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Identifying novel roles for microglia and astrocytes in neurological diseases

By

Laura Craciun

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

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of the

University of California, Berkeley

Committee in charge:

Professor Kaoru Saijo, Chair

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Professor Marla Feller

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by

Laura Craciun

Abstract

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University of California, Berkeley

Professor Kaoru Saijo, Chair

Over the past few decades, neuroscientists have been finding a growing number of roles for microglia and astrocytes in brain development and homeostasis. Consequentially, both of these glial cells are also now identified to be implicated in almost all neurological disorders. This thesis discusses work on two separate projects that each discovers novel roles for microglia or astrocytes in a specific disease context.

Chapter two discusses a project that explores the role of copper in microglial inflammation. We find that copper pools with microglial cell bodies increase when cells are presented with an inflammatory stimuli. Previous epidemiological data has identified copper as a risk factor for the development of neurodegenerative disorders, like Parkinson's Disease (PD). Interestingly, increased neuroinflammation is also prominently associated with all neurodegenerative diseases. Therefore, we have now created a mouse system to test whether or not inflammation-induced PD-like symptoms are impacted by copper levels.

Chapter three describes a project that discovers a role for transcription factor 4 (Tcf4) in astrocytes. We initially thought that Tcf4 could potentially be important for microglial inflammation, as we generated *Tcf4* knock-down microglia cell lines that exhibited impaired inflammation. However, we then found that astrocytes highly express Tcf4 in mouse brain, while microglia levels are undetectable. Lastly, we generated a mouse model in which *Tcf4* was specifically deleted in astrocytes. Intriguingly, these mice exhibited shortened life-spans, seizures, and anatomical brain abnormalities, none of which were observed in the control mice.

Together, this work demonstrates that both microglia and astrocytes are involved in neurological diseases in ways that were not previously known. As the etiology and treatment of both PD and epilepsy are relatively unknown, we hope that this work can contribute to the development of new therapeutic options.

Dedication

For my cat, Prince.

(Sorry, Mom and Dad... you're next in line though, I promise.)

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Chapter 1: Emergence of microglia and astrocytes in neuroscience

1.1: An introduction to glia

The contribution of glial cells to brain function has been a topic of much debate ever since their discovery. Despite early neuroanatomists of the nineteenth century meticulously characterizing the morphology of many different glial cell types, neurons were long the prime focus of studies seeking to understand the mechanisms that underlie normal brain development and function. Derived from the ancient Greek meaning for “glue,” glia were thought to be nothing more than the connective tissue of the brain. The first type of glia to be identified was the **astrocyte**, named for its star-like shape and discovered by Michal von Lenhossék in 1895 ^[1]. In the early twentieth century, Pío del Río Hortega was first to introduce the term for another glia type, **microglia**, which he noticed to change morphology and congregate at locations of brain injury ^[2]. This postulate that the cells function like macrophages in the immune system was later confirmed as it was discovered that microglia perform phagocytosis and antigen presentation ^[3]. These are two of the main glial cell types that fundamentally function as homeostatic regulators of the nervous system.

As organisms evolved, the centralization of the nervous system occurred in tandem with the appearance of glial cells. The most ancient nervous systems in Cnidarians (hydras and jellyfish) and Ctenophora (comb jellyfish) were made of solely neurons that connected to one another in evenly dispersed networks throughout their processes ^[4]. The most primitive glial-like cells are found in flatworms and nematodes, and host a number of functions that vary from simple supportive elements to functions that parallel those seen of astrocytes in the mammalian nervous system ^[5,6]. From here, ascending phylogeny finds new types of glial cells that appear and increase in number, with humans possessing unique glial cells with distinctive functions that are not seen in other organisms.

Neuroscientists theorize that increasingly intricate nervous systems of more evolved organisms present a need for higher glia numbers to account for the increased cognitive abilities, complex behaviors, homeostatic needs, and energy expenditure ^[7]. Therefore, it is thought that the evolution of the nervous system is roughly paralleled with an increase in the ratio of glial cells to neurons within an organism. It is estimated that the human brain is composed of about 50% glial cells and 50% neurons ^[8]. Of this ~50% of glial cells found in the brain, astrocytes are thought to compose about 40-50% and microglia about 5-10% ^[10,11]. Additionally, the glia-to-neuron is highly dependent on brain region. For example, the striatum has about 3.7 glial cells per neuron, whereas the cerebellum has about 0.1 glial cell per neuron ^[9].

The discovery that astrocytes and microglia make up such a significant portion of the brain was critical in shifting the neuron-centric doctrine of neuroscience to one that

also encompasses glial cells. Over the past two decades, the contribution of glia—specifically astrocytes and microglia—to nearly all neuropathological disorders has also started to be universally recognized. Understanding neuropathology ultimately requires in-depth analysis of glial pathological changes, which in turn may stimulate the development of novel, glia-specific therapies.

1.2: Microglia

It is thought that microglia appeared in the central nervous system (CNS) when barriers separating early brain tissue from circulation emerged. This presented the need for a resident innate immune cell to migrate and remain in the nervous system. In fact, microglia populate the brain before neurons or other glial cells develop ^[12-14]. They are the most populous cell type in the brain that is of mesodermal origin as they arise from yolk sac primitive macrophages known as hematopoietic cells ^[12-14]. In mice, this happens at about E9.5, after which the number of microglia rises and peaks during early post-natal brain development, and then falls to a steady density ^[15]. At this point, the microglia are now matured and distributed in non-overlapping regions throughout the CNS, where they maintain their numbers via self-renewal ^[16-18].

Microglia are now well-documented modulators of CNS development, homeostasis, and plasticity. Investigations of mice and humans lacking microglia show severe anatomical defects that can cause early death ^[19-22]. The appearance of microglia in the embryonic brain at E9.5 in mice also coincides with the start of neurogenesis ^[23-25]. In these early stages of development, microglia adopt an amoeboid morphology and a transcriptional profile that reflects highly active phagocytosis and proliferation ^[26-29]. Since microglia are key sensors of changes in their immediate surroundings, it is not surprising that single-cell transcriptomic analysis has revealed microglial heterogeneity to be at its highest during this time of normal physiological development ^[23,30]. These microglia regulate the number of neuronal progenitor cells (NPCs), and ultimately neurons, that are there in excess and undergoing apoptosis ^[31]. NPC and neuron proliferation, death, and degradation is an important component of development, and microglia are critical for cleaning up the debris. In later embryonic stages of cortical development, microglia also populate the brain in an “inside-out” manner alongside neurons, which means that each cell migrates past its predecessor to form a more superficial layer ^[32].

The ability of microglia to constantly survey and respond to the environment extends beyond the detection of apoptotic neuronal debris during early development ^[33,34]. Once neuronal cells are settled in their layers of the brain, circuits form as they start to develop synaptic connections with one another, which begins shortly before birth in mice ^[35,36]. These synapses are made in excess, from which strong activity-dependent connections can be maintained in proper neuronal networks that underlie neural processing and function ^[37,38]. During this time, microglia are contributing to the refinement of these connections, engulfing the excess synapses in an act known as

synaptic pruning [39]. This is supported by studies on microglial depletion that show increased neuronal connections and activity in the adult mouse brain [40–42].

There are currently two well established “eat-me” signals that microglia can detect these neuron fragments: phosphatidylserine (PS) and secreted complement proteins, the latter being repurposed classic innate immune proteins that all macrophages have [43,44]. PS is a phospholipid that flips to the outer plasma membrane when cells undergo stress, and it has been shown that neuronal processes and synapses can transiently expose PS [45]. PS can then be recognized by several different microglial receptors that are involved in phagocytosis, such as the MerTK (proto-oncogene tyrosine-protein kinase MER) pathway [46]. Synaptic pruning through complement proteins is thought to occur through complement factors C1q and C3, which are secreted by astrocytes to mark excess synapses for detection by microglial C3 receptors and engulfment by the microglial cell [47–49]. When either of these “eat-me” pathways are perturbed, there are severe consequences in synaptic connectivity and circuit function [46,50,51]. It is also believed that these pathways overlap, as studies have shown that exposed PS is present in isolated synaptosomes that have been targeted for elimination by C1q [52].

Microglia also have other methods of contributing to neuronal and synaptic brain development. For one, they can provide neurotrophic support—such as the secretion of insulin-like growth factor 1 (IGF-1) and brain derived neurotrophic factor (BDNF)—to developing and proliferating neurons [53–56]. Next, microglia can contribute to synaptogenesis and synaptic pruning throughout adolescence and adulthood in order to modify the strength and efficacy of neurotransmission in an activity-dependent manner; this is known as synaptic plasticity [42,47,57,58]. In postnatal zones of proliferation such as the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, microglia continue to engulf excess NPCs and neurons [59]. Similarly, microglia are also active in the development and maturation of oligodendrocyte progenitor cells (OPCs) and oligodendrocytes [60,61]. For example, they are found near OPCs to promote their development, also through IGF-1 secretion [62]. Their phagocytic capabilities also contribute to the regulation of mature oligodendrocyte number and myelin sheath production [60,61,63].

Microglia are perhaps still best known for their role in immunity. At CNS interfaces like the meninges and choroid plexus, other tissue macrophages are found, including: border-associated macrophages, dendritic cells, monocytes, T and B cells, and natural killer cells [64,65]. However, microglia are the only ones that remain in the brain parenchyma, in close proximity to neurons and with motile processes that are constantly surveying the environment [66]. They express similar immune response equipment as other peripheral macrophages, including Toll-like receptors (TLRs) [67]. TLRs are transmembrane innate immune system proteins that recognize structurally conserved molecules, such as endogenously-caused damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [68,69]. Like other tissue macrophages, such molecules will activate microglial TLRs and start an immune response that is characterized by increased phagocytosis, proliferation, and a production of proinflammatory molecules that contribute to tissue repair such as cytokines and

chemokines [70]. They also adopt an amoeboid-like shape to help better fulfill their roles as scavengers of damage signals and pathogens [33,70–72], reminiscent of their morphology in brain development. Additionally, phagocytic pathways involved in engulfment of NPC, neurons, and synapses are reused during this immune response [71–77]. Just as microglia have signals to start an immune response, there are also those that are known to dampen it, such as transforming growth factor beta (TGFβ), which induces microglia to adopt a ramified “steady-state” morphology and reduce secretion of proinflammatory factors [78,79].

The immune response of microglia to changes in the environment can happen at any point in an organism’s life. Neuroinflammation is now understood to be associated with a number of factors, including infection, dietary deficiencies, psychological stress, and environmental toxins [79–81]. Given the multitude of roles that microglia play in development, neural network formation, and homeostasis, exposures to such insults that impact microglia function can exert long-lasting impacts on brain health. For example, there is now strong evidence that exposure to TLR-activators during embryonic development of mice leads to changes in microglial molecular profile, density, morphology, and/or motility [67,75]. In turn, this has consequences on mouse behavior, neuronal wiring, and overall neural function during adolescence and adult life, which are reminiscent of behaviors that are observed in neuropsychiatric and neurodegenerative diseases [76,77,80,82–85]. In juvenile mice, exposure to early life stressors such as separation anxiety and dietary deficiencies results in similar deficits in behavior and neural circuit formation [86–93]. Normal aging also causes profound changes in the physiology of microglia that then likely impacts overall brain function. Transcriptomics data of microglia in the aged brain reveals extensive heterogeneity [94–96]. These studies show that compared to younger brains, aged microglia upregulate pro-inflammatory genes and antigen-presenting markers, and down-regulate anti-inflammatory factors. Other studies have identified aged microglia to show signs of oxidative stress, altered morphology, low motility, diminished reactivity and reduced phagocytic abilities [97]. Since age is a major risk factor for neurodegenerative diseases, this highlights the importance of thinking about microglia when considering the pathophysiology of such diseases.

1.3: Astrocytes

The way astrocytes are shaped and positioned in the brain reflects their importance in sustaining homeostasis and providing neuronal protection. Like most other cells types in the brain—besides microglia—astrocytes develop from the ectoderm germ cell layer [98]. The intricate morphology of this cell type, consisting of many thick branches that arborize into thousands of tiny filaments reaching out into the brain parenchyma, reveals not only why they are named the “star cell,” but also how well-suited they are for maintaining proper brain function. It wasn’t until the advancement of imaging techniques in the early 2000s that astrocytes were shown to ubiquitously tile the entire brain parenchyma in a relatively non-overlapping, tightly-packed manner [35,99,100]. In the mouse cortex, a single astrocyte can contact tens of neurons, hundreds of dendrites, and hundreds of thousands of synapses [11,98]. This number is even greater in human, as

human astrocytes are much larger, have ten-fold more processes, and can therefore contact close to two million synapses ^[11,101]. While it varies across brain regions, about 60-90% of synapses in the brain are ensheathed by astrocytic processes, contacts that are also dynamic and influenced by neuronal activity ^[102–105]. This intricacy in morphology mirrors the cell's complexity.

How neuroscientists have viewed the function of astrocytes has changed drastically throughout the decades. They were first thought to be inert cells that simply provide the structure and matrix for brain architecture ^[106,107]. In the early 2000s, discoveries were made highlighting the ways in which astrocytes support neuron function, such as with neurotransmitter uptake and recycling, ion buffering, and water homeostasis ^[98,107–110]. Current glial researchers now appreciate even more critical ways in which astrocytes regulate brain function throughout an organisms life, such as directly impacting synapse formation, synaptic pruning, synaptic plasticity, and even broader impacts on neuronal circuits and animal behavior ^[105,107,111–113].

Astrocytes are important contributors to the growth of synaptic connections between neurons. During late postnatal development, astrocytes arise from NPCs after an intrinsic, temporally-regulated shift from neurogenesis to astrogenesis ^[114–117]. Peak astrogenesis occurs right after birth and completing in the first postnatal week ^[118]. This maturation period, in which astrocytic processes extensively arborize, transpires during the same time that synaptogenesis occurs among neurons ^[36]. Seminal work from the Barres Lab found that adding astrocyte-treated media onto neuron cultures significantly induced the number and maturity of the synaptic connections ^[110,119]. Since then, extensive work in rodents has identified a number of astrocyte-secreted factors to contribute to synaptogenesis across nearly all brain regions and synapse types. Thrombospondins (TSPs), extracellular matrix proteins that help connect cells, were among the first proteins to be identified as one of these factors ^[120]. Specific subsets of TSPs (TSP1 and TSP2) are expressed in the CNS, and are greatly enriched in astrocytes compared to other cell types ^[121]. Peak expression of these TSPs occurs around P5-P7 when excitatory postnatal synaptogenesis begins ^[121]. As intracortical synapses, TSPs can interact with a calcium channel subunit on the gabapentin receptor to mediate the connection among neurons ^[122,123]. TSP3 and TSP4, however, are thought to be important for maintaining function and structure of synapses in the adult CNS, and thus are expressed by astrocytes later in life, but less is known about their molecular mechanisms ^[121,123].

Hevin is another astrocyte-mediated factor that has been found to regulate formation of a different neuronal connection: thalamocortical synapses ^[124,125]. Hevin is glycoprotein that is localized to synaptic clefts and expressed by astrocytes ^[122,126]. It works by interacting with postsynaptic neuroligins and presynaptic neurexins—the cell-adhesion molecules that make up a synapse—to stabilize the structure of the synapse ^[127]. Contrastingly, astrocytes can secrete factors that antagonize synapse formation, such as SPARC (secreted protein specific rich in cysteine), which blocks the formation of both thalamocortical synapses and cholinergic synapses ^[124,128].

