a disruption in neural network connectivity marked by increased average whole tissue correlation and decrease in modularity compared to control (non-implanted) microtissues (**Fig. 1.7, a-c**). Additionally, microtissues implanted with a microwire displayed a significant loss in neurite density around the wire. Neurite density was determined using the mRuby fluorescent reporter protein delivered to microtissues via AAV. Volume fraction of was calculated from binarized fluorescent volumetric images (**Fig. 1.7, d-f**). Significant decrease in volume fraction was observed between 50um and 400µm from the wire boundary (**Fig. 1.7, g**) indicative of loss in neurite density around the foreign body.

Finally, we evaluated use of “perpetual” antioxidant ceria nanoparticles to mitigate oxidative stress around the microwire implant and therefore protect neuronal function and structure from damaging oxidative stress released in response to the foreign body. Remarkably

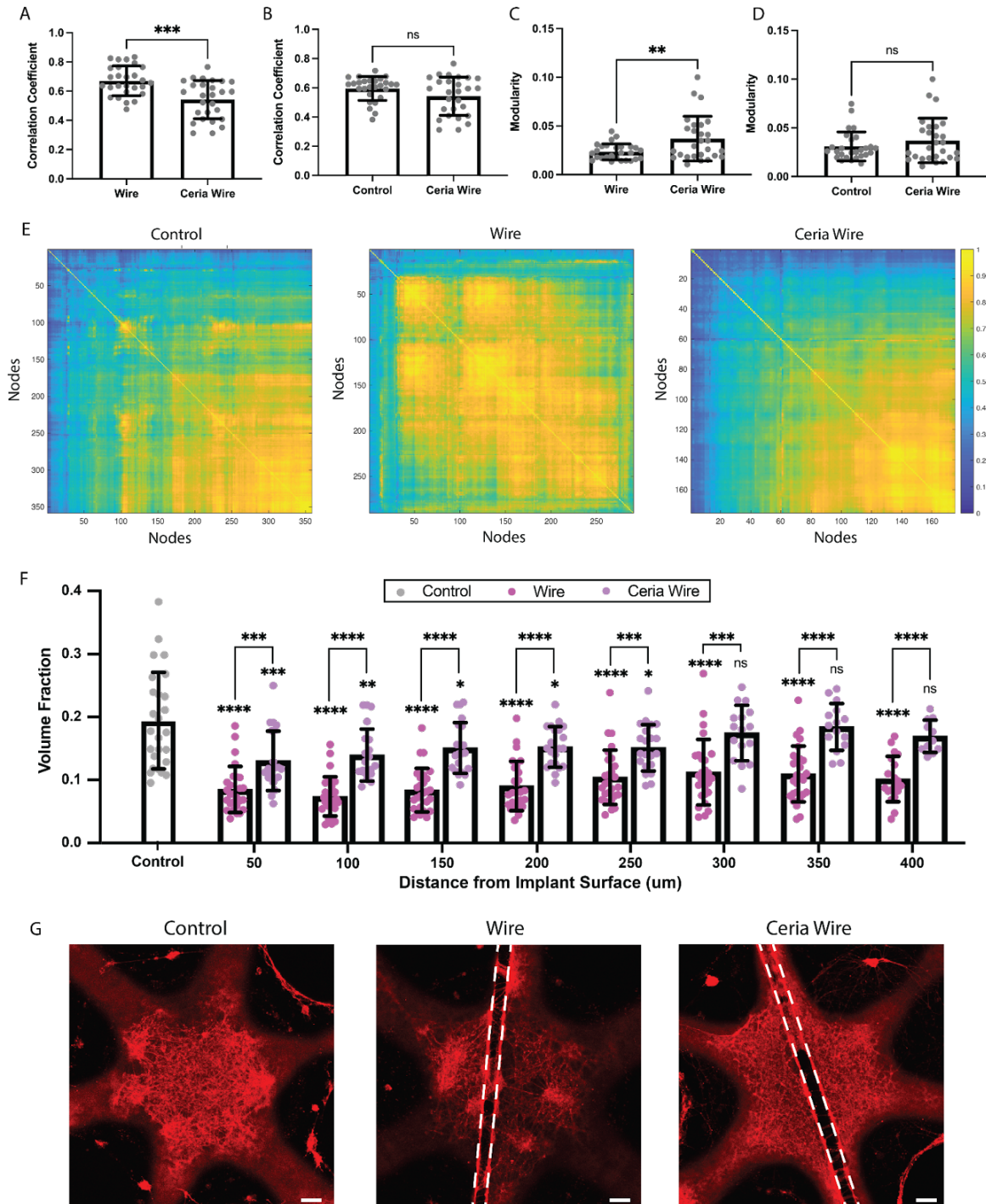


Figure 1.8 | Ceria treatment restores functional connectivity and protects neurite density loss around implants

a, Ceria treatment significantly reduced correlation ($p = 0.0002$) around wire implants. **b**, Correlation of ceria treated wire implanted microtissues is not significantly different from controls ($p = 0.0819$). **c**, Ceria treatment strengthened community structure through increased modularity ($p = 0.0056$). **d**, Ceria treatment restored modularity of the network in implanted tissues to control levels ($p = 0.2545$). **e**, Correlograms from representative (K) control, (L) wire implanted, and (M) ceria treated wire implanted microtissues. **f**, Neuronal volume fractions of wire implants (pink) were significantly lower than non-implanted controls (gray) (50 μm , $p < 0.0001$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p < 0.0001$; 300 μm , $p < 0.0001$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$, significance indicated above pink data points). Ceria treated wire implants (purple) exhibited significant increases in neuronal volume fractions over untreated wire implants (50 μm , $p = 0.0002$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p = 0.0001$; 300 μm , $p = 0.0003$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$; significance indicated above brackets), reducing neuronal density loss from 0-250 μm and restoring neuronal densities to that of non-implanted controls from 250-400 μm (50 μm , $p = 0.0007$; 100 μm , $p = 0.0044$; 150 μm , $p = 0.0124$; 200 μm , $p = 0.0141$; 250 μm , $p = 0.0126$; 300 μm , $p = 0.2662$; 350 μm , $p = 0.5218$; 400 μm , $p = 0.1270$; significance indicated above purple data points). **g**, The loss of neuronal density around wire implants and subsequent protection of neuronal density after ceria treatment can be seen visually in representative images of non-implanted controls, wire implants, and ceria-treated wire-implants. Scale bars = 100 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

we see significant reduction in average whole tissue correlation (**Fig. 1.8, a**) after 2 weeks of implantation with ceria antioxidant treatment. This restored connectivity levels towards that of non-implanted controls (**Fig. 1.8, b**). Similarly, modularity was significantly increased in ceria treated microwire implanted microtissues (**Fig. 1.8, c**) and was restored towards that of non-implanted controls (**Fig. 1.8, d**). The restoration of functional connectivity towards that of non-implanted controls can be visualized by the pairwise correlation matrices of non-implanted control, wire, and wire+ceria treatment microtissues following two weeks of culture (**Fig. 1.8, e**). Similarly, ceria treatment mitigated loss of neurite density around implanted wires (**Fig. 1.8, f-g**). Together these results demonstrated that antioxidant treatment mitigates the neurotoxic effects of oxidative stress seen in microtissues implanted with tungsten microwires. In this study, we developed a 3D *in vitro* model of the device-tissue interface and showed that microtissues exhibited symptoms of oxidative stress-mediated innate neuroinflammation following microwire implantation. We concluded by showing efficacy of antioxidant ceria nanoparticles in treating foreign body induced neuroinflammation and secondary oxidative stress.

These studies underscore the versatility and potential of 3D cortical microtissues as an advanced *in vitro* model for investigating neural dynamics and neuroinflammatory processes in a controlled setting. By leveraging genetic tools, live imaging, and functional network analysis, we have demonstrated the capacity of this system to capture both structural and functional alterations in response to inflammatory stimuli and implanted foreign bodies. Importantly, the ability to longitudinally track microglia-neuron interactions in a physiologically relevant microenvironment represents a significant advancement over traditional 2D cultures and transgenic rodent models. This work laid a strong foundation for my doctoral research, equipping me with the technical expertise and analytical framework necessary to investigate the spatiotemporal dynamics of microglia-neuron interactions in Alzheimer's disease. Building upon this foundation, I began by establishing a detailed protocol, described in Chapter 2, for designing and deploying a 3D cortical microtissue system compatible with longitudinal live imaging studies, wherein I provided a step-by-step framework for their development and application in studying microglial function in neurodegenerative disease. In Chapter 3, I demonstrate how microglia-mediated synaptic remodeling drives circuitry maturation in 3D cortical microtissues and that these processes are disrupted in an amyloidogenic microenvironment. In Chapter 4 I describe, for the first time, xenograft culture system wherein iPSC-derived microglia functionally integrate into 3D cortical microtissues, actively surveil the microenvironment for damage, and respond to neuroinflammatory stimuli.

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Chapter 2

A three-dimensional primary cortical culture system compatible with transgenic disease models, virally mediated fluorescence, and live microscopy

2.1 Preface

The ability to accurately model complex neural interactions *in vitro* is critical for advancing our understanding of both normal brain function and the pathophysiology of neurological disorders. Traditional two-dimensional cell cultures, while useful for controlled and repeatable experimentation, often fail to capture the intricate cellular architectures and dynamic interactions that define neural tissue *in vivo*. In response to these limitations, this chapter presents the development of a 3D primary cortical culture system designed to provide a more physiologically relevant environment for studying neuron-glia interactions.

Building upon previous methodologies, we optimized a protocol for generating self-assembled, heterogeneous 3D cortical microtissues from primary rodent cells. These microtissues, engineered into an equibiaxial “trampoline” geometry, provide a stable, optically accessible platform compatible with live confocal microscopy. By leveraging adeno-associated viral (AAV) vectors to induce fluorescence in specific neural cell types, we established a multimodal system for longitudinal imaging, enabling high-resolution visualization of cellular dynamics over extended time periods. This chapter details the development and validation of this culture system, emphasizing its potential for investigating neurodevelopmental processes, synaptic remodeling, and disease-related cellular dysfunction in a controlled *in vitro* setting.

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2.2 Abstract

In vitro cell culture models can offer high-resolution and high-throughput experimentation of cellular behaviors. However, *in vitro* culture approaches often fail to fully recapitulate complex cell processes involving synergistic interactions between heterogeneous neural cell populations and the surrounding neural microenvironment. Here, we describe the formation of a three-dimensional primary cortical cell culture system compatible with live confocal microscopy.

Key Words: Three-dimensional cell culture, live imaging, primary cell culture, *in vitro*

2.3 Introduction

Parkinson's disease, Alzheimer's disease and other dementias, multiple sclerosis, and epilepsy are among the large number of debilitating neurological disorders, many of which lack effective treatments. The paths towards successful therapeutic intervention for such disorders are often obstructed by a limited understanding of disease etiology and complex underlying mechanisms. Further, current diagnostic and clinical technologies used to study the human brain are typically non-invasive with limited spatial and temporal resolution, which hinders direct study of cellular mechanisms of disease. While *in vivo* animal models can provide a path to understanding neurological disorders, they are often defined by a slow iterative process and study conclusions are limited to only those pathways that are well conserved across species. Alternatively, *in vitro* modeling of the brain can provide repeatable, well-controlled, and environmentally isolated experimentation for effective molecular and cellular investigations of neural function in health and disease.

To date, myriad sources are used to form *in vitro* neural cell cultures including immortalized cell lines, induced-pluripotent stem cells, and human patient derived or animal primary tissue (Goshi et al. 2020; Anderl, Redpath, and Ball 2009; Lancaster et al. 2013; Clevers 2016; Zhao et al. 2020). In the case of primary rodent cultures, a tissue sample is collected from the host and then dissociated into a cellular suspension by means of enzymatic and mechanical manipulation. Embryonic or neonatal cells, having gone through fewer differentiated states, are more likely to survive in culture (Juurlink and Walz, 1999). For primary monolayer cultures, tissues are dissociated into a single cell suspension and seeded onto flat culture plates where they attach to the two-dimensional surface. However, when dissociated cells are left in suspension, they aggregate and reassemble into three-dimensional structures (DeLong, Robert DeLong, and Sidman 1970). Compared to two-dimensional cultures which physically constrain cell mechanics

and morphologies to the culture surface, three-dimensional cell cultures provide increased physiological relevance with cytoarchitecture and cell-cell interactions that are more akin to those found in the living brain (Juurlink and Walz, 1999; Irons et al. 2008; Zhuang et al. 2018; Fennema et al. 2013; Dingle et al. 2015; Xiang et al. 2017). Without specific selection for a unique cell type, dissociated cells will form heterogeneous neural cell cultures including both glial and neuronal subtypes with evidence of neuronal remodeling and formation of axonal networks with fully developed synapses and microcircuitry (Seeds 1973; Peng, Juurlink, and Hertz 1991; Trujillo et al. 2019; Severino et al. 2016; Quadrato et al. 2017; Lam et al. 2019; Atherton et al. 2021, 2022). Additionally, *in vitro* primary cultures can be formed from an array of transgenic animal models, which allows for study of disease phenotypes in an engineered tissue environment, with control over exact cell type concentrations and the ability to supplement or deplete specific cell types.

We have previously reported on the use of a three-dimensional, self-assembled, primary cortical cell culture platform for investigative studies of neuroinflammatory effects on functional neural network structures (Atherton et al. 2021, 2022). Briefly, we derived three-dimensional cortical cultures from primary rodent tissue utilizing a protocol adapted from the Laboratory of Dr. Hoffman-Kim whose previous work demonstrated neural cell subtype diversity and functionality of intra-cellular interactions in primary rodent cortical spheroids (Dingle et al. 2015; Boutin et al. 2018). We further optimized the protocol to seed cortical cells into an equibiaxial tension “trampoline” shaped microtissue, previously described by Schell et al., to create a culture geometry compatible with longitudinal microscopy studies (Schell et al. 2016). The trampoline shape provides a stable ~300 μm thick microtissue, consisting of a self-assembled extracellular matrix, and heterogeneous cell population (Atherton et al. 2021) (**Fig. 2.1**). Development of a custom injection mold allowed us to seed microtissues within 350 μm of the bottom of a glass bottom multi-well culture plate. The injection mold was devised in order to bring the microtissue within working distance of high-power objectives of an inverted confocal microscope. Concurrently we optimized methods to induce fluorescence in several key neural cell types within the 3D

microtissues via adeno-associated viral (AAV) vectors. Combined with our engineered plate set-up and unique microtissue geometry, we were able to use AAV-mediated fluorescence to perform high-resolution microscopy over several weeks, capturing longitudinal evolution of intricate cellular morphologies and functions at the single cell level. As this multimodal culture and live-imaging platform allows us to monitor the direct and indirect influence of cellular dynamics in and on the surrounding neural microenvironment, we highlight its application to study complex multicellular processes in both healthy and diseased states.

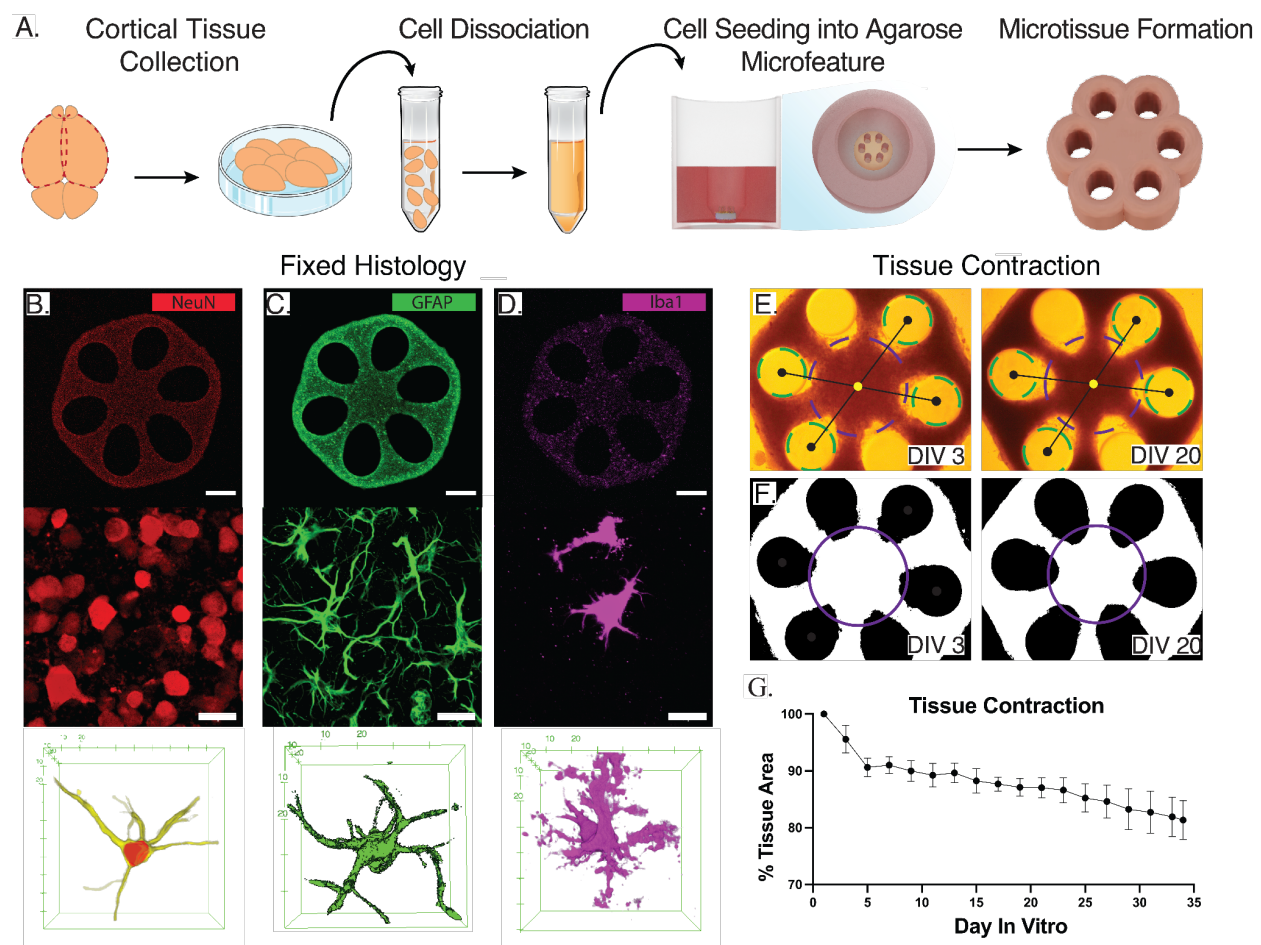


Figure 2.1 | Formation of three-dimensional primary cortical microtissue

Primary rat cortical tissue is collected at postnatal day 1-2 and dissociated by enzymatic means to form a single cell suspension prior to seeding into agarose-molded microfeature (A). At 24-hr post-seeding 3D microtissue forms and immunolabeling of (B) neurons (NeuN, red), (C) astrocytes (GFAP, green), and (D) microglia (Iba1, magenta) demonstrates heterogenous cell population with intricate 3D morphologies. Microtissues show macro-morphology changes characterized by tissue contraction over weeks (E). Contraction of the center of the microtissue was measured with custom MATLAB code by placing an ROI in the center of the tissue (purple), binarizing the image (F) so that the tissue is white and then measuring

the percent of white pixels within the ROI. (G) Microtissues contracted the most within the first 14 *days in vitro* (89.63% tissue area) and continued to contract through day 34 (81.34% tissue area). Reproduced from (Atherton et al. 2021) with permission from *Scientific Reports*.

2.4 Methods

2.4.1 Agarose-molded multi-well plate

1. Place sterilized stainless steel injection mold into a single well of a glass-bottom 24 well plate.
The injection mold will be suspended in place approximately ~200 μm from the bottom of the plate by the custom 3D-printed PLA-lift piece (see **Discussion-1**).
2. Add 1 g of Ultra-Pure agarose powder to a 500 mL autoclavable glass bottle and sterilize with high heat and pressure (121°C min for 30 min).
3. Add 50 mL of sterile PBS to cooled sterile agarose powder to create a 2 % agarose solution.
Add the PBS to agarose powder and gently swirl. Place bottle in laboratory microwave and heat in 30 second increments until agarose goes into solution and reaches a rolling boil (approximately 1.5 min).
4. Place the hot agarose into a biosafety cabinet for preparation of the agarose-molded multi-well plates. Using a sterile 5 cc syringe, inject 1.2 mL of hot agarose through the stainless-steel injection mold placed atop 1 well of the 24 well glass-bottom plate. Carefully remove the syringe, leaving the injection mold and PLA-lift in place. Place the plate on an ice pack for a minimum of 7 min to allow agarose to cool and solidify.
5. Carefully place the 2 mm diameter stainless-steel rod into the stainless-steel injection mold and scrape excess agarose away from the 6 peg holes. Steadily remove the injection mold and PLA-lift piece simultaneously, leaving behind the agarose-molded microfeature at the bottom of the well. To clean the injection mold before the next molding, use the plunger from a 0.5 cc syringe to force residual agarose out of the hollow injection mold.

6. Add 1 mL of CCM to keep agarose molds from dehydrating and place in 37°C incubator until needed for cell-seeding. (see **Discussion-4**)

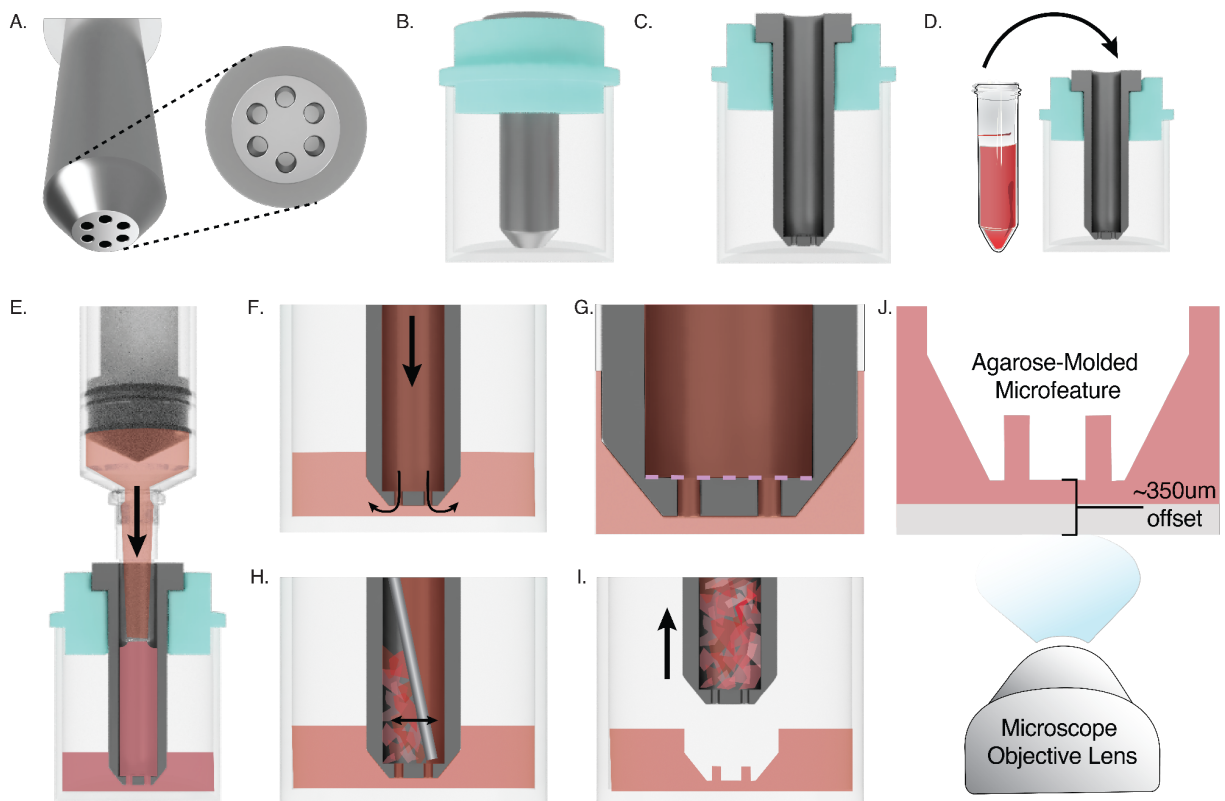


Figure 2.2 | Agarose-molded microfeatures on multi-well plate

A custom machined stainless steel injection mold (a) is placed atop a single well of a 24-well plate using a 3D-printed PLA-lift piece (b-c). Hot agarose is pushed through the injection mold with a luer lock syringe (d-e). Agarose moves from the injection mold into the well through the peg holes and is allowed to cool until agarose solidifies (f). To remove the mold from cooled solid agarose, the pegs need to be separated from the agarose inside the injection column using a sterile stainless-steel rod (g-h). Once separated, the mold is carefully removed (i) leaving behind the agarose-molded microfeature at the bottom of the well (j). Reproduced from (Atherton et al. 2021) with permission from Scientific Reports.

2.4.2 Neonatal cortical tissue collection

1. A whole litter of postnatal day 0-2 rat pups are collected and sexed. An equal number of male and female pups are pooled for use in a single dissociation experiment.
2. Following approved Institutional Animal Care and Use Committee (IACUC) protocols for non-CO₂ euthanasia of neonate rat pups, whole brains are then dissected away using sterile small

scissors and scooped spatula and placed into 35 mm petri dish containing 4 mL of HA-27 media (see **Discussion-5**). Carefully remove the thin, vascularized dura layer before dissecting away the cortex from the right and left hemisphere. Using forceps, gently transfer cortices into a new 35 mm sterile petri dish containing fresh HA-B7 media.

3. Once all cortices have been collected and pooled together, forceps are used to cut each cortex piece into smaller pieces. Each piece of cortex is cut into approximately one third of its original size.

2.4.3 Tissue dissociation and plate seeding

1. Prepare the papain solution by adding Hibernate A [minus calcium] to the sterile lyophilized papain powder, bringing final concentration to 2 mg/mL. Solution will be cloudy at first. Mix by gentle inversion, and once papain goes fully into solution, transfer the entire volume to sterile 15 mL conical tube. Place the 15 mL conical in 30°C water bath for 10 min.
2. Gently place each piece of cortex into the papain vial, being careful to add as little of the HA-B27 media as possible. (see **Discussion-6**)
3. Incubate cortices in papain solution at 30°C for 30 minutes, gently agitating every 5 min.
4. Following 30 min papain incubation, carefully remove supernatant without disturbing the pellet of tissue.
5. Add 2 mL of prewarmed (37°C) HA-B7 media to the cortices pellet and triturate using a P1000 pipette tip. Be careful not to overmix.
6. Transfer the solution to a sterile 40 µm filter placed over a sterile 50 mL conical tube and allow for passive diffusion through the filter before washing with another 3 mL of warmed HA-B7 (see **Discussion-7**).
7. Collect the flow-through from the 50 mL conical and transfer to a 15 mL conical for centrifugation. Centrifuge for 5 min at 150xg.

8. Carefully remove the supernatant and resuspend the pellet with 2 mL of warmed CCM. Transfer the solution to a clean 40 μ m filter placed over a new 50 mL conical tube. Allow for passive diffusion through the filter before washing with another 3 mL of warmed CCM.
9. Collect the flow-through from the 50 mL conical and transfer to a 15 mL conical for centrifugation. Centrifuge for 5 min at 150xg.
10. Repeat the filter, centrifuge step once more for a total of 3 filters. Following the final centrifugation step, remove the supernatant and add 5 mL of warmed CCM and resuspend the pellet for cell counting.
11. Take a small aliquot of cell suspension, add Trypan Blue at a 1:2 dilution to count cells by hemocytometer and assess live and dead cell populations to determine cell viability. If viability is lower than 90 %, it is not recommended to use for seeding and microtissue formation (see **Discussion-8**).
12. Calculate total number of cells (typically average of 5e6 cells per pup).
13. Centrifuge for 5 min at 150xg. Resuspend pellet in CCM to final concentration of 2.7e8 cells/mL (2.7e6 cells in 10 μ L of CCM per microtissue).
14. Remove agarose-molded plates from the incubator and remove all CCM from each well, including the liquid around the pegs while being careful to not touch the bottom of the agarose with the pipette tip.
15. Carefully add 10 μ L of cell suspension, dropwise, into the agarose microfeature.
16. Place the plate back into the incubator for 25-30 min to allow cells to settle.
17. After 30 min, remove the plate from the incubator and add 1 mL of CCM dropwise to each well, being careful to not disturb the cell pellet at the bottom of the microfeature. Place the plate back in the incubator at 37°C 5 % CO₂.
18. At 24 hrs post-seeding (DIV1), the cells will self-assemble into the trampoline shape, and begin to contract around the pegs. Change media on DIV1, and then every other day thereafter (see **Discussion-9**).

2.4.4 Adeno-associated viral transduction

1. Adeno-associated viral (AAV) transduction of microtissues can be performed at various experimental timepoints. For the purpose of this protocol, we describe AAV transduction at DIV1 for delivery of a genetically encoded calcium indicator (GCaMP6s) and imaging of spontaneous calcium transients and DIV14, 16, and 18.
2. Ready to use AAV1 particles produced from pAAV-hSyn1-mRuby2-GSG-P2A-GCaMP6s-WPRE-pA (Addgene, 50942) should be removed from the -80°C freezer and thawed on ice immediately prior to use. Dilute thawed viral particles in warmed CCM to a final concentration of 1×10^{10} vg/mL.
3. At DIV1, remove microtissue plates from the incubator and replace the 1 mL of CCM in each well with 500 μL of freshly prepared viral media.

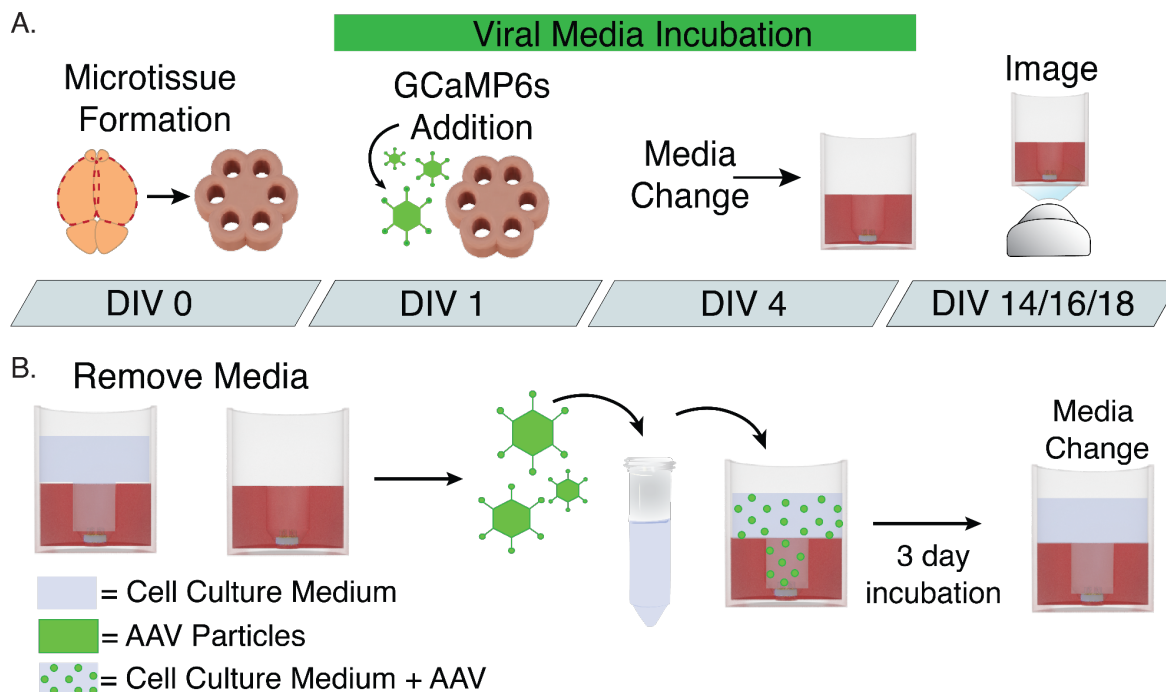


Figure 2.3 | Adeno-associated viral transduction of three-dimensional cortical microtissues

Three-dimensional cortical microtissues are transduced at DIV1 with AAV viral particles to induce fluorescent labeling of neural cell populations and subsequent longitudinal live imaging studies (a). AAV particles are diluted into cell culture media and allowed to incubate over microtissues for three days before viral media is removed and replaced with regular cell culture media (b).