Originally thought to be a neuron-intrinsic process, it is now well-documented that astrocytes secrete a number of factors that contribute to synapse maturation. During synapse formation, only N-methyl-D-aspartate (NMDA) receptors are found on the synapse; they remain immature and do not fire action potentials, and are therefore called “silent” synapses. Synapse maturation is classically viewed to occur when α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are inserted postsynaptically, and later a switch in the AMPA receptor subunits from GluA1 to GluA2 [129]. In the hippocampus and cerebellum, glypicans 4 and 6 (Gpc4 and Gpc6) were found to be highly expressed and secreted by astrocytes, with Gpc4 peaking between P6-P14 during initial synaptogenesis and Gpc6 between P14-P21 during synapse maturation [130,131]. Both glypicans can increase synapse formation and cause GluA1 insertion, therefore helping unsilence synapses [130,131]. Astrocytic TNF- α (tumor necrosis factor alpha) is also responsible for a homeostatic activity-dependent regulation of synaptic connectivity called synaptic scaling [133]. This is a uniform adjustment in the strength of the synapses to a neuron’s prolonged activity, which is necessary maintaining neural network stability. TNF- α released by astrocytes modulates the synaptic surface expression of AMPA receptors, and specifically subunit GluA1, on hippocampal neurons [132]. This change in AMPAR causes an increase mEPSCs (or mini excitatory postsynaptic currents) [132]. Thus, these mechanisms are not only in effect during development, but regulating synaptic strength to maintain circuits in adulthood.

Besides Gpcs, other astrocytic factors have been identified to contribute to synapse maturation. Enriched in and secreted by astrocytes, Chordin-like 1 (Chrdl-1) was found to increase GluA2 subunit expression in AMPA receptors at thalamocortical synapses, thus playing a critical role in synaptic circuit refinement [134]. More recently, a study identified pentraxin-3 (PTX3) as an astrocyte-secreted factor during late embryonic and early postnatal development [135]. PTX3 recruits AMPARs to developing synapses, which then increases both frequency and amplitude of neuronal mEPSCs without changing cell’s synapse number [135]. Lastly, cholesterol synthesis by astrocytes is another factor that is needed for synapse maturation, which must be synthesized in the brain as it cannot cross the BBB [136-138]. Cholesterol is a critical component of the cell membrane, and neurons require it at synaptic membranes to stabilize NMDARs and AMPARs [136-138].

While considerable progress has been made in the study of astrocytes and excitatory synapse formation, less is known about the connection to inhibitory synapse formation. Modulating inhibitory synaptogenesis is critical for maintaining a proper excitatory-inhibitory balance across neural networks. Similar to studies done on excitatory synapses, applying astrocyte-conditioned media to inhibitory neurons (also known as GABAergic neurons, as they express receptors that bind γ -aminobutyric acid, or GABA) leads to an increase in length and arborization of the axon, an increase in synapse number, and an increase in expression of GABA receptors [113,139,140]. However, the molecular mechanisms behind this are not known.

Despite not being known for their phagocytic capabilities, astrocytes are involved in synaptic pruning. This was initially described at the developing thalamocortical synapse

in mice, where astrocytes remove weak synapses using receptors MEGF10 (multiple epidermal growth factor-like domains 10) and MerTK ^[141]. This activity-dependent engulfment by astrocytes using these molecular pathways has additionally been documented in the adult hippocampus, where synapses are constantly formed and eliminated. Like with microglia, astrocytes can phagocytose during times of damage or pathogen invasion, adopting a reactive state that is known as reactive astrogliosis ^[142]. These astrocytes are capable of carrying out an immune response that includes moderate proliferation, hypertrophy of processes, and transcriptomic changes towards a more inflammatory profile ^[142,143]. This occurs because they have a number of innate immune receptors, including some TLRs, that can respond to diverse DAMPs and PAMPs derived from neurons, other glia, microbes, serum proteins, cell or protein debris, metabolic deficiencies, and more ^[144]. During this response, astrocytes can release various proinflammatory factors that can be damaging if overproduced, such as cytokines, reactive oxygen species (ROS), and other neurotoxic factors ^[145–147]. Astrocytes are also highly active when nervous system repair is needed, as they form a glial scar around the site of injury that is necessary for healing ^[148]. However, these reactive astrocytes can have reparative functions, such as upregulating trophic factors that help neuron growth and survival ^[149,150].

Astrocytes have many other homeostatic functions that are important for maintaining brain function. First, astrocytes are necessary for maintaining the extracellular environment in the brain. One way this is done is through the wide range of ion channels present on their membranes, such as potassium channels. During periods of high electrical activity, neurons release potassium, which in high quantities can cause overexcitation and excitotoxicity. Astrocytic potassium channels assist in clearing the excess accumulation of extracellular potassium, which is known as spatial potassium buffering ^[151]. Once potassium is in the astrocyte, it disperses to areas where it is lower in concentration and also to other astrocytes through gap junctions ^[152]. These ion channels also maintain the astrocyte's membrane potential, which regulates transporters that are sensitive to gradient changes ^[153]. It is also thought that the sodium-potassium pump is responsible for the astrocytic potassium uptake, while simultaneously maintains the cell's membrane potential ^[154]. Proper regulation of transporters is also important for maintaining a suitable extracellular environment. The uptake of glutamate by transporters on astrocytes is another main mechanism to prevent hyperexcitability ^[155–157]. Once inside, glutamine synthase converts the glutamate to glutamine for transport back to neurons to use again ^[158]. Maintaining the extracellular water balance is another way astrocytes prevent hyperexcitability, since water content impacts ion concentrations and membrane potentials. Astrocyte membranes are rich in aquaporin 4 (AQP4), a protein that transports water into the cell ^[159]. Water uptake needs to occur concomitantly with spatial potassium buffering so that astrocytic membrane potential can properly stimulate the uptake of potassium.

Not only do astrocytes have transporters for neurotransmitters, they can also generate and secrete them. This concept of “gliotransmission” is highly controversial in astrocyte biology; numerous studies have shown that these gliotransmitters—which include glutamate ^[160,161], D-serine ^[162], GABA ^[163], ATP ^[164], and glycine ^[165,166]—can

affect the activity of neurons, while others have contradicted this claim ^[167,168]. However, what is not debated is the calcium waves observed in astrocytes after local neuron activity, an observation first made in the early 1990s ^[169,170]. Though still not considered to be electrically excitable, these astrocytic calcium signals can propagate to other astrocytes through gap junctions, generating calcium waves. Whether or not this calcium impacts the release of gliotransmitters is still unknown. However, there is evidence of calcium-independent methods of some gliotransmitter release from astrocytes. For example, astrocytes can release ATP through gap junctions like pannexin-1 (panx-1) and connexin-43 (Cx-43) hemichannels, which can enhance the excitability of neurons ^[171,172].

Astrocytes have endfeet that ensheath the endothelial cells that make up the blood brain barrier (BBB), the physical barrier that protects the brain from harmful molecules in the blood stream ^[173–175]. They also secrete factors like vascular endothelial growth factor (VEGF) that regulates the proteins that make up the tight junctions of the endothelial cells, thus helping maintain the integrity of the BBB ^[175,176]. In addition, astrocytes control the exchange of nutrients with the bloodstream, such as glucose that is used to make ATP to fuel biological processes ^[177,178].

Astrocytes have other ways in which they are critical for metabolic support, such as helping meet local energy demands during periods of hypoglycemia or high neuronal activity ^[155,179]. They express glucose transporter 1 (GLUT1) that is necessary for glucose uptake, which is made into lactate for neurons to use as energy ^[179]. Glucose can also be delivered from astrocytes to neurons via gap junctions ^[180]. Alternatively, glucose can be converted into glycogen for storage in astrocytes until required ^[181,182].

1.4: Linking glia to neurological diseases

Even though early neuroanatomists correctly identified glia to be the most drastically altered in diseased and injured brains, neuron-centric views still dominated how scientists thought about neurological pathologies. Now with decades of research accumulating evidence that microglia and astrocytes are necessary for a healthy brain physiology, there is a greater need to understand how these cells malfunction and contribute to disease. Astrocytic and microglial failure to perform their functions create disease-permissive landscapes in which neurons also malfunction, die, and cause neurological deficits. Additionally, in response to neurological diseases—including neurodevelopmental, metabolic and psychiatric disorders, as well as in infectious diseases or toxic insults—both cell types have their own pathological changes as active responders to the brain environment. These responses can be both harmful and beneficial.

With processes constantly extending and retracting to scan the environment, it is no surprise that microglia are one of the first responders to disease or damage in the brain. Such challenges produce PAMPs and DAMPs that microglia are equipped to react to with a fine-tuned inflammatory response that includes: morphological changes, altered gene expression, release of proinflammatory factors (e.g. cytokines, chemokines, ROS),

increased or decreased phagocytosis, and production of trophic factors [68-70]. They also migrate to the site of damage to destroy the pathological insult and remove damaged or dying cells [33,34,183]. If the injury is acute, such as in the case of traumatic brain injuries, this response is generally neuroprotective, as engulfing cellular apoptotic debris is essential for healing [184,185]. However, during sustained injuries, infections, and chronic neurological diseases, these roles prove to be harmful. A prolonged response can lead to excessive neuron malfunction and eventual death, thus contributing to injury or disease progression. This is especially well documented in neurodegenerative diseases. For example, researchers have repeatedly reported higher levels of proinflammatory cytokines in patients with mild cognitive decline that went on to develop Alzheimer's [186,187]. In mouse model studies, the protein aggregates that often accompany these diseases—such as amyloid- β (A β) oligomers in Alzheimer's—are known to be surrounded by clusters of reactive microglia [188,189]. Additionally, some of the earliest signs of neurodegenerative diseases like Alzheimer's are inflammation followed by synapse loss [190-192]. There are now many studies showing microglia phagocytic pathways to be upregulated in Alzheimer's brains, such as TREM2 and the complement cascade [40]. Interestingly, these are the same pathways that are used for synapse elimination in development, as discussed. Additionally, the proinflammatory factors that microglia produce in this state can stimulate astrocytes to release their own neurotoxic factors, further exacerbating disease progression [145,147].

During a pathological condition, astrocytes can do one or all of the following to contribute to disease progression: 1) reactive astrogliosis, 2) astrodegeneration accompanied with astrocytic atrophy and loss of function, and 3) pathological remodeling. First, reactive astrocytes are characterized by morphological hypertrophy, transcriptomic changes, and loss of healthy functions [142,143]. They can be both neuroprotective and neurotoxic. Reactive astrocytes that are neuroprotective can help heal by secreting trophic factors, isolating injured areas by creating a glial scar, and aiding BBB repair [148, 175, 176]. These astrocytes are generally seen in acute brain injuries like focal ischemia [146, 149]. Suppressing this method of reactive astrogliosis exacerbates brain pathologies [146, 149]. However, reactive astrocytes can also be neurotoxic, especially during conditions of prolonged stress or damage, secreting damaging proinflammatory factors and enhancing synaptic and neuronal degeneration [145,147,193].

Nearly all neurological pathologies exhibit reactive astrocytes in one way. This reactive astrogliosis is especially prevalent in many neurodegenerative diseases, like Alzheimer's, Parkinson's, and Huntington's [193-198]. Second, astrodegeneration can also occur in neuropathologies, which is observed as morphological atrophy, increased astrocytic death, and overall decreased astrocyte numbers [199]. This form of astrocyte pathology is highly prevalent in neuropsychiatric diseases, like major depressive disorder and schizophrenia (SZ) [200-202]. Reducing astrocyte numbers can cause a failure in many homeostatic pathways that triggers abnormal neurotransmission and excitotoxic neuronal death. Lastly, astrocytes can pathologically remodel and acquire abnormal functions or molecular pathways. In these cases, astrocytes can lose important functions like providing trophic support or preventing excitotoxicity. For example, it is documented that in mesial temporal lobe epilepsy, the astrocytic expression of gap junctions and certain

potassium channels changes, which in turn impacts spatial potassium buffering and brain excitability ^[203]. Similar to microglia, a number of these astropathologies are also reported in the aging brain ^[204].

The contribution of microglia and astrocytes to neurological disease is likely to be not only a consequence of the pathologies, but also causative. Given the many developmental and homeostatic roles that these cells have, it is no surprise that there is a growing body of work highlighting misregulation of glial function to be one of the initiating factors for neurological disease. For example, underlying mechanisms of autism spectrum disorders (ASDs) are most likely associated with improper neuronal networks and aberrant neurotransmission that glia are involved in developing and maintaining ^[195,205,206]. There are now well-documented mouse-models of ASDs that are developed by perturbing embryonic microglia with an infectious insult ^[77,80]. Genetic manipulations of synaptic pruning mechanisms of microglia can also cause ASD-like behaviors in mice ^[42,47,84]. Neuropsychiatric disorders like SZ are similarly characterized by improper synaptic connections and neuronal activity, also implicating glia. Various studies have found a causal role of astrocytes in SZ, including the generation of a mouse mutant model specifically targeting one of the most commonly affected genes *DISC1* (Disrupted in schizophrenia 1) only in astrocytes ^[207]. Additionally, risk genes underlying a wide range of neurodevelopmental and neurodegenerative diseases are often exclusively expressed in astrocytes or microglia, suggesting that glia-specific malfunctions are responsible for the disease etiology ^[206]. Alexander's disease is a well-documented example of this; it is caused by mutations in the gene for glial fibrillary acidic protein (*GFAP*), which is highly expressed in astrocytes, that leads to many debilitating symptoms such as severe mental delays, abnormal brain anatomy, and seizures ^[208].

Whether glia pathology is primary or solely secondary to disease remains to be determined. However, the latter case is undisputed and can profoundly impact the progression of nearly all neurological diseases. The work in this thesis aims to strengthen the understanding of microglia and astrocyte function in two different neuropathological conditions, ultimately reasoning that they could be considered as cell-specific targets for novel disease prevention and treatment avenues.

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Chapter 2: Role of copper during microglial inflammation and Parkinson's Disease (PD)-like dopaminergic neuron death

In this chapter, we begin to explore the how copper is involved in neuroinflammation. We first use a copper probe to detect how astrocytes and microglia respond to specific inflammatory stimuli. We find that microglial copper strongly changes intracellular levels in response to LPS. Interestingly, previous work has indicated that neuroinflammation and copper levels are involved in increasing risk for PD development. Work in our lab is currently in progress, with the aim of better understanding this connection.

2.1: Background

2.1a: The physiological roles of copper

Transition metals are vital to the physiology of all forms of life. They act as structural and metabolic cofactors for many essential biomolecules, and can also actively regulate molecular processes such as enzymatic activity and cellular communication. Copper is the third most abundant transition metal in the human body ^[1]. It is obtained through diet and absorbed in the stomach and small intestine to be transported to the liver, one of the organs with the highest copper content ^[2–5]. From here, it enters the bloodstream to be transported to other organs on carrier proteins like ceruloplasmin and albumin ^[2–4]. Copper has many responsibilities in sustaining normal physiology, including: iron homeostasis, skin and hair pigmentation, maintaining connective tissue and blood vessel integrity, myelination, antioxidant defense, neurotransmitter synthesis, and cellular respiration ^[6–9].

Copper transporter 1 (CTR1), encoded by solute carrier family 31 member 1 (*SLC31A1*) gene, accounts for 70% of copper import into mammalian cells, where it is passed to chaperone proteins or small molecules to hold in the cytoplasm ^[10,11]. Mice lacking *Ctr1* have growth and developmental defects so severe that they die *in utero* mid-gestation, highlighting the essentiality of copper ^[12]. Copper has two physiologically relevant oxidative states—Cu(I) and Cu(II)—with the latter known to be the predominant form in the cytoplasm (**Figure 2.1A**) ^[13]. Because copper has a great capacity for gaining and donating electrons, it is both highly desirable as a cofactor for molecular pathways and also dangerous as a potential generator of free radicals that can be toxic. Therefore, copper metabolism needs to be tightly regulated to simultaneously enable utilization of and protection from this trace metal (**Figure 2.1B**). Intracellular copper is held by small molecules, like glutathione or metallothioneins, in the cytoplasm until mobilized for cellular use ^[4,7,14]. This is a dynamic pool of free copper that is involved with other homeostatic functions. Alternatively, it is transferred to specific copper chaperones to be delivered to copper-dependent biological processes. For example, Cu,Zn-SOD1 (superoxide dismutase 1), an enzyme that detoxifies superoxide by conversion to hydrogen peroxide,

utilizes copper chaperone for SOD1 (CCS) for copper delivery [15–17]. For this reason, copper has been traditionally thought of as a static metabolic cofactor used for protection against the generation of ROS. Copper is also important for the assembly of the cytochrome c oxidase (CCO), a mitochondrial enzyme that is last in the electron transport chain (ETC) and reduces oxygen into water to allow ATP production during cellular respiration [15,18,19]. The chaperones that deliver copper to the CCO include SCO1 (synthesis of cytochrome C oxidase 1), SCO2 (synthesis of cytochrome C oxidase 2), and COX17 (cytochrome C oxidase copper chaperone); supporting the importance of copper delivery to the CCO, inhibiting the function of any of these chaperones in mice results in impaired CCO activity and neonatal death [4,7,14]. ATOX1 (antioxidant 1 copper chaperone) is the chaperone that delivers copper to ATP7A (ATPase copper transporting alpha) and ATP7B (ATPase copper transporting beta) [20,21]. These ATPases are the main copper exporters that typically sit on the Golgi apparatus to sort copper into the lumen of the secretory compartment for loading onto cuproenzymes, such as ceruloplasmin [20,21]. ATP7B is highly prevalent in liver cells for loading copper onto ceruloplasmin to be sent to the rest of the body or to remove it by secretion into bile [22]. ATP7A is found in all other cell types, and can move to the plasma membrane for copper export when intracellular levels are high [23]. Although ATP7A and ATP7B perform similar functions, they are not biologically redundant [3,20,21].

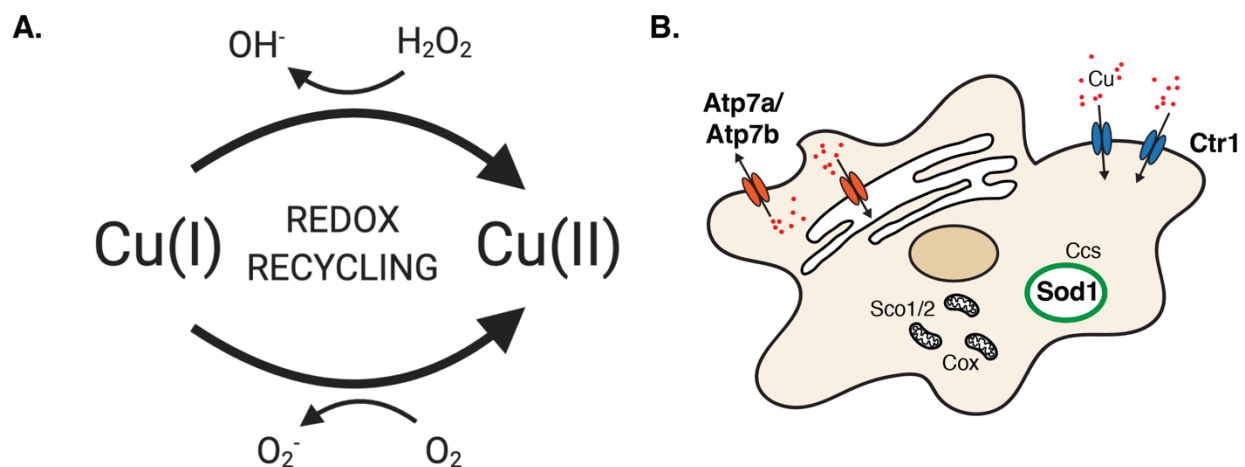


Figure 2.1. Copper is a component of many essential biological processes.

A) Schematic indicating redox activity of copper, which is utilized by physiological processes and produced harmful free radicals as byproducts. **B)** Schematic of cellular metabolism of copper in mouse microglia. Copper enters cells mainly through copper importer Ctr1. Inside cell, metallothioneins and glutathione maintain low free copper levels. Atp7a is a copper exporter that is responsible for incorporating copper onto cuproproteins at the Golgi apparatus or for exporting copper through the plasma membrane. Other cell types use Atp7b as well. Ccs is the copper chaperone that delivers to Sod1. Cox and Sco1/2 are chaperones that deliver copper to mitochondrial cytochrome c oxidase.

Copper is essential for normal brain function. The brain is one of the most energy-demanding organs in the body [6,8,13,19,24]. 95% of ATP is made in mitochondria where copper is essential for the final ETC step in cellular respiration [9,25]. The brain is also sensitive to oxidative stress due to this high metabolism. Copper acts as a cofactor for enzymes like SOD1 that are important for reducing superoxide production [9,17,26]. It is also

used as a cofactor for various enzymes that are used to synthesize neurotransmitters, like dopamine- β -hydroxylase that catalyzes the oxidative hydroxylation of dopamine into noradrenaline [27,28]. Copper has also been reported to be involved in neurotransmission. Early neurobiologists found that synapses release copper in an ATP7A-dependent manner upon neuronal depolarization [29,30]. With new ways to visualize copper, imaging studies have provided direct evidence for a dynamic redistribution of copper from the cell body to the dendrites of a neuron upon stimulation in a calcium-dependent manner [31,32]. In mouse studies, depleting copper from the cell using copper chelators impacted the spontaneous activity of the neurons [31,32]. This was replicated in genetic models of copper depletion by targeting Ctr1 in mice, also suggesting that copper pool levels in the brain are Ctr1-dependent [31]. Copper has been shown to have an effect on neurotransmission by interacting with many proteins, including GABA receptors, NMDA receptors, AMPA receptors, P2X receptors, and even voltage-gated sodium, potassium, and calcium channels [33–37]. In all these cases, copper was seen to impact synaptic strength, activity, and/or plasticity to influence neural output and behavior. Lastly, copper can modulate circadian rhythm and rest-arousal cycles [38,39]. These studies have found that the locus coeruleus (LC)—a brain region believed to be involved in sleep and arousal cycles—is ~10 times richer in copper than other brain regions and requires copper for LC function like rest-activity modulation [38,39].

CTR1, ATP7A, and ATP7B all work together to maintain copper levels in the brain. Copper in the blood stream enters endothelial cells lining the brain capillaries through CTR1, and is exported into the interstitial fluid of the brain parenchyma via ATP7A. In the case of copper leaving the brain, CTR1 again transports copper into endothelial cells from the CSF, and ATP7B exports it into the capillaries. Genetic diseases that cause deficiencies in copper homeostasis by targeting ATP7A and ATP7B have harmful effects on the CNS. ATP7A was first discovered because mutations in the gene caused Menkes disease [23,40]. Since ATP7A is responsible for the delivery of copper from the digestive tract into the bloodstream (and across the BBB into the brain), this causes a severe copper deficiency in serum, the liver, and the brain. The consequences are detrimental, including seizures, infantile-onset neurodegeneration, brain atrophy, and subsequent early death [20,21,23,41,42]. Mutations in ATP7B are the cause of Wilson's disease [2,21,43–45]. This genetic disorder causes copper to build up all over the body, causing liver dysfunction, weakness, fluid buildup in the abdomen, swelling of legs, yellowish skin, and itchiness [45]. Patients form Kayser-Fleischer rings around the irises of their eyes, which are made of copper deposits and are often important for diagnosis. In terms of other brain-related symptoms, patients are reported to have psychosis, anxiety, personality changes, trouble speaking, and seizures. Intriguingly, copper build-up in the brain seems to be specifically in the basal ganglia, which if left untreated can cause neuronal death and parkinsonian-like behaviors such as tremors and muscle stiffness [22,40,42,45,46]. Thus, the neuropathological consequences of copper deficiency (Menkes) and copper excess (Wilson's) evidence how important copper is for brain development.

The antimicrobial effects of copper have been known since the time of ancient Egypt and Rome [47]. Even today, copper is used in hospital surfaces and medical devices to prevent infections [48]. Therefore, it is no surprise that the mammalian immune system

uses copper to stop microbial growth and prevent infection [49,50]. Macrophages, the main innate immune cells, kill microbes by internalizing them into vesicles called phagosomes that are acidified and filled with ROS and lytic proteins [49,50]. Macrophages increase CTR1 activity to grow intracellular copper and also move ATP7A to phagosomal membranes in response to microbial or immune stimuli, suggesting that copper is pumped into the phagosome to assist in the immune response [6,49,50]. Interestingly, bacteria lacking copper exporters are less able to survive the internalization within macrophages, indicating that bacteria have evolved protective methods against the high copper concentrations that exist within the phagosome [49]. This is supported by the elevated levels of copper serum and ceruloplasmin that is reported during inflammatory responses [51]. Patients who are copper depleted due to Menkes disease have frequent and severe infections [51–53].

2.1b: Parkinson's Disease (PD)

PD is the most common, long-term neurodegenerative motor disorder to impact the elderly population [54]. The most prominent neuropathologies of PD are the progressive loss of dopaminergic (DA) neurons in the substantia nigra, decreased dopamine content in the caudate putamen where DA neurons have projection terminals, and accumulation of Lewy bodies in surviving cells that are predominately made of aggregated α -synuclein protein [55–58]. As the disease develops, patients start to present core motor symptoms that lead to a diagnosis: resting tremor, slow movement (bradykinesia), rigidity, difficulty walking, and postural instability [55–58]. There are non-motor symptoms that are often observed first: cognitive decline, anxiety, impaired sleep, olfactory deficits, and depression [55,57,59,60]. These symptoms emerge slowly and worsen as the disease progresses. To this day, there is no cure for PD; the treatments that exist only aim to reduce the effects of symptoms, which eventually become ineffective [61–64]. An example of this is the administration of L-DOPA, a precursor for dopamine that is meant to supplement the pathological loss of dopamine that occurs from the DA neuron death [56].

The etiology of PD is still unknown. Only about 5-10% of PD cases are caused by known inherited risk genes, leading researchers to believe that disease onset involves other aging and environmental factors [56,57,64–67]. Environmental risk factors that have been identified include: toxins, heavy metals, pesticides, traumatic lesions, and bacterial or viral infections [56,57,64–67]. To study PD *in vivo*, animal models have been generated to recapitulate the phenotypes using genetics, neurotoxins, inflammation, and pharmacology [56,57,64–67]. Researchers have been trying to develop genetic mouse models that replicate all the core neuropathologies of PD by targeting the few identified risk genes [57,65,66]. These mutated genes that cause familial PD include: *LRK2* (leucine rich kinase 2), *SNCA* (synuclein alpha), and *PARK7* (parkinsonism associated deglycase) [57,65,66]. While these studies can provide interesting mechanistic perspectives on genes associated with PD, many of the models do not display clear degeneration of DA neuron or motor deficits. Neurotoxin-based models are also widely used. Exposing rodents to 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), or a pesticide called rotenone can cause progressive degeneration of DA neurons in the SN

[57,65,66]. While many of these models exhibit significant DA neuron loss, motor deficits, and even Lewy body formation, there are none that have all key phenotypes. Additionally, while DA neuron death does occur, it is often very rapid and not representative of how PD progresses in human patients.

Studies of genetic and neurotoxic models have helped researchers understand the role of oxidative stress, mitochondrial dysfunction, and defective protein clearance in PD [57,65,66]. However, like with other neurodegenerative disorders, neuroinflammation is another key neuropathology of PD that is seen in these PD models and human patients. In fact, microgliosis was first noted by early neuroanatomists studying brain tissues from deceased PD patients [61,63,64]. Advances imaging techniques like positron emission tomography (PET) have been able to show in human patients that microgliosis is an early and sustained response of PD [61,63,64]. Proinflammatory mediators like TNF- α , interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β) are detected in elevated levels in patient's cerebral spinal fluid (CSF) [57,65,66]. Genome-wide associate studies (GWAS) of PD also continue to implicate immune pathways and microglia-specific genes in disease risk [63,67]. Interestingly, epidemiology studies have observed that PD onset is associated with many viral pathogens, including: influenza, Ebola virus, human immunodeficiency virus, herpes simplex virus-a1, and varicella-zoster virus [63]. These observations have inspired the development of an inflammatory model of PD. Administration of lipopolysaccharide (LPS), a TLR4 agonist, is one example of such a model. This model was generated after a laboratory worker accidentally exposed themselves to 10 μ g of LPS through an open wound and developed PD-like phenotypes characterized by tremors, bradykinesia, and rigidity [56,64]. Since then, intracranial, intranasal, and systemic methods of LPS delivery to rodents have all been used to model PD [64]. Intracranial stereotaxic injections of LPS into the substantia nigra have specifically been very successful in replicating all of the core PD phenotypes, including progressive degeneration of dopaminergic neurons in the SN, motor dysfunction, and α -synuclein pathology [64]. Microglia levels are 3-4 times greater in the substantia nigra than other brain regions, which potentially explains the very specific dopaminergic neuron death observed in these LPS models [61,63]. It is known that proinflammatory microglia can induce apoptotic neuronal death, likely through the production of cytokines (e.g. TNF- α), chemokines, and/or ROS [61,63].

Whether this neuropathological inflammation is a cause or a consequence remains to be determined. Nevertheless, it is undisputed that factors within the PD brain environment lead to a persistent inflammatory response from microglia. Secreted DAMPs from damaged and dying neurons bind to TLRs on microglia and can initiate inflammation [68,69]. Microglia are also stimulated by α -synuclein [61,62,67,70–72]. The microgliosis observed across PD models includes engulfing of protein aggregates, recruiting peripheral monocytes for an immune response, producing inflammatory cytokines, and releasing ROS [61–63,67,72]. All of these actions are harmful to neuron health, creating a vicious cycle that progresses PD pathology. Interestingly, aggregates of α -synuclein have been found at presynaptic terminals in PD [62,70,72]. Given the recent discoveries that microglia and their innate immune pathways are involved in synaptic pruning and that they are known to engulf protein aggregates like α -synuclein, this suggests that microglia could be contributing to the resulting synaptic degradation. Indeed, phagocytosis of α -synuclein is

done through a complement-dependent pathway, similar to synaptic pruning in development [67,72]. It is also well-documented that proinflammatory microglia signal onto astrocytes, which in turn produce their own neurotoxic factors; inhibiting this microglia-astrocyte crosstalk significantly alleviates synaptic and neuronal loss in PD mouse models [58,62,71,73,74].

2.1c: Connecting copper, neuroinflammation, and PD

Due to the existence of diseases like Menkes and Wilson's, it is clear that extreme copper levels have deleterious consequences, resulting in neurological impairments, neurodevelopmental deficits, and eventual neuronal degeneration and death. Copper is additionally linked to other neurodegenerative diseases—such as Alzheimer's, amyotrophic lateral sclerosis (ALS), and Huntington's—which are also characterized by malfunction of copper-bound proteins and changes in metal homeostasis in the brain [75–78]. Therefore, there is a growing body of evidence linking disrupted brain copper homeostasis and copper-associated pathways to neurodegeneration, such as that seen in diseases like PD [27,79–82]. The role of copper PD has yet to receive much attention, and therefore understanding its mechanistic role in neuropathologies could pave a new avenue for developing protective therapies.