4. Following a 3-day viral media incubation, a media change is performed at DIV4.
5. Microtissues are cultured until live imaging sessions begin at DIV14, with normal media changes every other day (see **Discussion-9**).

2.4.5 Live confocal imaging

1. On imaging day, the microscope room was prepped by first turning on the stage top incubator at least 15 min prior to imaging to warm to 37°C and pre-equilibrate with pre-mixed 5% CO₂. Additionally, sterile H₂O was carefully added by syringe to the humidifying chamber surrounding the plate holder.
2. Microtissue plates were transferred from the cell culture room to the microscopy room for live imaging sessions. Plates were transported in a heated and cushioned container, note this may not be necessary if the transport distance is minimal.
3. For 4 min calcium video acquisition, the microtissue plate was placed on the stage top incubator with the multi-well plate adaptor. Using the 10x objective and an aperture setting of 800 µm, the 488nm laser and EGFP detector were used to visualize fluorescence in individual microtissues. The objective was centered over the microtissue and brought within the focal plane, maximizing the number of fluorescent cells in the field of view. Time-lapse videos were recorded at 15 frames per second.
4. For high-resolution volumetric imaging, the microtissue was centered over the 30x oil immersion objective. Using both the 488 nm and the 560 nm laser, fluorescent signal from the GCaMP and the mRuby reporters were captured in a single microtissue. Due to the inability to clear the live tissues, a z-stack of approximately 80 µm was taken from the middle of the ~150 µm thick microtissue.

2.4.6 Immunohistochemistry

1. At the termination of the experiment, remove microtissue plates from the incubator, transfer to a certified fume hood, and aspirate culture media from each well, leaving behind only the agarose mold and the microtissue. Carefully add 1.5 mL of a 4% paraformaldehyde 8% sucrose fixative solution (see **Supplementary materials**) to each well and incubate at room temperature for 2 hours before removing fixative solution and replacing with 1.5 mL of 1xPBS. Perform 3 separate 2 hour washes with 1xPBS prior to removing microtissue plate from the fume hood.
2. Remove the agarose gels, including the microtissues, from each well and transfer to a clear surface (see **Discussion-10**). Using a clean razor blade, carefully cut away the excess agarose from around the microtissue, taking care not to cut into the microtissue. Once the surrounding agarose has been cut away as much as possible, transfer the microtissue in the remaining agarose to a glass scintillation vial filled with warmed PBS. Ensure that the agarose and microtissue are fully submerged in the PBS and place the vial on a hotplate at 200°C for approximately 10 min. Gently agitate the vial every minute until the agarose has melted away and the microtissue is floating free. Once the fixed microtissues are floating freely, transfer to a 35 mm petri dish and wash several times with warmed PBS to ensure any residual agarose is melted away from the microtissue (see **Discussion-11**).
3. Once microtissue have been harvested from the agarose molds, transfer to a 48-well tissue culture plate and wash with a final 500 μ L of room temperature PBS.
4. Remove PBS solution from each well and replace with 300 μ L of Blocking Solution (see **Supplementary materials**). Place plate on shaker with gentle agitation and incubate for 2 hrs in blocking solution.

5. Remove blocking solution and replace with 300 μ L of primary antibody cocktail (see **Supplementary materials**). Parafilm the plate and return to shaker for overnight incubation, again with gentle agitation.
6. On the following day, remove the primary antibody solution and replace with 500 μ L of 1x PBT solution (see **Supplementary materials**) and return the plate to the shaker for a 2 hr wash. Repeat 2 hr PBT wash step once more before removing PBT and adding 300 μ L of blocking solution to each well and incubating for additional 2 hours on the shaker. After 2 hour blocking, replace each well with 300 μ L of secondary antibody cocktail (see **Supplementary materials**). Parafilm the plate and return to shaker for overnight incubation.
7. On the following day, remove secondary antibody solution and replace with 500 μ L of 1x PBT solution to each well. Perform 4 separate 1 hour washes with 500 μ L of PBT to each well. Following the washes, add 300 μ L of DAPI solution to each well and place on the shaker for a 1-hour incubation. Remove DAPI solution and replace each well with 500 μ L of PBS. Store plate at 4°C until needed for clearing and imaging.
8. On the day of histology imaging, remove the plate from 4°C refrigeration and remove PBS from each well. Transfer microtissues to 1.5 mL microcentrifuge tubes and add a minimum of 500 μ L of clearing solution #1 (see **Supplementary materials**) and place on the shaker for 15 minutes (see **Discussion -12**).
9. Remove clearing solution #1 and replace with a minimum of 500 μ L of clearing solution #2 (see **Supplementary materials**), place on the shaker for 5 min incubation.
10. Remove 500 μ L of clearing solution #2 and replace with a fresh 500 μ L clearing solution #2 and place back on the shaker for 30-60 min (microtissue should no longer be opaque).
11. Microtissues can then be imaged for immunohistochemical labeling of cell type-specific antigens of interest.

2.5 Discussion

1. We chose to 3D print the lift piece in PLA, however, we note that other common printing filaments would be sufficient as well. The piece was designed using AutoDesk Fusion360 software. The well dimensions (inner well diameter and inner well depth) were taken from the plate manufacturer and used to determine the diameter of the lift piece as well as the lift height however this must be optimized if using different plate manufacturers or different printing filaments. Several iterations of the print were necessary due to the limited printing resolution of the extrusion printer used (Prusa i3mk3). Before agarose molds were made, the PLA-lift pieces were lightly sprayed with 70% ethanol, allowed to air dry in the biosafety cabinet, and then placed under UV light for 20 min to decontaminate before use.
2. The Tokai Hit STX stage top incubator was selected due to its compatibility with the Olympus FV3000 Confocal microscope. Depending on the microscope manufacturer, a different environmental chamber can be used for live imaging. However, it is recommended that for any imaging session lasting 30 minutes or longer, temperature and CO₂ should be provided to the plate to ensure microtissue health. We observed distinct differences in whole tissue firing rates of microtissues that were imaged at room temperature and ambient oxygen levels for periods longer than 30 minutes.
3. Depending on the specific antibodies selected for immunolabeling, we suggest doubling the concentration recommended by the vendor for 2D immunofluorescent labeling. We provide specific concentrations used for a triple-antibody primary cocktail used to label neuronal nuclei, astrocytes, and microglia.
4. Media should be changed a minimum of 2 times prior to seeding the cells into the agarose molds. Once the agarose molds have been made, the plate needs to be stored in a warm, humid environment (preferably a sterile autoclave). We have found that evaporation of the

media causes a precipitate to form on the agarose mold thus we recommend storing in the incubator until cell seeding.

5. Moving as quickly as possible when collecting the cortical tissue is important. We have found the most efficient workflow is to collect whole brains from the entire litter into 35 mm petri dishes (2 brains per 35 mm dish with 4 mL of HA-B27 media), then move on to dissect away the cortices. It is best to use the scissors for midline incision along the scalp and skull before scooping out the whole brain with the scooped spatula. To remove the dura, pinch with the forceps to make a gentle incision into cortical tissue. Then using the reverse dull side of the forceps, gently tease away the dura, followed by dissection of the cortex from each hemisphere.
6. Prepare the Papain solution after all the whole brains have been collected into 35 mm petri dishes and before the cortical dissection begins. Be sure to wait for the Papain to fully go into solution before transferring to a sterile 15 mL conical tube and placing in the 30°C water bath. We typically use 6 mg of papain for 8-10 sets of cortices (whole brains).
7. If performing a large dissociation (10 or more pups) it is suggested to use two filters for the first filtration step as there will be more cell debris to clog the filter. It is fine to use more than 5 mL of HA-B27 to wash the filters during this first step. At this point, now that the cells are in a single cell suspension, we recommend using only a p1000 tip for transfer of liquids and cells as the wider-bore opening of the tip will minimize shearing stress to the cells and help to maintain viability.
8. To perform cell count, we recommend a 1:4 dilution of cell suspension into CCM, then an additional 1:2 dilution into trypan blue. The total dilution factor for cell counting is then 1:8.
9. To perform a media change, remove all media from the top of agarose and replace. Do not remove any media that remains within the microfeature inner well. We recommend feeding microtissues every other day. However, allowing the microtissues to sit an extra day (e.g., over the weekend) and be put on a MWF feeding schedule has not proven harmful to the

microtissue health and overall viability. Additionally, it is recommended that microtissues are not fed immediately before a live imaging session. If microtissues need to be fed on an imaging day, we suggest feeding after the imaging session is complete.

10. Using curved forceps, carefully pierce the sides of the agarose, taking care not to disturb the microtissue at the center, and using a “corkscrew” motion gently lift upward to release the agarose mold from the bottom of the well. Transfer to a clean surface to cut away the excess agarose prior to melting.
11. For transfer of free-floating microtissues, use a clean razor blade to cut the end of a p1000 pipette tip, then use the pipette with cut tip as normal. The opening should be just larger than the microtissue diameter.
12. Due to the high viscosity of the clearing solutions, it is difficult to achieve adequate movement of the microtissue in the solution while on the plate shaker. To mitigate this, we have found it best to use 1.5 mL centrifuge tubes and to tape them together in a round sphere shape, packed with foil. This foil ball, containing the microcentrifuge tubes at the center, will be free to roll around the plate shaker tray and allow the clearing solution to adequately slosh around the microtissues in each tube.

2.6 Author Contributions

SB, EA and DB conceptualized the protocol. SB and EA wrote and worked the protocol. SB and EA made the figures. SB wrote the first draft of the protocol chapter.

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2.9 Supplementary Information

Materials

Agarose injection molds

1. Agarose solution: 1 g Ultra-Pure agarose powder, autoclaved in 500 mL glass bottle, 50 mL sterile 1x Dulbecco's phosphate buffered saline (PBS)
2. Stainless Steel Injection Mold: machined stainless-steel mold with 6 concentric peg holes of 600 μm diameter at one end of a 24.3 mm long hollow cylinder with a 3 mm diameter circular base.
3. Custom 3D-printed PLA-lift piece: 3D-printed piece to "lift" the injection mold approximately 200 μm from the bottom of the glass culture plate. The lift is a circular riser that press fits temporarily into the inner diameter of a single well on a 24 well glass-bottom plate. (see **Note 1**)
4. 24 well culture plate with #1.5H coverslip glass bottom (0.17 ± 0.005 mm) (CellVis, P241.5HN)
5. 5 cc sterile syringe
6. 11.43 cm long and 2 mm diameter stainless-steel rod
7. 0.5 cc sterile syringe with $\frac{1}{2}$ " 29 gauge needle
8. Ice or ice pack

Animals

1. Timed pregnant Sprague Dawley female rats were purchased from Charles River laboratory. Rats arrive between embryonic day 15 and 17 and they are subsequently monitored everyday thereafter until pups are born. Pups are collected no later than postnatal day 2.

Tissue dissociation

1. Hibernate® A-B27 Solution (50 mL recipe): 48.9 mL Hibernate® A media (Transnetyx Tissue Inc), 0.125 mL GlutaMAX™ (Thermo Fisher Scientific), 1 mL 1x B27 Neuronal supplement

(Thermo Fisher Scientific). Solution can be made in advance but recommended that B-27 supplement is added on the day of dissociation.

2. Papain Solution: 6mg of lyophilized papain powder (Transnetyx Tissue Inc.), 3 mL Hibernate®

A minus Calcium (Transnetyx Tissue Inc.). Add 3 mL of cold Hibernate® A minus Ca to lyophilized papain, mix well by inversion. Wait until solution becomes clear then place vial into 30°C water bath 10 min prior to use.

3. Complete Cortical Media (CCM) (50 mL recipe): 48.4 mL Neurobasal™-A medium, minus phenol red (Thermo Fisher Scientific), 0.125 mL GlutaMAX™, 0.5 mL Penicillin Streptomycin, 1 mL 1x B27 Neuronal supplement.
4. Tissue Dissection kit: sharp-pointed delicate dissection scissors, two curved precision tip forceps, stainless steel laboratory spatula with scoop, sterile gauze pads, sterile razor blades.
5. Tissue Processing Supplies: sterile 40 µm filters, sterile 50 mL and 15 mL conical tubes, hemocytometer, sterile 35 mm Petri dish, serological pipettes, p1000 and p200 pipettes and tips, trypan blue dye.

Adeno-associated viral transduction

1. Adeno-associated viral particles are stored in -80 °C in small-volume aliquots. For day-of transduction, virus is thawed on ice and diluted into CCM to reach the final desired viral genome per milliliter concentration.
2. Ice

Live confocal microscopy

1. Olympus FV3000 confocal laser scanning microscope
2. Stage Top Incubator (Tokai Hit STX stage top incubator) (see **Note 2**)

Immunohistochemistry

1. Fixative (50mL recipe): 4 g sucrose, 45 mL 1xPBS, 5 mL formalin to create an 8% sucrose, 4% paraformaldehyde solution

2. Blocking solution (10 mL recipe): 0.4g bovine serum albumin, 8.9 mL 1xPBS, 1 mL normal goat serum, 100 μ L TritonX-100.
3. PBS with 0.2% TritonX -100 (1xPBT) solution (500 mL recipe): 500 mL of 1x phosphate buffered saline, 100 μ L of TritonX-100.
4. Primary Antibodies: mouse anti-NeuN (Milipore, MAB377), chicken anti-GFAP (Milipore, AB5541), Rabbit anti-Iba1 (FujiFilm, 019-19741). Dilute antibodies to desired concentration in blocking solution. (see **Note 3**)
5. Secondary Antibodies: anti-mouse CY3 (JacksonImmuno ImmunoResearch, 115-165-068), anti-chicken AlexaFluor 488 (Thermo Fisher Scientific, A11039), anti-rabbit AlexaFluor 635 (Thermo Fisher Scientific, A31577), DAPI (Thermo Fisher Scientific, D1306). Dilute antibodies to desired concentration in blocking solution. (see **Note 3**)

Clearing Solutions

1. 50 % formamide (2mL recipe): 1 mL 100 % formamide solution, 1 mL PBS
2. 10 % Polyethylene glycol (PEG)/25 % Formamide (2 mL recipe): 1 mL of 20 % PEG solution, 1 mL 50 % formamide
3. 20 % PEG/50 % formamide (2 mL recipe): 1 mL of 40 % PEG solution, 1 mL of 100 % formamide.

Chapter 3

A 3D cortical microtissue model uncovers microglia-driven synaptic remodeling and disruption in Alzheimer's disease

3.1 Preface

Designed as dynamic regulators of essential brain function, microglia surveil and modulate the surrounding microenvironment in unique spatiotemporal patterns throughout development and in disease. Despite decades of evidence implicating their role in neurodegenerative disease, the pathophysiological basis of microglia in surveillance, phagocytosis, and synaptic remodeling in Alzheimer's disease (AD) remains unclear. This chapter presents the application of a novel 3D primary cortical microtissue platform to study microglia-driven synaptic remodeling under both healthy and AD-relevant conditions. By leveraging a custom-engineered scaffold-free culture system, we enable long-term *in vitro* modeling of neuron–glia interactions with high-resolution imaging and computational tools for quantitative analysis of synaptic maturation and pruning.

Our findings demonstrate that early neuronal network connectivity within the microtissue environment is shaped by microglia-mediated synaptic pruning. However, in AD-derived microtissues generated from TgF344-AD rats, we show that this process becomes dysregulated, contributing to synaptic loss and neuronal impairment. Further by using primary cortical cells from the TgF344-AD rat model (expressing human APP and PS1 mutations) we demonstrate that AD-derived microtissues recapitulate hallmark AD pathology including amyloid beta aggregation and microglia activation—offering new insights into the early mechanisms of disease progression. By integrating advanced imaging techniques with real-time functional assays, this system offers new

insights into the spatiotemporal dynamics of microglial-mediated synaptic dysfunction in AD. Moreover, this platform provides a valuable tool for exploring a multitude of neurodevelopmental and neurodegenerative disease mechanisms in addition to evaluating potential therapeutic interventions aimed at preserving synaptic integrity in neurodegenerative disorders.

Below is the submitted manuscript

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3.2 Abstract

Synaptic pruning by microglia refines neural circuits that underpin learning, memory, and cognitive function. Yet, the rapid and dynamic interplay between neurons and glial cells—and how these interactions are disrupted in inflammatory conditions such as Alzheimer's disease (AD)—remains poorly understood due to the absence of physiologically relevant models of disease and

analytical tools to interpret the results. Here, we present a novel microphysiological system enabling long-term culture of 3D primary cortical microtissues, fabricated using a custom injection mold generating scaffold-free tissues. The platform design enables real-time, high-resolution imaging of cell-type specific morphology and neuronal calcium activity while integrating custom computational tools for automatic analysis of synaptic maturation and pruning. Our methodology faithfully recapitulates the intricate, tightly controlled processes of neuronal circuitry formation observed under normal developmental conditions. In AD-relevant microtissues derived from rats harboring hAPP and PS1 mutations, we observed significant aberrations in synaptic pruning driven by microglial dysfunction. These AD mutant microtissues also mimic amyloid pathology by forming extracellular A β aggregates, which trigger microglial activation that led to synaptic failure and neuronal loss. Moreover, our data demonstrate that early glial reactivity combined with excessive synapse elimination disrupts network maturation, offering new insights into the pathogenetic mechanisms underlying synaptic loss and cognitive impairment in AD. Importantly, this platform can be readily disseminated and adapted for broader applications, offering a versatile tool for investigating glial-neuronal interactions and testing therapeutic interventions across a range of neurodevelopmental and neurodegenerative disorders.

Keywords

Microphysiological system (MPS); 3D culture model; Cortical microtissues; Synaptic pruning; Microglia; Calcium imaging; Alzheimer's disease; Neuron-glial interactions

3.3 Introduction

The precise organization of neuronal networks during brain development and their maintenance throughout life relies on coordinated interactions between neurons and glial cells. Microglia are highly dynamic glial cells that play a critical role in shaping synaptic architecture by actively surveilling the extracellular environment, engaging in synapse remodeling by pruning

excess or weak synapses to refine neural circuits, and responding to pathological stressors (Paolicelli et al. 2011; Filipello et al. 2018; Wu et al. 2015; Schafer et al. 2012). However, when microglial function is disrupted, as observed in Alzheimer's disease (AD) and other neurodegenerative disorders, aberrant synaptic pruning can lead to synapse loss and cognitive decline (Hong et al. 2016; Dejanovic et al. 2022; Rajendran and Paolicelli 2018; Sun et al. 2023). Understanding how microglia maintain or disrupt synaptic architecture is therefore a critical step in deciphering both the physiological mechanisms of brain maturation and the pathogenesis of neurodegenerative diseases.

Current knowledge of microglia-mediated synaptic pruning highlights several molecular pathways, including complement signaling, which tags supernumerary synapses for removal by microglia (Filipello et al. 2018; Stevens et al. 2007; Paolicelli et al. 2011; Soteros and Sia 2022). Studies have linked abnormal reactivation of these pathways to excessive synaptic loss in AD, pointing to a central role for complement proteins, cytokines, and other inflammatory mediators in regulating AD-associated microglia-synaptic dysfunction (Hong et al. 2016; Wen, Bi, and Shen 2024; Filipello et al. 2018; Dejanovic et al. 2018; 2022). However, the precise interplay between microglial states, neuroinflammatory signaling, synaptic targets, and neuronal activity in both development and disease remain poorly understood. A key challenge lies in capturing the rapid, dynamic nature of these interactions in a physiologically relevant setting. Existing *in vitro* and *in vivo* models often lack the ability to simultaneously track high-resolution structural changes and real-time functional readouts of microglia-driven synaptic remodeling over extended periods.

To address these limitations, we utilized 3D microphysiological primary cortical culture model that enables the long-term co-culture of neurons and glial cells in a scaffold-free environment. By combining a custom micro-molding culture technique that is compatible with advanced imaging platforms, this model enables real-time, high-resolution monitoring of both morphological and functional parameters of a 3D primary cortical culture (Brown, Atherton, and

Borton 2023). Crucially, it preserves the complex architecture and cellular diversity necessary for microglial involvement in synaptic remodeling. This approach creates an environment that closely resembles the complex composition of brain tissue, thus allowing for nuanced investigations into how microglia regulate synaptic connectivity under both healthy and disease-relevant conditions.

Using this 3D culture system, our findings show that we faithfully recapitulate the tightly controlled processes of neuronal circuitry formation observed under normal developmental conditions. In AD-relevant microtissues derived from the TgF344-AD rat model, we observed significant aberrations in synaptic pruning driven by microglial dysfunction. These TgF344-derived microtissues also mimic amyloid pathology by forming extracellular A β aggregates and microglial activation that ultimately led to synaptic failure and neuronal loss. Moreover, our data demonstrate that early glial reactivity and excessive synapse elimination disrupts network maturation, offering new insights into the pathogenetic mechanisms underlying synaptic loss and cognitive impairment in AD. Importantly, this platform provides a versatile tool for investigating glial-neuronal interactions and testing therapeutic interventions across a range of neurodevelopmental and neurodegenerative disorders.

3.4 Results

3.4.1 3D primary cortical microtissues contain diverse glial cell populations that mature over the first 8 weeks *in vitro*

In this study, we sought to investigate microglia influence in mediating synaptic circuits within a 3D primary cortical culture system. Using a previously established primary cortical cell culture protocol (Brown, Atherton, and Borton 2023) we created 3D cortical microtissues from primary tissue collected from the postnatal rat brain and a scaffold-free agarose micro-mold culture system. Upon seeding into the agarose micro-mold, cells will not adhere to the agarose but rather

within 24 hour they begin to secrete ECM proteins such as laminin and collagen and self-assemble in the space around the pegs forming a unique “trampoline-like” geometry that is approximately 800um wide at the center and 300um thick (**Fig. 3.1, a**). The unique microtissue geometry promotes equibiaxial tension of cellular forces and is characterized by cellular organization and extracellular matrix fiber alignment that is uniform in all directions (Schell et al. 2016). The 6 upright pegs oriented at the bottom of the agarose micro-molds prevent tissue contraction into a free-floating spheroid, physically constraining the microtissues within 150um of the glass bottom of the multi-well plate and thus enabling compatibility with longitudinal live-imaging assays using inverted confocal microscopy.

Using this 3D *in vitro* cortical model, we sought to characterize glial cell-type diversity and morphological maturation in microtissues cultured to 2, 7, 28, and 60 days *in vitro* (DIV). We began by assessing astrocyte populations using immunohistochemical staining for glial fibrillary acidic protein (GFAP) (**Fig. 3.1, b**). Although astrocyte immunoreactivity for GFAP is known to be lower in rat CNS tissue through postnatal day 7, we observe a distinct population of GFAP+ cells at both DIV2 and DIV7 without a significant change in the number of cells between the two time points (**Fig. 3.1, f**). However, we did observe distinct morphological maturation and arborization of astrocytes between DIV7 and DIV28 that coincides with a significant increase in cell number ($p=0.0057$) (**Fig. 3.1, f**). Due to limited GFAP expression in fine processes of astrocytes (Sofroniew and Vinters 2010), we confirmed astrocyte-arbor morphogenesis using AAV-mediated expression of a GFP reporter protein to visualize fine processes (**Supplemental Fig. 3.12.1**). We then characterized the microglia populations by assessing immunoreactivity to ionized-calcium binding adaptor molecule 1 (Iba1) (**Fig. 3.1, c**). No significant change in Iba1+ microglia cells was observed between DIV2, 7, 28 and 60 (**Fig. 3.1, g**). However, a significant shift in microglia morphological state from reactive and amoeboid to homeostatic and ramified occurred between DIV2 and DIV28. Sholl analysis indicated significant reductions in the number of processes at

radii between 15µm and 45µm away from the cell soma in DIV2 microglia compared to DIV28 ($p < 0.0001$) indicative of amoeboid and less ramified morphologies (**Supplemental Fig. 3.12.2**).

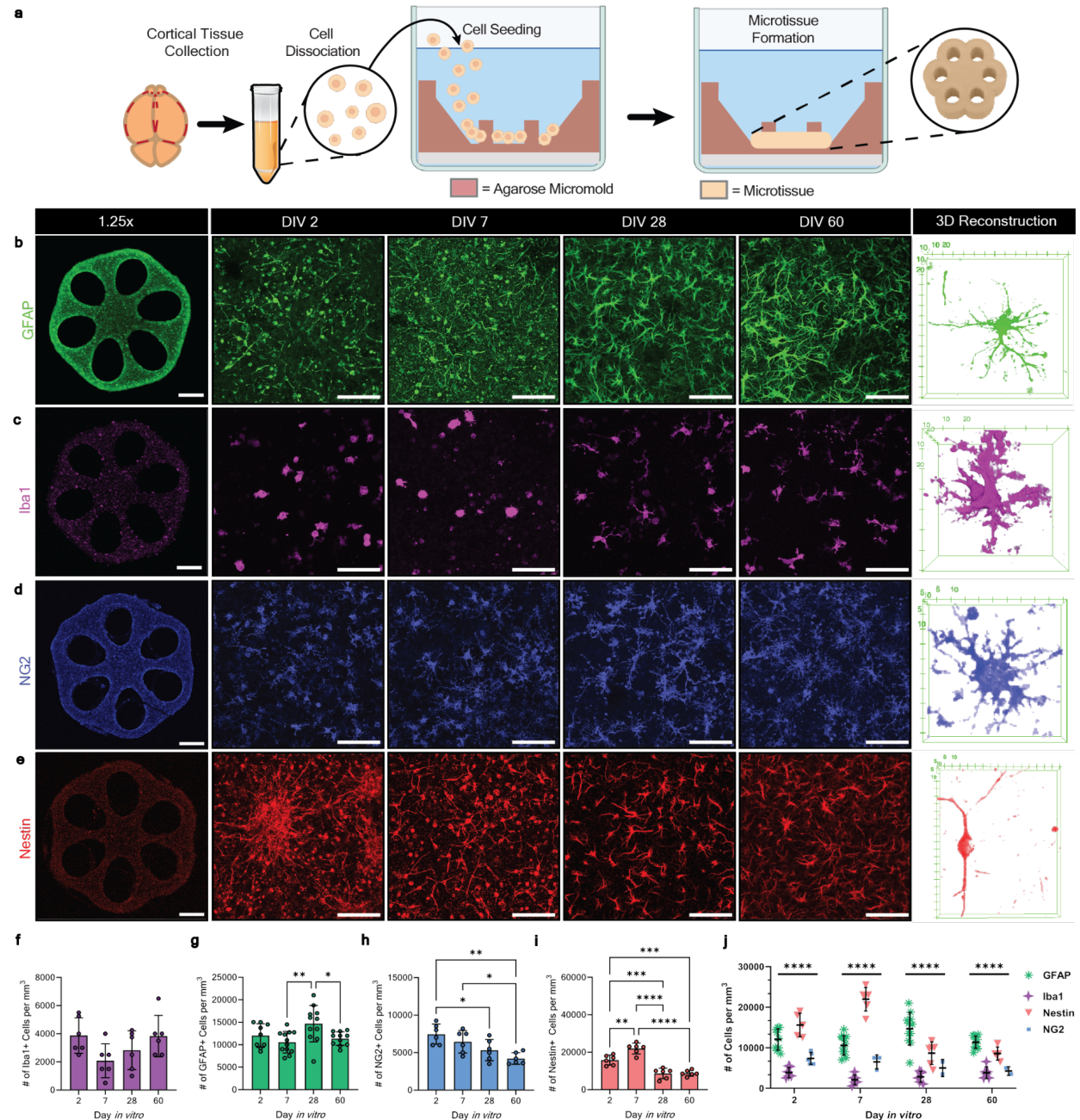


Figure 3.1 | Microtissues exhibit cellular diversity and morphological maturation across 2 months *in vitro*

a, Microtissue formation from postnatal rat cortical tissue. Seeding of single cell suspension into agarose micro-molds. Immunofluorescent images of **(b)** GFAP+ astrocytes, **(c)** Iba1+ microglia, **(d)** NG2+ OPCs, and **(e)** nestin+ neural precursor cells. Left most panel immunofluorescent images of whole microtissue taken at 1.25x (scale bar = 500µm) followed by maximum intensity projection images taken at 30x between DIV2, 7, 28, & 60 (scale bar = 100µm). Example 3D rendering of individual cells in right most panel. f-i,

Average number of **(f)** GFAP+, **(g)** Iba1+, **(h)** NG2+, and **(i)** Nestin+ cells per mm³ with one-way ANOVA followed by Tukey's post-hoc comparison: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. **j**, Two-way ANOVA comparison of number of cells per mm³ shows significant effect across cell types at DIV2, 7, 28, & 60 ****p<0.0001. Data is represented at mean $\pm$ standard error of the mean with an n $\geq$ 6 microtissues for each time point.

Oligodendrocytes are an equally important glial cell that, despite being highly implicated in white matter abnormalities, neurodegeneration, and cognitive decline associated with AD, are often excluded from 3D neural culture systems due to their complex differentiation process, long maturation time, and challenges in achieving functional myelination *in vitro*. Therefore, we sought to characterize the presence of oligodendrocytes in the microtissues. As the chondroitin sulphate proteoglycan neural/glial antigen 2 (NG2) is a type I membrane protein expressed in the developing and adult rat CNS by oligodendrocyte precursor cells (OPCs), we characterized microtissue OPC populations by NG2 immunoreactivity. Populations of OPCs were detected as early as DIV2 with no significant change in cell numbers detected through DIV60 (**Fig. 3.1, d&h**). Remarkably, the OPCs maintained a stellate morphology with ramified processes throughout the first 2 months of culture. We further investigated whether OPCs differentiated into mature oligodendrocytes and found that, by DIV28, a myelin basic protein (MBP+) and NG2- population emerged. Strikingly, this population emerged with evidence of MBP along neurites (**Supplemental Fig. 3.12.4**), suggesting possible signs of early myelination sheath formation.