Like other environmental toxins, epidemiological studies have linked risk of PD onset to exposure of heavy metals like copper [19,82]. For example, long-term exposure to copper from occupational work increases PD risk, as does living in urban populations that have more emissions of copper [27]. The development of Parkinsonian symptoms and dopaminergic neuron death in patients with Wilson's disease is another intriguing correlation of how copper can potentially contribute to PD development and progression. In fact, while copper is found throughout the brain, there are much higher levels in the substantia nigra in healthy brains [81,83]. Some studies have also reported that copper concentrations in CSF are higher in PD patients than in controls [27,84]. Injecting copper in high doses into the SN of rodents was found to replicate certain PD phenotypes, including decreased dopamine, increased oxidative stress, and loss of dopaminergic neurons [82,85]. While this increase in copper levels is associated with PD risk, this is still controversial as recent studies are reporting the opposite. For one, total copper levels in substantia nigra of PD patients have been reported to be decreased by 35-50%, while levels are unaffected in other brain regions [81–83]. Other studies have shown that PD patients have lower blood concentrations of copper, ceruloplasmin, and its oxidase activity [27,84]. Confoundingly, therapeutic approaches targeting either excess copper or decreased copper in the brain are both showing promising results in clinical trials. 8-hydroxyquinoline-2-carboxaldehyde isonicotinoyl hydrazone (INHHQ) is a metal-binding protein that efficiently competes with α -synuclein for copper, therefore inhibiting protein aggregation *in vitro* [86]. Toxin and genetic mouse models of PD have shown that copper-containing compound diacetylbis(N(4)-methylthiosemicarbazonato) copper(II) (Cu^{II}-at-sm) seems to rescue dopaminergic cell death and improve motor dysfunction [87,88]. In fact, Cu^{II}-at-sm is currently successfully being used as a therapeutic strategy for ALS, as it is thought to restore the functionality of copper-deficient SOD1, the protein affected in the

disease [88–90]. For these reasons, Cu^{II}-atsm is undergoing phase 1 of a clinical trial for PD patients [NCT04082832].

The role of oxidative stress and mitochondrial dysfunction in PD progression is undisputed. Post-mortem brains even reveal increased levels of oxidative damage on proteins, DNA, and lipids [91]. If copper levels aren't properly maintained, its redox activity can increase oxidative stress through production of highly reactive hydroxyl radicals [91]. These radicals can be damaging to cells and even cause death, while also promoting inflammatory reactions in surrounding tissue. Both animal and cell culture models of copper deficiency support this, as key mitochondrial and oxidative proteins like SOD1 and COX are altered to produce features of a more apoptotic and stressed cellular state [92–94]. Furthermore, SOD1 aggregates have been found in PD brains that are independent from α -synuclein Lewy bodies and neurites; intriguingly, these aggregates are much higher in the SN and exhibit decreased incorporation of copper into the protein [95]. Another potential way copper can impact oxidative damage is with DJ-1, a protein encoded by gene *PARK7* that is known to be associated with inherited and sporadic PD and is also thought to deliver copper to SOD1 [80,96]. Mice that are deficient in DJ-1 are susceptible to MPTP-induced dopaminergic cell death following a failure to upregulate SOD1 expression [93,94,96,97]. High levels of DJ-1 have also been reported in brains of human PD patients [80,96,98].

Copper could also be involved in PD through its role in dopamine oxidation. After synthesis, dopamine is stored almost completely inside vesicles due to its susceptibility to oxidation [99,100]. Despite being stabilized by the low pH of the vesicular lumen, dopamine can still autoxidize into ROS and dopamine quinones (DAQs). DAQs and DAQ-modified proteins then polymerize to form neuromelanin as a protective mechanism to prevent neurotoxicity [99,101,102]. Copper has been shown to be a major source of dopamine oxidation, which leads more neuromelanin, but also more harmful DAQs and hydroxyl radicals [99,101,102]. Interestingly, neuromelanin levels are highest in the substantia nigra, just like copper [102]. Next, copper has been found within aggregates of α -synuclein. Copper has been described to accelerate α -synuclein aggregation even at physiological concentrations [103–105]. *In vitro* data using a human neuroblastoma cell line found that depleting copper results in reduced α -synuclein aggregates, which was reversed when copper was added back [105]. In fact, α -synuclein deposition occurs first in the SN, the brain region in which copper levels are also highest [64,85,106]. Additionally, it has been shown that reducing α -synuclein-bound Cu²⁺ to Cu¹⁺ can increase production of ROS [103–105]. Lastly, copper could potentially be contributing to neuroinflammation, which is a known mechanism to impact PD development and progression. For example, altered copper levels within microglia could be impacting their inflammatory response, thus producing proinflammatory factors and ROS that are damaging to neuron health. Copper exposure in mice has led to significantly elevated neuroinflammation [107,108]. This phenomenon has been shown in other tissues as well; for example, LPS-induced vascular inflammation in rodents was reduced when treating with copper chelator TTM (tetrathiomolybdate), suggesting that reduced copper is potentially anti-inflammatory [109]. Additionally, reduced metallothioneins, or proteins that are critical for buffering intracellular copper, are reported to enhance inflammatory cell response, potentially due

to the resulting increased free copper enhancing ROS and cellular stress [27,100]. This notion is also debated, as studies on ALS mice with copper decreased the proinflammatory status when treated with Cu^{II}-atsm to increase intracellular copper, suggesting that increased copper is anti-inflammatory [88–90].

The cause of PD is still being debated and is generally considered to be multifactorial: a combination of genetics, aging, and various environmental factors. Therefore, it is necessary to explore all mechanisms that are contributing to damage to better understand the disease. In this chapter, we explore the role of copper in neuroinflammation, and then seek to understand its role in a neuroinflammatory LPS-induced model of PD. While the role of copper has been thoroughly studied in neurons, little is known about how it contributes to microglia activity. Given what is known about copper in the inflammatory response of macrophages and also the role of microglial inflammation to PD, we are interested in exploring whether or not microglia could be impacted by changes in copper levels and the potential contribution this has on PD development. More specifically, we hypothesize that microglia copper levels within the SN are higher in LPS-induced dopaminergic cell death in mice, thereby furthering the proinflammatory response of microglia that then exacerbates DA neuron death. With copper therapies advancing through clinical trials in other neurodegenerative disease like ALS, understanding the different ways that copper impacts PD progression can open new approaches for developing therapies.

2.2: Results

2.3a: Inflammatory stimuli cause copper pools to change within microglia and astrocytes

While significant work has been done trying to understand the biological roles of copper in neurons, little is known about its function in glial cells. Given what is known about the copper's contribution to inflammation in the immune system, we hypothesized that copper could also be critical for immune function in the brain, specifically in cells that are equipped to respond, such as astrocytes and microglia. To address this hypothesis, we first used a copper probe to image how the transition metal moves intracellularly when glial cells are undergoing an inflammatory response. CD649 is a copper-directed acyl imidazole fluorescent probe that contains a thioether NS2 receptor, which increases electrophilicity upon copper binding for nucleophilic attack and covalent labeling of proximal proteins with the fluorescent dye [110]. This probe was synthesized by researchers from Dr. Christopher Chang's lab in the Department of Chemistry at the University of California, with whom we are collaborating with on this project. It overcomes a major challenge of small-molecules diffusing away from their target upon binding; instead, the covalent bond formation between the fluorescent reporter and proximal proteins preserves the spatial information of localized copper hotspots. Another advantage of the CD649 copper probe is that it can also detect copper ions alone. For

these reasons, we used the probe to detect changes in labile copper status in astrocytes and microglia after inflammatory stimuli.

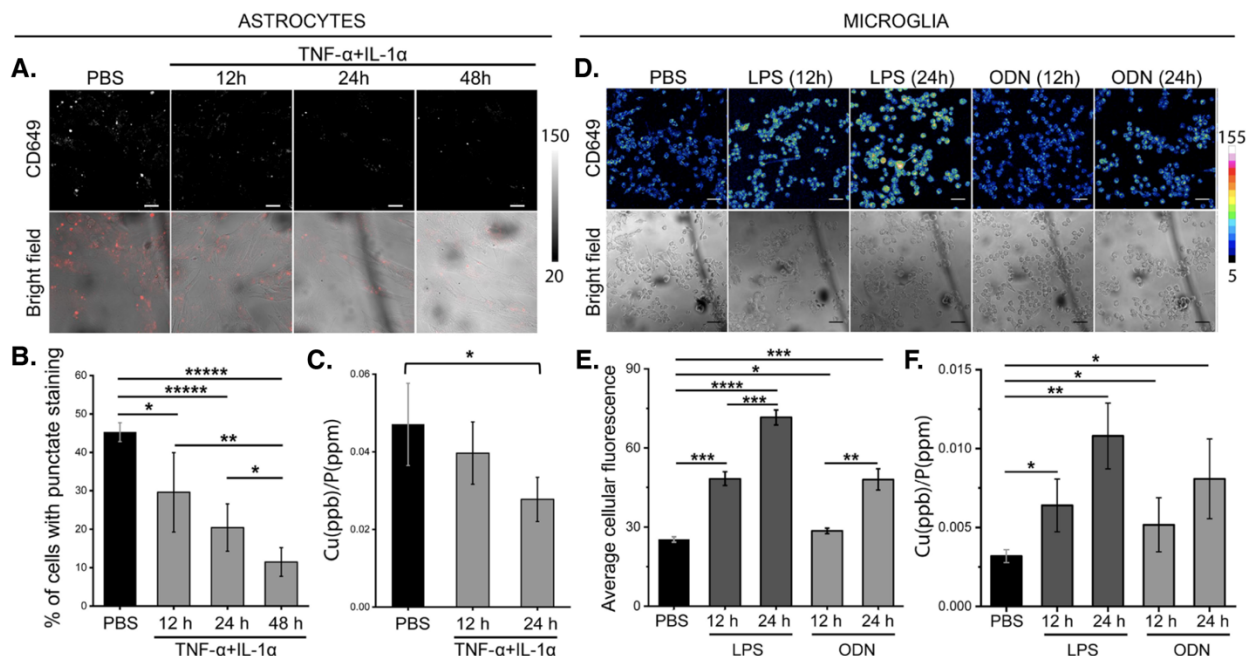


Figure 2.2. Fluorescence imaging with copper probe CD649 in human astrocytes (HA) and SIM-A9 microglial reveal changes in copper levels under proinflammatory stimuli.

A) Confocal fluorescence imaging of HA treated by PBS or combination of TNF- α and IL-1 α (both as 20ng/mL) up to 48h and stained with 5 μ M CD649 in HBSS for 30min, fixed and imaged. Scale bar = 25 μ m. **B)** Average fluorescence intensity of CD649 from experiment A performed in triplicate. Error bars denotes standard deviation (SD; n=3). **C)** ICP-MS measurement to determine total cellular ^{63}Cu levels in astrocytes under TNF- α and IL-1 α stimuli (with normalization of different cell numbers by total cellular ^{31}P level). Error bars denote SD; n=2. **D)** Confocal fluorescence imaging of SIM-A9 microglia stimulated by PBS control, LPS (0.1 μ g/mL) or ODN (5 μ g/mL). Give μ M CD649 in HBSS was incubated subsequently for 30 min and then imaged after fixation. Scale bar = 25 μ m. **E)** Average cellular fluorescence intensity of CD649 determined from experiments in D performed in triplicate. Error bars denote SD; n=5 different images from the triplicate experiments. **F)** ICP-MS measurement to determine total cellular ^{63}Cu levels in SIM-A9 microglia under LPS or ODN inflammatory stimuli (with normalization of different cell numbers by total cellular ^{31}P level). Error bars denote SD; average of technical triplicates. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

It is reported that astrocytes can acquire, store, and export copper as they are highly resistant to copper toxicity [77]. To induce an inflammatory response in astrocytes, we used tumor necrosis factor alpha (TNF- α) and interleukin-1 alpha (IL-1 α) to stimulate human astrocytes (HA) [ScienCell Research Laboratories], as they are known factors to induce inflammation in astrocytes [73,111]. CD649 reveals that labile copper pools are localized within punctate patterns in human astrocytes when in their resting state (**Figure 2.2A**). Upon inflammatory stimulus, we observed over several time points (12h, 24h, and 48h) that these puncta disseminate and there is an overall loss of fluorescence, indicating that copper is decreasing in the cells. **Figure 2.2B** quantifies the percent of cells with puncta staining at each timepoint, showing that the decrease in puncta

staining is significant. This is further supported by ICP-MS (inductively coupled plasma mass spectrometry) measurements of total cellular copper to phosphorus ratios, which indicate that TNF- α and IL-1 α decrease copper content in astrocytes (**Figure 2.2C**). Copper biology in microglia has yet to be investigated. Therefore, we were intrigued to find that inflammation caused a different copper response in mouse microglia SIM-A9 cells [112], compared to what was observed in HA. To stimulate SIM-A9 cells, we used two different TLR stimulants that are known to cause a proinflammatory response in microglia: lipopolysaccharide (LPS; TLR4 agonist) and oligodeoxynucleotides (ODN; TLR9 agonist). In **Figure 2.21D**, we show that treating microglia with either LPS or ODN both induced an increase in their labile copper pools at 12h and 24h, compared to basal state. This is quantified in **Figure 2.2E**, which shows that at each timepoint there is a significant increase in copper content within SIM-A9 cells when treated with LPS or ODN. These increases were supported by complementary ICP-MS measurements of total copper (**Figure 2.2F**). Taken together, these data show that neuroinflammation changes intracellular copper levels within astrocytes and microglia.

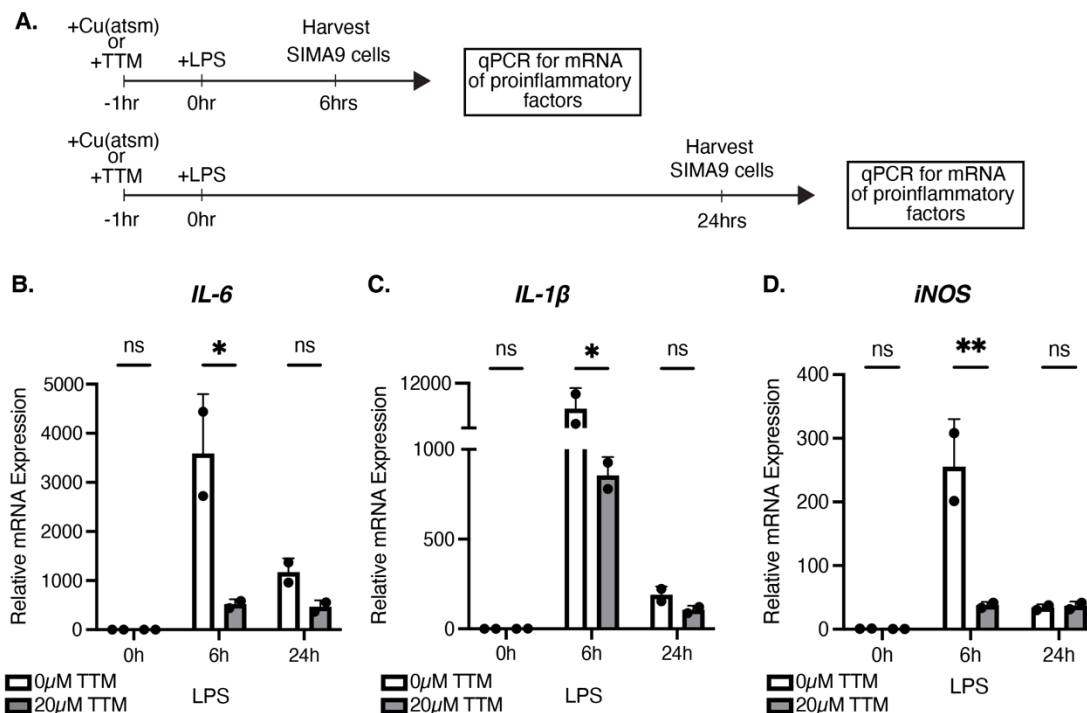


Figure 2.3. Treatment of SIM-A9 cells with TTM decreases inflammatory response to LPS at 1h.