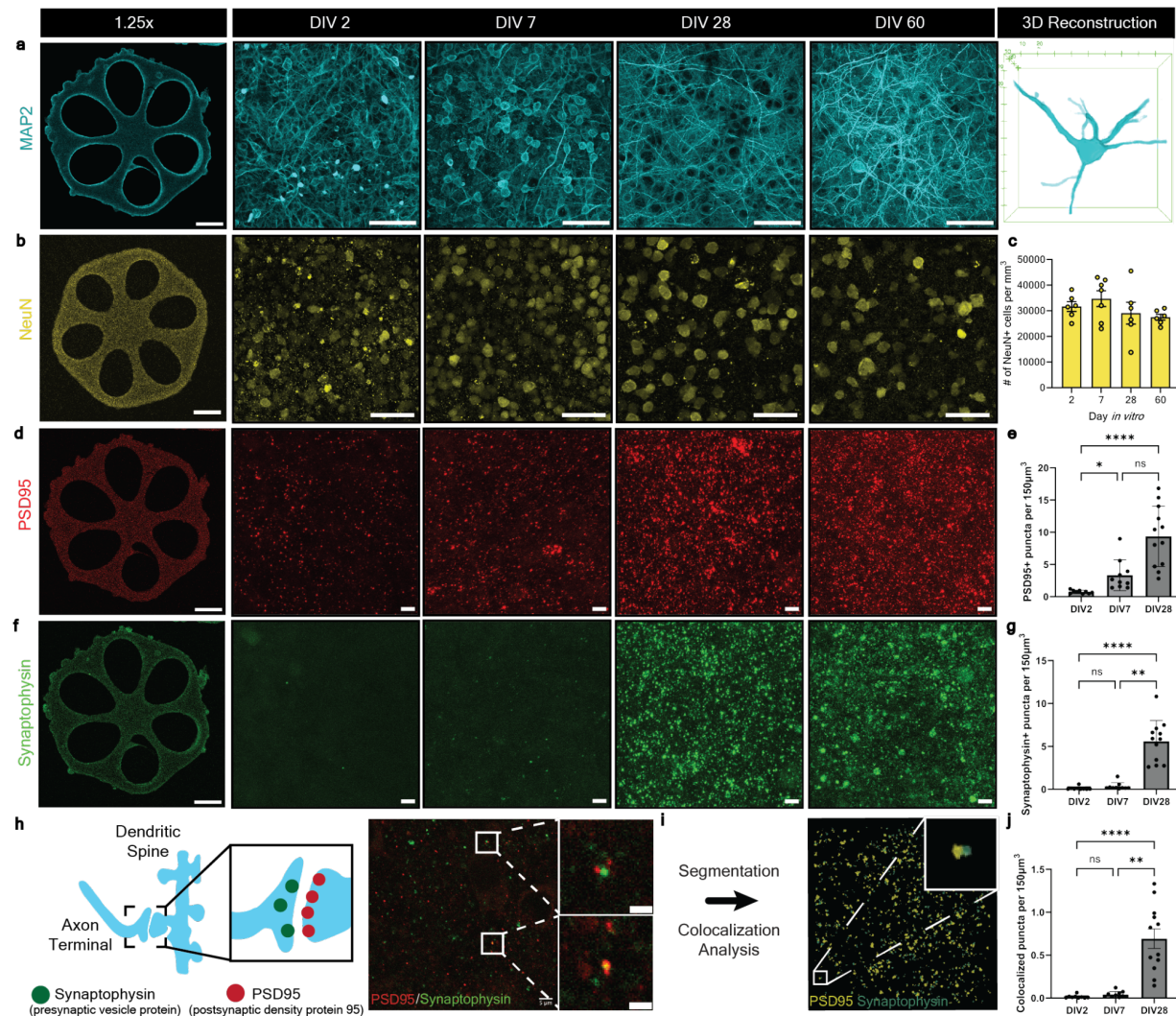
Finally, we characterized populations of nestin+ neural progenitor cells (NPCs) (**Fig. 3.1, e**). In the embryonic (E15-18) developing rat cortex, nestin is primarily expressed in proliferating NPCs within the cortical neuroepithelium and in radial glial in early postnatal days (Lendahl, Zimmerman, and McKay 1990; Frederiksen and McKay 1998; Rao 1999; Hendrickson et al. 2011). As NPCs differentiate into mature neurons and astrocytes, nestin expression gradually decreases in the cortex. We observed early colocalization of GFAP and nestin expression in cells at DIV2 and DIV7 (**Supplemental Fig. 3.12.3**) with significant decrease in nestin+ cells as microtissues matured from DIV7 to DIV28 (p<0.0001) and DIV60 (p<0.0001) (**Fig. 1i**). This loss in nestin+ cells, coincident with the increase in GFAP+ astrocytes, follows a trend of progenitor-to-glial

differentiation and proliferation that is seen in the first postnatal month *in vivo* (Landry, Ivy, and Brown 1990; Miller and Gauthier 2007; Levison and Goldman, 1993). Together, the data show that microtissues contain diverse glia populations including astrocytes, microglia, and oligodendrocyte precursor cells that morphologically mature over the first 28 days *in vitro*.

3.4.2 Cortical microtissues exhibit neuronal and synaptic maturation

After characterizing glia cellular diversity and morphological maturation in the microtissues, we then sought to quantify the neuronal and synaptic protein densities from DIV2 to DIV60. We determined the neuronal cell populations via immunohistochemical labeling of microtubule-associated protein 2 (MAP2) and neuronal nuclear protein (NeuN) and (**Fig. 3.2, a&b**). In the developing rat cortex, MAP2 is primarily localized in the soma and dendrites and plays critical roles in stabilizing microtubules in dendritic arbors, as well as promoting neurite outgrowth and synaptic development. As neurite outgrowth is a defining feature of neuronal morphological maturation and the structural foundation of neural connectivity, we quantified numbers of NeuN+ cells and qualitatively assessed MAP2-defined morphological features at between DIV2 and DIV60. Importantly, we did not see significant changes to the number of neuronal nuclei across 2 months in culture (**Fig. 3.2, c**). However, we did observe noticeable neurite outgrowth between DIV2 and DIV60 (**Fig. 3.2, a**), suggesting neuronal survival and maturation in the microtissue microenvironment. We then measured the densities of the postsynaptic density protein 95 (PSD95), which serves as a scaffolding protein to anchor AMPA and NMDA receptors in glutamatergic synapses and has been spatially and temporally associated with dendritic spine morphogenesis (Chen et al. 2015; Pak et al. 2001; Cane et al. 2014). Along with PSD95, the presynaptic vesicle protein synaptophysin density was quantified from DIV2 to DIV28 using custom analysis software (see methods) to segment and count the number of synaptic puncta from immunolabeled images. A significant increase in postsynaptic density

occurred between DIV2 and DIV7 ($p=0.0292$) and between DIV2 and DIV28 ($p<0.0001$) with no significant change between DIV7 and DV28 ($p=0.0806$) (**Fig. 3.2, e**). Additionally, a significant increase in the presynaptic density was observed between DIV7 and DIV28 ($p=0.0056$) as well as DIV2 and DIV28 ($p<0.0001$) but no significant change between DIV2 and DIV7 ($p=0.2572$) (**Fig. 3.2, g**) reflecting the temporal patterns of pre and postsynaptic protein expression changes characterized in the early *in vivo* postnatal rat cortex (Pinto, Jones, and Murphy 2013).



by maximum intensity projection images taken at 30x (MAP2, NeuN) and 60x (PSD95, Synaptophysin) between DIV2, 7, 28, & 60 (scale bar = 50µm for MAP2 and NeuN images and scale bar = 5µm for PSD95 and Synaptophysin). **h**, spatial colocalization of pre and postsynaptic puncta can be easily detected in single z plane of confocal fluorescent image (scale bar = 2µm). **i**, application of open-source puncta segmentation and colocalization software allow for automated quantification of apposed synaptic puncta in x, y, and z coordinate system of densely labeled confocal fluorescent volumetric images. **j**, spatially colocalized synaptic puncta in microtissues at DIV2, 7, and 28. Kruskal-Wallis followed by Dunn's post-hoc comparison: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ for number of synaptic puncta (individual or colocalized) per 150µm³. Data is represented at mean ± standard error of the mean with an $n \geq 6$ microtissues for each time point.

Formation of functional synapses requires the close alignment of both pre- and postsynaptic compartments. In the developing rat cortex, functional synapses begin to appear in the first postnatal week, with rapid formation occurring during the second postnatal week coinciding with eye opening (Pinto, Jones, and Murphy 2013). Both *in vitro* and *in vivo* studies have focused on the colocalization of pre and postsynaptic markers as a readout of mature synapse formation (**Fig. 3.2, h**) (Flavell et al. 2006; Nieland et al. 2014; Paradis et al. 2007; Schätzle et al. 2012; Sharma et al. 2013; Verstraelen et al. 2020; 2018; Savage et al. 2024; Verschuuren et al. 2019). Following quantification of both synaptophysin+ (presynaptic) and PSD95+ (postsynaptic) puncta from a single multichannel image, the two segmented channels are merged to identify spatially colocalized pre and postsynaptic puncta by using a seed point–to–seed point distance threshold of 0.6µm (**Fig. 3.2, i**) (**see methods and Supplemental Fig. 3.12.5**). A significant increase in the number of colocalized pre and post synaptic puncta occurred between DIV7 and DIV28 ($p = 0.0036$) and between DIV2 and DIV28 ($p < 0.0001$) (**Fig. 3.2, j**).

3.4.3 TgF344-AD derived microtissues exhibit glial reactivity that precedes increased amyloid pathology and synaptic dysfunction

We next sought to create an AD-relevant 3D microenvironment by incorporating familial AD mutations in the amyloid-β precursor protein (*APP*) and presenilin 1 (*PSEN1*) genes. Previous stem-cell based approaches have demonstrated that expression of *APP* and *PS1* in neural progenitor or mixed neuron-glia cultures exhibit amyloid plaque-like formations by 6 weeks

(Essayan-Perez et al. 2019; Lomoio et al. 2023; Choi et al. 2014; Park et al. 2018) and phosphorylated tau aggregation by 10 weeks (Choi et al. 2014). To this end we generated amyloidogenic primary cortical microtissues from the double-mutant humanized TgF344-AD rats (Cohen et al. 2013) (**Fig. 3.3, a**) which carry human *APP* and *PSEN1* mutations and demonstrate amyloid plaques by 4 months (Fang et al. 2023a; Fang et al. 2023b) as well as phosphorylated tau and neuronal loss by 16 months *in vivo* (Cohen et al. 2013). Additionally, several studies have reported gliosis of astrocytes and microglia, blood-brain barrier decoupling (Fang et al. 2023b; Bac et al. 2023; Van den Berg et al. 2022; Fang et al. 2023a; Cohen et al. 2013; Joo et al. 2017; Chaney et al. 2021) and synaptic dysfunction, including altered cortical excitatory-inhibitory balance, enhanced long-term potentiation at medial perforant path synapses, and increased excitability in dentate granule cells (Van den Berg et al. 2022; Joo et al. 2017; Smith and McMahon 2018; Muñoz-Moreno et al. 2018; Bazzigaluppi et al. 2018). However, these studies offer limited insight into the structural synaptic landscape that coincides with cognitive deficits (Saré et al. 2020) as well as the temporal dynamics and possible interplay between glial activation, amyloid pathology, and synaptic dysfunction.

TgF344-AD derived microtissues began to exhibit activation of astrocytes, microglia and OPCs as early as DIV28 (**Fig. 3.3, b**). Specifically, glial reactivity was marked by astrocytic hypertrophy and increased, albeit insignificant, GFAP mean fluorescence intensity (MFI) ($p=0.4320$) (**Fig. 3.3, d**) and ameboid-shaped Iba1+ microglia with significantly fewer distal processes ($p<0.0001$) (**Fig. 3.3, e**). Astrocyte and microglia reactivity in TgF344-AD microtissues persisted at DIV80 compared to wildtype (**Fig. 3.3, c** and **Supplemental Fig. 3.12.6**). However, we additionally saw a significant decrease in the number of OPC cells at DIV80 ($p=0.0026$). We note that although morphological classification of OPC reactivity in AD is not fully understood, evidence of OPC hypertrophy, increased process branching, and a significant reduction in cell density has been shown in APP/PS1 mouse models (Dong et al. 2018; Li et al. 2013; Chacon-

De-La-Rocha et al. 2020). Interestingly, no significant differences in GFAP+ and Iba1+ cell densities were detected between wildtype and TgF344-AD microtissues at either DIV28 or DIV80 (Supplemental Fig. 3.12.6). Although glial reactivity appeared as early as DIV28, we did not observe a loss of NeuN+ cells until DIV80 ($p=0.0005$) (Fig. 3.3, g).

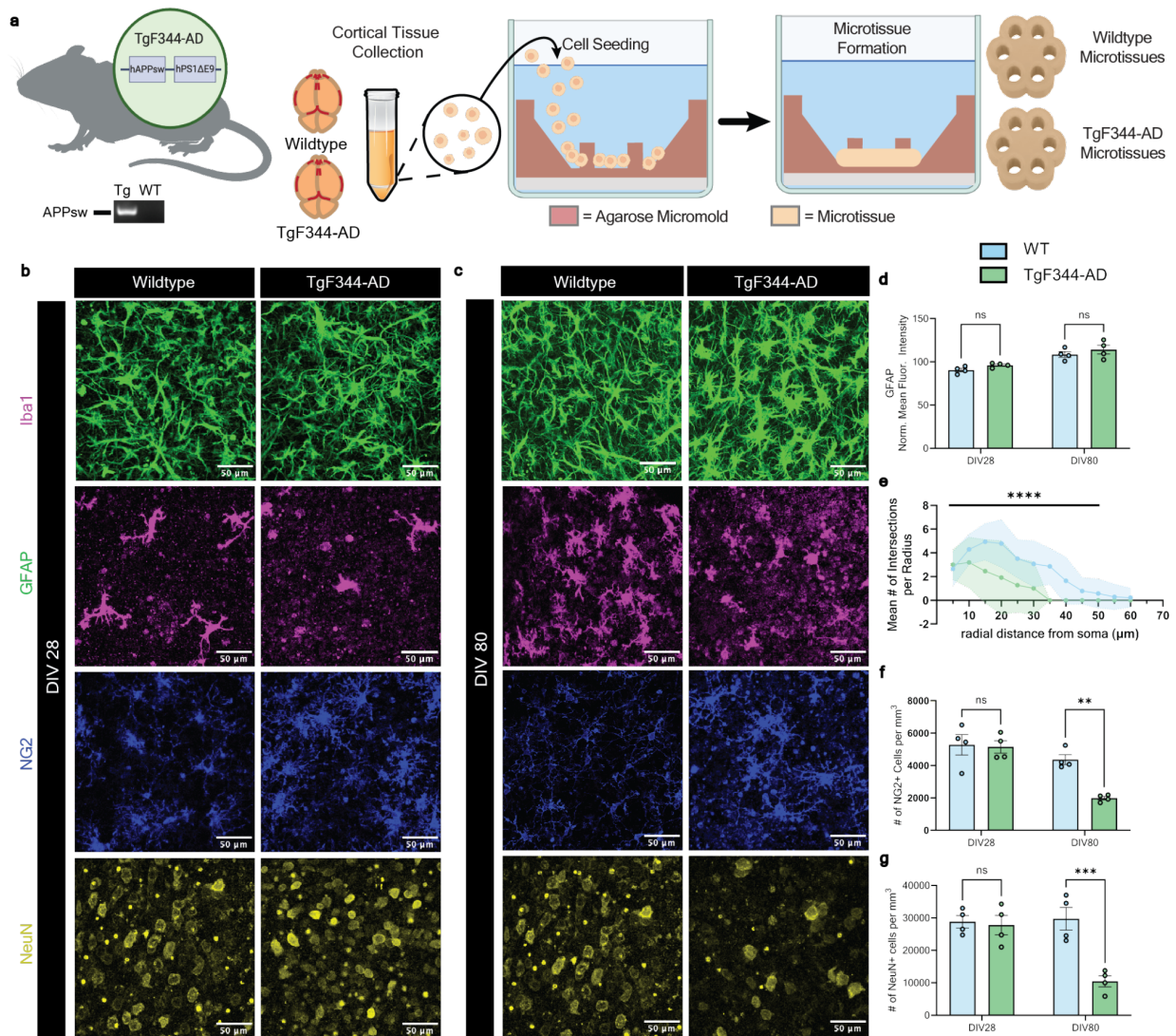


Figure 3.3 | AD-relevant microtissues exhibit early glial reactivity

a, Wildtype and TgF344-AD microtissue formation. **b**, Confocal fluorescent images of Iba1+ microglia, GFAP+ astrocytes, NG2+ OPCs and NeuN+ neurons collected from wildtype and TgF344-AD microtissues at DIV28. **c**, Confocal fluorescent images of Iba1+ microglia, GFAP+ astrocytes, NG2+ OPCs and NeuN+ neurons collected from wildtype and TgF344-AD microtissues at DIV80. **d**, Mean GFAP fluorescence intensity from maximum intensity projection confocal fluorescent 30x images of wildtype vs TgF344-AD microtissues. **e**, Sholl analysis from individual microglia shows average number of branch intersections over concentric circles at increasing radii in wildtype vs TgF344-AD microtissues at DIV28. Shaded region delineates standard deviation with $n=5$ individual microglia per microtissues with $n=3$ microtissues per

genotype for each time point. Following significant effect by two-way ANOVA, Sidaks' post-hoc comparison was completed where **** $p < 0.0001$. **f**, Average number of NG2+ cells per mm^3 in wildtype vs TgF344-AD microtissues at DIV28 and DIV80. **g**, Average number of NeuN+ cells per mm^3 in wildtype vs TgF344-AD microtissues at DIV28 and DIV80. Two-way ANOVA followed by Tukey's post-hoc comparison: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ for mean GFAP fluorescence intensity and number of NG2+ and NeuN+ cells per mm^3 . Data is represented at mean $\pm$ standard error of the mean with an $n \geq 4$ microtissues per genotype for each time point.

In conjunction with significant neuronal loss, we detected amyloid-plaque-like features in TgF344-AD microtissues at DIV80 ($p < 0.0001$) which closely resemble plaques detected in the cortex and hippocampus of TgF344 rats at 4 months (Fang et al. 2023a) (**Fig. 3.4, a&c**). These plaque-like depositions of aggregated A β 42 were found to be in physical contact with Iba1+ microglia (**Fig. 3.4, b**) suggesting a resemblance to the pro-inflammatory microenvironment surrounding amyloid plaques (Keren-Shaul et al. 2017; Clayton et al. 2021; Grubman et al. 2021; Plescher et al. 2018). We note that gliosis appeared as early as DIV28 and coincided with an

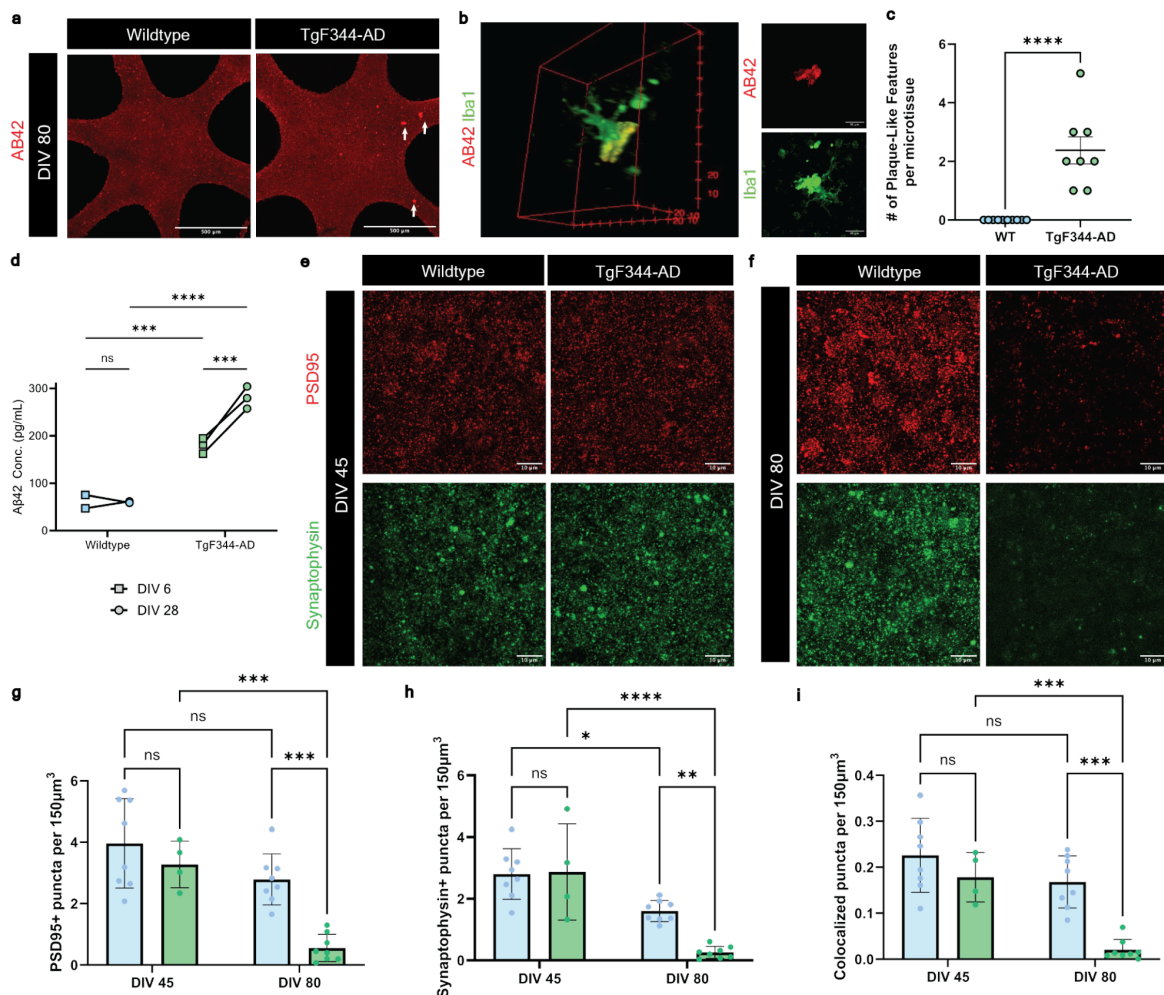


Figure 3.4 | AD-relevant microtissues display amyloid and synaptic pathology by 80 days *in vitro*
a, DIV80 TgF344-AD microtissues display amyloid plaque-like features (white arrows) (scale bar = 500µm). **b**, Iba1+ microglia sequestered around Aβ-42+ plaques (scale bar = 20µm). **c**, number of plaque-like features based on immunofluorescent staining with Aβ-42 antibody in wildtype vs TgF344-AD microtissues (Unpaired T-test, ****p<0.0001) n≥8 microtissues per genotype. **d**, secreted Aβ-42 concentration detected by ELISA in culture supernatants from Wildtype and TgF344-AD microtissues at DIV6 and DIV28 (two-way ANOVA followed by Tukey's post-hoc comparison: ****p<0.0001, ***p<0.001) supernatant from n=3 microtissue per genotype was collected for each time point and run in triplicate. Data is represented as the mean ± standard error of the mean. **e&f**, Representative confocal fluorescent images of synaptic puncta PSD95 and synaptophysin in wildtype vs TgF344-AD microtissues at **(e)**DIV45 and **(f)**DIV80 (scale bar = 10µm). **g-i**, Quantification of number of synaptic puncta per 150µm³ in wildtype vs TgF344-AD microtissues at DIV45 vs DIV80 (two-way ANOVA followed by Tukey's post-hoc comparison: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05) n ≥ 4 microtissues per genotype for each timepoint. Data is represented as the mean ± standard error of the mean.

increase in secreted Aβ42 (p=0.0002) (**Fig. 3.4, d**), suggesting glial reactivity in the TgF344-AD microtissues may be triggered by small changes in *APP* expression within the 3D microenvironment that precede larger amyloid-plaque deposition.

We further investigated whether early glial and amyloid pathology led to synaptic deficits by quantifying pre- and postsynaptic densities in DIV45 and DIV80 microtissues (**Fig. 3.4, e-i**). At DIV45, there was no detectable difference in the density of pre- or postsynaptic proteins, nor in the amount of colocalized puncta (**Fig. 3.4, g-i**). However, significant synapse loss was detected by DIV80 marked by loss of PSD95+, synaptophysin+, and colocalized puncta (p=0.0007, p=0.0079, p=0.0002) (**Fig. 3.4, g-i**). TgF344-AD glial reactivity preceded plaque-like features and synapses loss, suggesting early glial-dysfunction may drive downstream pathology in the microtissue culture system.

3.4.4 Microglia depletion disrupts neuronal network maturation and functional connectivity in developing microtissues

Given that microglia play a critical role in sculpting the synaptic microenvironment and have been implicated in AD-associated synaptic dysfunction, we posited that removing microglia from the system would negatively impact the functional connectivity of neurons. We thus investigated the neuronal network connectivity in microglia-depleted microtissues throughout the

first month of culture (**Fig. 3.5, a**). Sustained microglia depletion was achieved through inhibition of microglial receptor to colony stimulating factor 1 (CSF-1R). CSF-1 signaling is essential for microglia survival and both pharmacological and genetic inhibition of CSF-1R are widely used *in vitro* and *in vivo* to selectively ablate microglia (Spangenberg et al. 2019; Henry et al. 2020; Pridans et al. 2018; Rosin et al. 2018; Coleman et al. 2020; Wallace et al. 2020; Elmore et al. 2015). We assessed the efficacy of the CSF-1R inhibitor molecule PLX5622 (Spangenberg et al. 2019) in DIV21 microtissues by spiking culture media with 2uM PLX5622 media for 1 week. Immunohistochemical analysis revealed highly selective depletion of Iba1+ microglia compared to DMSO-treated controls ($p<0.0001$) with no significant effect on general morphology and cell number of GFAP+ ($p=0.2553$) and NeuN+ ($p=0.7196$) cells (**Supplemental Fig. 3.12.7**).

To image functional activity in neuronal populations, we introduced a genetically encoded calcium indicator, GcaMP (T. W. Chen et al. 2013), delivered via an adeno-associated viral vector to record spontaneous temporal dynamics of intra-neuronal calcium concentration (calcium transients) between DIV14 and DIV35. The selected viral vector was a bicistronic construct that encoded GCaMP6s followed by a P2A peptide-fused untargeted red fluorophore (mRuby) to allow co-expression of calcium indicator and mRuby fluorophore in neuronal cells under the same human Synapsin promoter (**Fig. 3.5, b**). Viral transductions were performed at DIV1 in either PLX5622-treated or dimethyl sulfoxide (DMSO) treated control microtissues. Stable fluorescence onset was observed by DIV7, and spontaneous calcium transients were detected by DIV14 (**Fig. 3.5, b**). Microtissues were imaged every other day for the following 3 weeks using an inverted multi-laser scanning confocal microscope with a 10x objective [1.27mmx1.27mm field of view (FOV)] at 15Hz in 4 min recording sessions. Time-series fluorescence data from each ROI [individual cell per ROI; >60 cells per FOV] of each sample was analyzed using the open-source software packages FluoroSNNAP (Patel et al. 2015) and reported as changes in fluorescence intensity normalized to the baseline fluorescence F0.

Example dF/F0 traces from individual cells in control (DMSO) and PLX5622-treated samples at week 2, week 3, and week 4 in culture show altered calcium patterns between DMSO and PLX (**Fig. 3.5, d**). We then implemented a widely used mathematical graph-based approach to studying neural network functionality (Atherton et al. 2021; 2022; Dingle et al. 2020; Zippo et al. 2013; Van Den Heuvel et al. 2010; Bullmore and Sporns 2009; Muldoon, Bridgeford, and Bassett 2016; Newman 2006; Chiappalone et al. 2006) in order to analyze microcircuit connectivity differences in control and microglia-depleted microtissues. Briefly, each node in the graph was represented by an individual cell region of interest (ROI) and each node was connected by an edge. Each node pair was characterized by a connecting edge weight, defined as the Pearson linear correlation of between the single-cell calcium traces associated with each node. The average edge weights were extracted from each labeled node in the microtissue using the FluoroSNNAP software and binned by imaging week (week 2: DIV14-DIV20, week 3: DIV22-DIV26, week 4: DIV28-32). Control microtissues displayed a decrease in average edge weight between week 2 and week 4 ($p < 0.0001$) (**Fig. 3.5, e**). A visual representation of this network-wide connectivity can be seen in the Pearson correlation matrices between week 2 and week 4 (**Fig. 3.5, f**). Higher whole tissue average edge weights observed in week 2 correspond with the significant increase in synaptic densities shown in (**Fig. 3.2, d-g**), suggesting that the initial formation of new synapses during early development drives network-wide connectivity wherein all nodes are highly interconnected. Similar findings were reported in a study using primary embryonic rat cortical cells cultured on multi-electrode arrays where gradual decrease in whole-network average correlation occurred between DIV21 and DIV35 (Chiappalone et al. 2006). Our findings mirror the observed temporal pattern of gradual decreasing whole-tissue edge weight seen in our developing microtissues and suggest a rewiring and active sculpting of network circuitry to remove excess synapses and strengthen synaptic connections between sub-groupings of cells.