A) Timeline of experiment. **B)** mRNA expression of *IL-6*, **C)** *IL-1 β* and **D)** *iNOS* in SIM-A9 cells either treated with TTM for 1h prior to start of experiment, or no treatment as a control. Stimulation experiments were repeated in three separate biological replicates. Data shown from one representative biological sample. qPCR was conducted in technical duplicate. Data are shown as an average of technical duplicates. Two-way ANOVA followed by Tukey's multiple comparisons. All data are mean $\pm$ SD. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001.

2.3a: In vitro depletion of copper availability decreases LPS-induced inflammatory response

Since LPS is known to be associated with dopaminergic neuron death and PD-like phenotypes, we were particularly interested by the copper changes observed in microglia upon LPS stimulation, and decided to explore this further. Since we observed an increase in copper content in microglia during inflammation, we hypothesize that copper could be critical for microglia to carry out an inflammatory response. We first used copper chelator TTM to decrease copper content within cells. An hour after TTM application, cells were stimulated with LPS and harvested at 6h and 24h timepoints (**Figure 2.3A**). Next, we evaluated the inflammatory response of these cells by assessing for proinflammatory factors: tumor necrosis factor alpha (*Tnf-α*), interleukin 1 beta (*Il-1β*), and inducible nitric oxide synthase (*iNOS*). **Figure 2.3B** shows that at 6 hours, there is a significant decrease in *IL-6* expression in the cells treated with TTM compared to control. The same decrease at 6 hours was observed with *Il-1β* and *iNOS* (**Figure 2.3C** and **Figure 2.3D**). Likely due to the effects of TTM wearing off, there were no significant differences observed in any of the factors at 24 hours. These data indicate that copper is required for activation of inflammatory responses in microglia upon LPS stimulation.

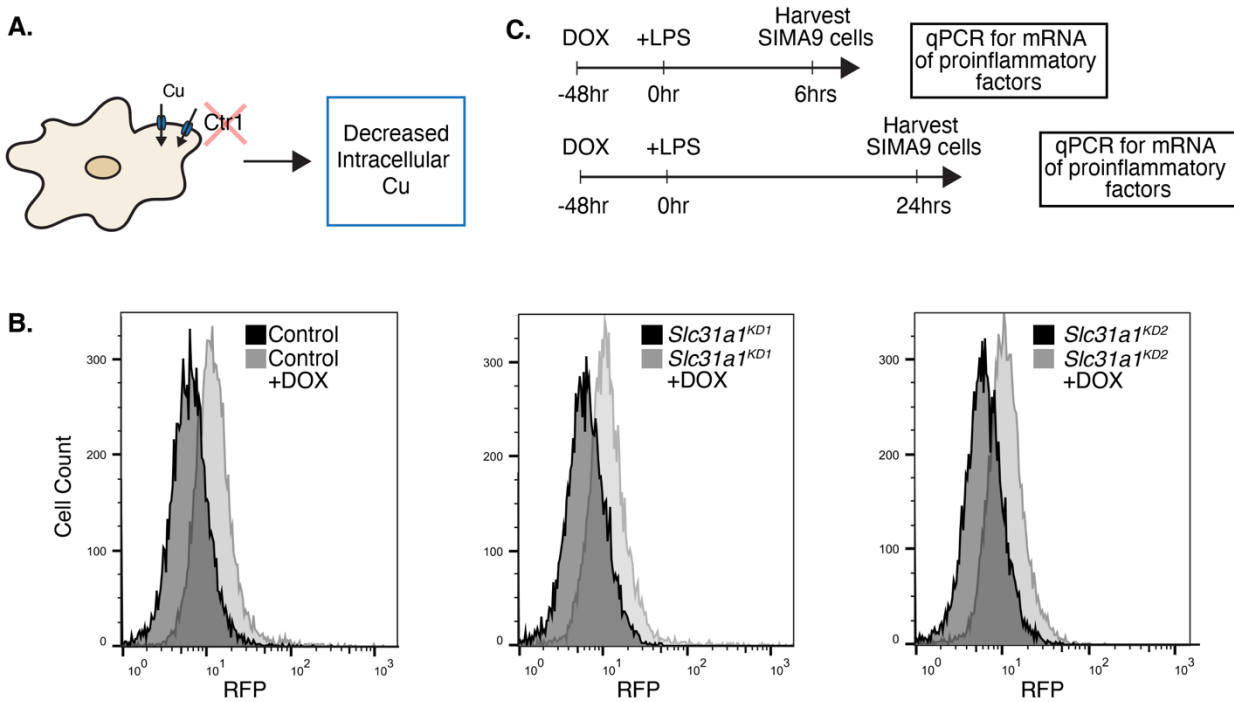


Figure 2.4. Generation of *Slc31a1* knock-down SIM-A9 cells using pTRIPZ lentiviral system.

A) Schematic indicating knock-down of gene *Slc31a1* that codes for Ctr1 copper transporter will result in decreased intracellular copper. **B)** FACS histogram plots of cells transfected with shRNAs containing control (left), *Slc31a1*^{KD1} (middle), and *Slc31a1*^{KD2} sequences. Cells treated with DOX show shift in RFP expression. **C)** Timeline of experiment.

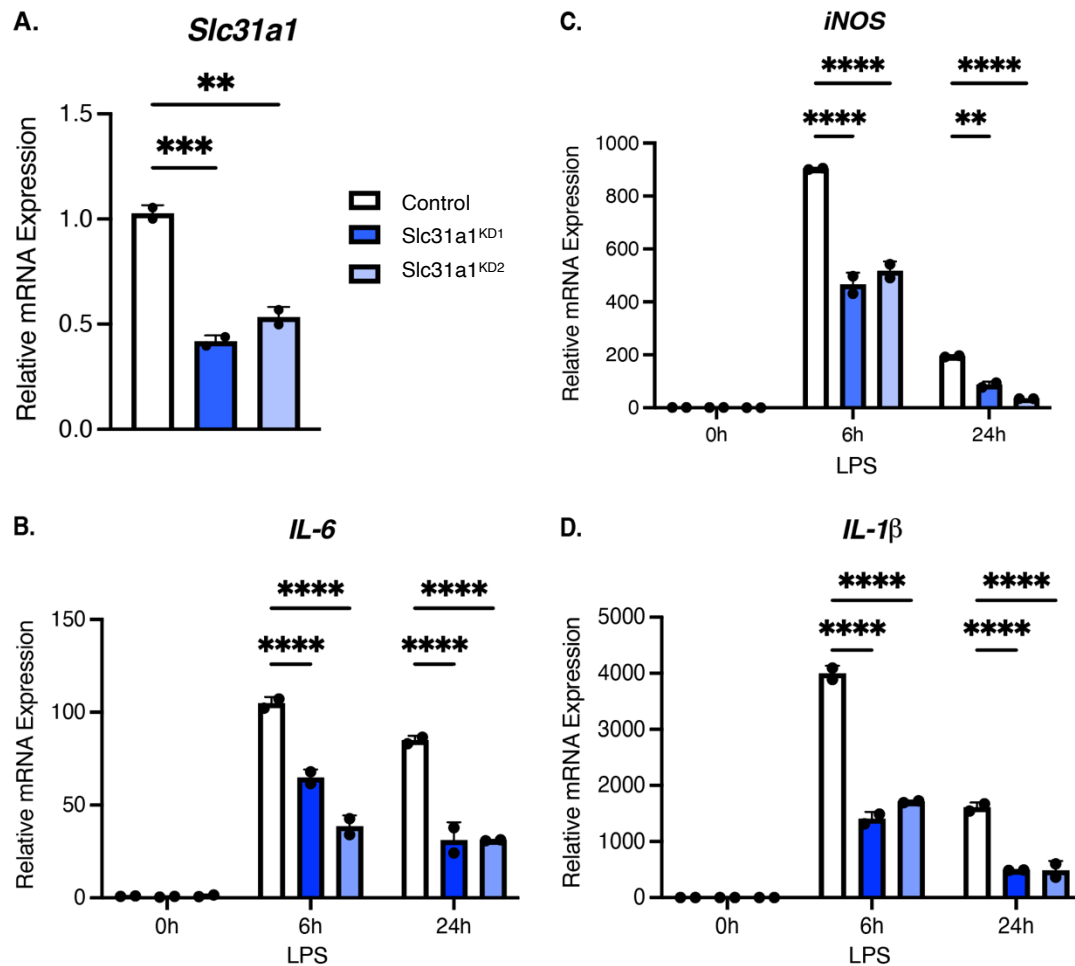


Figure 2.5. *Slc31a1* knock-down SIM-A9 show decreased inflammatory response to LPS.

A) shRNA-containing lentivirus was delivery to generate *Slc31a1*^{KD1} and *Slc31a1*^{KD2} SIM-A9 cells lines and the knock-down efficiency is shown. One-way ANOVA. Dunnett's Multiple comparisons: Control vs. *Slc31a1*^{KD1} $p=0.0019$, Control vs. *Slc31a1*^{KD2} $p=0.0005$. **B)** mRNA expression of *IL-6*, **C)** *iNOS* and **D)** *IL-1β* in SIM-A9 cells either treated with TTM for 1h prior to start of experiment, or no treatment as a control. Stimulation experiments were repeated in three separate biological replicates. Data shown from one representative biological sample. qPCR was conducted in technical duplicate. Data are shown as an average of technical duplicates. Two-way ANOVA followed by Tukey's multiple comparisons. All data are mean $\pm$ SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

To validate these results using a different method, we used a genetic approach to decrease copper content within cells and retest our hypothesis. As the main importer of copper, we targeted *Ctr1* for knock-down to generate a cell line with decreased copper levels (**Figure 2.4A**). We used two shRNA constructs that were cloned into the pTRIPZ lentiviral vector to knock down *Slc31a1*, the *Ctr1* gene. We opted to use a pTRIPZ vector, which induces expression of the shRNA when cells are treated with doxycycline (DOX), because *Ctr1* is essential for cellular function and cells will not survive long without it (*data not shown*). To confirm that the cells were successfully transfected and express the shRNA-containing plasmid, we used flow cytometry to detect red fluorescent protein

(RFP) that was also included in the plasmid (**Figure 2.4B**). Following validation, we proceeded with the experiment by treating SIM-A9 cells with DOX for 48 hours, and then treated the cells with LPS with be harvested at 8 and 24 hour time points (**Figure 2.4C**). RNA was then isolated from these cells, which we used to confirm that *Slc31a1* expression is reduced in both shRNA cell lines, both around a 50% decrease (**Figure 2.4A**). Expression levels of proinflammatory factors *Il-6*, *iNOS* and *Il-1 β* were also evaluated. **Figure 2.5B** shows that knock-down of *Slc31a1* lead to a decreased *Il-6* expression at both 6 and 24 hours. The same decrease was observed in *iNOS* and *Il-1 β* at both timepoints (**Figure 2.5C** and **Figure 2.5D**). Taken together, these data show that both chemically and genetically decreasing copper content suppress the inflammatory response of microglia.

2.3c: LPS induced PD-like dopaminergic neuron death

If copper is involved at a cellular level in neuroinflammation, we expect copper to be involved in neurological diseases that are associated with neuroinflammation, as well. More specifically, given the epidemiological data that implicates copper with PD development, we hypothesize that copper excess in microglia leads to an increased inflammatory response that contributes to PD progression. To test this, we opted to use a mouse model of DA neuron death that is induced by stereotaxic LPS injection into the SN (**Figure 2.6A**). This is a neuroinflammatory model of PD that specifically kills DA neurons in the SN; other PD pathologies have also been reported in this model, such a motor dysfunction and α -synuclein build-up. We first confirm that the model sufficiently induced DA neuron death. LPS was injected into the SN of an adult mouse using a stereotax; after 21 days, the mouse brain was harvested for sectioning (**Figure 2.6B**). We use PBS injections as a control. **Figure 2.6C** shows a representative image of LPS and PBS injections into adult male mice. Sections are stained for Th (tyrosine hydroxylase, which is expressed by DA neurons specifically). Given the extent to which LPS is causing DA neuron death, we are now proceeding to the next step of the experiment in which we are turning to our collaborators in Dr. Chang's lab once more. We aim to use this model of DA-neuron death to assess how copper levels within the SN change, and also pinpoint the cell types in which copper levels are changing. The Chang lab is leader in the field of transition metal signaling, specifically in developing tools to better image transition metals in biology. With our LPS- and PBS-injected sectioned mouse brains, collaborators in the Chang lab will be using laser ablation ICP-MS to detect changes in copper concentrations across the SN. We will couple this with immunohistochemistry to fluorescently label microglia and DA neurons, therefore allowing us to map the copper changes at a cellular level. It is known that neuroinflammation is one of the main pathologies of PD. Additionally, our data shows that copper content within microglia increases during inflammation. We expect that our data from the Chang lab shows that microglia in our LPS-injected mice contain higher levels of copper compared to control PBS injections.

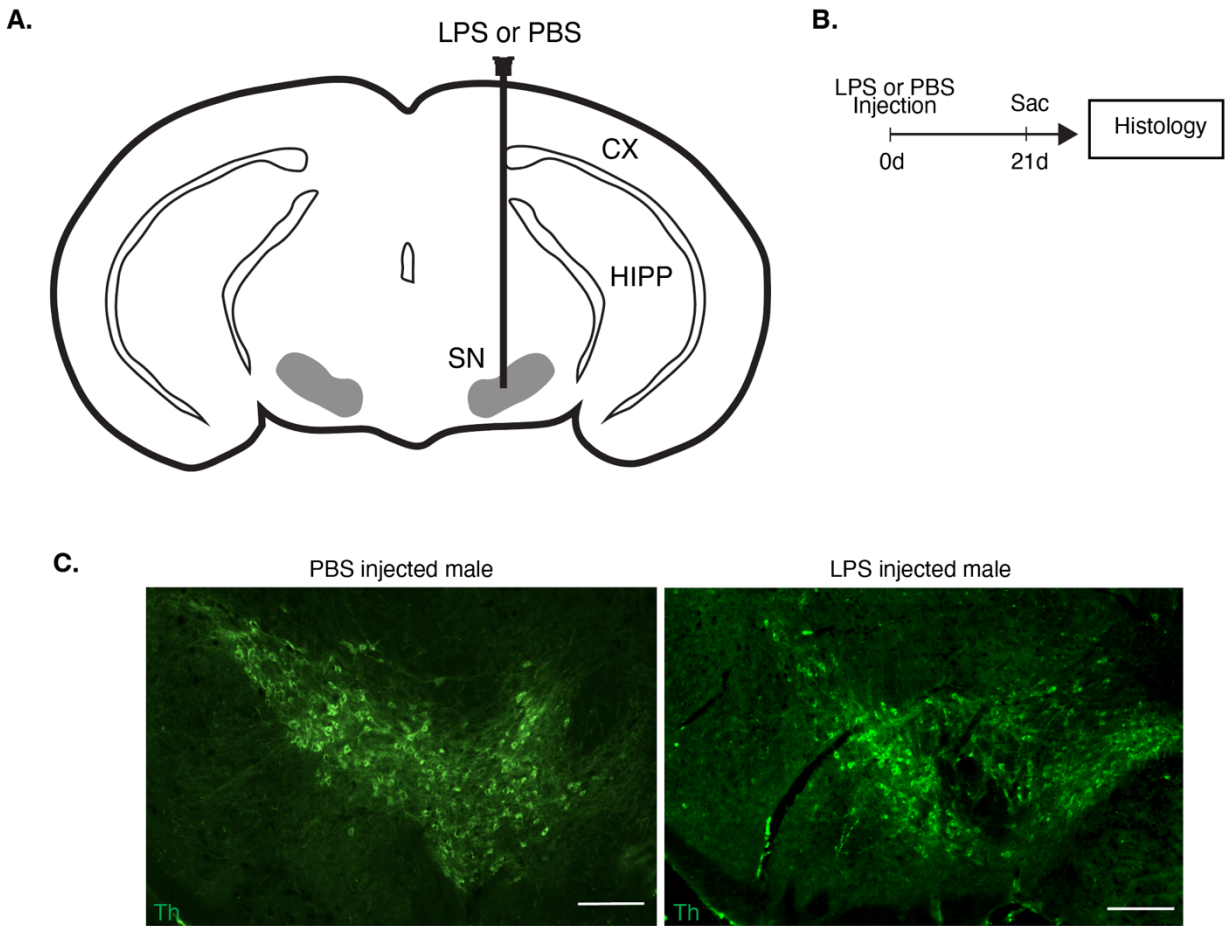


Figure 2.6. Injection of LPS in mouse SN successfully causes death of DA neurons.

A) Schematic of LPS or PBS injection into substantia nigra (SN) of mouse brain. HIPP = hippocampus. CX = cortex. **B)** Timeline of experiment. **C)** Representative images of SN from PBS injected male versus of LPS injected male. Green indicates Th+ cells. Scale bar 100 μ m.

2.3: Discussion and Future Directions

In this chapter, we report a novel role for copper during the inflammatory response of astrocytes and microglia using a CD649 copper indicator. In astrocytes, copper is localized to puncta that disseminate when introduced to an inflammatory stimulus. It is already known that astrocytes can efficiently store copper and are highly resistant to its toxicity [77,113]. They also express glutathione and metallothioneins at high levels [77,113]. This supports our data that indicate copper pools to be sequestered into puncta when cells are at rest. During an inflammatory response, the puncta disappear, thus copper could be a necessary cofactor for certain proteins and enzymes that are involved in inflammatory molecular processes, or that copper is being exported for deliver to other cells for use. Interestingly, the opposite is observed in SIM-A9 microglia. At rest, copper was spread out through the cell body, followed by an increase in copper when cells were stimulated with TLR ligands LPS or ODN. This suggests that copper is necessary for inflammation in microglia, which matches what is known about the function of copper in other macrophages. During an inflammatory response, macrophages phagocytose pathogens and cellular debris to be sequestered into and destroyed by phagosomes that are filled with ROS and lytic proteins [50,51]. These phagosomes are also filled with copper—which is also harmful to microbes—therefore, macrophages increase Ctr1 expression in order to bring in more copper to be pumped into the phagosome and assist in the immune response [50,51]. Atp7a is also translocated to these phagosomes to export copper from the cytoplasm into the lumen of the phagosome [49]. Though this has never been shown in microglia, as the brain's resident macrophage we expect that a similar mechanism is in place given the large increase in intracellular copper that we observe. In the future, we aim to assess for changes in Ctr1 and Atp7a mRNA levels, and image to see if protein expression of these transporters as well.

We next demonstrate that decreased free copper within microglia cells suppressed inflammatory transcriptional responses to LPS. We used both a chemical and genetic methods to show this, with TTM and shRNAs targeting *Slc31a1*, respectively. This supports our hypothesis that copper is necessary for microglia to carry out an inflammatory response. Though our work has currently only looked at three specific inflammatory cytokines—IL-6, IL-1 β , and iNOS—more work is necessary to understand how copper is more broadly impacting this inflammatory response. RNA-sequencing experiments are in the works in our lab to assess for overall changes in the transcriptomic profile. From here, we can identify other inflammatory factors that could be impact by copper levels. This will be important for translating how this data will work *in vivo*. For example, it is known that inflammatory factors secreted by microglia are not only neurotoxic on their own, but also bind to receptors on astrocytes and cause them to release even more neurotoxic factors as well [73,111]. If copper levels can change the inflammatory response of microglia, there will be greater overall consequences in the brain that can result in reduced neuron death when in the presence of injury, pathogenic factors, and other harmful factors.

By injecting LPS into the SN of mice, we've established a reliable system of specific dopaminergic neuron death that is reflective of PD pathology. Using this system, we can test whether or not copper levels within the SN change during a significant neuroinflammatory response. The Chang will utilize LA-ICP-MS that will be able to detect copper levels on mouse brain sections that were given to the lab to be analyzed. Immunohistochemistry will be used concurrently to label Th (DA neurons) and Iba1 (microglia) expressing cells. Overlaying the results of these two will allow us to assess cell specific changes in copper levels. We hypothesize that under these PD-like conditions, microglia copper levels will be higher in the LPS injected mouse SNs compared to PBS controls. PD studies have resulted in conflicting results as to how PD progression and copper levels are related. Though epidemiological data seems to suggest that increased copper levels are a risk factor for PD development ^[100], data from post-mortem brain tissue suggests that copper levels within the SN is decreased compared to healthy brain ^[81,83,106]. However, this decrease could be due to the death of DA neurons, which are high in copper. Having fewer cells that contain copper will lead to an overall decrease in copper levels within the SN. To better understand the role of copper, we need to see how levels are impacted at a cellular level.

If data from our collaborators support our hypothesis that copper levels within microglia in PD-like LPS-induced model of DA neuron death, this data could be used to inspire novel PD treatments. Our data also shows that decreasing copper content within microglia decrease the inflammatory response. Since neuroinflammation is a critical component of PD progression, targeting copper levels within microglia can suppress inflammatory responses of PD brains. Though not a cure, the search for methods of suppressing inflammation in neurodegenerative diseases like PD is highly sought-after as it is thought to be a way of slowing disease advancement. To test this in mice, future experiments will be needed to repeat our LPS injections in mice with decreased copper content in brains. Examples of this include mice that are heterozygous for Ctr1, or injection shRNAs for Ctr1 in SN of the mouse brain. Alternatively, TTM treatment could also be used as it is already being used for treatment of other neurodegenerative diseases ^[100]. TTM is capable of crossing the BBB, therefore it can not only be delivered orally or intraperitoneally, but also potentially considered for larger therapeutic options.

2.4: Methods

2.4a: In vitro experiments

Cell Culture

SIM-A9 (murine microglial cell line, kindly provided by Dr. Nagamoto-Combs) ^[114] cells were maintained with DMEM/F12 (Corning) supplemented with 10% fetal bovine serum (FBS; Hyclone GE Healthcare Life Sciences), 5% horse serum (HS; Hyclone GE Healthcare Life Sciences) and 1% penicillin/streptomycin (pen/strep; Gibco) (Complete medium). HEK 293T cells were cultured in DMEM (Corning) supplemented with 10% FBS and 1% pen/strep. All cells were cultured in an incubator at 37°C and 5% CO₂.

TTM treatment of immune stimulated SIM-A9 cells

For all immune stimulation time course experiments, SIM-A9 cells were seeded in 24-well plates at a density of 10^5 cells/well, in triplicate, and stimulated with lipopolysaccharide (LPS; $0.1\mu\text{g/ml}$; Sigma-Aldrich) for 6, 12 or 24 hours. No stimulation controls received an equivalent volume of sterile 1x DPBS (Invitrogen). For cells treated with copper chelator tetrathiomolybdate (TTM; $20\mu\text{M}$) to decrease intracellular copper, cells were given TTM one hour before start of immune stimulation experiment. Following completion of experiment, cells were lysed and stored at -80°C for RNA isolations.

RNA extraction and RT-qPCR

Total RNA was isolated from SIM-A9 cells using the quick-RNA Miniprep kit (Zymo Research). Primers were designed using Primer-BLAST and are listed in Supplementary Table 1. Complementary DNA was reverse transcribed using SuperScript III (Invitrogen) and qPCR was performed using SYBR green master mix (Roche) on a Quant Studio6 qPCR machine (ThermoFisher). All data collected by QuantStudio Real Time PCR Software v1.3 were normalized to housekeeping gene hypoxanthine phosphoribosyltransferase (*Hprt*) using the delta-delta Ct method. Primers used in this assay were designed using Primer-BLAST and are listed in Table 2.1. Biological and technical replicates were indicated in the figure legend.

CD649 staining and imaging

Copper levels following each experiment were checked using CD649 probe. CD649 was synthesized and provided by Chang lab ^[110]. SIMA9 cells were plated onto 8 well-chamber slides, and inflammation was induced by addition of LPS, as described. After inflammation for the indicated time interval passed, cells were washed with PBS, incubated with $1\mu\text{M}$ CD649 in HBSS (0.9mM MgCl_2 ; Gibco) for 30 minutes and fixed by 4% paraformaldehyde (PFA) for 5 minutes at room temperature. The cells were then washed with PBS and kept at 4°C until imaging. Confocal fluorescence imaging was performed with Zeiss laser scanning microscope 710 AxioObserver using a 20x objective lens and Zen 2021 software (Carl Zeiss; UC Berkeley Molecular Imaging Center). CD649 was excited using 633 diode laser and emission collected with META detector between 650 and 750nm. Image analysis and quantification was performed using FIJI (National Institutes of Health).

shRNA-mediated knockdown of Ctr1 in SIMA9 cells

pTRIPZ inducible lentiviral shRNA (Dharmacon) was engineered to use the third generation Tet-On system and tightly regulate the induction of shRNA expression targeting *Slc31a1* (Ctr1), or induction of transgene sequence for luciferase as a control, in the presence of doxycycline (Sigma). Sequences are listed in Supplementary Table 2.1. Lentivirus was packaged via co-transfection of each pTRIPZ shRNA plasmid with pCMV-VSV-G (Addgene plasmid #8454) and pCMV-dR8.2 (Addgene plasmid #8455) into HEK 293T cells using Lipofectamine 3000 reagent (Life Technologies) according to the manufacturer's instructions. Viral supernatant was harvested after 48h and added to SIM-A9 cells. 24h post-transduction, cells were changed into complete medium. 24h after,

medium of all transduced cells was changed daily to fresh medium containing puromycin (8 μ g/ml) to select for cells expressing transgene of interest. Cell death was observed for 5 days, after which remaining cells were expanded in complete medium. Successful transduction of the plasmid was checked by: 1) presence of RFP+ cells via FACS on an Aria Fusion (BD Biosciences, UC Berkeley Cancer Research Laboratory); 2) RFP fluorescence was detected by confocal imaging with Zeiss laser scanning microscope 710 AxioObserver using a 20x objective lens and Zen 2010 software; and 3) percent knockdown determined via RT-qPCR. Following confirmation, each cell line was expanded and subcultured for immune stimulation experiments, as described.

2.4b: *In vivo* experiments

Animals

C57BL/6J wild-type male and female mice (aged four to six months) were used in this study. These mice were obtained from the Jackson Laboratory, then bred and housed in the specific pathogen- free (SPF) animal facility of the Li Ka Shing building at University of California, Berkeley. All animals were weaned around three weeks postnatally, housed in cages of 2-5 same-sex mice, and maintained on a 12-hour/12-hour light/dark cycle (on 7:00am; off 7:00pm) with *ad libitum* access to food and water. During the COVID-19 pandemic, the facility light cycle was changed to a 14-hour/10-hour light/dark cycle (on 6:00am; off 8:00pm). All animal care and procedures were approved by the University of California, Berkeley Institutional Animal Care and Use Committee and Office of Laboratory Animal Care.

Stereotaxic injections into mouse substantia nigra

Adult male and female mice were anesthetized with 2% isoflurane (vol/vol) in oxygen, and placed on a heating pad and in a stereotaxic frame (Kopf Instruments). 0.1ml bupivacaine was injected subcutaneously at the incision site as a local anesthetic before surgery, and general anesthesia was maintained at 2% isoflurane during surgery. An incision was made down the middle of the scalp and a small hole was drilled over the injection site, which was into the right SN with coordinates from bregma: AP -3.3mm, ML -1.2mm, and DV -4.6mm. A 1 μ l injection of 5 μ g of LPS (Sigma) or 1 μ l of PBS was delivered over a two minutes period into the right SN via microinjection using a Nanoject II injector (Drummond Scientific Company). Meloxicam (10mg/kg) was injected subcutaneously after completion of the surgery, and animals were returned to original housing once awake from anesthesia.

Immunofluorescence (IHC) of fixed tissue

Approximately 21 days after stereotaxic LPS or PBS injections in SN, mice were transcardially perfused with phosphate buffer solution (PBS) PBS and 4% PFA in 0.1M PBS under anesthesia. Brains were harvested and post-fixed in 4% PFA overnight, followed by transfer into 30% sucrose (in PBS) before freezing in Optimal Cutting Temperature mounting medium (OCT; Tissue Tek). Sections were obtained coronally at 30 μ m using a cryostat (CM1950; Leica Biosystems) directly onto Superfrost slides (ThermoFisher). Sections were blocked by incubating in PBST (0.1% Triton-X-100 in

PBS) containing 5% normal donkey serum, and then incubated with primary antibodies (Supplementary Table 2) for 48h at 4°C. After washing, brain sections were incubated with corresponding secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 594 (Supplementary Table 1) overnight at 4°C. Sections were then stained with iamidino-2-phenylindole (DAPI; 1:10000) to label nuclei and mounted in fluoromount-G mounting media (SouthernBiotech). Images were acquired using Zeiss Axio ScanZ1 slide scanner using a 20x objective lens and Zen Blue 3.1 software (Carl Zeiss; UC Berkeley Molecular Imaging Center).

2.4c: Statistical Analysis

Whenever possible, quantification and analyses were masked to treatment or genotype. Statistical analysis was performed using GraphPad Prism 9 software. A one-way ANOVA with Dunnett's multiple comparison test was used to compare the means of three or more group (mRNA expression level of shRNA KD). Two-way ANOVAs with Tukey's multiple comparisons were used to compare differences between groups for experiments with two independent variables (mRNA expression level of cytokines). *P* values were corrected and indicated as post analyses by Tukey's multiple comparisons. Data are represented as mean ± SD, unless otherwise indicated. Significance was set in the figure panels as asterisks: **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

2.4d: Table 2.1

RT-qPCR Primers	
<i>Hprt</i>	F: TCAGTCAACGGGGGACATAAA R: GGGGCTGTACTGCTTAACCAG
<i>IL-6</i>	F: TAGTCCTTCCTACCCCAATTTCC R: TTGGTCCTTAGCCACTCCTTC
<i>iNOS</i>	F: GTTCTCAGCCCAACAATACAAGA R: GTGGACGGGTCGATGTCAC
<i>IL-1β</i>	F: GCAACTGTTCTGAAGTCAACT R: ATCTTTTGGGGTCCGTCAACT
shRNA Sequences	
<i>Slc31a1</i> shRNA 1	atTGCTGTTGACAGTGAGCGCAAGGTGTTTGCTGAAGTTCT ATAGTGAAGCCACAGATGTATAGAAGTTCAGCAAACACCTT ATGCCTACTGCCTCGGA _c
<i>Slc31a1</i> shRNA 2	atTGCTGTTGACAGTGAGCGAAACAGCGTTGTTACTAGTGA ATAGTGAAGCCACAGATGTATTCAGTAACAACGCTGTT CTGCCTACTGCCTCGGA _c
Antibodies	
Tyrosine Hydroxylase	https://www.labscoop.com/p/ImmunoStar-Tyrosine-Hydroxylase-Antibody/4188114#pnlSpec
Iba1	https://www.thermofisher.com/antibody/product/IBA1-Antibody-Polyclonal/PA5-18039

Alexa Fluor® 488	https://www.thermofisher.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11006
Alexa Fluor® 594	https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32744

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Chapter 3: Hetero- and homozygous astrocyte-specific deletion of *Tcf4* cause seizure activity in mice

In this chapter, we sought to understand the role of transcription factor 4 (Tcf4) in microglial inflammation. However, we instead found that the expression of Tcf4 is much higher in astrocytes, and therefore became interested in the function of Tcf4 in this other glial cell type. When we selectively deleted Tcf4 in astrocytes in mice, the animals went on to develop seizures and to have shortened lifespans.