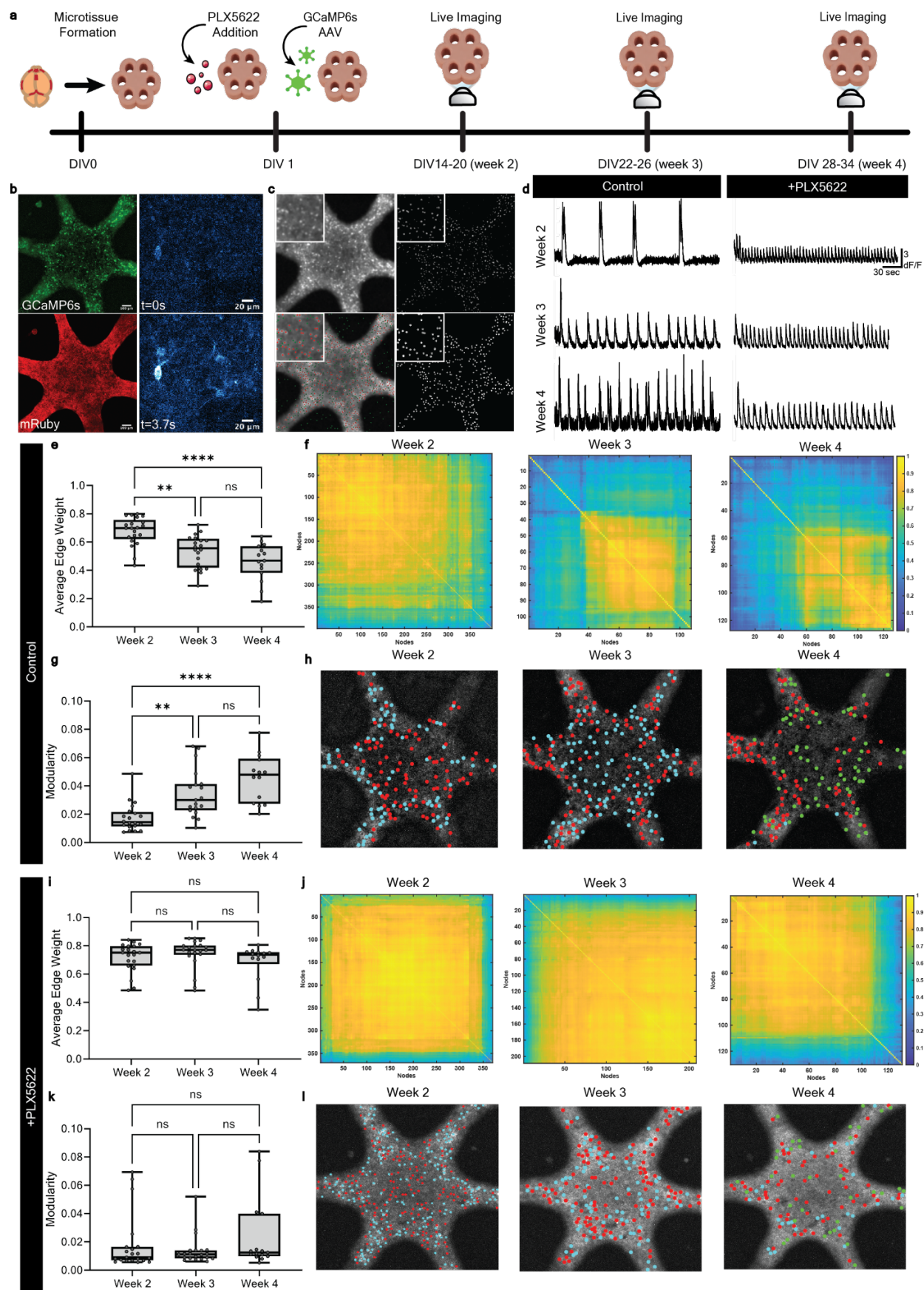


Figure 3.5 | Microglia depletion disrupts functional network connectivity

a, Schematic of experimental timeline of longitudinal calcium imaging in control and PLX5622-treated microtissues **b**, Representative confocal fluorescent images of GCaMP6 and mRuby expression throughout the microtissue and example of neuronal GCaMP6 expression during calcium influx ($t=0$ vs $t=3.7$ seconds later) (left panel scale bar = $100\mu\text{m}$; right panel scale bar = $20\mu\text{m}$). **c**, Representative images of semi-automated ROI detection. Top panel shows maximum intensity projection images processed with Gaussian filter ($\sigma = 2.0$) and binarized image after thresholding based on fluorescence intensity of local neighborhoods. ($\text{threshold_block_size} = 5$, $\text{threshold_fraction_above_mean} = 0.2$). Lower panel shows output from manual interactive correction of ROIs using the napari visualizer (red=correctly labeled cell, green = not a cell), and masked image with individual cell ROIs overlaid onto greyscale maximum intensity projection image of microtissue. **d**, Representative single cell dF/F_0 traces from individual cells in control (DMSO) and PLX5622-treated samples at week 2, week 3, and week 4. **e & i**, Average edge weights of **(e)** control and **(i)** PLX5622-treated microtissues at week 2, 3, and 4 show significant changes to whole tissue correlation during 3 weeks of culture that is entirely negated in PLX5622-treated microtissues. **f & j**, Node pair edge weight Pearson correlation matrices of individual **(f)** control and **(j)** PLX5622-treated microtissues at week 2, 3, & 4. **g & k**, Whole-tissue modularity values from **(g)** control and **(k)** PLX5622-treated microtissues at week 2, 3, and 4 reveal increasing modularity coincides with microtissue maturation in control tissues and is negated in PLX5622-treated groups. **h & l**, Representative images of separate modules (red, blue, green = modules) overlaid onto maximum intensity projection images of **(h)** control and **(l)** PLX5622-treated microtissues at week 2, 3, & 4. Kruskal Wallis Test followed by Dunn's post-hoc comparison: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n \geq 14$ microtissues per condition for each weekly timepoint. Data is represented in box plots displaying the median, interquartile ranges, and minimum/maximum whiskers extending to $[1.5 \times \text{IQR} / \text{minimum-maximum range}]$.

Sub-groupings or “modules” are formed as groupings of cells become more densely connected to each other than to other cells of the network. Modularity is a metric of community structure wherein higher degrees of modularity coincide with mature network structures (Newman 2006; Bullmore and Sporns 2009) that is disrupted in disease states including schizophrenia, fAD, and mild cognitive impairment (MCI) (Alexander-Bloch et al. 2010; Voevodskaya et al. 2018; Rosales Jubal et al. 2021). Remarkably, microtissue cortical networks exhibited significant rise in modularity between week 2 and week 3 ($p=0.0029$) which continued into week 4 ($p<0.0001$) (**Fig. 3.5, g**). This increase in modularity corresponds with a higher total number of modules within the microcircuit (**Fig. 3.5, h**). Additionally, the microtissues exhibited greater intra-modular correlation coinciding with reduced inter-module correlation and an overall decline in average whole-network correlation (**Supplemental Fig. 3.12.8**). Together, dynamic synaptic densities and the formation of sub-networks provides insight to changes in network structure and connectivity mediated by alterations in the synaptic landscape. To further probe the relationship between the synaptic densities and network connectivity readouts, we quantified calcium activity of neuronal populations in PLX5622-treated microtissues devoid of microglia-mediated synaptic pruning and

formation. Interestingly, across four weeks of microtissue culture, we observed no network maturation in microglia-depleted microtissues, marked by no significant changes to average edge weights or modularity across weeks (**Fig. 3.5, i-l**). These results highlight the role of microglia in sculpting neuronal networks in the developing microtissue environment.

3.4.5 Microglia-synapse engulfment increases during healthy microtissue development and persists in aged TgF344-AD microtissues

To corroborate microglia as a key modulator in synaptic alterations underpinning neuronal network connectivity changes, we quantified the engulfment of synaptic material by microglia in developing microtissues. Although the molecular machinery and signaling responsible for instructing microglia-mediated synapse removal and formation is still not fully understood, synaptic engulfment assays have been widely deployed as a readout of microglia-induced synapse elimination or pruning (Vainchtein et al. 2018; Rueda-Carrasco et al. 2023; Werneburg et al. 2020; Schafer et al. 2012; Hong et al. 2016; Wilton et al. 2023; Stevens et al. 2007; Favuzzi et al. 2021). A variety of techniques have been used to assess microglia-synaptic engulfment, including genetic encoded fluorescent labeling and live imaging, immunohistochemistry, electron microscopy, and fluorescence activated cell sorting. Fluorescent super-resolution and confocal microscopy are commonly applied as they are usually more accessible and can be applied to visualize colocalization of synaptic proteins within microglia across a variety of *in vivo* and *in vitro* models. We packaged several commonly used fluorescence-based cell segmentation and spatial overlap algorithms into a python-based open-source software pipeline to reliably segment Iba1+ labeled microglia and synaptophysin+ presynaptic puncta and quantify internalized synaptic material from multichannel fluorescent 3D confocal images (**Fig. 3.6, a-c**).

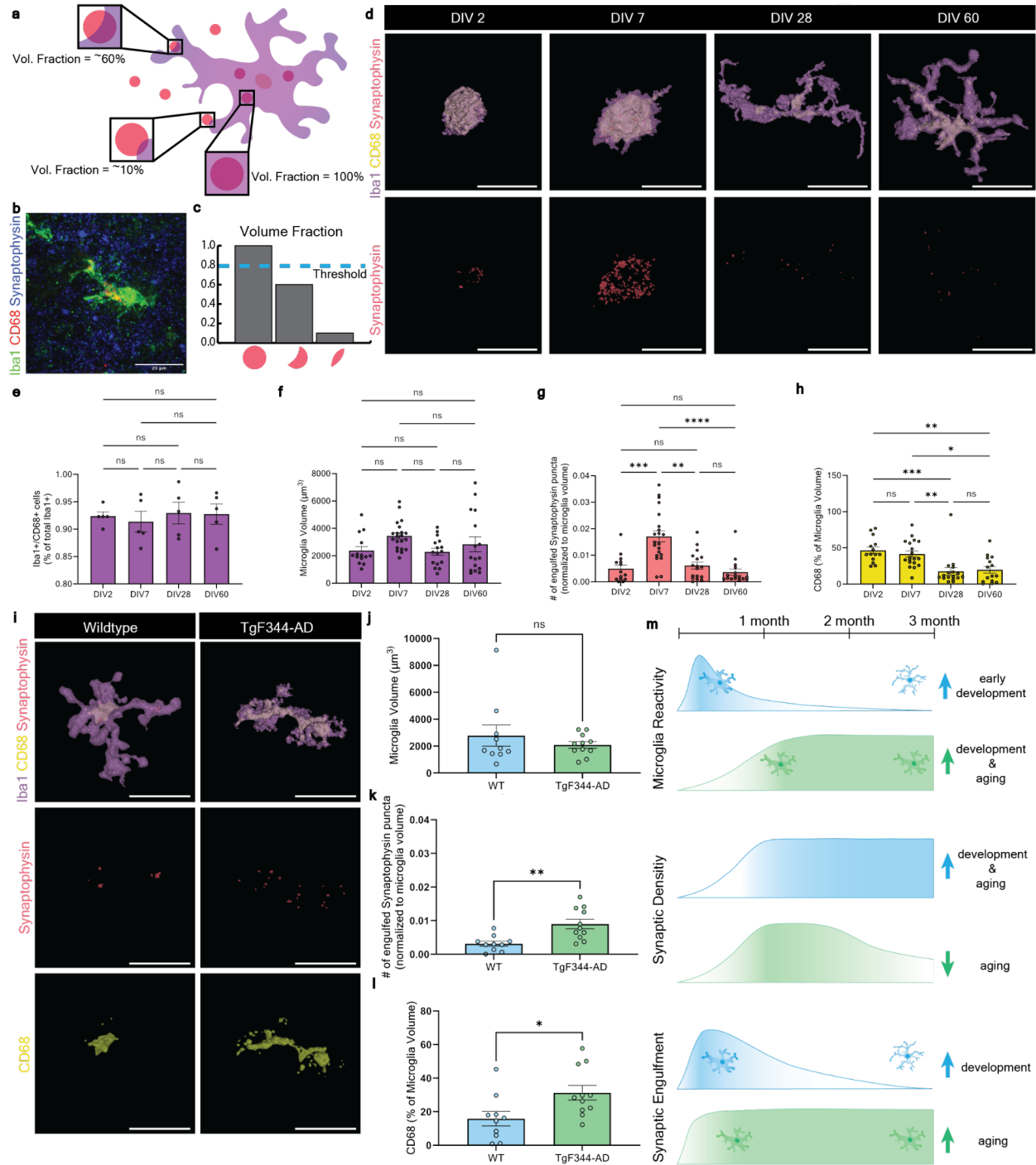


Figure 3.6 | Microglia-synapse engulfment increases during healthy microtissue development and persists in aged AD-relevant microtissues

a, Microglia synaptic engulfment assay **b**, Representative confocal fluorescent image of Iba1+ microglia (green), CD68 lysosome (red), and synaptophysin puncta (blue) (scale bar = 20µm). **c**, schematic showing threshold of internalized synaptic puncta where engulfed puncta are characterized at being 80% interior to Iba1+ microglia. **d**, Example 3D renderings of segmented Iba1+ microglia, CD68+ lysosomes, and synaptophysin+ presynaptic puncta using custom python-based software package. Synaptophysin channel is isolated in lower panel (scale bar = 20µm). **e**, average number of CD68+/Iba1+ cells at DIV2, 7, 28, &

60, shown as percent of total Iba1+ cells counted per mm³. **f**, No significant change in microglia volume (μm^3) in microtissues at DIV2, 7, 28, & 60. **g**, number of engulfed synaptophysin+ puncta normalized to Iba1+ microglia volume at DIV2, 7, 28, & 60. **h**, Volume of CD68+ lysosome normalized to Iba1+ microglia volume. Kruskal-Wallis followed by Dunn's post-hoc comparison: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). For (e) $n = 5$ microtissues per time point. For (**f-h**), $n = 5$ microglia per microtissue with $n = 3$ microtissues for each time point. **i**, Example 3D renderings of segmented Iba1+ microglia, CD68+ lysosomes, and synaptophysin+ presynaptic puncta from wildtype and TgF344-AD microtissues at DIV80. Top panel shows merged channels, middle and bottom panel show synaptophysin+ and CD68+ segmentations alone (scale bar = $20\mu\text{m}$). **j**, No significant change in microglia volume (μm^3) from wildtype vs TgF344-AD microtissues at DIV80. **k**, number of engulfed synaptophysin+ puncta normalized to Iba1+ microglia volume from wildtype vs TgF344-AD microtissues at DIV80. **l**, Volume of CD68+ lysosome normalized to Iba1+ microglia volume from wildtype vs TgF344-AD microtissues at DIV80. Mann Whitney test: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). For (**j-l**), data is represented at mean $\pm$ standard error of the mean with $n \geq 5$ microglia per microtissue with $n = 2$ microtissues per genotype. **m**, schematic representation of microglia reactivity, synaptic engulfment, and synaptic densities in healthy control vs TgF344-AD derived microtissues.

Example 3D renderings of Iba1+/CD68+ microglia with internalized synaptophysin+ puncta (seen separately in lower panel of **Fig. 3.6, d**, **Supplementary Fig. 3.12.9**) were sampled from DIV2, DIV7, DIV28 and DIV60 (**Fig. 3.6, d**). We did not see a significant change in the number of Iba1+/CD68+ cells, nor in the average microglia volume between DIV2, DIV7, DIV28 and DIV60 (**Fig. 3.6, e-f**) despite a distinct morphological shift from round and ameboid at DIV2 and DIV7 to increasingly ramified by DIV60 (**Fig. 3.6, d**). The number of engulfed synaptic puncta, normalized to microglia volume) peaked between DIV2 and DIV7 ($p = 0.0006$) and decreased by DIV28 ($p = 0.0036$) (**Fig. 3.6, g**). Increased synapse internalization DIV7 was paralleled by lysosomal activation, marked by elevated ratio of CD68+ lysosome volume to microglia volume ($p = 0.0014$) (**Fig. 3.6, h**). We note that the ameboid morphology seen at DIV2 did correspond with increased CD68 volumes but not with an increase in synaptic engulfment. This was likely the result of microglia phagocytosis of cellular debris following the tissue digestion and seeding.

After establishing microglia engulfment of synapses in healthy microtissues, we sought to quantify microglia-synapse engulfment in TgF344-AD derived microtissues (**Fig. 3.6, i**) to probe whether the previously observed decline in synaptic densities at DIV80 coincided with elevated microglia engulfment activity. Synaptic engulfment analysis of TgF344-AD+ microglia at DIV80 revealed an increased number of engulfed synapses (normalized to microglia volume) compared

to wildtype samples ($p=0.0015$) (**Fig. 3.6, k**). To our surprise, there was no significant change in microglia volume ($p=0.9022$) between wildtype and TgF344-AD microglia (**Fig. 3.6, j**), despite changes in morphology. However, TgF344-AD+ microglia did have a significant increase in CD68+ lysosomal volume compared to wildtype microglia ($p=0.0151$) (**Fig. 3.6, l**). Increased CD68 expression has been commonly used as a marker of microglia activation, in particular with AD-associated microglia activation and gliosis surrounding amyloid plaques in the human brain as well as in transgenic rodent models including the TgF344-AD rat (Hong et al. 2016; Hopperton et al. 2018; Park et al. 2018; Grubman et al. 2021; Bac et al. 2023). Compared to healthy microtissues, increases in microglia reactivity, amyloid pathology, and synaptic engulfment, correlated with decreased synaptic densities, suggest a maladaptive response of microglia in the TgF344-AD microtissue, leading to excessive synapse removal (**Fig. 3.6, m**).

3.5 Discussion

In this study, we set out to investigate microglia-driven synaptic remodeling under both healthy and disease-relevant conditions, with a particular focus on dysfunction in AD. To this end, we developed a 3D primary cortical microtissue platform that captures the complexity of neuron–glia interactions in a long-term, physiologically relevant microenvironment. Our findings demonstrate that, during early microtissue development, microglia engage in tightly regulated synaptic pruning, facilitating the formation and maturation of functional neuronal circuits. However, in primary cortical microtissues derived from TgF344-AD rats expressing human *APP* and *PS1*, we observed aberrant microglial activation and the emergence of amyloid-beta aggregates (**Fig. 3.3 & 3.4**), which together drive excessive synapse elimination (**Fig. 3.6**) and ultimately lead to neuronal loss (**Fig. 3.3**). By integrating advanced imaging modalities and computational tools, we quantitatively tracked these processes in real time, revealing key insights into the temporal and spatial dynamics of microglial-mediated synaptic dysfunction in AD. The 3D

culture system not only offers a powerful model for dissecting fundamental mechanisms of neuron–glia crosstalk but also lays the groundwork for therapeutic exploration targeting microglial pathways in neurodegenerative disorders.

To better understand microglial influence on microtissue neuronal connectivity and network maturation, we compared control and microglia-depleted microtissues during the first month of development (**Fig 3.5**). Calcium imaging and graph theoretical analyses of neuronal functional connectivity revealed that microglia are essential for shaping neuronal circuits, as microglia-depleted (PLX5622-treated) microtissues exhibited a pronounced lack of network maturation. While synapse formation and removal remain central to sculpting neuronal circuitry, microglia—along with astrocytes—are known to modulate synaptic plasticity through glutamate and cytokine signaling (Gao et al. 2023; Pascual et al. 2012; Paixão and Klein 2010; Perez-Catalan, Doe, and Ackerman 2021; de Ceglia et al. 2023). Although our focus here was on structural aspects of microglial modulation, future work investigating molecular drivers of plasticity in TgF344-AD microtissues—both before and after amyloid pathology onset—could further clarify how glial dysfunction precipitates synaptic deficits. Notably, by generating 3D primary cortical microtissues from TgF344-AD rats, we overcame key challenges associated with modeling chronic pathology and real-time neuron-glia interactions in an *in vitro* setting. Within 3 months, TgF344-AD derived microtissues displayed hallmark features of AD pathology, including glial reactivity, progressive amyloid accumulation, and neuronal loss (**Fig. 3.3 & 3.4**). Interestingly, glial activation emerged by DIV28—preceding neuronal loss, heightened microglia-synaptic engulfment, and amyloid plaque formation at DIV80—suggesting that early glial dysfunction may predispose synapses to degeneration, supporting a glia-driven hypothesis of AD pathogenesis.

We show that microglia exert a strong regulatory influence on neuronal circuitry development by pruning supernumerary synapses. Quantification of microglia-mediated synapse engulfment peaked between DIV7 and DIV28 (**Fig. 3.6**), coinciding with heightened lysosomal

activity and aligning with critical phases of neuronal network refinement. Notably, we detected differences in microglial engulfment of presynaptic material in both healthy developing microtissues and aged ($\geq$ DIV80) AD microtissues, reinforcing the central role of microglia in synaptic function in the TgF344-AD rat model. Future investigations into complement signaling and microglia-mediated removal of postsynaptic elements could further elucidate the synaptic dysfunction underlying cognitive and memory deficits in the TgF344-AD rat model (Van den Berg et al. 2022; Joo et al. 2017; Smith and McMahon 2018; Muñoz-Moreno et al. 2018; Bazzigaluppi et al. 2018; Saré et al. 2020).

Remarkably, synaptic sculpting persisted in our microtissues despite the absence of sensory stimuli—a factor typically associated with early, activity-dependent synaptic remodeling *in vivo* (Chung et al. 2013; Bernardinelli et al. 2014; Huttenlocher 1979; Wu et al. 2015; Schafer et al. 2012; Stevens et al. 2007; Paolicelli et al. 2011). This finding points to intrinsic, sensory-independent mechanisms of glial-synapse interactions within the 3D neural environment. Indeed, our data show that depleting microglia disrupts neural network connectivity, while in an amyloidogenic context, overactive microglial pruning emerges. Furthermore, TgF344-AD microtissues display a maladaptive microglial state, marked by ameboid morphology, heightened phagocytic activity, and increased synaptic engulfment, that precedes visible amyloid protein aggregation and may hasten synaptic loss. These observations align with previous work in APP/PS1 rodent models and human AD brain tissue, where aberrant microglial activation has been implicated in pathological synaptic pruning and impaired amyloid-beta clearance, underscoring the central role of microglia in AD pathogenesis.

A key limitation of our current approach is that, although the TgF344-AD rat model exhibits robust tau pathology *in vivo* by around 16 months (Cohen et al. 2013), we did not detect phosphorylated tau in our microtissues cultured up to 80 days. It is possible that an extended culture period is necessary to recapitulate the later-onset tau pathology observed *in vivo*.

Nonetheless, the current experimental timeframe is sufficient to model amyloidosis-driven pathogenic changes and synaptic deficits. Moving forward, we aim to cultivate these microtissues for longer durations to investigate the emergence of tau aggregates and potential synergistic interactions with amyloid pathology. Additionally, future studies will focus on monitoring microglial calcium activity in both wildtype and AD microtissues. Altered microglial calcium dynamics have been linked to AD progression, yet its real-time interplay with neuronal networks remains underexplored. By integrating calcium imaging of microglia, we hope to gain deeper insights into how microglial calcium dynamics influence, and are influenced by, the neuronal microenvironment in health and disease.

Our findings establish a 3D primary cortical microtissue platform as a robust, physiologically relevant model for elucidating the complex interplay between microglia and neurons in both healthy and disease states. By capturing hallmark AD-like phenotypes—ranging from amyloid accumulation to microglial reactivity and synaptic loss—our system highlights the pivotal role of microglia in shaping synaptic architecture and underscores the glia-driven processes that can precipitate neuronal vulnerability in Alzheimer’s disease. The versatility of this approach provides an avenue for extending culture durations and incorporating additional functional assays to explore later-onset events. This primary microtissue model helps advance our mechanistic understanding of microglial involvement in AD and may aid in the development of targeted therapies aimed at preserving synaptic integrity in the aging and diseased brain.

3.6 Materials and Methods

3.6.1 Animals

All animal experiments were approved by the Brown University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institute of Health

Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). Purchased timed-pregnant Sprague Dawley rats were monitored for arrival of litter and pups which were subsequently collected at postnatal day 1. Heterozygous TgF344-AD rats with dual mutant human APP KM670/671NL Swedish and PS1 Δ exon9 mutations driven by a mouse prion promoter on a Fischer (F344) genetic background were used in development of TgF344-AD microtissues. The TgF34-AD rat model has been previously described in detail (Cohen et al. 2013). Pups were collected at postnatal day 1, sexed, and genotyped. For genotyping the TgF344-AD rat pups the following primer sequences were used: mouse prion protein gene (mPrp) Forward Sequence: 5'-CCT CTT TGT GAC TAT GTG GAC TGA TGT CGG-3', mPrp Reverse 5'-GTG GAT ACC CCC TCC CCC AGC CTA GAC C-3', APP Forward 5'-CCG AGA TCT CTG AAG TGA AGA TGG ATG-3', and PS1 Forward 5'-CAG GTG GTG GAG CAA GAT G-3'. Once transgene-positive and transgene-negative pups were identified following PCR and gel electrophoresis, equal numbers of TgF344-AD male and female pups were pooled for cortical tissue collection. Similarly, an equal number of male and female wildtype littermates were pooled for cortical tissue collection and formation of wildtype microtissues.

3.6.2 Agarose gels

Following our previously published protocol (Brown, Atherton, and Borton 2023) agarose micro-molds were created following previously described methods in Brown et al 2023. Briefly, custom stainless-steel injection molds were placed within each well of a 24-well glass-bottom culture plate (Cat. # P241.5HN, CellVis). The injection mold was held in place approximately 150 μ m above the bottom of the plate using a custom aluminum milled single well insert that fits into each well. A 2% (w/v) agarose (cat # 16500, Invitrogen) solution is prepared in Dulbecco's phosphate buffered saline and heated for 30 second intervals in a laboratory microwave until agarose is in solution and mixture reaches a steady boil. Hot agarose was injected into the

stainless-steel molds using a 10 mL sterile syringe. The syringe was removed and the plate placed on ice for 15 minutes to allow for the agarose to solidify before carefully removing the injection mold. 1 mL of complete cortical media (CCM) containing 2% neuronal supplement B-27 (Cat. # 17504-044 Invitrogen), 1% Penicillin-Streptomycin (Cat. #15140122, Thermo Scientific), and 0.25% GlutaMAX (Cat. # 35050061, Thermo Scientific) was added to each well to keep the agarose microwells hydrated and plates were placed at 37 C 5% CO₂ until primary cell seeding.

3.6.3 Three-dimensional primary cortical cultures

Primary cortical cultures were made based on previously described methods (Brown, Atherton, and Borton 2023). Briefly, an equal number of males and females collected at postnatal day 1 and deeply anesthetized before euthanasia. Whole brains were collected and placed in cold Hibernate A media (Cat. # HA-500, Transnetyx Tissue) containing 1% B-27 neuronal supplement (Cat. # 17504-044 Invitrogen). Cortices were dissected away and enzymatically digested in a 2 mg/mL Papain and Hibernate A-minus Calcium (Cat. #PAP & #HACA, Transnetyx Tissue) solution for 30 minutes. Following digestion, tissue was triturated using a large-bore (1000 µl) pipette and a single cell suspension was added to a 40 µm cell strainer. Cells were washed 3 times with cold Hibernate A media containing 1% B27 neuronal supplement. Suspension was centrifuged at 150 g for 5 min and supernatant removed before the pellet was resuspended in a fresh 5 mL of CCM. Two additional filtration and centrifugation steps were completed before cells were counted and assessed for >95% viability. The pellet was resuspended to a final concentration of 2.7e8 cells/mL and 10 µl of cells were carefully added dropwise to the center of the agarose-molded microwells. Prior to cell seeding all media was removed from the microwells. Following seeding, plates were placed at 37C, 5% CO₂, 95% humidity and allowed to incubate for 20 min before 1mL of warmed complete cortical media was carefully added to each well. Media was subsequently changed every other day thereafter. For PLX-treatment groups, PLX5622 compound (Sellekchem Cat. #S8874) was diluted to 2µM in complete cortical media and added

to microtissues. Control samples were dosed with 2 μ M DMSO as a vehicle control. PLX5622 inhibitor or vehicle control were added during complete media changes every three days.

3.6.4 Immunohistochemistry

Microtissues were fixed in 4% (w/v) paraformaldehyde (Cat. #F79-500, Milipore Sigma), 8% sucrose (Cat. # S7903, Milipore Sigma) solution for 2 hours followed by 3, 2 hr washes with 1x dulbecco's phosphate buffered saline (1xDPBS) (Cat. #D1408, Milipore Sigma). Agarose-microwells containing microtissues were carefully removed from each well and placed in fresh 1xDPBS and heated to 200C on a hotplate until agarose melted away leaving free-floating microtissues. Microtissues were transferred to a 48-well plate and washed with fresh 1xDPBS before replacing with 300 μ l blocking solution consisting of 4% (w/v) bovine serum albumin (Cat. # A7030, Milipore Sigma), 10% normal goat serum (Cat. # 005-000-121, JacksonImmunoResearch), and 1% TritonX-100 (Cat. #T8787, Milipore Sigma) in 1x DPBS. Microtissues were placed on an orbital shaker and allowed to incubate at room temperature in blocking solution for 2 hrs before replacing with 200 μ l of primary antibody cocktails diluted in blocking solution and incubated overnight with gentle orbital shaking. The following primary antibodies and dilutions were used: chicken anti-GFAP, 1:200, (cat. #AB5541, Milipore), rabbit anti-Iba, 1:200 (cat #019-19741, WAKO Chemicals), rabbit anti-NG2, 1:250 (cat. #AB5320, Milipore Sigma), mouse anti-nestin, 1:100 (cat. #MAB353 Milipore Sigma), rat anti-MBP, 1:250 (cat #MCA409S, BioRad), mouse anti-BetaIII Tubulin, 1:500 (cat. #AB78078, Abcam), rabbit anti-laminin, 1:25 (cat. #L9393, Milipre Sigma), rabbit anti-collagen IV, 1:150 (cat. #ab6586, Abcam), chicken anti-MAP2, 1:250 (cat. # 18806, Synaptic Systems), mouse anti-NeuN, 1:500 (cat #MAB377, Milipore Sigma), mouse anti-PSD95, 1:50 (cat. #MAB1596 , Milipore Sigma), rabbit anti-Synaptophysin, 1:250 (cat. #5461S, Cell Signaling Technology), mouse anti-amyloid beta peptide (A-beta 40/42), 1:50 (cat. #M-1586-100, Biosensis), mouse anti-CD68, 1:100 (cat. #

MCA341GA, BioRad). We note that the anti-Amyloid beta monoclonal antibody does not detect amyloid precursor protein APP and has been previously described to be highly selective for AB42 compared to AB40 with detection of AB40 at antigen concentrations of 2.5 pmol but detects unaggregated, oligomeric and fibrillar AB42 at antigen concentration as low as ~0.1 pmol (Youmans et al. 2012). Following overnight primary antibody incubation, microtissues were subsequently washed with phosphate buffered saline containing 0.2% TritonX-100 (1xPBT) 2 times over 4 hrs with gentle orbital agitation. Microtissues were placed in 300 µl of fresh blocking solution for 2 hrs on shaker before overnight incubation with secondary antibodies. Secondary antibodies included goat anti-mouse CY3, 1:800 (cat. #115-165-068, Jackson ImmunoResearch), goat anti-chicken Alexa Fluor 488, 1:800 (cat. #A11039, Invitrogen), goat anti-rabbit Alexa Fluor 635, 1:800 (cat. #A31577, Invitrogen), and goat anti-rat Alexa Fluor 647, 1:800 (cat. #A21247, Invitrogen). Following overnight incubation, secondary antibody solutions were removed and microtissues were washed 4 times over 4 hours with 1xPBT. Finally, microtissues were stained with a dsDNA marker DAPI at 1:1000 (cat. #D1306, Invitrogen) for 1 hr prior to imaging. Samples were removed from a 48-well plate and plated onto a glass bottom petri dish (Mattek, cat #P35G-1.5-14-C) with minimal 1 x DPBS. Immunofluorescent images were collected on an Olympus FV3000 multi-laser scanning confocal microscope, using resonant and sequential scanning to collect volumetric images with 3.91, 0.74, and 0.45 µm z-axis resolution using 10x, 30x, and 60x objectives respectively. In some cases, multi-area image acquisition was used to obtain 4 separate high resolution 30x images (141.42 µm x 141.42 µm each) that were then stitched together using Olympus FluoView software to create a single high-resolution image covering of large area in the microtissue center.