3.1: Background

3.1a: TCF4 and Pitt-Hopkins Syndrome

TCF4 is a basic helix-loop-helix (bHLH) transcription factor (TF) that binds to Ephrussi box (E-box) DNA motifs (CANNTG) when homo- or heterodimerized with other TFs [1–3]. The bHLH sequence is key for recognizing other factors to dimerize and bind to E-box sequences [2,3]. Human *TCF4* is found on chromosome 18 and contains 41 exons [4]. While expressed in a variety of organs, it is most highly expressed in the CNS and across most brain structures [4–6]. It is also developmentally regulated, as its expression is highest during early neurodevelopment in humans and across model organisms [7–10].

Autosomal dominant mutations resulting in haploinsufficiency of *TCF4* cause a rare congenital autism-spectrum disorder called Pitt-Hopkins Syndrome (PTHS), first identified in 1978 [11]. Reports indicate PTHS prevalence to be between 1 in 34,000 and 1 in 41,000 births [12]. Patients usually start to present symptoms at early ages, which includes recurrent **seizures**, autistic behaviors, intellectual disabilities, motor delays, breathing abnormalities, absent or limited speech, gastrointestinal dysfunctions, and craniofacial dysmorphisms [12–16]. Imaging of PTHS patients' brains have identified an underdevelopment of the corpus callosum, smaller hippocampi, enlarged caudate nuclei, and cerebellar and vermis hypoplasia [6,13,14,17,18]. There is currently no specific treatments for this condition [11]. The mutations are almost all *de novo* within the *TCF4* locus [5,19]. Thus far, missense, nonsense, point, and truncation mutations have all been identified to cause haploinsufficiency of *TCF4* PTHS [5,14,19,20]. Interestingly, most of the mutations occur in the bHLH domain that is required for TCF4 dimerization and DNA binding [19]. Mutations in the 5' end of the gene are also associated with mild to moderate forms of intellectual disability, without the severity of other typical PTHS symptoms [21,22]. Apart from PTHS, genome wide association studies have implicated common variants within *TCF4* introns with a diagnosis of schizophrenia [23–25], post-traumatic stress disorder [26], and bipolar disorder [27]. A partial duplication of the gene was also found in DNA obtained from the brain of patients with major depressive disorder [28].

Many animal models have been developed over the years to study the consequences of TCF4 dysfunction; from this body of work, significant progress has been

made in understanding TCF4 function in neurons, specifically. Of note, conventional homozygous deletion is embryonically lethal due to developmental defects [29], therefore most of this work attempting to understand Tcf4 function only uses heterozygous knockout mouse models, mimicking PTHS patients. First, given that peak Tcf4 expression coincides with cortical neurogenesis, migration, and neuronal maturation, it is no surprise that much work has sought to understand its role in regulating these processes. In *Drosophila*, loss-of-function of daughterless (Da), a Tcf4 ortholog, disrupts neural development and leads to a complete loss of peripheral neurons [30–32]. In mouse, Tcf4 has been shown to regulate neural progenitor cell (NPC) proliferation and lineage specification of pyramidal neurons in the developing cortex [8,9,33]. Other mouse work has shown disrupted neuronal migration in the cortex [9,34,35] and hippocampus [34,36]. These studies then show abnormal layers of cortical pyramidal neurons, reduced cortex size, hypoplasia of hippocampus, deformities of the corpus callosum, and smaller brain volume. *Tcf4* overexpression in developing rat prefrontal cortex caused an atypical distribution of cortical neurons within columns; this was dependent on Ca²⁺-mediated neuronal activity through NMDARs [37,38].

The impact of Tcf4 on neuronal development is also seen at a cellular level. For example, studies found that Tcf4 is critical for normal neurite length development in the mouse cortex and olfactory bulb [30]. Additionally, *Tcf4* loss-of-function has been shown to decrease the number of synapses in neuron cultures [9,34,38–40] and increase in *Drosophila* neuromuscular junctions [30]. Second, Tcf4 can regulate intrinsic excitability, synaptic transmission, and synaptic plasticity. Embryonic KO of *Tcf4* led to downregulation of Kcna1, a voltage gated potassium channel. Knock down of *Tcf4* in neurons of the prefrontal cortex led to a downregulation of other ion channels Kcnq1 and Scn10a, which resulted in abnormal membrane excitability [5]. Several Tcf4 loss-of-function mouse models led to enhanced hippocampal long-term synaptic potentiation and evoked synaptic transmission due to elevated NMDA receptor function [41,42]. Lastly, the cellular and developmental phenotypes observed in all these studies on Tcf4 loss in various animal models also resulted in specific behavioral deficits. Interestingly, they also recapitulate different aspects of the human syndrome. Kennedy and colleagues first showed that Tcf4 haploinsufficiency cause hyperactivity and deficits in social interactions, vocalizations, prepulse inhibition, and learning and memory, all characteristic behavioral phenotypes of autism [42]. Thaxton et al characterized four different mouse models of PTHS and observed hyperactivity, reduced anxiety, and deficits in spatial learning [41]. A third study additionally reported abnormal nesting, increased self-grooming, and reduced grip strength [43].

As a transcription factor, there is a need to identify the genes that Tcf4 regulates to explain the mechanisms behind its involvement in brain development. This can also provide better insights into *Tcf4*-related neurological diseases, especially PTHS. Depending on its binding partner, Tcf4 can act as a transcription repressor or activator. Chip-seq studies on human SH-SY5Y neuroblastoma cells identified over 10,000 TCF4-binding sites across the genome that could be assigned to over 5,500 genes [44]. Enrichment analysis identified genes associated with nervous system development, neurogenesis, and ion transport. *TCF4* knockdown in the same neuroblastoma cell line

found over 1,000 differentially expressed genes (DEGs) involving in TGF- β signaling, epithelial to mesenchymal transition, and apoptosis; this also included genes associated with neurodevelopmental disorders like UBE3A, FRM1 and MEF2C [45]. TCF4 knockdown in human NPCs found DEGs associated with the cell cycle [46]. Other studies in mouse neurons with altered Tcf4 expression have also identified DEGs, including *Wnt7b* [36], *Nrxn1* [30,47], *Gadd45g* [37], and ion channels *Scn10a* and *Kcnq1* [5]. Several ChIP-seq in neurons have become to identify direct binding targets of TCF4, including *Bmp7* [35], *Nrxn1* [30], *Gadd45g* [37], *Plp1* [48], and *Gjb2* [48]. Overall, these studies find that Tcf4 regulates a number of neuronal genes that are important for brain development, differentiation, intrinsic excitability, synapses, and survival. However, *TCF4* is widely expressed in many cells types in the brain—particularly astrocytes—and datasets are needed to improve our understanding *TCF4* regulation in other cellular contexts.

3.1b: Epilepsy and the role of astrocytes

Epilepsy is a chronic condition characterized by recurrent seizures. It impacts about 1-2% of the population, making it the most common neurological disorder in the world [49]. Seizures are widely accepted to occur due to an aberrant firing of neuronal populations from an imbalance of excitatory and inhibitory currents [49]. Most research seeking to understand the underlying causes of epilepsy has focused on neurons. This work has found that brain hyperexcitability can occur due to changes in neuronal signaling via impaired neurotransmitter receptors, ion channels, and ion transporters. While there are many approved anti-epileptic drugs (AEDs), a large number of patients are known to have severe side effects or even fail to responds to them at all, indicating that new targets need to be developed for epilepsy treatment [50-51]. However, there is recent evidence indication that non-neuronal cells are contributing to epilepsy development.

Astrocytes are critical for maintaining the brain environment, and specifically for protecting against hyperexcitability [50-53]. Therefore, there are many reasons to believe that they can be contributing to epilepsy development. For one, reactive astrogliosis is prominent in all forms of epilepsy [50-53]. While this gliosis is unequivocally an integral part of the pathology, whether it is a cause of consequence of epileptic activity is still up for debate. Interestingly, in the case of trauma-associated epilepsy, gliosis precedes the development of epilepsy and could therefore be causative [54]. Robel and colleagues then demonstrated that an astrocyte-specific deletion of the $\beta 1$ integrin gene, *Itgb1*, in mice showed widespread gliosis and developed spontaneous, recurring seizures [55,56]. Another study showed that virally induced astrogliosis in the hippocampus was sufficient to cause neuron hyperexcitability [57]. Astrocyte-specific deletion of *Tsc1*—the gene that causes tuberous sclerosis complex—causes abnormal neuronal organization, astrogliosis, and seizure activity [58]. Alexander's disease, which is caused by a mutation in the astrocyte-specific *GFAP* gene that is commonly impacted in reactive astrocytes, is characterized by excitotoxic neuronal loss and seizures [59].

Astrocytes are critical for maintaining ion and water homeostasis. The extracellular space is very small in volume, therefore modest ion fluxes can the change concentrations considerably, thus impacting neuronal activity. This is especially important with potassium

buffering; failure of astrocytes to adequately buffer is sufficient to induce epileptiform activity in mouse and human hippocampus [60,61]. Multiple models of acquired epilepsy and tissue from human patients with mesial temporal lobe epilepsy also report downregulation of potassium channels in astrocytes [62]. Distribution of chloride ions is important for normal neuronal activity as well, as it governs the function of GABA type A receptors [55,63,64]. Loss-of-function mutations in chloride channels are known to increase risk of epilepsy in humans [65]. Another way that astrocytes could be impacting extracellular ion concentrations is through water transport. Astrocytes highly express aquaporin 4 (AQP4), and changes in this transporter's expression has been observed in cases of animal and human epilepsy [66,67]. Finally, astrocytes are all connected by gap junctions composed of connexin proteins [68]. These gap junctions are critical for intercellular movement of ions and small molecules between astrocytes. This assists in the ion and glutamate buffering, thus any change in gap junction coupling could contribute to epilepsy [69]. Both increases and decreases of connexin expression levels have been reports in animal models and humans with epilepsy [68,70].

One of the most widely accepted roles of astrocytes in normal brain physiology is their clearance of glutamate via Na⁺-coupled electrogenic excitatory amino acid transporters, EAAT1 and EAAT2 (Glast and Glt1 in mice, respectively) [71,72]. Rapid astrocytic clearance of excess glutamate in the synaptic cleft terminates action potentials, therefore controlling excitatory neurotransmission and preventing hyperexcitability [73,74]. Indeed, studies of *Glt1* KO mice have severe spontaneous seizures, and *Glt1* overexpression causes resistance if compound-induced seizures [75]. Once glutamate is taken up by astrocytes, it is degraded by conversion to α -ketoglutarate via glutamate dehydrogenase, which then enters the tricarboxylic acid (TCA) cycle to serve as an energy source [76–78]. Other glutamate is recycled by conversion into glutamine via glutamine synthase to be released back into the extracellular space to be imported back into the neurons for glutamate synthesis [77]. These processes all impose a large energetic burden on astrocytes, triggering the need for glucose uptake from blood vessels using glucose transporter GLUT1 [78,79]. Astrocytes oxidize glucose into pyruvate through glycolysis, which is then converted into lactate that gets shuttled to neurons, a process that seems to be correlated with high neuronal activity due to their high energy expenditure [77,78]. Any perturbations to these metabolic pathways can impact neuronal excitability, and ultimately increase risk for epileptic seizures [76].

Like many other neurological diseases, epilepsy can chronically alter synaptic structure and functions that underlie neural circuits. It is also known now that astrocytes influence these circuits by regulating synaptogenesis, synaptic pruning, and synaptic activity. The role of astrocytes in synaptogenesis is particularly prominent, as they secrete many factors that are known to impact this, such as BDNF, TSPs, cholesterol, glypicans, and hevin, among others. Interestingly, after injury, reactive astrocytes are reported to secrete excessive TSPs, which have been shown to promote aberrant synaptogenesis and alter neuronal circuitry to promote excessive excitability, as is observed during epileptic activity [52,76]. Additionally, it has been found that gabapentin disrupts astrocyte-secreted TSPs and attenuates epileptiform activity, suggesting that reactive astrocytes could be a potential mechanism for increasing network hyperexcitability [80,81]. Along with

microglia, astrocytes use the complement component pathway to regulate synaptic pruning; mice deficient in this pathway show enhanced synaptic connectivity and eventually develop seizures [82,83]. Additionally, studies on neurodevelopmental disorders that are characterized by seizures also report aberrant synaptogenesis and synaptic pruning due to dysfunctional glia, like fragile X syndrome and Rett syndrome [52,84–86]. Astrocytic release of TNF- α and cholesterol is a mechanism by which these cells can contribute to synaptic strength by regulating trafficking of glutamate and GABA receptors [87,88]. While the molecular characterization of this hasn't been done in epilepsy research, this could be another potential mechanism by which astrocytes impact network excitability, and thus contribute to epilepsy development. Finally, gliotransmission is thought to impact synaptic activity, as well. Astrocytes are capable of releasing many molecules that can potentially modulate neuronal excitability, including: ATP, glutamate and adenosine [76,89–91]. Research on malignant glia provide compelling evidence for this being a driver of epilepsy. Astrocyte-derived tumors cause seizures in over 70% of patients, even before official diagnosis [92]. The seizures caused by these tumors are also often drug resistant [92,93]. In both human patients and mouse models of these gliomas, there is an excessive release of glutamate that causes a chronic elevation of extracellular glutamate, inducing excitotoxicity on surrounding neurons [94].

3.1c: Role of *Tcf4* in glial cells

Neurodevelopmental conditions (NDCs), such as ASDs like PTHS, arise from altered brain development that results in aberrant neural circuitry and atypical behaviors. Since microglia and astrocytes are heavily involved in the modulation of critical processes that leads to proper neuronal wiring and activity that ensure these behaviors, there is a growing interest in understanding how these glial cells contribute to NDCs. Indeed, alterations in glial cell function and gene expression have been identified in many NDCs, especially ASDs. It is also possible that glial cell dysfunction can be leading factors that contribute to NDC development. For example, maternal and/or patient infections seem to increase risk of disease [95,96]. This inspired the generation of a maternal immune activation animal models of certain NDCs; treating pregnant dams with an innate inflammatory stimuli like LPS or Poly(I:C) causes pups to have altered behaviors in their adult life that are reminiscent of NDCs [96]. The function of *Tcf4* in glial cells is largely unexplored. To date, there are only two studies that show *Tcf4*'s involvement in OL differentiation and/or survival in the CNS [7,48]. PTHS mouse models exhibit a cell autonomous reduction in mature OL numbers and in myelination. No work has been done examining the role of *Tcf4* in microglia or astrocytes.