3.6.5 Cell counting, mean fluorescence intensity, and Sholl analysis

Stitched 30x magnification images were used for determining the number of cells per mm³. These images were input into ImageJ and the channels were split. A single ROI (186.17 x 199.98 μ m) with a designated and constant position encompassing the center of the tissue was placed in each z stack for every stitched image. Next, the DAPI channel was re-merged with each corresponding channel of interest to count individual cells of various fluorescent channels that overlay with DAPI-labeled nuclei. This process was repeated twice per microtissue, with $n \geq 6$ microtissues from $n = 3$ biological replicates (biological replicate=litter of pups with equal number of male and female animals used for formation of microtissues) for each time point. To determine the mean fluorescence intensity (MFI) for GFAP-labeled images, 30x .oir multichannel images were opened in ImageJ and the GFAP channel was isolated and converted into an RGB Color Image then the MFI was calculated using (Analyze>Measure), with the measurements set to include the “Mean gray value.” 2D Sholl analysis was performed on individual Iba1+ microglia from max intensity projections of 30x z stacks in ImageJ using the Microglia Morphometry and SNT ImageJ plugins (Ferreira et al. 2014; Martinez et al. 2023). Upon opening the Microglia Segmentation and Tracking plugin, user-defined parameters were set for intensity, threshold, minimum cell size, and maximum cell skeleton length. the minimum cell size and maximal cell skeleton length were manually adjusted based on measurements and approximations from the previously saved Maximum Projection images. Using the Microglia Mophometry plugin a skeletonized image was created from the segmentations created in Microglia Segmentation and Tracking plugin. The skeletonized image was opened in ImageJ, and the ROI manager was used to select individual microglia skeletons for further analysis. Cells with merged or overlapping skeletons were excluded from analysis. The SNT plugin from Neuroanatomy was opened, and the Sholl analysis tool was selected. The center of the active ROI was set and adjusted so it overlaid the nucleus (DAPI and Iba-1), and the “preview” option was selected to facilitate the

adjustment of the following parameters. The start radius and step size were determined to be 5 μm and maintained constant across all skeletons analyzed; however, the end radius was customized to the skeleton, depending on the length of its extensions and its proximity to neighboring cells, the average radius used was 30 μm for DIV2 samples and 60 μm for DIV28 microtissues. In addition, only full shells were used for this analysis. The remaining parameters were unchanged from the default settings. Finally, the outputs provided both linear and normalized plots and a detailed table and summary. The results were saved in the corresponding tissue and skeleton. This whole process was repeated for an $n=18$ cells from $n=6$ microtissues across 3 biological replicates per time point.

3.6.6 Enzyme-linked immunosorbent assay

Supernatants were collected from TgF344-AD and wildtype microtissues starting at DIV6 and stored at -20°C . Supernatants collected from DIV6 and DIV28 WT and TgF344-AD tissues ($n=3$ for both genotypes per time point run in triplicate) were assessed for concentration of insoluble $\text{A}\beta$ -42 by ELISA. Solutions were serially diluted to known concentration and a standard curve was generated to calculate the amount within the unknown samples.

3.6.7 Synaptic puncta counting and colocalization analysis

To quantify synaptic puncta from volumetric multichannel fluorescent images, the image data was processed using segmentation and spatial colocalization analysis pipelines developed in house (https://github.com/neuromotion/AD_synapse_counting_colocalization_engulfment) in a series of jupyter notebooks. Briefly, using 3D multichannel fluorescent images, pixel segmentation was performed by systematically training pixel classifiers for the pre- and postsynaptic fluorescence channels using the open-source Ilastik (<https://www.ilastik.org/>) software. Pixel classification was performed using 25 user-selected pixel features including Gaussian smoothing,

Laplacian of Gaussian, Gaussian gradient magnitude, difference of Gaussians, structure tensor eigenvalues, and hessian of Gaussian eigenvalues, all with sigma of 0.3, 0.7, 1.0, 1.6, and 3.5. Following input of user-defined pixel features, a Random Forest classifier was trained to perform pixel classification of “cells” or fluorescent puncta vs background “non-cell” fluorescence. Pixel segmentation was then performed in the analysis pipeline by communicating via command line with the Ilastik program and associated trained classifier files. Following pixel segmentation, instance segmentation was performed using a dual connected component analysis (CCA) and gradient descent (GD) region labeling method. This method was developed to handle touching yet distinct puncta which CCA alone groups together, while guaranteeing smaller puncta were still included in the analysis. Briefly, CCA was applied to the entire pixel-segmented image stack and the COM of each component was calculated and designated a Connected Component Center. Separately, the distance transform of the pixel segmentation was computed, and seed points were randomly placed at 25% of the voxel locations. Gradient descent over an interpolated surface was used to find local maxima of the distance transform from the seed points. The DBSCAN algorithm was used to cluster the final positions of the seed points into local maxima. Any local maxima under the threshold of 1.2 voxels was eliminated from the analysis, then the connected component centers were merged with the centers from gradient descent. These center points were then used as seed points for watershed analysis to perform Instance segmentation. The center of mass of the Instance Segmented objects were considered as the centers of the synaptic puncta. This analysis was performed on both the pre and post synaptic channels. Puncta center points were added to a K-D tree data structure, one for pre and one for post synaptic channels. Pre and post synaptic puncta locations were then compared to one another. Pre and post synaptic puncta were classified as being co-localized if their centers were within $0.6\mu\text{m}$ of each other.

3.6.8 Calcium imaging

Following previously published protocol (Brown, Atherton, and Borton 2023), microtissues were transduced at DIV1 with ready-to-use AAV1 particles produced from pAAV-hSyn1-mRuby2-GSG-P2A-GCaMP6s-WPRE-pA (Addgene Cat # 50942) at 1×10^{10} vg per 1 mL of complete cortical media. Microtissues were incubated with viral media for three days prior to complete media change with non-viral CCM. Microtissues were imaged every other day between DIV14 and DIV35 in 4 min recording sessions on an Olympus FV3000 multi-laser scanning confocal microscope. During imaging sessions, multiwell plates were placed in a temperature, humidity, and CO₂ controlled stage-stop incubator (Tokai Hitt STXF) and imaged at 37°C, 5% CO₂ for the duration of the recording session. Using the 488nm laser and EGFP filter, microtissues were imaged with 10x objective (1.27mmx1.27mm FOV) at 15Hz for 4 min with the aperture set to 800 μ m to capture maximum fluorescence from single z-plane of tissue with large number of cell bodies visible. Plates were only imaged on days where media was not changed. Additionally, plates were allowed to equilibrate in the stage-stop incubator for 30 minutes prior to imaging. Recordings (.oir format) were loaded into FIJI using the Olympus Viewer plugin (<https://imagej.net/OlympusImageJPlugin>) and converted to tiff stacks. A maximum intensity projection of the time-series data was created to identify any cell bodies expressing GCaMP6s-associated fluorescence during the video. Maximum intensity projection images of each microtissue were then saved as .tiff files for semi-automated ROI detection.

3.6.9 Semi-automated ROI detection and calcium signal extraction

Semi-automated ROI detection of individual active cells was completed using code previously described in (Atherton et al. 2021) (<https://github.com/neuromotion/calcium-roi-analysis>). Once the labeled ROIs were subsequently verified by the user through the Napari interactive interface, individual ROI coordinates were saved as .npz files for future processing. A

masked image was created from curated ROIs .npz file and overlaid onto each frame from the 4min time-series recording (<https://github.com/neuromotion/CalciumMask>). Time-series fluorescence data from each ROI (>60 cells per FOV) of each sample were extracted and analyzed using the open-source software package FluoroSNNAP(Patel et al. 2015) and reported as changes in fluorescence intensity normalized to the baseline fluorescence F_0 . Using the segmentation graphical user interface in the FluoroSNNAP application, masked videos (tiff stacks) were segmented by setting an intensity threshold of 0.01 with a minimum ROI radius of 3. FluoroSNNAP analysis preferences were set to the following parameters: “Choose analysis modules”, select “convert raw fluorescence”, “detect calcium transients”; “Acquisition frame rate”, set to 15Hz. $\Delta f/f$ traces were created by detecting the baseline fluorescent signal, calibrated from the 20th percentile of the signal across a 10 second time window. Calcium events of individual ROIs were detected with a template-based method using a threshold of 0.7 and a minimum amplitude of 0.01. Calcium traces were extracted by running the “Batch single-cell and network analysis”. The output “processed_analysis.mat” file was renamed to include the experiment name.

3.6.10 Graph theory analysis

Functional connectivity analysis was performed using a graph-based approach and MATLAB scripts previously described (Atherton et al. 2021; Dingle et al. 2020). Calcium Signal Processing code (<https://github.com/neuromotion/CalciumSignalProcessing>) was used to create the Pearson cross correlation matrix from single cell calcium traces (ΔF_{Cell}) extracted from the FluoroSNNAP output file (processed_analysis.mat). The whole tissue average edge weight was calculated as the mean correlation coefficient of each sample correlation matrix. The whole tissue firing rate was also calculated in the Calcium Signal Processing code by extracting the whole tissue event count (Spikes_whole) from the FLUOROSNNAP output file (processed_analysis.mat) and dividing by the 4 min recording time. Modularity and module

composition were calculated using network-analysis-master code from Dingle et al 2015 (<https://github.com/yutingdingle/network-analysis>). Briefly, the correlation matrix computed in Calcium Signal Processing code was used as the undirected weighted matrix A in the example.m file. Modularity was calculated based on Newman's Spectral Optimization method (Newman et al 2006) where modularity is defined as the sum of edges converging into a module minus the sum of expected edges in a random network of the same size. Module composition is determined based on maximizing network modularity.

3.6.11 Synaptic engulfment analysis

To quantify the number of synapses engulfed within Iba1+ microglia, we developed a python-based analysis pipeline to automate cell segmentation and synapse localization (https://github.com/neuromotion/AD_synapse_counting_colocalization_engulfment). The inputs to the analysis pipeline are pixel segmented images from relevant fluorescent channels of a 60x multichannel fluorescent images. Following initial conversion of the .oir file into .hdf5 format, Z-stacks were initially processed in Ilastik (<https://www.ilastik.org/>) to train three separate pixel classifiers – one for each channel of the volumetric image. The three fluorescent channels were associated with the three primary antibodies used in this assay: Iba1 (microglia), CD68 (lysosomal membrane), and Synaptophysin (presynaptic vesicle protein). Pixel classification was performed using 25 user-selected pixel features including Gaussian smoothing, Laplacian of Gaussian, Gaussian gradient magnitude, difference of Gaussians, structure tensor eigenvalues, and hessian of Gaussian eigenvalues, all with sigma of 0.3, 0.7, 1.0, 1.6, and 3.5. Following input of user-defined pixel features, a Random Forest classifier was trained to distinguish pixels containing “cells” or fluorescent puncta vs from background or “non-cell” fluorescence. Pixel segmentation was then performed via a command line interface to ilastik from within our custom pipeline by invoking the trained classifiers. Instance segmentation of synaptic puncta, microglia, and

lysosomal compartments were performed using CCA. From the instance segmented images, a volume overlap matrix was computed between synapses and microglia, and a separate overlap matrix was computed between microglia and lysosomal compartment. An overlap threshold of 0.8 was set to quantify synaptic puncta as being interior to microglia, meaning that if 80% the volume of a segmented synaptic puncta was interior to a microglia, it was classified as being engulfed. Similarly, an overlap threshold of 1.0 was used to select microglia that were positive for internal CD68 lysosomes to be used in subsequent volume ratio analysis. Once synaptic puncta and lysosomes were identified as interior to microglia and verified by the user in the Napari GUI, normalized metrics such as number of synapses per microglia volume were computed.

3.6.12 Statistical analysis

Statistical analyses were performed using JMP and GraphPad Prism statistical and graphing software. Individual cell and synaptic puncta counts were taken from several microtissues from n=3 biological replicates (exact n information is reported in each figure caption). A biological replicate was determined as a single litter of pups with equal number of male and female pups pooled to collect cortices and seed an average of 16 microtissues per litter. In data where two groups were compared at a single time point (synapse engulfment, microglia and CD68 volume, AB42-plaque counts, cell counts in PLX5622-treated vs control tissues or wildtype vs TgF344-AD samples), parametric (two-tailed unpaired Student's T-test) was used for datasets of Gaussian distribution and non-parametric (Mann-Whitney) was used when parametric tests were not applicable. For secreted A β 42 concentration assay, two-tailed paired Student's t-test was used to normalize variances of concentration on different days. In data where single variable changes were detected across several time points, (cell counts, astrocyte MFI, microglia Sholl analysis, synaptic puncta density, network connectivity metrics including whole-microtissue average edge weight and modularity, synaptic engulfment, microglia and cd68 volume) statistical

significance was tested by parametric (one-way ANOVA with Tukey's post-hoc analysis) for datasets with Gaussian distribution or by non-parametric (Kruskal-Wallis with Dunn's post-hoc analysis) where parametric tests were not applicable. In data where multiple variable analysis was required (cell counts, astrocyte MFI, microglia Sholl analysis, synaptic densities) across several time points in either PLX5622-treated, DMSO control, wildtype, or TgF344-AD microtissues, parametric (two-way ANOVA with Tukey post-hoc analysis) was used. Normality was determined by Shapiro Wilks test and in the even where n was too low quantile-quantile plots were used to visually inspect distribution. Significance level of $\alpha < 0.05$ was determined (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). All error bars represent either the standard deviation from the mean or the standard error of the mean and is denoted in the figure captions.

3.7 Data Availability

All data used to generate the results of this manuscript will be made publicly available upon publication and can be made available upon reasonable request.

3.8 Author Contributions

S.B., Y.A.H, and D.A.B. conceptualized the study; S.B. designed, performed, and analyzed experiments; A.D. performed and analyzed the experiments; K.S. generated experimental data; S.B., A.D., and K.S. performed image acquisition and data analysis; S.B. and A.D-E. conceptualized the software tool requirements, A.D-E. implemented the tools and S.B. analyzed the data; S.B. wrote the first draft of the manuscript; All authors reviewed and edited the final manuscript.

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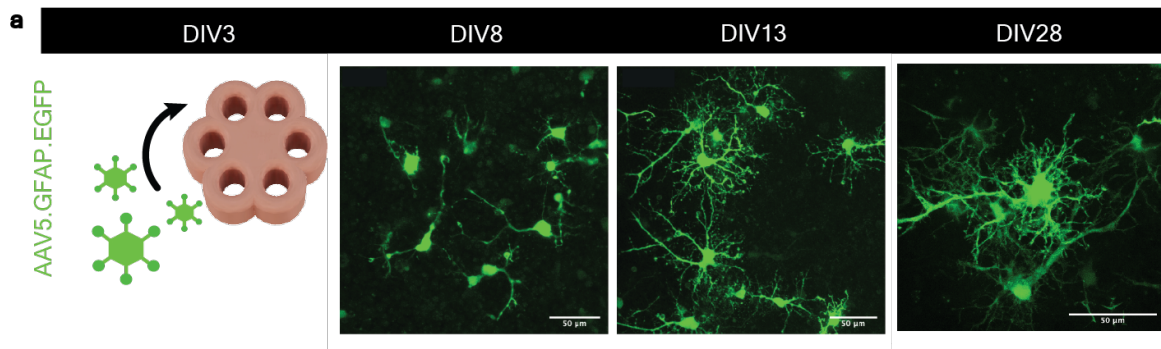
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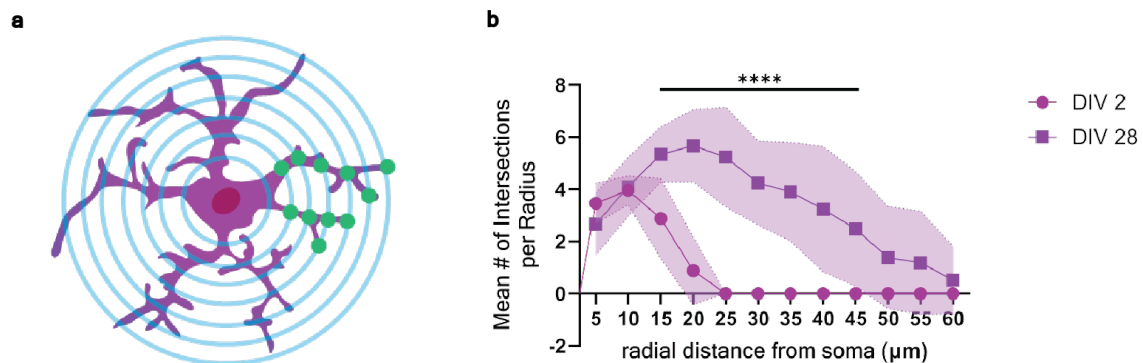
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3.12 Supplementary Figures



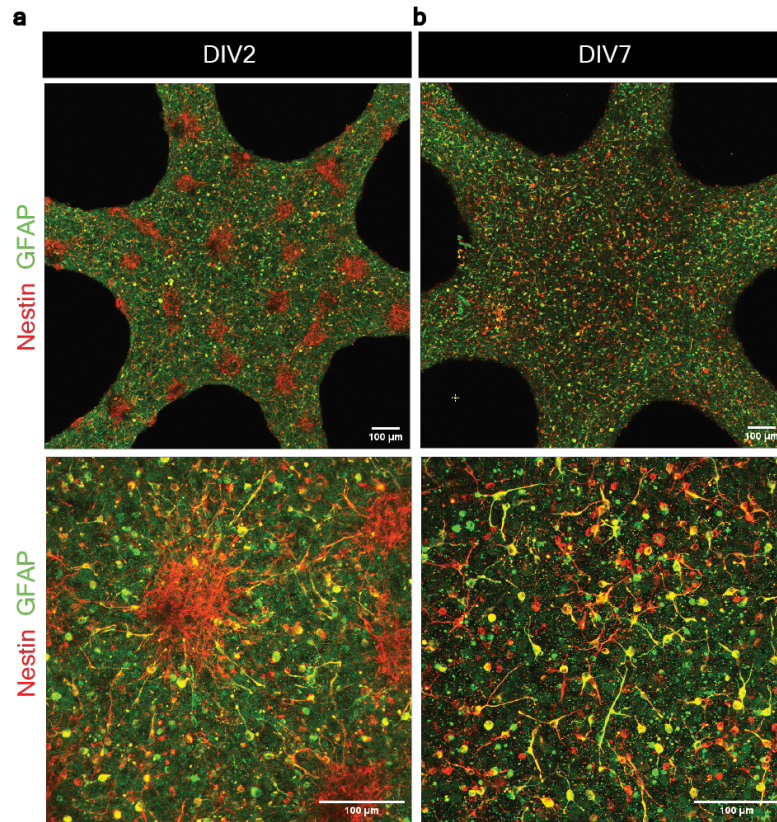
Supplemental Figure 3.12.1

a, Microtissues transduced at DIV3 with AAV5.GFAP.EGFP adeno-associated viral particles (5.2×10^6 vg/mL). Representative live confocal images of GFP+ astrocytes taken between DIV8 and DIV28 show morphological maturation including arborization with fine processes.



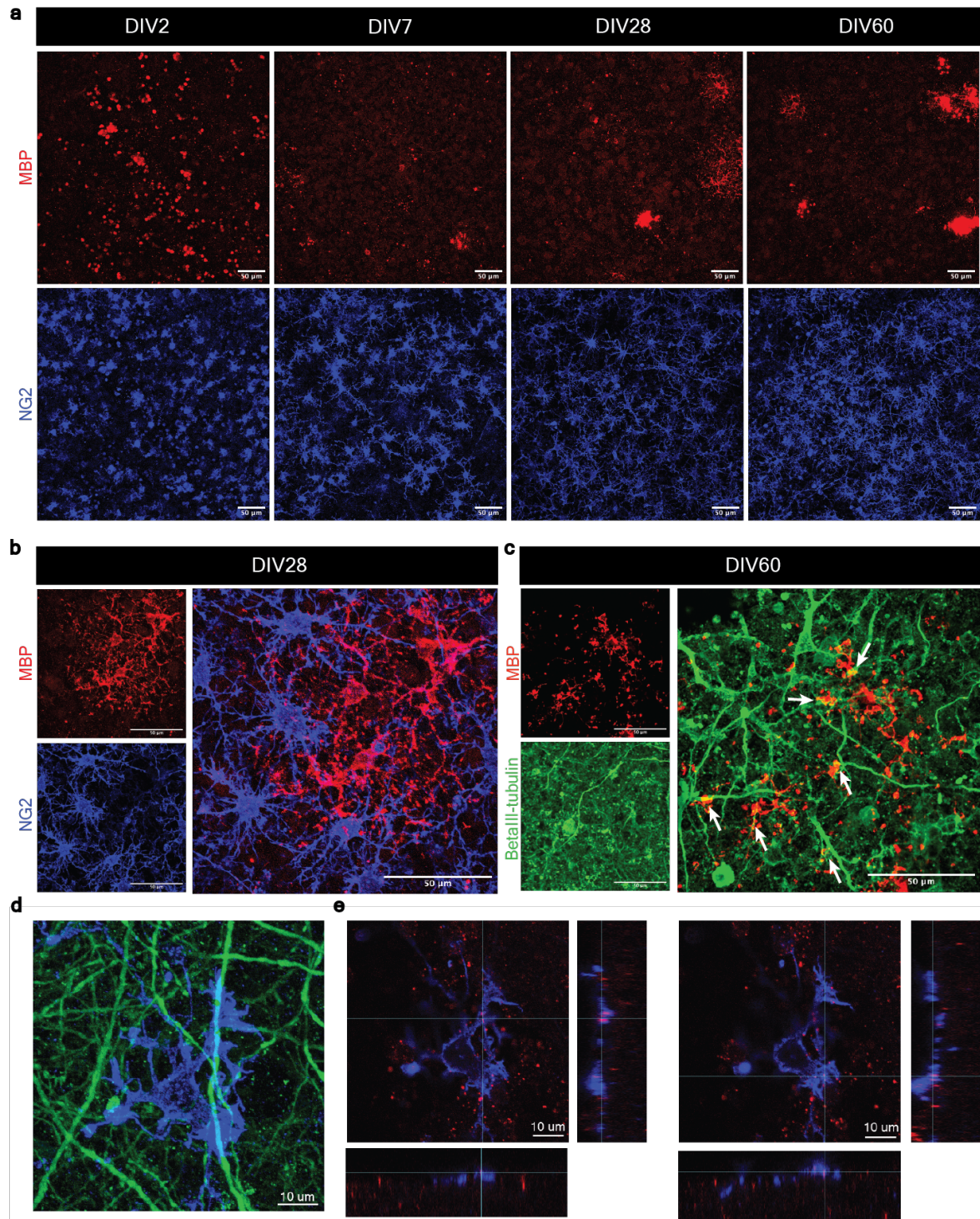
Supplemental Figure 3.12.2

a, Schematic of microglia Sholl analysis with concentric circles set at increasing radial distances from soma center. Green dots mark branch point intersection. **b**, Sholl analysis from individual microglia shows number of branch intersections over concentric circles at increasing radii in microglia from DIV2 vs DIV28 microtissues. Shaded region indicated standard deviation. Significant increase in number of branch points at more distal radii ($15\mu\text{m}$ - $45\mu\text{m}$) in DIV28 microglia compared to DIV2 indicative of a more ramified and branched morphology. $n \geq 21$ individual microglia from $n=6$ microtissues for each time point. Following significant effect by two-way ANOVA, Sidaks' post-hoc comparison was completed where **** $p < 0.0001$



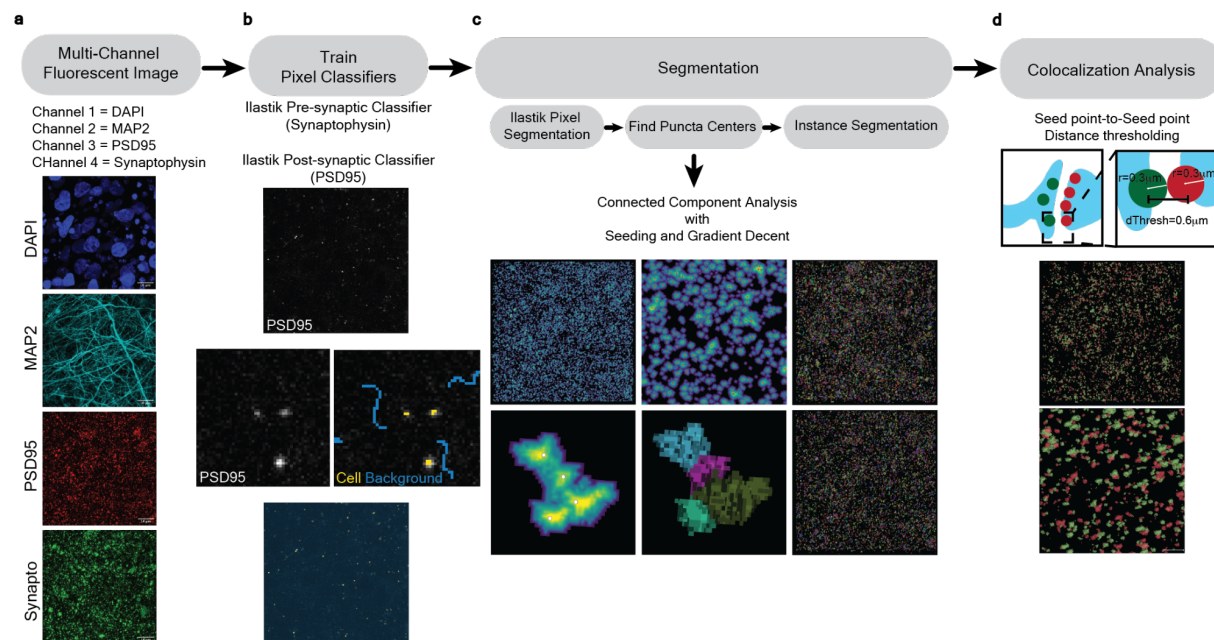
Supplemental Figure 3.12.3

Representative confocal fluorescent images of GFAP+ and Nestin+ cells from microtissues at **(a)** DIV2 vs **(b)** DIV7. Top panel taken at 10x, lower panel taken at 30x (scale bar = 100μm). Nestin+ clusters of cells noted at DIV2 that disperse by DIV7. GFAP and Nestin signal overlap suggesting early astroglia differentiation from nestin+ precursor cells.



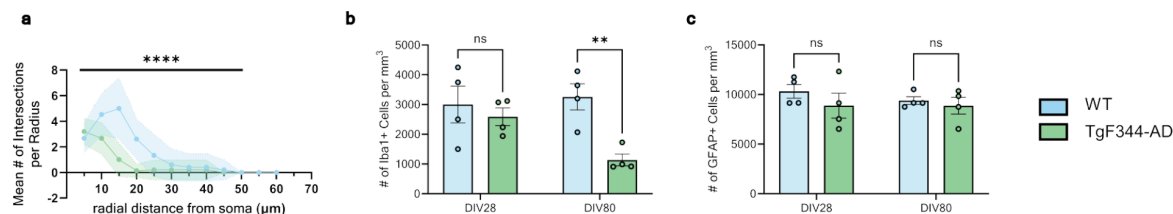
Supplemental Figure 3.12.4

a, Representative confocal fluorescent images of MBP+ staining at DIV2, 7, 28, and 60. Punctate staining at DIV2 suggesting labeling of MBP+ debris following the enzymatic tissue dissociation. **b**, MBP+ cells at DIV28 are negative for NG2. **c**, MBB+ cells at DIV60 colocalize along Beta III tubulin-labeled axons. **d**, NG2+ OPC cells localize along MAP2+ neurites and **(e)** appear to contain PSD95+ puncta.



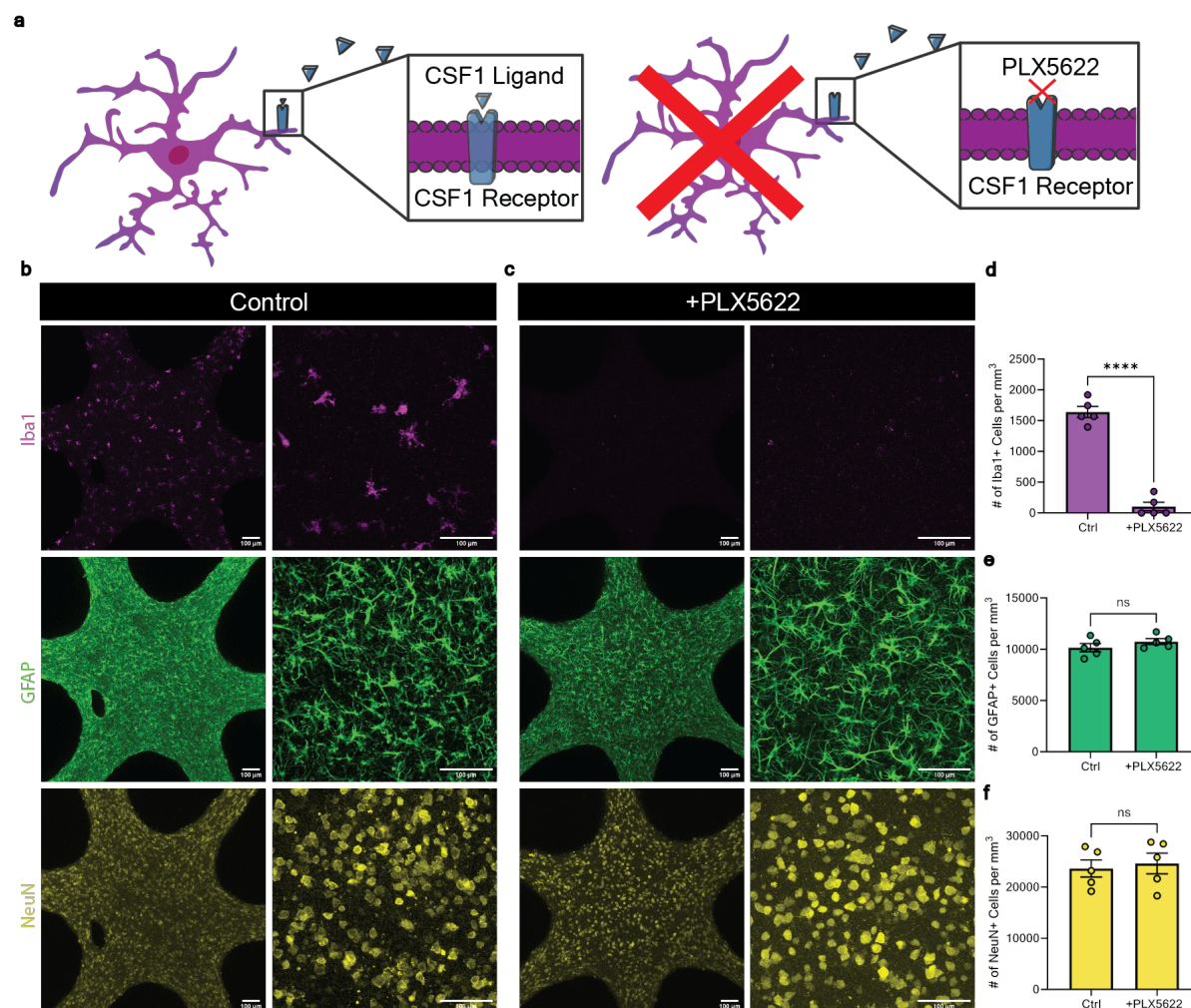
Supplemental Figure 3.12.5

a, Schematic overview of custom synaptic puncta counting and colocalization analysis pipeline. **a**, multichannel fluorescent images are initially converted from .oir to .hdf5 file for downstream analysis in Ilastik pixel segmentation software. **b**, multichannel fluorescent .hdf5 files are opened in Ilastik software as greyscale z stacks and pixel classifiers are trained for each channel of the image. This requires manual labeling of a minimum of 10 objects for “cell” and 10 objects or areas as “background”. Pixel classification was performed using 25 user-selected pixel features including gaussian smoothing, laplacian of gaussian, gaussian gradient magnitude, difference of gaussians, structure tensor eigenvalues, and hessian of gaussian eigenvalues, all with sigma of 0.3, 0.7, 1.0, 1.6, and 3.5. Following input of user-defined pixel features, a Random Forest classifier was trained to perform pixel classification of “cells” or fluorescent puncta vs background “non-cell” fluorescence. **c**, Instance segmentation was performed using a dual connected component analysis (CCA) and gradient descent (GD) region labeling method. This method was developed to handle touching yet distinct puncta which CCA alone groups together, while guaranteeing smaller puncta were still included in the analysis. Following CCA and GD analysis to determine individual puncta centers. These center points were then used as seed points for watershed analysis to perform Instance segmentation. The center of mass of the Instance Segmented objects were considered as the centers of synaptic puncta. This analysis was performed on both the pre and post synaptic channels. Puncta center points were added to a K-D tree data structure, one for pre and one for post synaptic channels. **d**, Pre and post synaptic puncta locations were then compared to one another. Pre and post synaptic puncta were classified as being co-localized if their centers were within 0.6μm of each other.



Supplemental Figure 3.12.6

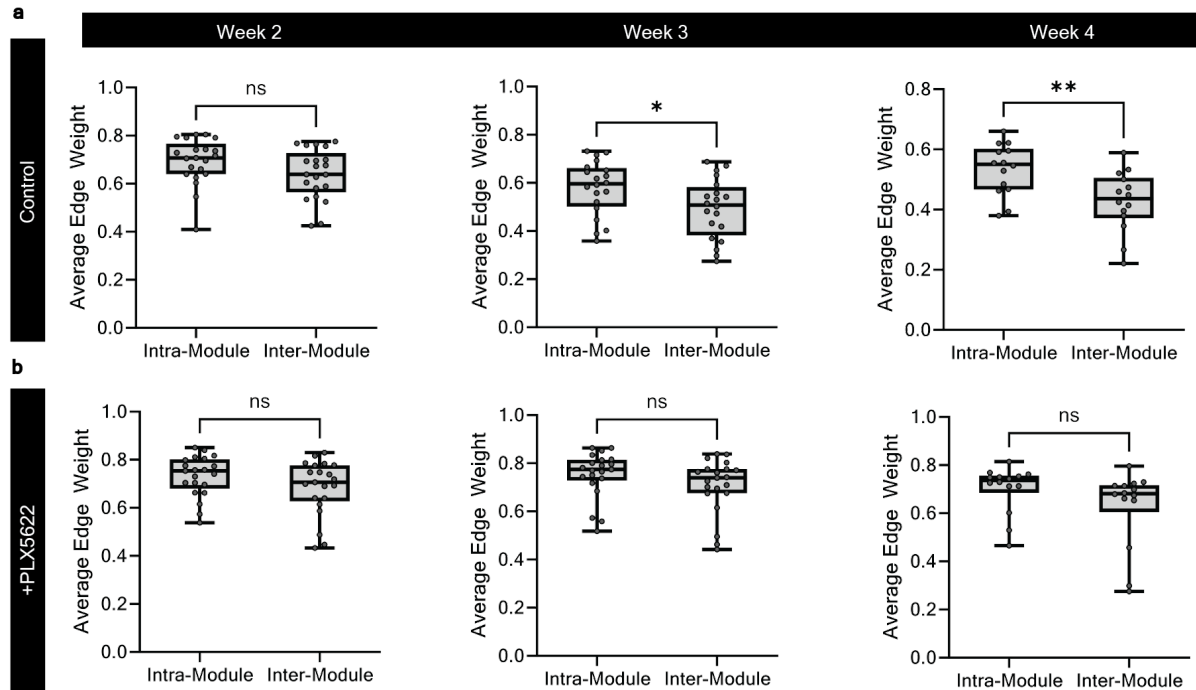
a, Sholl analysis from microglia shows average number of branch intersections over concentric circles at increasing radii in wildtype vs TgF344-AD microtissues at DIV80. Shaded area delineates standard deviation. Significant effect by Two-way ANOVA, **** $p < 0.0001$. $n = 5$ individual microglia per microtissues with $n = 3$ microtissues per genotype for each time point. Number of **(b)** Iba1+ cells and **(c)** GFAP+ cells per mm^3 in wildtype vs TgF344-AD microtissues at DIV28 and DIV80. Data is represented as mean $\pm$ standard error of the mean with Two-way ANOVA followed by Tukey's post-hoc comparison: * $p < 0.05$.



Supplemental Figure 3.12.7

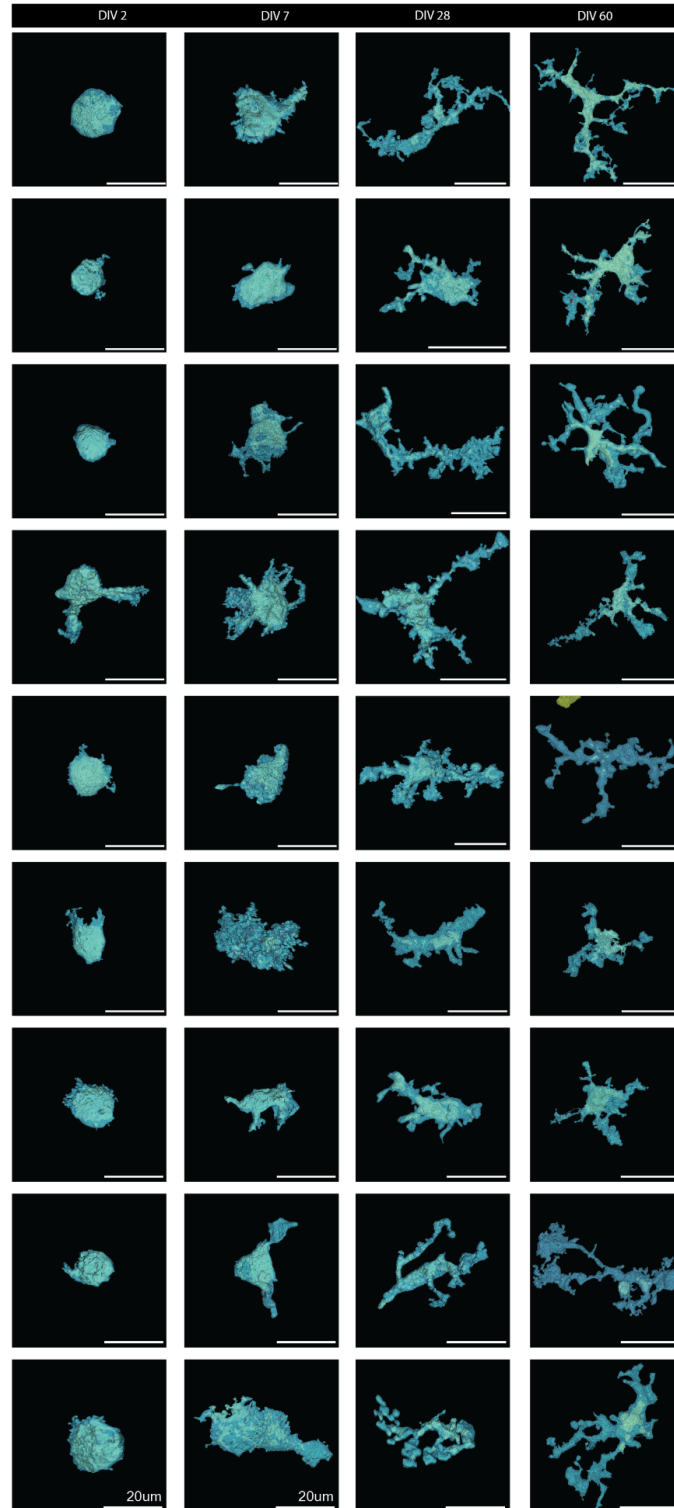
a, Microglia ablation from microtissues via addition of a CSF1R inhibitor PLX5622. **b-d**, Microtissue treatment with PLX5622 for one week followed by tissue fixation at DIV21 and subsequent IHC shows no off-target effect of PLX5622 on neuronal or astrocyte populations including no change in number of **(e)**

GFAP+ and (f) NeuN+ cells per mm³ while (d) significantly diminishing the Iba1+ microglia population. Data is represented as mean $\pm$ standard error of the mean with unpaired T test: ****p<0.0001 for n=5 microtissues per condition.



Supplemental Figure 3.12.8

Average intra-module edge weights vs average inter-module edge weights in (a) DMSO control and (b) PLX5622-treated microtissues at week 2, 3, & 4. Significant decrease in inter-module correlation compared to intra-module correlation as microtissues mature across weeks indicating enhanced connectivity of individual nodes within modules compared to node connectivity between modules. PLX5622-treatment entirely negates this effect. Mann Whitney test: **p<0.01, *p<0.05) n $\geq$ 6 microtissues per condition for each weekly timepoint. Data is represented in box plots displaying the median, interquartile ranges, and minimum/maximum whiskers extending to [1.5 $\times$ IQR / minimum-maximum range].



Supplemental Figure 3.12.9

Example 3D renderings of segmented Iba1+ microglia, CD68+ lysosomes, and synaptophysin+ presynaptic puncta using custom python-based software package from DIV2, 7, 28, & 60 (scale bar = 20μm)

Chapter 4

iPSC-derived microglia functionally integrate into a 3D xenograft culture system

4.1 Preface

Microglia play an essential role in maintaining brain homeostasis, yet their dysfunction is increasingly recognized as a key contributor to neurodegenerative diseases such as Alzheimer's disease. While induced pluripotent stem cell (iPSC)-derived microglia provide a promising tool for studying human microglia biology, recent studies have highlighted that due to their highly plastic nature and innate ability to sense the surrounding environment, microglia undergo dramatic shifts in morphology and gene expression when cultured *in vitro*. This chapter presents the development of a 3D xenograft culture system designed to overcome these challenges by enabling integration of iPSC-derived microglia into a physiologically relevant *in vitro* 3D neural environment.

We demonstrate that iPSC-derived microglia successfully engraft into cortical microtissues, adopting morphological and functional properties akin to their *in vivo* counterparts. Our findings reveal that prolonged culture within this environment enhances microglial maturation, improving their capacity for immune surveillance and injury response. Additionally, we show that engrafted microglia exhibit a robust response to systemic inflammatory cues signaling their ability to sense and functionally respond to the surrounding microenvironment. These results validate the 3D xenograft culture system as a powerful platform for studying human microglia in health and disease, offering new opportunities to investigate microglial dysfunction in Alzheimer's disease and related disorders. Moving forward, this system holds significant promise for

therapeutic screening and for exploring microglial interactions in complex neuroinflammatory conditions.

4.2 Abstract

Microglia, the resident immune cells of the central nervous system, play essential roles in maintaining brain homeostasis and responding to injury. Induced pluripotent stem cell (iPSC)-derived microglia provide a valuable platform for studying microglial function in human disease models. However, the innate ability of microglia to rapidly respond to the surrounding environment poses a unique challenge in maturing iPSC-derived microglia towards homeostatic brain-specific cell states *in vitro*. Here, we demonstrate that iPSC-derived microglia functionally integrate into a 3D xenograft culture system, adopting morphological and transcriptional profiles consistent with *in vivo*-like microglial states. Using this system, we investigate iPSC-derived microglia surveillance and response to damage in a 3D brain-like microtissue environment. Our findings reveal that engrafted iPSC-derived microglia morphologically mature within the 3D microtissues and functionally respond to systemic neuroinflammatory cues. Additionally, we show that iPSC-derived microglia response to laser-induced tissue lesioning is dependent on cell maturation with immature states correlating with impaired ability to detect and respond to extracellular ATP signals associated with tissue injury. These results demonstrate the utility of a 3D xenograft culture system for promoting mature brain-specific iPSC-derived microglia cell states and in the future could provide insight into possible *APOE* isoform-dependent deficits in microglial motility and damage response. Together, this work validates a 3D xenograft model as an advanced platform for studying human microglia dysfunction which may contribute to neurodegenerative processes in Alzheimer's disease and other neurological disorders, offering new opportunities for therapeutic development.

4.3 Introduction

Despite decades of research, Alzheimer's disease (AD) remains a severe public health crisis, affecting nearly 6.7 million Americans in the year 2023 and accounting for 60-80% of dementia cases worldwide. The confounding and complex nature of possible mechanisms behind AD-associated pathologies has limited the ability to identify successful targets for therapeutic intervention. Growing evidence implicates microglia as a key player in the neuroinflammatory processes that underlie a number of neurodegenerative diseases, including Alzheimer's Disease and AD-related dementia (ADRD) (Barres 2008; Liddel et al. 2017; S. Hong et al. 2016; De Strooper and Karran 2016). The development of novel diagnostic research tools including single-cell RNA sequencing (scRNAseq), genome-wide association studies (GWAS), advanced gene editing, and differentiation of human neural cells from induced pluripotent stem cells (iPSCs) has improved our understanding of microglia mechanisms in health and disease (Galatro et al. 2017; Muffat et al. 2016; Brownjohn et al. 2018; Olah et al. 2018; H. Park et al. 2019; Mertens et al. 2018; Guttikonda et al. 2021; Abud et al. 2017; Perry, Nicoll, and Holmes 2010; Santiago et al. 2017). However, the exact mechanisms and conditions by which microglia contribute to neurodegeneration in AD are not well understood.

The iPSC-derived human microglia (iMG), with highly similar properties as their *in vivo* counterparts, have emerged as a rigorous platform to investigate neuroinflammation and AD-linked genetics (McQuade et al. 2018; Muffat et al. 2016; Brownjohn et al. 2018; Guttikonda et al. 2021; Abud et al. 2017; Douvaras et al. 2017; Haenseler et al. 2017; M. Xu et al. 2019). A recent transition from 2D to 3D culture systems further matures the *in vitro* development of iMG (Olah et al. 2018; Abud et al. 2017; Haenseler et al. 2017) and reconstitutes a more AD-relevant niche (Choi et al. 2014; J. Park et al. 2018). Additionally, recent studies describe the engraftment of iMG into the mouse brain to form a xenograft model, wherein the resulting xenotransplanted iMG, morphologically and transcriptionally resemble adult human microglia found in the brain (Svoboda

et al. 2019; Fattorelli et al. 2021; Hasselmann et al. 2019; Abud et al. 2017; R. Xu et al. 2020). Despite such advances, technical barriers remain for the existing 3D iMG models: 1) Most organoid protocols lack cells of non-neuronal or non-ectoderm origin, including oligodendrocytes and mesodermal-derived vascular and microglia cells (Zhang et al. 2022; Porciúncula et al. 2021; Sloan et al. 2017; Eichmüller and Knoblich 2022; Lin et al. 2018). 2) Integration of human microglia into iPSC-derived neural organoids remains a significant challenge as there are few protocols and most have several experimental obstacles such as delicate timing of separate microglia differentiation in 2D culture prior to integration into organoids for 3D culture, high variability in cell recovery and low reproducibility (Popova et al. 2021; Abud et al. 2017; Ormel et al. 2018; Bodnar et al. 2021; Sabate-Soler et al. 2022; Y. Hong et al. 2023). 3) the latest chimera approaches by xeno-engraftment into immune-deficient mouse brains (Svoboda et al. 2019 ; Hasselmann et al. 2019; R. Xu et al. 2020; Rongvaux et al. 2014) *in vivo* have inherently low throughput as they rely on heavy use of animals and additionally have minimal ease of experimental manipulation (Svoboda et al. 2019; Fattorelli et al. 2021; Hasselmann et al. 2019). Such challenges highlight the need for novel culture systems that permit the study of human microglia in a brain-like environment. Here, we utilize a primary 3D cortical microtissue model that offers enhanced cellular diversity and functionality to engraft human iPSC-derived microglia thus creating a high-throughput xenograft culture system.

4.4 Results

4.4.1 Generation of human iPSC-derived microglia through patterned hematopoiesis

iMG were generated following a previously published protocol which outlines a simplified approach to generate robust populations of human microglia from iPSC (Abud et al. 2017). Briefly,

the protocol describes differentiation of iPSCs on a KOLF2.1 background toward a mesodermal, hematopoietic lineage using commercially available media (StemCell Technologies) (**Fig. 4.1**).

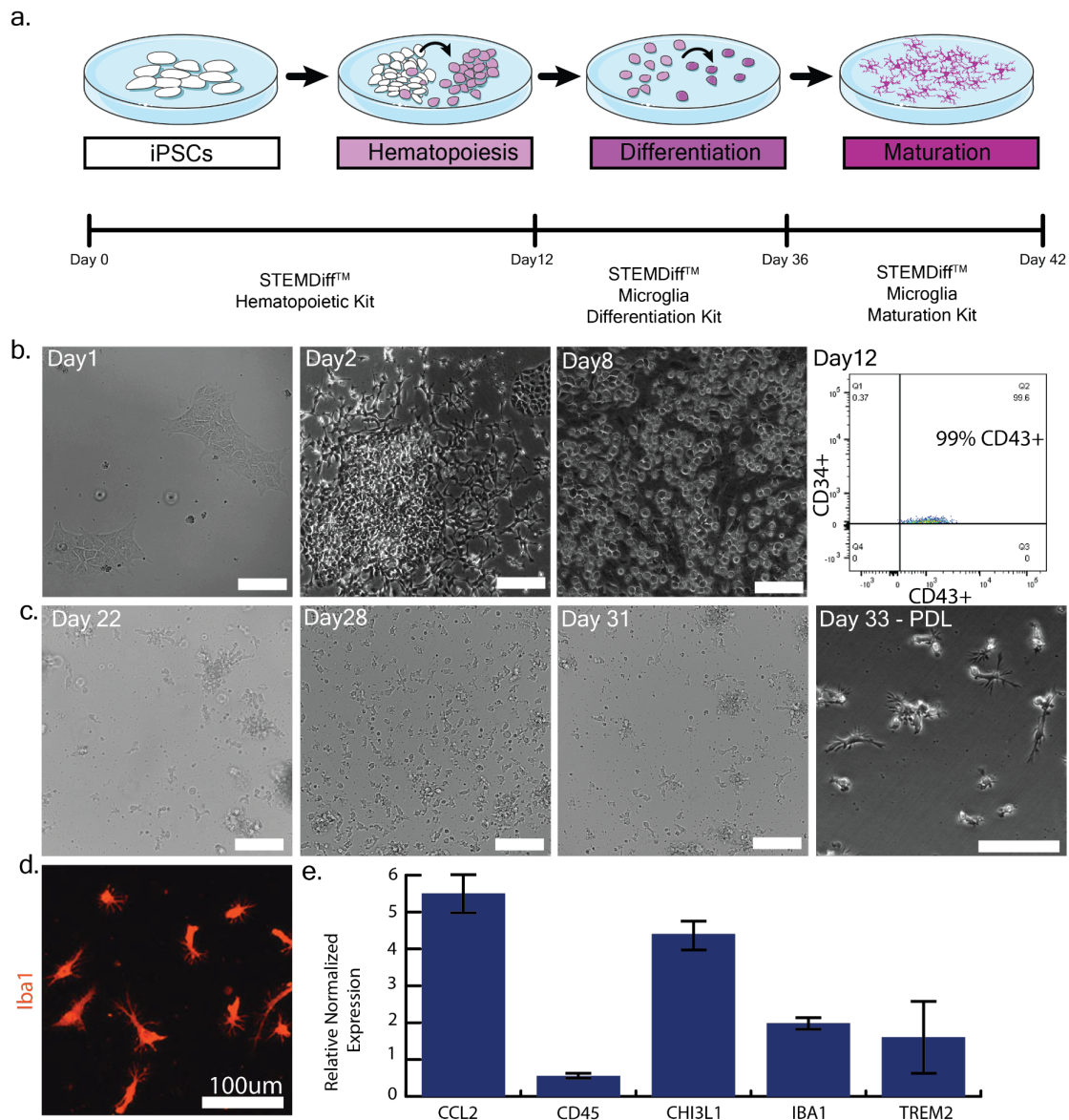


Figure 4.1 | Generation of microglia from human iPSCs

a, iMG differentiation following patterned hematopoiesis using STEMDiff hematopoietic and STEMDiff Microglia Differentiation kits. **b**, brightfield images from day 1-8 of hematopoietic progenitor cell differentiation. At day 2 HPCs begin to differentiate in semi-suspension from dense colonies. By day 8 rounded morphology of HPCs cultured in suspension. Day 12 FACS sorting for CD43+/CD35+ population. **c**, Brightfield images show morphological maturation of iMG during differentiation from the HPC population between day 22-31. iMG cultured on PDL-coated plates at day 33 show robust microglia-like morphology. **d**, iMG plated on PDL-coated plates are stained for immunoreactivity to microglia marker IBA1. **e**, qPCR analysis from iMG collected at day 32 show expression of microglia markers Iba1 and Trem2, macrophage marker CD45, and neuroinflammatory markers CCL2 and CHI3L1 (scale bars = 100µm)

The hematopoietic kit generates KDR+CD235A+ primitive hematopoietic hemangioblasts through

activin-nodal signaling and inhibition of the Wnt- β -catenin pathway (Guttikonda et al. 2021). Addition of hematopoietic cytokines push the primitive hematopoietic hemangioblast population toward CD43⁺ CD235a⁺CD41⁺ primitive HPCs. Following 10 days of culture to promote hematopoiesis, non-adherent, CD43⁺ hematopoietic progenitor cells (HPCs) can be collected and replated for continued differentiation toward microglia. HPC identity (CD34⁺/CD43⁺) was confirmed by flow cytometry on a sampling from the collected non-adherent population (CD34⁺ gate = %13.7 total cells; CD43⁺ gate on CD34⁺ cells = 99% (**Fig. 4.1, b**).

HPCs were placed into differentiation media containing IL-34, Macrophage Colony Stimulating Factor (MCSF), and Transforming Growth Factor β (TGF β) cytokines for 24 days. Following the 24 days of differentiation, microglia are then placed into maturation media containing additional neuronal-derived CD200 and CX3CL1 for at least 4 days before collecting for engraftment into 3D cortical microtissues. Distinct classical microglia morphologies were observed following plating onto poly-d-lysine (PDL) coated plates at day 33 (**Fig. 4.1, c**) but confirmation of microglia identity was assessed by qPCR and immunofluorescence. Microglia plated on PDL plates were subsequently fixed and assessed for iMG immunoreactivity to ionized calcium binding adaptor molecule 1 (Iba1) (**Fig. 4.1, d**). qPCR analysis of cell lysates sampled from iMG collected at day 33 confirmed expression of key microglia genes including Trem2, Iba1, and CD45 (**Fig. 4.1, e**).

4.4.2 Generation of a xenograft culture system

To incorporate iMG into 3D cortical microtissues we developed two separate engraftment approaches: 1) engrafting into microtissues at DIV0 (eDIV0) during initial rat cortical cell seeding and 2) engrafting into fully established microtissues at DIV21 (eDIV21) (**Fig. 4.2**). Prior to engraftment, iMG were transduced with a lentiviral vector (FUGW) to induce expression of a GFP reporter under the ubiquitin-C promoter (**Fig. 4.2, a**). iMG engrafted at eDIV0 were gently mixed into the single cell suspension of primary rat cortical cells prior to seeding into the agarose micro-

molds at a ratio of 1:14. At 24 hours post seeding, GFP+iMG were detected within the microtissue

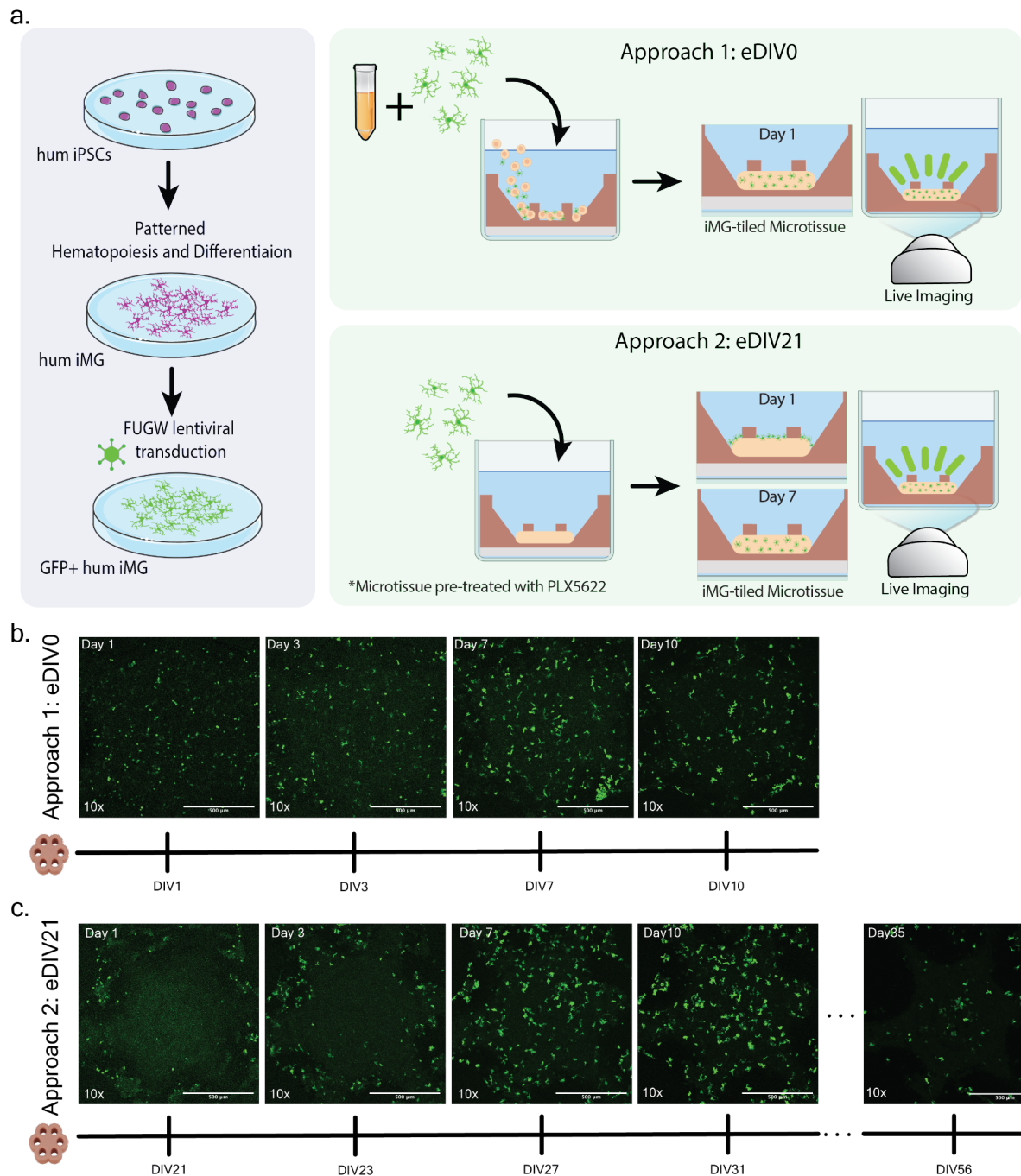


Figure 4.2 | Engraftment of iMG into 3D cortical microtissues

a, Schematic overview of strategies to engraft iMG into 3D cortical microtissues. Following differentiation of human iMG from hemaotpoietic progenitor populations, iMG are transduced with FUGW lentiviral particles approximately 3 days prior to engraftment to provide stable GFP fluorescence. iMG engrafted in approach 1 and gently mixed with primary rat cortical cells prior to seeding into agarose micro-molds. iMG engrafted in approach 2 are seeded over the top of DIV21 microtissues that have been pre-treated

with CSF1R inhibitor PLX5622 to deplete endogenous rat microglia populations. **b**, iMG engrafted at eDIV0 are evenly distributed throughout the microtissue and acquire increasingly ramified morphology between DIV3 and DIV10. **c**, iMG engrafted at eDIV21 are seen at tissue boundaries at 8 hrs post-engraftment and gradually tile into the microtissue. By day 6 post-engraftment iMG display more ramified morphologies and are evenly distributed throughout the microtissue.

(**Fig. 4.2, b**) but did not exhibit ramified morphologies. iMG morphological maturation appeared between day 7 and day 10 post engraftment (**Fig. 4.2, b**). By day 12 post engraftment, iMG populations had significantly decreased suggesting a possible negative host response from the endogenous rat microglia. To mitigate this effect, we developed a secondary engraftment approach wherein iMG are seeded on top of existing microtissues that do not contain endogenous rat microglia populations.

GFP+ iMG engrafted at eDIV21 were added to microtissues by gently seeding over the top of DIV21 old microtissues (**Fig. 4.2, c**). Prior to seeding, microtissues were treated with a small-molecule inhibitor to microglia receptor to colony stimulating factor 1 (CSF-1R). Ablation of endogenous rat microglia prior to iMG engraftment is a widely used approach in iPSC-xenograft studies as it mitigates a possible negative immune response from host immune regulators such as microglia. CSF-1 signaling is essential for microglia survival and both pharmacological and genetic inhibition of CSF-1R are widely used *in vitro* and *in vivo* to selectively ablate microglia (Spangenberg et al. 2019; Henry et al. 2020; Pridans et al. 2018; Rosin, Vora, and Kurrasch 2018; Coleman, Zou, and Crews 2020; Wallace et al. 2020; Elmore et al. 2015). We have previously assessed the efficacy of the CSF-1R inhibitor molecule PLX5622 (Spangenberg et al. 2019) in DIV21 microtissues by spiking culture media with 2uM PLX5622 media for 1 week and seen highly selective depletion of Iba1+ microglia compared to DMSO-treated controls ($p < 0.0001$) with no significant effect on general morphology and cell number of GFAP+ ($p = 0.2553$) and NeuN+ ($p = 0.7196$) cells. At 8 hours post seeding, very few GFP+iMG can be seen in the middle of the tissue (**Fig. 4.2, c**) but instead can be seen accumulating along the microtissue boundary. However, by day 2 post-engraftment, GFP+iMG can be seen migrating into the middle of the microtissue and by day 6 post-engraftment, the whole microtissue center is densely populated

and iMG morphological maturation begins to appear. Remarkably, iMG populations remained stable for up to four weeks post engraftment (**Fig. 4.2, c**). Compared to eDIV0 engrafted microtissues, eDIV21 microtissues, void of endogenous rat microglia, promote stable culture of GFP+iMG for 1 month post engraftment.

4.4.3 Engrafted iPSC-derived microglia functionally respond to neuroinflammatory stimulation

Neuroinflammation in the brain can be initiated in response to a variety of endogenous and exogenous stimuli including invading pathogens or infection, injury, and even abnormal protein aggregation. As the tissue-resident immune cells of the brain, microglia are central to these processes. With their motile processes, microglia survey the parenchyma and can respond to perturbations via their pattern recognition receptors (PRRs) to elicit both proinflammatory and anti-inflammatory signaling. This functional plasticity correlates with dynamic shifts in cell morphology and gene expression patterns. Under a pro-inflammatory or “activated” state microglia morphology will transform from highly ramified with small soma and numerous branches to a less ramified more amoeboid shape with a larger soma and fewer branches. These distinct morphological changes are widely utilized as a biomarker to quantify microglial activation across experimental platforms as both cell shape and motility represent a fundamental aspect of the cell identity and can be highly predictive of function (Colombo et al. 2022; Madore et al. 2013; Hansen et al. 2022; Vidal-Itriago et al. 2022). Capturing and quantifying changes in microglia morphology is essential to deciphering their functional response in the environment. Therefore, we next sought to assess the functional response of engrafted iMG to systemic inflammatory cues.

To achieve this, we dosed iMG-engrafted microtissues with bacterial cell-wall endotoxin lipopolysaccharide (LPS) (**Fig 4.3, a**). LPS is a common neuroinflammatory agent used both *in vitro* and *in vivo* to elicit robust microglia activation through microglia-specific toll-like receptor

(TLR) PRR signaling (Vance, Isberg, and Portnoy 2009; Akira and Takeda 2004; Ciesielska, Matyjek, and Kwiatkowska 2021; Stewart et al. 2010). Microtissue culture media was spiked with LPS at 10ug/mL or a PBS vehicle control. At 24 hours post LPS-addition, we observed a shift in iMG morphology with cells exhibiting more rounded and ameboid morphologies in LPS-treated (**Fig. 4.3, c**). Conversely, the control, non LPS-treated iMG displayed smaller somata and more ramified processes that are characteristic of homeostatic microglia (**Fig. 4.3, b**). Our 3D xenograft culture system is compatible with longitudinal live imaging using an inverted confocal microscope thus enabling continuous monitoring of individual cell features such as motility and surface area to volume dynamics. With this approach can capture swift changes iMG undergo, as seen in the transitions of a single cell from ameboid to ramified morphologies and vice versa in a 3 hr time window (**Fig. 4.3, d**) thus underscoring the capability of this model system to investigate the fast and dynamic shifts between cellular states. Using an open-source image analysis package (MotiQ) high-resolution volumetric recordings of individual iMG were recorded over 3 hours. Briefly, the 4D datasets (3D volume images taken at multiple time points) are processed for automatic cell identification and 3D segmentation. From 3D reconstructions of single cells we can track dynamics over time such as cell ramification index, number of extensions and retractions, and dynamic vs static cell areas which have become a standard parameter in microglia morphology analyses (Hansen et al. 2022; Zhao et al. 2025; Bolasco et al. 2018; Milior et al. 2020) (**Fig. 4.3, e-j**). LPS-treated iMG display a significantly lower ramification index across a 60 min recording compared to PBS-treated control groups ($p=0.0041$) (**Fig. 4.3, e**). This is representative of the classical ameboid morphology associated with microglia under LPS stimulation (Nakamura, Si, and Kataoka 1999; Ye et al. 2020; Lively and Schlichter 2018; Jung et al. 2023). Additionally, the LPS-treated iMG exhibits significantly fewer branch extensions ($p=0.0079$) (**Fig. 4.3, f**) and retractions ($p=0.0079$) (**Fig. 4.3, g**) reflecting diminished surveillance activity associated with reactive microglia phenotypes.

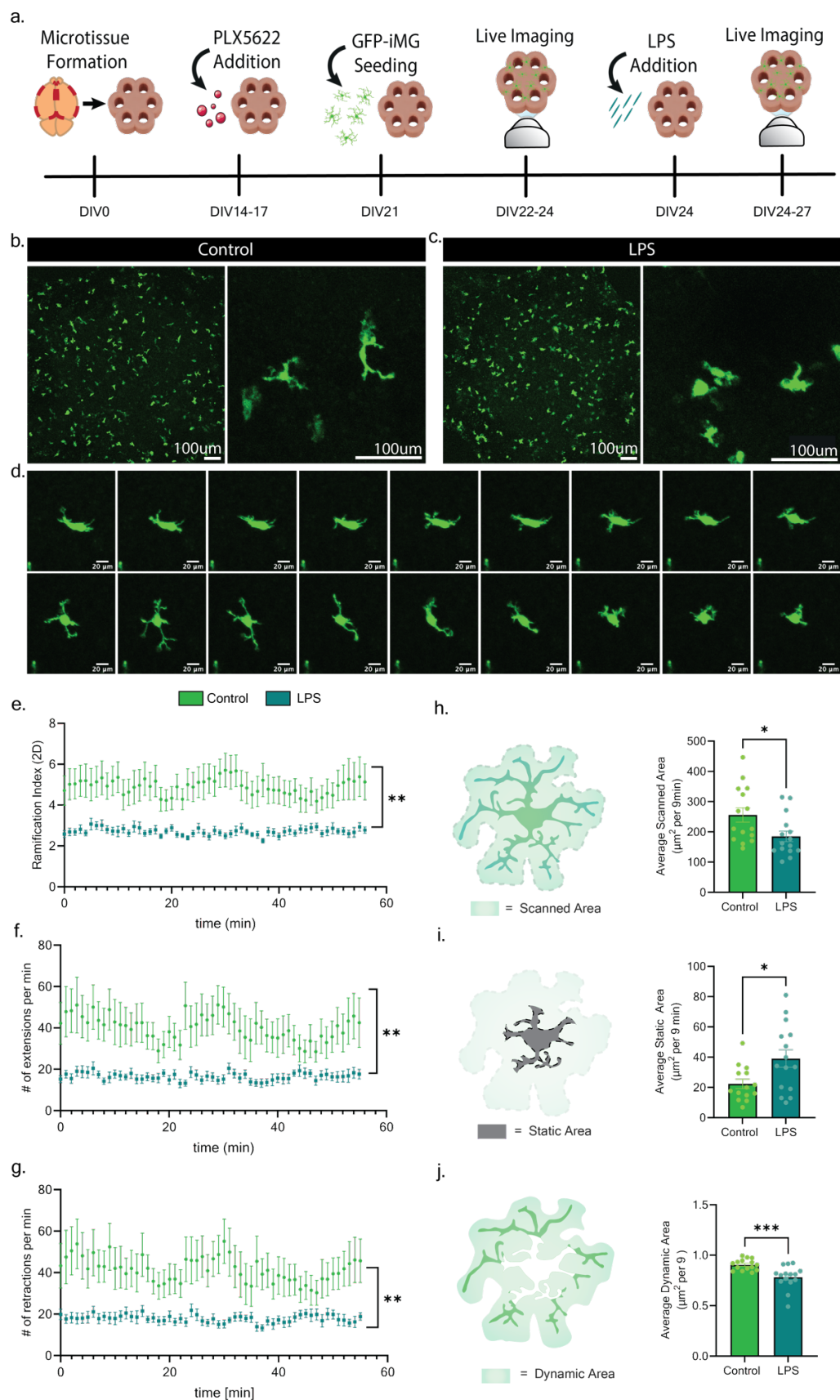


Figure 4.3 | Engrafted iMG functionally respond to neuroinflammatory stimuli

a, Schematic of experimental timeline for LPS-stimulation of engrafted iMG. Culture media is spiked with 10ug/mL of LPS at DIV24 following engraftment of iMG at DIV21. **b-c**, at 24 hrs iMG from LPS-treated microtissues display morphological shift toward more amoeboid and round cell shape compared to iMG from the control (PBS-treated) microtissues. **d**, multiple frames of a single cell across a 3 hr recording period show dramatic shifts in morphology highlighting an advantage of this platform to image, in real time, dynamic behaviors of cells within the 3D neural microenvironment. **e**, Ramification index of iMG from LPS-treated microtissues is significantly smaller compared to iMG from control microtissues as shown over a 1 hr recording. Significantly fewer (**f**) number of extensions per min and (**g**) number of retractions per min in iMG from LPS-treated microtissues compared to iMG from control microtissues. Significant main column effect by two-way ANOVA: $^{**}p<0.01$ ($n=10$ microglia from 3 microtissues per condition) Data is represented as the mean $\pm$ standard error of the mean. From the (**h**) total area scanned, the (**i**) static area, and (**j**) dynamic area were calculated across a 9 min recording window and averaged across the hour long recording for iMG from LPS-treated microtissues compared to iMG from control microtissues. Significance was detected by Mann Whitney test: $^{***}p<0.001$, $^{*}p<0.05$ ($n=15$ microglia from 3 microtissue per condition) Data is represented as the mean $\pm$ standard error of the mean.

To further probe possible differences in iMG surveillance behavior we assessed the distribution of static and dynamic cell features within each recorded cell from LPS and control conditions (**Fig. 4.3, h-j**). The total area “scanned” by an iMG treated with LPS was significantly less compared to the control group ($p=0.0164$) (**Fig. 4.3, h**). Within the total scanned area, the percentage occupied by static features of the cell body was higher in the LPS group ($p=0.0328$) (**Fig. 4.3, i**). Further, the percent of the scanned area not associated with static cell features but rather was transiently covered by moving cell processes, or the “dynamic” area was greater in the control groups ($p=0.0002$) (**Fig. 4.3, j**). These findings confirmed that iMG, when cultured in the 3D microtissues can perform immune-sensing behaviors in response to systemic inflammatory cues and actively surveil the surrounding microenvironment in a manner that is characteristic of human microglia. This supports use of a 3D xenograft culture system with functional integration of human iMG to enable further investigations of cell-type specific microglia function. Our model allowed us to obtain functional iMG that operated within a human-brain-like environment, thereby enabling us to investigate human-specific microglia functions under both physiological and pathological conditions *in vivo*.

4.4.4 Engrafted iPSC-derived microglia response to laser ablation injury is culture-time dependent

One of the key responsibilities of microglia during development and maintenance of the brain parenchyma throughout life is to continuously survey their environment for signs of injury or dysfunction. Their ability to rapidly detect and respond to damage is critical for maintaining brain homeostasis and function. A key component of this response is the detection of ATP released from injured cells, which acts as a damage-associated molecular signal that binds to microglia P2Ry12 that activates microglia motility and recruitment to the injury site. Efficient microglial surveillance and directed migration toward ATP gradients are essential for clearing debris, modulating inflammation, and promoting tissue repair. Therefore, assessing the responsiveness of iMG engrafted into a 3D microtissue environment provides a critical measure of their functional integration and ability to support neural health.

In the present study, iMG exhibited a time-dependent enhancement in their response to laser-induced injury within the 3D microtissue environment, suggesting a progressive adaptation to their tissue surroundings. Quantitative analysis revealed that iMG cultured for 4 weeks in the microtissue environment displayed a more robust response to tissue damage compared to iMG cultured for 1 week in the microtissues (**Fig 4.4**). iMG motility was quantified using the open-source plugin to FIJI TrackMate which can measure the displacement, speed, and traveled area of individual iMG following focal laser-induced tissue lesioning. At 4 weeks post-engraftment, iMG exhibit a robust response to tissue injury marked by distinct change in morphology and migration of cells towards the lesion site (**Fig. 4.4, a-c**). Motility traces of 4wk old iMG (**Fig. 4.4, d**) exhibit significantly greater motile properties in a 3 hour window following laser-injury compared to pre-laser injury motility. Specifically, iMG displayed an increase in track displacement ($p=0.0007$) (**Fig. 4.4, e**), meaning the increase in the distance between the xy coordinates of the iMG center of mass (COM) at time=0 and time=203min post laser injury. Further the total distance covered by

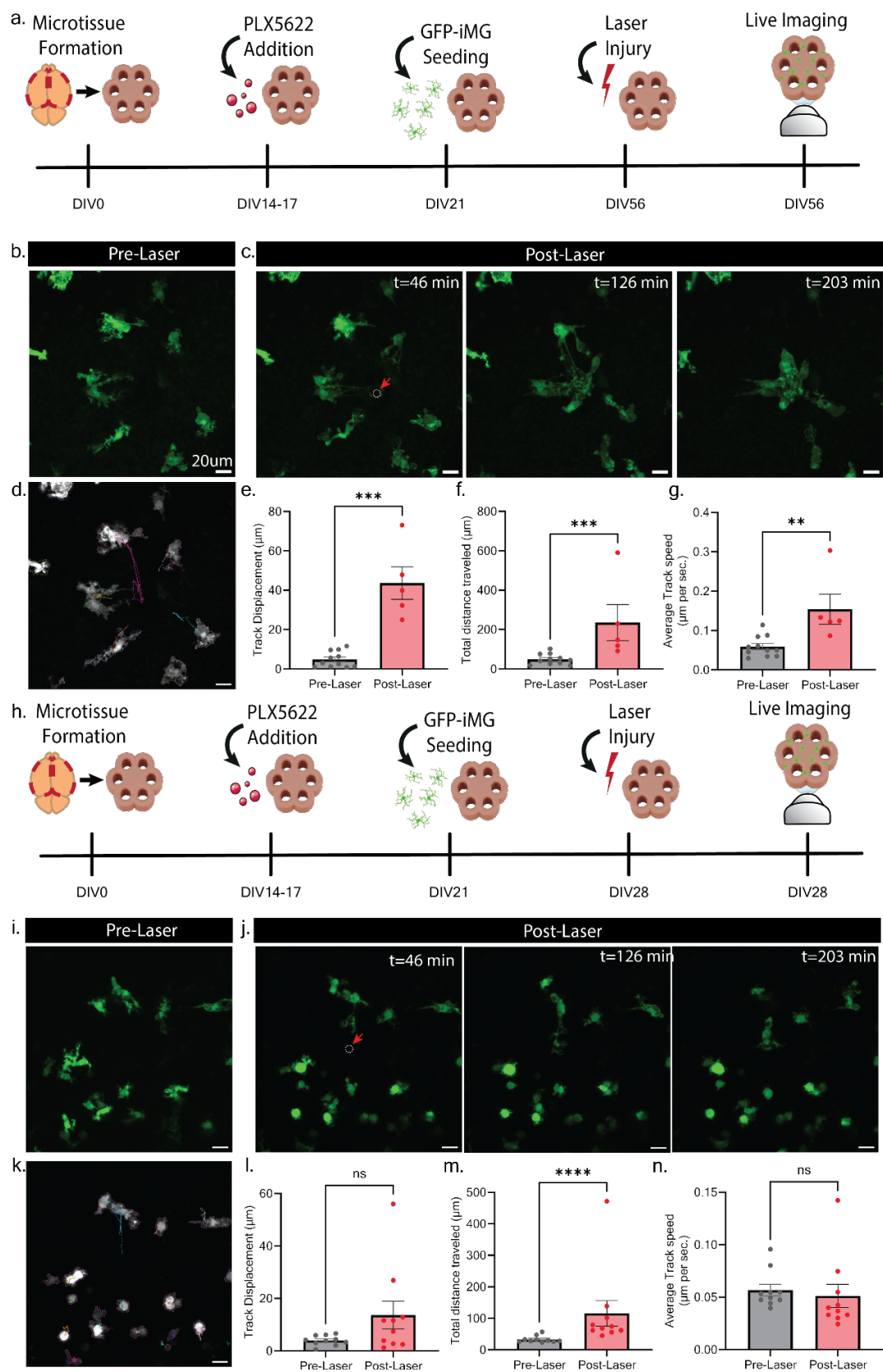


Figure 4.4 | Engrafted iMG respond to laser-induced tissue lesion

a, Schematic of experimental timeline for laser-induced tissue injury at DIV56 following engraftment of iMG at DIV21. **b-c**, Comparison of iMG morphology and location (**b**) prior to laser injury and (**c**) for 203 min after injury. **c**, iMG processes begin to extend toward area of laser stimulation (red arrow) at 46 min post injury and total cell migration occurs by 203 min post injury. **d**, Tracking of single cell COM during 3 hr post-injury recording. Individual cell traces shown in pink, blue, blue, white, yellow and orange. **e**, Compared to pre-injury iMG recordings, tracking of COM showed significant displacement post injury. **f**, Significant increase in total distance traveled of each cell COM in post-injury recording. **g**, Significantly higher average cell speeds (um/sec) post injury compared to pre-injury. **h**, Schematic of experimental timeline for laser-induced tissue injury at DIV28 following engraftment of iMG at DIV21. **i-j**, Comparison of iMG morphology and location (**i**) prior to laser injury and (**j**) for 203 min after injury. **j**, only a single iMG begins to extend processes toward the area of laser stimulation (red arrow) at 46 min post injury. At 203 min post injury only 1 cell migrated towards the injury site. **k**, Tracking of single cell COM during 3 hr post-injury recording. Multicolored traces of individual cell traces shown. Compared to pre-injury iMG recordings, tracking of COM showed (**l**) no significant displacement and (**n**) no significant change in average cell speed (um/sec) in recordings of iMG post injury. **m**, Interestingly there was a significant increase in total distance traveled of each cell COM in post-injury recording compared to pre. Significance was detected by Mann Whitney test: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) ($n \geq 5$ microglia from 1 microtissue per condition) Data is represented as the mean $\pm$ standard error of the mean.

the iMG COM was significantly greater post injury ($p=0.0009$) (**Fig. 4.4, f**) suggesting iMG were in a highly motile state. Additionally, the average speed of iMG was greater in the post-injury recording window ($p=0.0027$) (**Fig. 4.4, g**).

Conversely, iMG response to laser injury following 1 week of culture in the microtissue environment was markedly diminished (**Fig. 4.4, h-j**). iMG displayed a shift in morphology towards more ameboid or activated state but displayed limited motile response with fewer cells migrating towards the lesion site (**Fig. 4.4, j**). Individual tracing of cell motility (**Fig. 4.4, k**) revealed only a slight increase in track displacement of cells post-injury compared to baseline pre-injury recordings ($p=0.1431$) (**Fig. 4.4, l**). Interestingly, the total distance traveled of individual cell COMs was higher in the post-injury recordings ($p < 0.0001$) possibly reflecting the change in cell morphology towards a more ameboid shape which would cause the COM to shift (**Fig. 4.4, m**). However, compared to post-injury cells at 4 weeks post engraftment, the total distance traveled of post-injury cells at 1 week post engraftment was significantly lower ($p=0.0280$) (data not shown). Additionally we did not observe any significant change to average cell motility speed in post-injury cells at 1 week post engraftment compared to pre-injury ($p=0.0892$) (**Fig. 4.4, n**).

The enhanced motility seen at week 4 post-engraftment suggests that prolonged culture supports the functional maturation of engrafted microglia. This is likely resultant from limited expression of mature microglia purinergic receptors P2RY12 and P2X7, which are required for process motility in detection and response to ATP release from injured cells (Tewari, Michalski, and Egan 2024; Gómez Morillas, Besson, and Lerouet 2021; Ohsawa et al. 2007; Janks, Sharma, and Egan 2018). Recent studies have shown that P2RY12 expression is absent during *in vitro* culture of iMG, even when cultured in an organoid environment and is rescued upon engraftment into the rodent brain (Schafer et al. 2023). Their findings suggest that the lack of these mature microglia-specific genes *in vitro* points to either insufficient acquisition of a brain-like environment-specific signature or an incomplete transition to a mature homeostatic state. Together, our analysis of iMG response to tissue lesioning at 1 versus 4 weeks post-engraftment in the xenograft culture system indicate that microglial functionality within the microtissue improves over time and suggests the brain-like microtissue environment is sufficient in inducing maturation of iMG with brain-like environment-specific gene signatures.

4.5 Discussion

In this study, we established a three-dimensional (3D) xenograft culture system to investigate the functional integration and responses of human induced pluripotent stem cell-derived microglia (iMG) in a physiologically relevant environment. Our findings demonstrate that iMG successfully engraft into 3D cortical microtissues, adopt *in vivo*-like morphological states, and functionally respond to inflammatory stimuli and tissue injury.

Our results indicate that iMG are capable of stable integration into 3D cortical microtissues, with optimal survival and maturation occurring in microtissues where endogenous rat microglia were depleted prior to engraftment (eDIV21 condition). The enhanced stability of iMG populations in eDIV21 conditions suggests that host microglia may present a competitive or

inhibitory influence on the engrafted human microglia, consistent with previous reports of xenograft-host interactions in mixed-species systems. Additionally, the observed time-dependent maturation of iMG within the microtissue environment underscores the necessity of a supportive cellular and molecular niche for proper microglial development.

We further validated the functional capacity of iMG by assessing their response to systemic inflammatory cues. Engrafted iMG exhibited classical morphological transitions from a homeostatic, ramified state towards an activated, ameboid state in response to LPS treatment, consistent with established microglial activation paradigms. Image-based quantification of cell motility and process dynamics revealed that LPS-treated iMG displayed significantly reduced process ramification, fewer extensions and retractions, and a shift towards a more static phenotype. These findings confirm that our 3D xenograft culture system supports microglial immune surveillance and activation, reinforcing its relevance for studying neuroinflammatory processes in human disease models.

To probe whether engrafted iMG would acquire functional response to tissue damage and injury we quantified iMG motility following laser-induced lesioning. We show that iMG motility and recruitment to injury sites is enhanced with prolonged culture time of iMG within the 3D microtissues. While iMG engrafted for one week exhibited limited migratory response, iMG cultured for four weeks demonstrated significantly greater motile properties, including increased displacement, speed, and total distance traveled post-injury. These results suggest that prolonged culture within the 3D microtissue environment facilitates functional maturation of iMG, potentially through acquisition of brain-like gene expression signatures. The delayed upregulation of key microglial receptors such as P2RY12 may be a contributing factor to the observed time-dependent improvements in injury response. These findings highlight the importance of culture duration when utilizing iMG for modeling microglial behavior *in vitro* and highlight the utility of a 3D xenograft culture system to promote acquisition of brain-like cell states of iMG.

The establishment of a high-throughput xenograft culture system for human iMG provides a valuable tool for studying microglial biology in both health and disease contexts. Our model enables controlled investigations of microglial responses within a complex cellular environment, offering advantages over traditional 2D cultures and animal xenograft approaches. Future studies leveraging this platform could focus on elucidating the molecular mechanisms underlying previous reports of microglia-specific APOE4-associated deficits in P2Ry12 mediated response to injury and amyloid plaques (Victor et al. 2022; Sepulveda et al. 2024; Liu et al. 2023). Additional areas of continued work would include, screening of potential therapeutic compounds targeting microglial dysfunction and further optimizing the culture system to enhance microglial maturation and longevity. In conclusion, our study demonstrates that iMG can successfully integrate into a 3D xenograft culture system, exhibit functional immune responses, and recapitulate brain-like tissue surveillance and injury response. These findings underscore the utility of a xenograft culture model for advancing our understanding of human microglia in neurodegenerative diseases and for identifying novel therapeutic strategies targeting microglial dysfunction in AD and related disorders.

4.6 Materials and Methods

4.6.1 Animals

All animal experiments were approved by the Brown University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institute of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). Purchased timed-pregnant Sprague Dawley rats were monitored for arrival of litter and pups which were subsequently collected at postnatal day1. An equal number of male and female littermates were pooled for cortical tissue collection and formation of microtissues.

4.6.2 Agarose gels

Following our previously published protocol¹³ agarose micro-molds were created following previously described methods in Brown et al 2023. Briefly, custom stainless-steel injection molds were placed within each well of a 24-well glass-bottom culture plate (Cat. # P241.5HN, CellVis). The injection mold was held in place approximately 150 μ m above the bottom of the plate using a custom aluminum milled single well insert that fits into each well. A 2% (w/v) agarose (cat # 16500, Invitrogen) solution is prepared in dulbecco's phosphate buffered saline and heated for 30 second intervals in a laboratory microwave until agarose is in solution and mixture reaches a steady boil. Hot agarose was injected into the stainless-steel molds using a 10 mL sterile syringe. The syringe was removed and the plate placed on ice for 15 minutes to allow for the agarose to solidify before carefully removing the injection mold. 1 mL of complete cortical media (CCM) containing 2% neuronal supplement B-27 (Cat. # 17504-044 Invitrogen), 1% Penicillin-Streptomycin (Cat. #15140122, Thermo Scientific), and 0.25% GlutaMAX (Cat. # 35050061, Thermo Scientific) was added to each well to keep the agarose microwells hydrated and plates were placed at 37 C 5% CO₂ until primary cell seeding.

4.6.3 Three-dimensional primary cortical cultures

Primary cortical cultures were made based on previously described methods¹³. Briefly, an equal number of males and females were collected at postnatal day 1 and deeply anesthetized before euthanasia. Whole brains were collected and placed in cold Hibernate A media (Cat. # HA-500, Transnetyx Tissue) containing 1% B-27 neuronal supplement (Cat. # 17504-044 Invitrogen). Cortices were dissected away and enzymatically digested in a 2 mg/mL Papain and Hibernate A-minus Calcium (Cat. #PAP & #HACA, Transnetyx Tissue) solution for 30 minutes. Following digestion, tissue was triturated using a large-bore (1000 μ l) pipette and a single cell suspension was added to a 40 μ m cell strainer. Cells were washed 3 times with cold Hibernate

A media containing 1% B27 neuronal supplement. Suspension was centrifuged at 150 g for 5 min and supernatant removed before the pellet was resuspended in a fresh 5 mL of CCM. Two additional filtration and centrifugation steps were completed before cells were counted and assessed for >95% viability. The pellet was resuspended to a final concentration of 2.7×10^8 cells/mL and 10 μ L of cells were carefully added dropwise to the center of the agarose-molded microwells. Prior to cell seeding all media was removed from the microwells. Following seeding, plates were placed at 37°C, 5% CO₂, 95% humidity and allowed to incubate for 20 min before 1 mL of warmed complete cortical media was carefully added to each well. Media was subsequently changed every other day thereafter. For PLX-treatment groups, PLX5622 compound (Sellekchem Cat. No.S8874) was diluted to 2 μ M in complete cortical media and added to microtissues. Control samples were dosed with 2 μ M DMSO as a vehicle control. PLX5622 inhibitor or vehicle control were added during complete media changes every three days.

4.6.4 Generating iPSC-derived microglia

iMG were generated following our previously published protocol (Mika's protocol paper) using hematopoietic STEMdiff™ and Microglia STEMdiff™ kits and medias (Catalog #05310, #100-0019). Briefly, human iPS cells colonies are seeded as small aggregate in STEMFlex™ media in a 6-well plate. After one day, STEMFlex™ medium is replaced with 2 mL per well Medium A (STEMdiff™ Hematopoietic Basal Medium containing Supplement A) to begin inducing the cells towards a mesoderm-like state (day 0). On day 2, another 1 mL of Medium A is added to each well. On day 3, a complete medium change is performed with 2 mL of Medium B (STEMdiff™ Hematopoietic Basal Medium containing Supplement B). At day 5, 7, and 9 1 mL of Medium B is added in half-medium changes. HPCs in suspension are harvested on day 10. HPCs are then centrifuged at 300xg for 5 min and resuspended with STEMdiff™ Microglia Differentiation Medium to perform cell count and assess viability via hemocytometer. HPCs are seeded at 1×10^5 – 2×10^5 cells per well of a Matrigel®-coated 6-well plate containing 2 mL STEMdiff™

Microglia Differentiation Medium (cell density 1.1×10^4 – 2.2×10^4 cells/cm²). Plates are then incubated at 37 °C and 5% CO₂. 3. Add 1 mL STEMdiff™ Microglia Differentiation Medium to each well every other day. On day 12, transfer the cell suspension to a 15 mL conical tube. Centrifuge at 300 × g for 5 min. Resuspend in 1 mL conditioned media and add to a new well containing 1 mL fresh STEMdiff™ Microglia Differentiation Medium+cytokines. Add 1 mL STEMdiff™ Microglia Differentiation Medium+ cytokines to each well every other day until day 24.

On day 24, transfer the cell suspension to a 15 mL conical tube. Centrifuge at 300 × g for 5 min. Resuspend in 1 mL STEM-diff™ Microglia Maturation Medium, and count by hemocytometer. Add 1e6 cells to one well of a Matrigel®-coated 6-well plate to contain a total of 2 mL STEMdiff™ Microglia Maturation Medium. Incubate at 37 °C and 5% CO₂. 3. Add 1 mL of STEMdiff™ Microglia Maturation Medium+cytokines every other day. 4. Microglia are mature starting at day 28 and can be harvested until day 34.

4.6.5 Lipopolysaccharide challenge

Microtissues engrafted with iMG were divided into the following groups: i) PBS (control) and ii) LPS group (10ug/ml). Media was removed from microtissues and replaced with 1mL of LPS+ or PBS+ media. Microtissues were allowed to incubate with LPS or PBS+ media for up to 48 hrs. Live imaging of iMG response to LPS was performed as early as 24 hrs post treatment.

4.6.6 Live Imaging of engrafted iMG

To perform longitudinal live imaging of engrafted iMG, maturing iMG cells are transduced with FUGW lentiviral particles approximately 3 days prior to cell seeding. 5ul of lentiviral particles were added to each well of the 6 well plate. Following engraftment, microtissues were imaged as early as 24 hours post engraftment on an Olympus FV3000 multi-laser scanning confocal microscope. During imaging sessions, multiwall plates were placed in a temperature, humidity, and CO₂ controlled stage-stop incubator (Tokai Hitt STXF) and imaged at 37C, 5% CO₂ for the

duration of the recording session. Using the 488nm laser and EGFP filter, microtissues were imaged with 10x objective (3.164mm x 3.164mm FOV) and 20x objectives (636.42umx636.42um FOV) for LPS recordings. Plates were allowed to equilibrate in the stage-stop incubator for 30 minutes prior to imaging. Individual z-stacks were acquired with z-step resolution of 3.61um and 0.97um for the 10x and 20x objective respectively. For recordings of cell activity following LPS and PBS treatment, the Multi Area Time Lapse feature on the FluoView software was used. Each well location and exact XYZ coordinates of where each z stack was to be acquired were defined in a multi-area mapping. 204 imaging cycles were acquired over 3.4 hrs in an XYZT fashion. Recordings (.oir format) were loaded into FIJI using the Olympus Viewer plugin (<https://imagej.net/OlympusImageJPlugin>) and converted to tiff stacks. A maximum intensity projection across the z stack was created for 2D analysis of iMG motility. Using the open-source MotiQ plugin (<https://github.com/hansenjn/MotiQ/tree/master>) was used to perform quantitative analysis of individual cell from a larger FOV. Individual ROIs were drawn over 5 individual cells per treatment condition and processed in the MotiQ plugin.

4.6.7 Laser-induced tissue lesioning

Focal laser lesions were performed following a minimum baseline recording period of 20 min, during which z-stacks (with 3.83um z-step resolution) were acquired as described above using the 488nm laser at 10x objectives (265.16umx265.16um FOV). To induce focal lesions, the Olympus FluoView FV3000 confocal microscope was set up for a fluorescence recovery after bleaching (FRAP) experiments using the 10x objective. Briefly, a CAV tornado ROI (~5µm in diameter) at a focal plane approximately ~50 µm into the microtissue surface was set. Location of the lesion ROI was determined based on location of surround iMG. Area with relatively concentric spread of iMG around the ROI was chosen. Using the LSM Stimulation window in the FluoView software, the pre-stimulation window was set to 1 min if 20 min baseline recordings

were previously acquired. If a baseline recording was required, the pre-stimulation recording window was set to 20 min. The stimulation window was set to 45 seconds (non-continuous) and the stimulation source was set to be the 405nm laser at 100% power. The post-stimulation window was set as desired using the same 488nm laser and recording parameters as during the baseline recording period. Recordings (.oir format) were loaded into FIJI using the Olympus Viewer plugin (<https://imagej.net/OlympusImageJPlugin>) and converted to tiff stacks. A maximum intensity projection across the z stack was created for 2D analysis of iMG motility. Using the open-source TrackMate plugin (<https://github.com/trackmate-sc>) was used to perform quantitative analysis of groupings of cells near the lesion ROI. Briefly, the pixel segmentation was performed first in the Ilastik software (<https://www.ilastik.org/>) and the TrackMate detector was implemented to use ilastik pixel classifiers as detector to create objects to track (<https://github.com/trackmate-sc/TrackMate-Ilstik>).

4.6.8 Immunohistochemistry

iMG seeded on PDL-plates were fixed in 4% (w/v) paraformaldehyde (Cat. # F79-500, Milipore Sigma), 8% sucrose (Cat. # S7903, Milipore Sigma) solution for 2 hours followed by 3, 2 hr washes with 1x dulbecco's phosphate buffered saline (1xDPBS) (Cat. #D1408, Milipore Sigma). DPBS was aspirated and replaced with 1mL blocking solution per well consisting of 4% (w/v) bovine serum albumin (Cat. # A7030, Milipore Sigma), 10% normal goat serum (Cat. # 005-000-121, JacksonImmunoResearch), and 1% TritonX-100 (Cat. #T8787, Milipore Sigma) in 1x DPBS. Plate was placed on an orbital shaker and allowed to incubate at room temperature in blocking solution for 2 hrs before replacing with 1mL of primary antibody (rabbit anti-Iba, 1:200 (cat #019-19741, WAKO Chemicals)) diluted in blocking solution and incubated overnight with gentle orbital shaking. Following overnight primary antibody incubation, well were subsequently washed with phosphate buffered saline containing 0.2% TritonX-100 (1xPBT) 2 times over 4 hrs

with gentle orbital agitation. Microtissues were placed in 1mL of fresh blocking solution for 2 hrs on shaker before overnight incubation with secondary antibody (goat anti-rabbit Alexa Fluor 635, 1:800 (cat. #A31577, Invitrogen). Following overnight incubation, secondary antibody solution were removed and wells were washed 4 times over 4 hours with 1xPBT. Finally, microtissues were stained with a dsDNA marker DAPI at 1:1000 (cat. #D1306, Invitrogen) for 1 hr prior to imaging. Immunofluorescent images were collected on an Olympus FV3000 multi-laser scanning confocal microscope using a 20x objective. Images were loaded into FIJI using the Olympus Viewer plugin (<https://imagej.net/OlympusImageJPlugin>).

4.6.9 Statistical analysis

Statistical analysis was performed using JMP and GraphPad Prism statistical and graphing software. Individual cell counts were taken from several microtissues from n=3 biological replicates (exact n information is reported in each figure caption). A biological replicate was determined as a single litter of pups with equal number of male and female pups pooled to collect cortices and seed an average of 16 microtissues per litter. In data where two groups were compared at a single time point, parametric (two-tailed unpaired Student's T-test) was used for datasets of Gaussian distribution and non-parametric (Mann-Whitney) was used when parametric tests were not applicable. In data where single variable changes were detected across several time points, statistical significance was tested by parametric (one-way ANOVA with Tukey's post-hoc analysis) for datasets with Gaussian distribution or by non-parametric (Kruskal-Wallis with Dunn's post-hoc analysis) where parametric tests were not applicable. In data where multiple variable analysis was required across several time points in either LPS-treated or PBS-treated control microtissues, parametric (two-way ANOVA with Tukey post-hoc analysis) was used. Normality was determined by Shapiro Wilks test and in the even where n was too low, quantile-quantile plots were used to visually inspect distribution. Significance level of $\alpha < 0.05$ was

determined (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). All error bars represent either the standard deviation from the mean or the standard error of the mean and is denoted in the figure captions.

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Chapter 5

Summary, Future Directions, and Conclusions

In this thesis I have developed and deployed novel research tools to advance the study of microglia cells and their roles in neuroinflammatory mechanisms that underlie neurodegeneration and disease. In Chapter 2, I developed a protocol for generating and maintaining 3D primary cortical microtissues. In this approach I demonstrated application of the 3D cortical microtissue model in longitudinal live-imaging studies that enabled real-time visualization of cell-cell interactions in a 3D brain-like microenvironment. In Chapter 3, I deployed the 3D microtissue model to investigate how microglia modulate the microtissue microenvironment during development and in amyloidogenic conditions. In Chapter 4, I demonstrated that human iPSC-derived microglia can be engrafted into 3D microtissues forming a xenograft culture system that can be utilized to study human-specific features of microglia mechanisms during neuroinflammatory processes.

5.1 Development of a 3D primary cortical culture system

Chapter 2 establishes a protocol for generating 3D primary cortical cultures with a stable equibiaxial geometry, optimized for live imaging studies and adeno-associated viral fluorescence labeling. This protocol provides a detailed methodology for creating a heterogeneous multicellular microenvironment *in vitro* using dissociated cortical tissue from postnatal rats. In this approach I showcase the system's compatibility with transgenic disease models, enabling the controlled study of neurodegenerative processes in an *in vitro* setting that closely mimics the brain microenvironment with enhanced cellular diversity, ECM support, and cell-cell interactions.

Further I validate the use of adeno-associated viral fluorescence to perform real-time functional imaging of neuronal activity via genetically encoded calcium reporters. This chapter emphasizes the utility of a 3D microtissue model for use in real-time tracking of dynamic cellular processes and clearly outlines how it can be deployed in parallel to advanced microscopy techniques for longitudinal analysis of neuronal and glial interactions. Overcoming the limitations of traditional cross-sectional studies, this methodology provides a powerful platform for investigating dynamic cellular processes underlying both healthy and diseased brain function.

5.2 Microglia-driven synaptic remodeling in Alzheimer's disease

In Chapter 3, I investigated microglia-driven synaptic remodeling under both healthy and AD-relevant conditions. To achieve this, I utilized the 3D primary cortical microtissue platform, capable of replicating neuron–glia interactions in a physiologically relevant *in vitro* environment, over an experimental timeline spanning several months. The results revealed that, under healthy developmental conditions, microglia engaged in tightly regulated synaptic pruning, facilitating the formation and maturation of functional neuronal circuits. However, in microtissues derived from TgF344-AD rats—which express human *APP* and *PS1*—I observed evidence of early microglial activation and the eventual formation of A β aggregates by the third month *in vitro*. Notably, I demonstrate that the pathological environment which develops in TgF344-AD microtissues within the first 2 months ultimately led to excessive synapse elimination and significant neuronal loss by the third month.

By integrating advanced imaging techniques and computational analyses, I tracked microglia modulation of neuronal networks in real time, providing key insights into the spatiotemporal dynamics of how circuitry is shaped by microglia-mediated synaptic pruning in this 3D *in vitro* setting. Importantly, disrupted network maturation that occurred following microglia depletion, underscored the essential role of microglia in regulating neuronal connectivity and

circuit stability in the first month of culture. However, in aged ($\geq$ DIV80) TgF344-AD microtissues, microglia exhibited a maladaptive state, characterized by heightened phagocytic activity, excessive synaptic pruning, and ameboid morphology—all of which paralleled amyloid plaque formation. These findings support a glia-driven hypothesis of AD pathogenesis, suggesting that early microglial dysfunction may contribute to excessive removal of synapses. Collectively, these findings established the 3D primary cortical microtissue model as an advanced multicellular culture system for studying intricate microglia–neuron interactions in both normal and AD-relevant states.

5.3 Functional integration of iPSC-derived microglia into 3D xenograft culture system

In Chapter 4, I developed a 3D xenograft culture system to examine the functional integration and immune responses of human iPSC-derived microglia within a 3D microtissue environment. My findings demonstrated that iMG successfully engrafted into the 3D cultures, tiled the cortical microtissues, adopted *in vivo*-like morphological characteristics, and exhibited functional responsiveness to inflammatory stimuli and tissue injury. Notably, iMG integration and survival were optimized when endogenous rat microglia were depleted before engraftment (eDIV21 condition). Additionally, time-dependent maturation of iMG within the microtissue environment reinforced the necessity of a supportive and cellularly diverse 3D microenvironment to promote proper microglial development and brain-like phenotypic signatures.

The functional capacity of iMG was assessed by examining their response to systemic inflammatory cues and a localized tissue injury. In response to LPS treatment, iMG exhibited hallmark activation patterns, transitioning from homeostatic, ramified states to ameboid, reactive morphologies—a shift accompanied by reduced process dynamics and increased static behavior.

Injury-response assays revealed that iMG motility and recruitment to laser-induced lesions was more robust in mature iMG compared to those only cultured in the microtissue environment for 1 week. With prolonged maturation, iMG exhibited increased displacement, speed, and total migration distance. These findings suggest that extended culture within the 3D microtissue model facilitates functional maturation, likely via the acquisition of brain-like gene expression signatures, including the upregulation of key microglial receptors such as P2RY12. Collectively, these results underscore the utility of 3D xenograft culture models for advancing our understanding of human microglia in neurodegenerative diseases and guiding the development of novel therapeutic strategies for disorders such as AD.

5.4 Future directions

This thesis presents the development and application of novel research tools to study neuron-glial interactions in both healthy and disease states. However, there are several key areas of research that, although outside the scope of this thesis, I feel are exciting and would be critical to the continuation of the discoveries and progress made thus far. Namely there are 5 main areas I would be excited to pursue in future work. First and foremost, performing functional imaging studies of neuronal connectivity with the TgF344-AD derived microtissues across several months *in vitro*. In parallel to these studies, I would suggest continuing the investigation of possible tau phosphorylation at later culture time points. In addition to these assays, I believe that characterizing the molecular complement pathways in the 3D microtissue system would be a critical next step in elucidating microtissue-specific mechanisms of microglia-mediated synaptic pruning. Further, validation of the xenograft culture model—including looking at possible APOE-isoform dependent modulation of engrafted iMG functional behaviors—would be a logical next step in expanding Chapter 4. I believe it is imperative to better understand the transcriptional readouts of microglia, among other cell types, that are developed and matured in the 3D microenvironment. Not only would RNA-seq analysis deepen our understanding of this unique *in*

vitro environment in terms of a complete characterization of present cell types but it could potentially highlight areas for new research programs. In particular, I would be curious to explore the expression profiles of pattern recognition receptors (such as Toll-like receptors) and subsequent regulation of antigen-presenting proteins such as major histocompatibility complex (MHC) class II in endogenous rat microglia when cultured in this microtissues system that is decoupled from systemic immune influence. Findings here could offer insight into how innate immune behaviors of the brain, namely microglia, mature and develop in the absence of adaptive immune factors (e.g. T-cells, B-cells, natural killer cells, and other circulating monocytes). Finally, given that this culture system is uniquely suited to perform longitudinal live imaging assays, I believe that implementing new tools for continued functional imaging of microglia via genetically encoded calcium reporters in parallel with chemigenetic voltage-sensors expressed in the neuronal population could offer profound new insights to the field as it is a highly understudied area.

5.4.1 Investigating complement-mediated signaling and neural connectivity in TgF344-AD microtissues via functional imaging

Continuing functional imaging of neuronal activity in the TgF344-AD derived microtissues would be essential in further validating the findings of Chapter 3. Interestingly, in a pilot study I performed functional calcium imaging in TgF344-AD microtissues between DIV14-DIV35 and detected abnormal network maturity compared to wildtype microtissues (**Fig. 5.1**). This abnormal network maturity was defined by a large increase in modularity by the fourth week *in vitro* (**Fig. 5.1, h**). Several previous studies, including a calcium-imaging based study of microcircuit connectivity in the 5XFAD mouse, have classified hyper-segregation of network modules and increased modularity as a phenotype of AD and MCI network dysfunction (Rosales et al. 2021; Pereira et al. 2016). However, given that we did not observe abnormal decrease in synaptic

densities in TgF344-AD microtissues until DIV80, I would be excited to further investigate possible causes of the disrupted network connectivity beyond structural changes in synaptic densities such as possible changes in synaptic long-term potentiation and depression which have also been linked to dysfunctional circuitry in the TgF344-AD model (Smith and McMahon 2018; Goodman et al. 2021). Additionally, given that the TgF344-AD microtissues displayed an increase in whole tissue firing rate—which has been linked to increased CD47 expression in neurons (Ding et al. 2021)—I would be curious to investigate the complement protein densities at these times to see if there was a possible compensatory mechanism occurring during the early stages TgF344-AD microtissue amyloidosis.

I hypothesize there is a biphasic paradigm of microglia dysfunction in the TgF344-AD microtissue model dependent on amyloidosis and the presence of oligomeric vs fibrillar A β species. It is possible that oligomeric A β secretion induces the first phase of “early” dysfunctional microglia phenotype that leads to impaired “normal” synaptic pruning. Under this condition I would expect higher CD47-SIRP α “don’t eat me” signaling (Lehrman et al. 2018; Zhang et al. 2015) that inhibits microglia pruning of synapses, maintaining high synapse densities which concurrently alters LTP (Matsuzaki et al. 2004; Zhou, Homma, and Poo 2004; Bastrikova et al. 2008) to result in high levels network modulation that results not from structural synapse remodeling but rather from upward plasticity changes caused by abnormal synapse refinement (Diniz and Crestani 2023; Stampanoni Bassi et al. 2019). Then the subsequent accumulation of fibrillar A β would induce an “aged” dysfunctional microglia phenotype that causes excessive synaptic pruning and breakdown of network connectivity (Pan et al. 2011). In this secondary phase, the accumulation of fibrillar amyloid species, causing sustained microglia reactivity and triggering chronic inflammation and increase in complement C1q, C3 and CR3 “eat me” signaling would lead to hyper-active pruning of synapses by microglia.

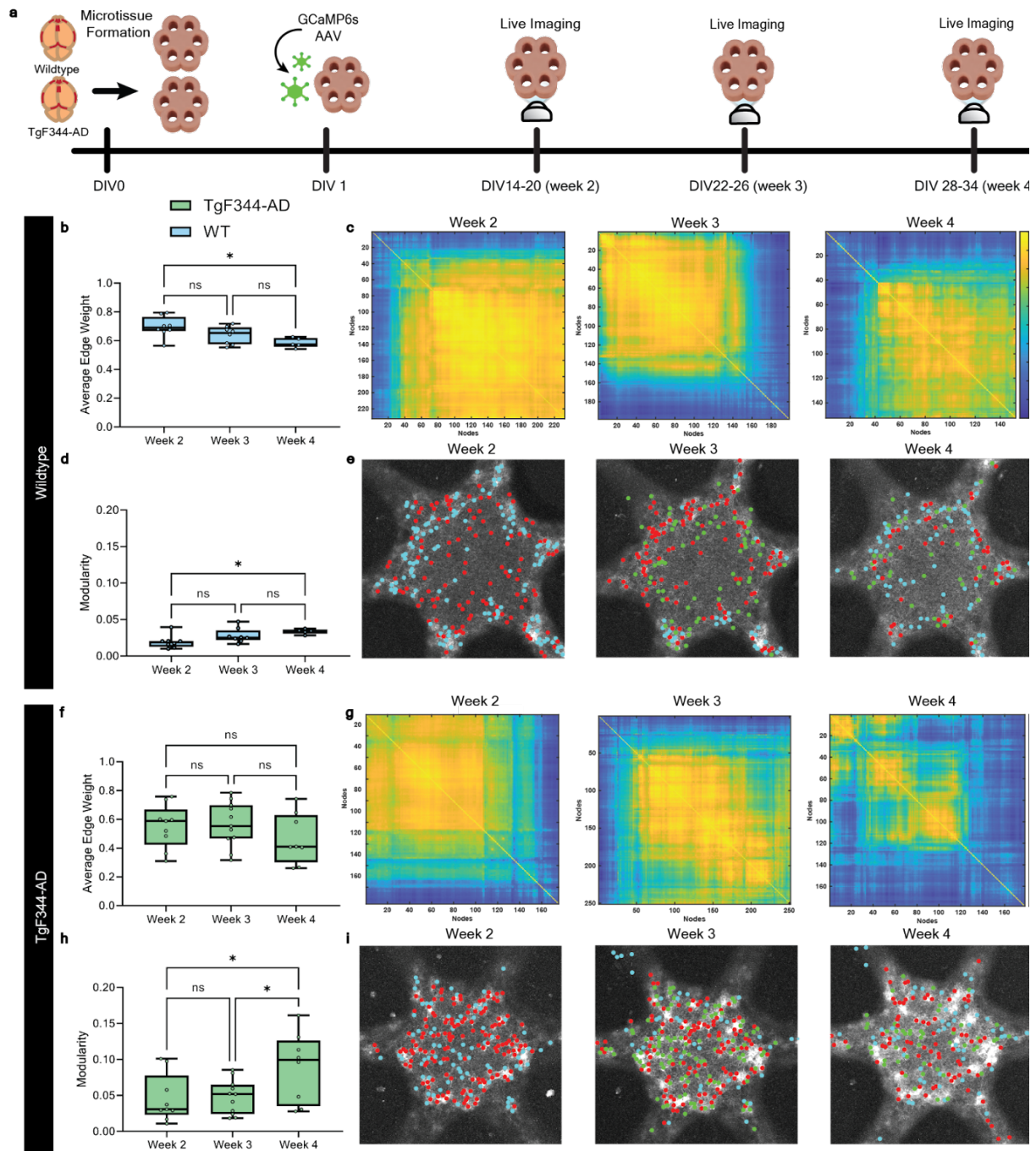


Figure 5.1 | Functional imaging of neuronal activity in TgF344-AD microtissues via genetically encoded calcium indicator

a, Schematic of experimental timeline of longitudinal calcium imaging in wildtype and TgF344-AD microtissues **b & f**, Average edge weights of **(b)** wildtype and **(f)** TgF344-AD microtissues at week 2, 3, and 4 show significant changes to whole tissue correlation during 3 weeks of culture that is absent in TgF344-AD microtissues. **c & g** Node pair edge weight Pearson correlation matrices of individual **(c)** wildtype and **(g)** TgF344-AD microtissues at week 2, 3, & 4. **d & h**, Whole-tissue modularity values from **(d)** wildtype and **(h)** TgF344-AD microtissues at week 2, 3, and 4 reveal increasing modularity coincides with microtissue maturation in control tissues but is dramatically higher in TgF344-AD microtissues. **e &**

i, Representative images of separate modules (red, blue, green = modules) overlaid onto maximum intensity projection images of (e) wildtype and (i) TgF344-AD microtissues at week 2, 3, & 4. Kruskal Wallis Test followed by Dunn's post-hoc comparison: **** $p < 0.0001$, with microtissue maturation in control tissues but is dramatically higher in TgF344-AD microtissues. e & i, Representative images of separate modules (red, blue, green = modules) overlaid onto maximum intensity projection images of (e) wildtype and (i) TgF344-AD microtissues at week 2, 3, & 4. Kruskal Wallis Test followed by Dunn's post-hoc comparison: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n \geq 5$ microtissues per condition for each weekly timepoint. Data is represented in box plots displaying the median, interquartile ranges, and minimum/maximum whiskers extending to $[1.5 \times \text{IQR} / \text{minimum-maximum range}]$.

Additionally, because we did not detect tau phosphorylation in DIV80 microtissues I would strongly suggest a continued investigation wherein microtissues are cultured to 4 or 6 months and reassessed for abnormal tau phosphorylation. Previous studies have detected several NFT-like structures in close proximity to amyloid plaques following the Gallyas method (Lamy et al. 1989; Cohen et al 2013) wherein 6 and 16 mo old TgF344-AD brain sections were impregnated with silver and imaged. In addition, CP13 immunostaining revealed teardrop-shaped structures morphologically consistent with NFTs in 16-month-old TgF344-AD rats (Cohen et al. 2013). Additionally, researchers analyzed brain homogenates from TgF344-AD and wildtype rats using a custom antibody made against a peptide fragment of pathogenic tau containing the pS396/404 epitope (pTau-PADRE). Performing western blots for total tau brain homogenates using Tau-PADRE antibody the study found six bands (~48–62 kDa in size) correlating with six isoforms of rat tau which the authors suggested were similar to those present in humans (Hanes et al. 2009). After confirming antibody specificity for human pathological tau through peptide neutralization experiments using AD patient brain sections, these pTau-PADRE western blots revealed increased immunoreactivity in aged TgF344-AD versus wildtype rats (Cohen et al. 2013). Their quantitative analysis revealed higher levels of insoluble tau and abnormal tau precipitation in TgF344-AD rats and (using pTau-PADRE), particularly at 6 and 16 months of age. Further several groups have shown tau pathology in TgF344-AD rats as early as 6 months of age using several well-validated pathogenic tau antibodies including the AT8, PHF1, MC1, pS396, and pS199/202 antibodies (Cohen et al. 2013, Bac et al. 2023). Given these findings I would suggest that

extending the culture of TgF344-AD microtissues may be necessary to detect phosphorylated tau.

5.4.2 Investigation of APOE isoform-dependent functional responses of engrafted iMG

In a continuation of the work described in Chapter 4, I believe that performing a comparative analysis of ApoE3 vs ApoE4 iMG functional response in the 3D xenograft culture system would prove most interesting and ultimately complete chapter 4 for final manuscript submission. Given recent findings highlighting APOE-induced deficits in microglia functional behaviors (Victor et al. 2022; Blumenfeld et al. 2023; Liu et al. 2023), this model system would be ideally suited to probe cell-type specific effects of APOE genotype on microglia function. By experimentally controlling for influence of pathogenic expression of ApoE4 in neurons or astrocytes (by only engrafting ApoE4 iMG), we would be poised to challenge the effect of ApoE4 on microglia in the presence or absence of amyloid pathologies (by engrafting into TgF344-AD or wildtype tissues). This would allow for a unique investigation of the intrinsic (i.e. genotype) vs extrinsic (i.e. environment) factors of microglia dysfunction. We have previously completed pilot studies for generating ApoE4 iMG but were unable to obtain high-enough yield to perform engraftment into the microtissues and perform subsequent imaging assays. However, the ongoing work in collaboration with Dr. Huang's lab and the team of graduate students that have been brought onto the project lend to an increased likelihood of completing these experiments.

5.4.3 Transcriptomic Analysis

Beyond repeating the imaging assays described in Chapter 4 in an analysis of ApoE3 vs ApoE4 iMG functional responses I would also recommend that an RNA-seq analysis be done in parallel. Although the live-imaging assays and immunohistochemical processing of microglia

remain very high-throughput and are classical tools used in the validation of microglia identity, RNA-seq may prove a more sensitive readout of the iMG phenotypes acquired under various experimental conditions. In a proof-of-concept study we successfully purified GFP+iMG out of microtissues for RNA extraction by qPCR. Microtissues were gently dissociated into a single cell suspension and FACS sorted for harvest of the GFP+iMG population. I believe that addition of transcriptomic analysis of engrafted iMG at various time points (1 week post engraftment vs 4 weeks post engraftment) as well as iMG cultured in microtissues under various conditions (LPS stimulation or TgF344-AD derived amyloidosis) would further elevate the manuscript outlined in Chapter 4.

RNA-seq in developing microtissues would not only prove useful in deciphering microglia phenotypes but in elucidating the true cellular diversity within the microtissue system. I would be eager to investigate whether the microtissues contain populations of endothelial cells, pericytes, and other CNS-like macrophages and how expression patterns seen in our neuron, synapse, astrocyte, microglia, and OPC IHC datasets compare to those from an RNA-seq analysis. Although we have made significant progress in the characterization of the microtissue system using traditional immunofluorescent assays, RNA-seq datasets would greatly enhance the validation of these cultures for use in basic and translational neuroscience research. By investigating transcriptomic profiles at various timepoints across microtissue development, we could identify pathways of cell maturation and proliferation that may be unique to this microenvironment. Further, by comparing to in vivo datasets we can provide a clearer understanding of the potential for translational work.

5.4.4 Decoupling innate vs adaptive immune influence in disease-associated neuroinflammation

This model offers the unique ability to investigate how innate immunity in the brain (i.e. microglia) drive neuroinflammatory mechanisms without influence from systemic and adaptive immune factors. Our evolving understanding of microglia as the resident immune cell in the brain and the recent discovery of anatomical niches of the CNS in which immune cells are compartmentalized has opened a great deal of conversation regarding the complex brain-immune network. This network involves both local and peripheral immune mechanisms and plays influential roles during development, adulthood, aging, and in various pathologies. Such anatomical interfaces where immune cells—specifically non-brain resident microglia—are compartmentalized are often described as being located along the borders the brain and include the meninges, the perivascular spaces, choroid plexus, skull bone marrow, and cervical lymph nodes. Under normal or healthy conditions, immune cells within these anatomical niches can influence CNS function from a distance by releasing various mediators, such as cytokines and neurotrophic factors (Ribeiro et al. 2019; Derecki et al. 2010; Baruch et al. 2013; Castellani et al. 2023). Alternatively, in response to chemotactic signals triggered by injury, they can enter the CNS parenchyma and directly modulate immune activity, providing additional support to resident microglia (Ito et al. 2019). While earlier studies have indicated that CNS-associated macrophages may play a harmful role in mouse models of neuroinflammation and neurodegeneration (Park et al. 2017; Thanopoulou et al. 2010; Jordão et al. 2019; Faraco et al. 2016), the specific mechanisms by which these border-associated immune cells create a communication network with brain-resident microglia and contribute to disease progression remain unclear and warrant further research.

I believe that use of the microtissue system, which maintains cellular diversity of the brain but is lacking influence from border-associated immune niche's, could offer great insight into the possible mechanisms of innate immunity—in the absence of crosstalk with systemic and adaptive immunity—and how those mechanisms might contribute to neuroinflammation. In a recent study using spatial ageing clocks and deep learning, researchers found that T cells increasingly infiltrate the brain with age and exert a pro-ageing effect on neighboring cells (Sun et al. 2023). This finding, in my view, clearly motivates a deeper investigation into how the innate and adaptive arms of the CNS-immune network interact to drive both normal and pathological aging. In particular, elucidating the extent to which microglia alone can potentiate neuroinflammatory processes in the brain will be paramount for identifying new therapeutic targets, especially in diseases like Alzheimer's, where immune dysfunction plays a central role.

5.4.5 Implementation of calcium and voltage sensing genetic tools

Finally, to further propel this research project in a new and exciting direction, I would propose an investigation of transient calcium flux within microglia populations under various experimental conditions. Recent studies have confirmed a functional role for calcium channels in human microglia. These studies have linked extracellular ATP-evoked calcium transients to fluctuations in membrane conductance in an inflammation-dependent mechanism and suggest that during brain inflammation, calcium-mediated microglial responses to purinergic signaling may be reduced (Palomba et al. 2021; Umpierre et al. 2020). This was later confirmed in a separate study that demonstrated diminished calcium-mediated microglia response to ATP-dependent purinergic signaling in ApoE4 iMG compared to ApoE3 iMG (Victor et al. 2022). Given the compelling evidence from these studies, I would be keen to record microglial calcium activity in engrafted iMG. We are ideally suited to this type of experimentation given that the 3D microtissue culture system is designed to be compatible with high-resolution fluorescent imaging in live

microtissues. Further, it would be pertinent to expand the continued functional imaging experiments to include use of the chemigenetic voltage-sensor Voltron (Abdelfattah et al. 2019) to capture membrane voltage changes in neuronal populations. This would be a superior approach compared to our previously used functional neuronal imaging that relied on changes in neuronal calcium concentration gradients that inherently occur at a much slower rate and are a proxy for neuronal activation. By using a voltage sensor we would be suited to capture dynamic network activity with higher sensitivity and temporal resolution.

5.5 Conclusion

The research described in this thesis first and foremost advances 3D culture systems, establishing a versatile and physiologically relevant platform for investigating neurodegenerative diseases with high translational potential. By enabling long-term, dynamic observation of neuron-glia interactions, these models overcome limitations of traditional 2D cultures and provide a controlled environment to study disease mechanisms in real time. The findings on microglia-driven synaptic remodeling reinforce the important role of microglia in synaptic maintenance in both developing microcircuits and during neuroinflammatory states. Importantly, the disruption of these mechanisms in an amyloidogenic microenvironment highlights the potential for targeting microglia-mediated synaptic dysfunction as an early biomarker of AD pathogenesis. The observed dysregulation of microglial pruning in AD-derived microtissues supports the hypothesis that early glial dysfunction may be triggered by pre-plaque amyloid deposition, and chronic microglia activation then contributes to synaptic loss and neuronal vulnerability.

Additionally, the human iMG xenograft model marks a significant step toward modeling human-specific microglial responses that strongly mimic those found in vivo while maintaining the experimental control of an in vitro setting. Here, I highlight the potential of this model to decipher cell-type specific dysfunction of AD risk gene in microglia, particularly in the context of APOE

genotype-associated dysfunction in responses to tissue damage and debris clearance. However, I do believe that this xenograft culture system, in lieu of continued experiments to further validate its use, could easily be adapted to study a wide range of disease models which particular focus of glia-neuron interactions. Due to the avascular nature of this primary cortical culture model, future work would be uniquely set-up to probe glial-mediated dysfunction in both healthy and disease states without influence from border-associated immune niche's. Given the challenges is separating innate vs adaptive immune influence on neuroinflammatory conditions in the brain, this model could prove useful in determining whether microglia alone can drive neuroinflammatory mechanisms underlying diseases such as AD or if the innate-adaptive immune crosstalk is required to drive pathological behaviors. Collectively, these models lay the groundwork for future therapeutic development, providing a platform to identify and test potential interventions aimed at restoring microglial homeostasis and preserving synaptic integrity. Future studies leveraging these systems will be crucial for developing targeted strategies to mitigate neurodegeneration and promote brain health in aging and disease.

In conclusion, this thesis presents a novel 3D microtissue culture system and provides new insights into microglia biology, with a particular focus on microglia dysfunction associated with neuroinflammatory mechanisms of AD. By improving microglial maturation, supporting complex neuronal circuitry, and enabling real-time imaging of microglia-neuron interactions, we demonstrate the utility of 3D microtissue cultures to investigate dynamic microglia-neuron interactions in physiological and pathological conditions. Furthermore, the development of a novel xenograft culture system to probe cell-type specific mechanisms of human microglia biology in an environmentally controlled yet physiologically relevant setting represents a significant step toward understanding neuroinflammatory mechanisms of AD. Together, these advancements establish a powerful platform for dissecting the cellular and molecular mechanisms underlying microglia-neuron interactions in the brain and offer great promise for guiding the development of targeted

therapies aimed at mitigating microglia-driven pathology in AD and other neurodegenerative disorders.

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