In the immune system, it is well-documented that *Tcf4* is a regulator of plasmacytoid dendritic cell (PCD) development [97–99]. These are immune cells that specialize in type I interferon (IFN α) secretion in response to viral nucleic acids, and *Tcf4* loss-of-function completely prevents the production of PDCs and IFN α . This phenotype is also observed in PTHS patients, whose blood samples revealed significant reduction in the number of PDCs and IFN α production [97]. Together with evidence that microglia can contribute to NDCs that are similar to PTHS, we hypothesized that *Tcf4* is necessary for maintaining normal microglia function, without which the risk for NDC development

would increase. To do this, we first generated murine microglia SIM-A9 cell lines in which *Tcf4* is either knocked-down. We then assessed the ability of these cells to respond to inflammatory stimuli as a read-out for how these cells are altering their function due to changes in *Tcf4* levels. We found that knocking-down *Tcf4* led to a decrease in expression proinflammatory factors in response to innate immune stimuli LPS or Poly(I:C). However, these results were questioned when we turned to characterize *Tcf4* in mouse brain, highlighting the importance of accounting for differences between *in vitro* and *in vivo* work. We found that *Tcf4* was not detectable in microglia, but instead noticed high expression in astrocytes. To this date, there are still no known studies on *Tcf4* function in astrocytes, therefore we shifted our work to focus on astrocytes. Next, we generated mice with either a complete or heterozygous knock-out of *Tcf4* in astrocytes exclusively. With these mice (but not the controls), we stumbled upon a fascinating phenotype in which the mutant mice exhibited severe seizures and premature death. So far, these mice also trend towards exhibiting reactive astrogliosis and brain anatomy malformations, which has not been observed in any of the controls. Overall, these results demonstrate that while *Tcf4* could be impacting microglial inflammation, the role in astrocytes is more critical for normal mouse function and could explain how patients with PTHS develop seizures.

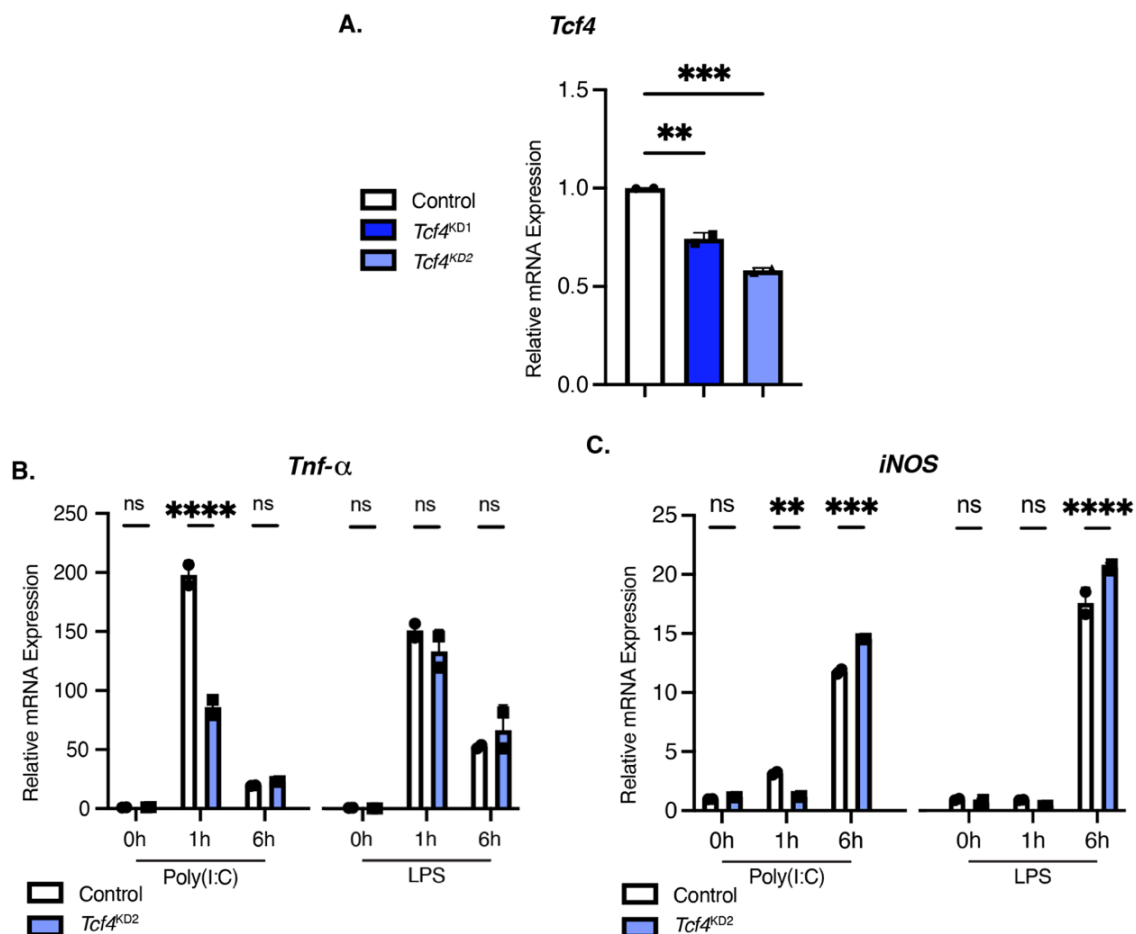


Figure 3.1. *Tcf4*^{KD2} SIM-A9 microglia exhibit decreased expression of proinflammatory factors *iNOS* and *TNF-α* in response to LPS.

A) shRNA-containing lentivirus was delivery to generate *Tcf4*^{KD1} and *Tcf4*^{KD2} SIM-A9 cells lines and the knock-down efficiency is shown. One-way ANOVA. Dunnett's Multiple comparisons: Control vs. *Tcf4*^{KD1} $p=0.0019$, Control vs. *Tcf4*^{KD2} $p=0.0005$. **B)** mRNA expression of *Tnf-α* and **C)** *iNOS* in control or *Tcf4*^{KD2} SIM-A9 cells stimulated with Poly(I:C) or LPS for 0,1, or 6 hours as determined by RT-qPCR. Stimulation experiments were repeated in three separate biological replicates. Data shown from one representative biological sample. qPCR was conducted in technical duplicate. Data are shown as an average of technical duplicates. Two-way ANOVA followed by Tukey's multiple comparisons. All data are mean $\pm$ SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

3.2: Results

3.2a: shRNA knock-down of *Tcf4* in SIM-A9 cells decreases inflammatory responses

To do this, we manipulated *Tcf4* expression in a murine microglial cell line, SIM-A9. We used two shRNA constructs that were cloned into the pGIPZ lentiviral vector to establish a cell line in which *Tcf4* mRNA was knocked-down; from the two, we selected the shRNA that induced the most significant knockdown to move forward with the rest of the experiments (*Tcf4*^{KD2}) (**Figure 3.1A**). To assay for the functional consequences of reduced *Tcf4* expression in microglia, we treated *Tcf4*^{KD2} cells with known inflammatory stimuli and assessed changes in proinflammatory factors after 0-, 1-, or 6-hours of treatment. Polyinosinic-polycytidylic acid (Poly(I:C)) is a TLR3 agonist that mimics viral infections via TRIF-dependent pathways^[100,101]. LPS is a TLR4 agonist that mimics gram negative bacterial infections and acts via TRIF- and MyD88-dependent pathways^[100,101]. At each timepoint, we assayed for canonical downstream targets of these pathways: *Tnf-α* and *iNOS*. In response to Poly(I:C) treatment, *Tcf4*^{KD2} cells has suppressed expression of both *iNOS* and *Tnf-α* at the 1-hour timepoint compared to control cells (**Figure 3.1B** and **3.1C**). In contrast, *Tcf4*^{KD2} cells treated with LPS showed no changes in *iNOS* and *Tnf-α* expression at the 1-hour timepoint (**Figure 3.1B** and **3.1C**). Additionally, *iNOS* expression levels at the 6-hour timepoint were elevated in response to both stimuli (**Figure 3.1C**). This suggests that *Tcf4* has a potentially signal-dependent and time-dependent transcriptional control of inflammation in microglia.

3.2b: *Tcf4* is strongly expressed in neurons and astrocytes, but not microglia

Based on our *in vitro* results, we hypothesized that *Tcf4* could be transcriptionally controlling microglial inflammation in mice. With previous research showing unregulated inflammation to be a risk factor for the development of ASDs^[95], we proposed that this could be a risk for PTHS as well. However, we first had to confirm that mouse microglia indeed express *Tcf4*, as that the time we started the experiment, it was unknown. To do this, we performed immunohistochemistry on *Cx3cr1*-GFP reporter mice^[102] to see if *Tcf4* is expressed in mouse microglia. *Cx3cr1* is the gene that codes for chemokine (C-X3-C motif) receptor 1, which binds fractalkine in a signaling mechanism that is thought to be important for migration of microglia into the CNS^[103]. Within the brain, resident

macrophages including microglia are the main cell type that express *Cx3cr1* and therefore it is a useful target for identifying microglia. Specifically, the mice used in this experiment have an enhanced green fluorescent protein (GFP) sequence replacing the first base pairs of the coding exon 2 of the *Cx3cr1* gene, thus labeling microglia in the brain with GFP. Adult *Cx3cr1*-GFP mice were sacrificed, and brain were harvested and fixed for sectioning and immunohistochemistry staining for Tcf4. We decided to focus our initial imaging on the hippocampus, given that neuronal Tcf4 has already been shown to be important for normal hippocampal development [34,36]. These studies show severe deficits in hippocampus structure. Additionally, PTHS is characterized by severe intellectual disability, a phenotype that is replicated in PTHS mouse models and is likely a consequence of the anatomical abnormalities observed in the hippocampi of these mice.

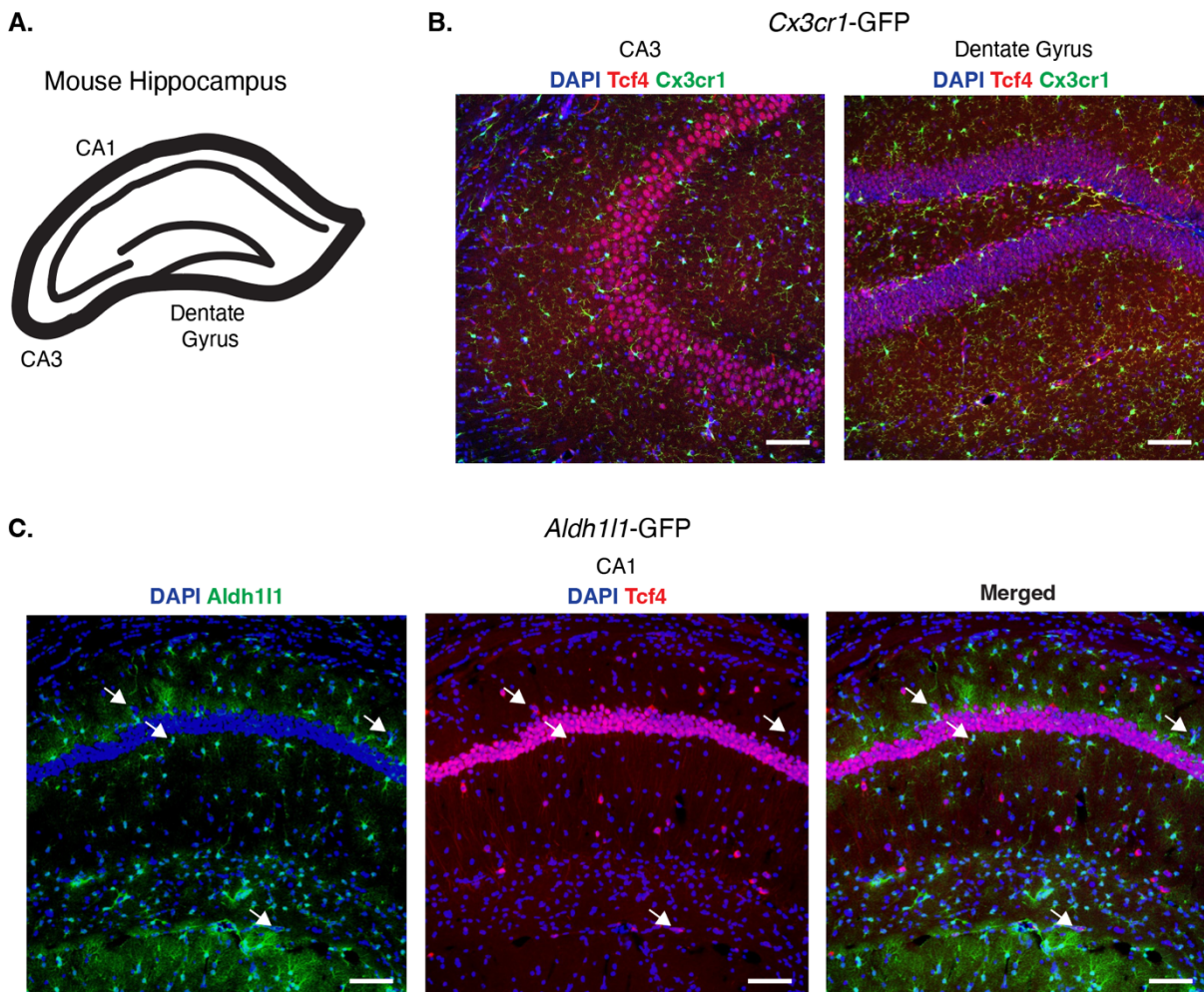


Figure 3.2. Wild-type mouse hippocampus sections indicate Tcf4 is expressed in neurons and astrocytes, but undetectable in microglia.

A) Schematic indicating parts of mouse hippocampus, as viewed coronally. **B)** CA3 (left panel) and dentate gyrus (right panel) of hippocampus from *Cx3cr1*-GFP mouse. Microglia (green) show no overlap with cells that express Tcf4 (red). Scale bar 100µm. **C)** CA1 of hippocampus from *Aldh1l1*-GFP mouse. Astrocyte marker *Aldh1l1* (green) shown in cells that also are labeled by Tcf4 (red). Arrows indicate cells show overlapped signals. Scale bar 100µm.

After staining *Cx3cr1*-GFP mouse brain for Tcf4, we used confocal microscopy to image various sections of the hippocampus (**Figure 3.2A**). While we saw high expression of Tcf4 in the hippocampus (n=3), none of these were additionally labeled with GFP, indicating that *Cx3cr1*-expressing microglia have undetectable levels of Tcf4 in their cells. **Figure 3.2B** depicts representative images from CA3 and the dentate gyrus of the hippocampus. Microglia are in green, labeled by GFP that is tagged to the microglia-specific *Cx3cr1* gene, and Tcf4 is in red. There is no colocalization of Tcf4 with cells GFP-labeled microglia cells, indicating that microglia may not actually express Tcf4 at detectable levels. However, it is evident that the Tcf4 staining is still very prominent in other cell types within the hippocampus. For one, the imaging indicates that the pyramidal neurons of the hippocampus are all highly expressing Tcf4 (**Figure 3.3B**). Additionally, we noticed astrocyte-shaped cells also expressing Tcf4. This finding peaked our interest, so we sought to repeat Tcf4 staining in a different mouse line, *Aldh1l1*-GFP. The *Aldh1l1* gene is specifically expressed in astrocytes in the brain, therefore this mouse line is labeling astrocytes with GFP [104]. **Figure 3.2C** depicts representative images of the CA1 of the hippocampus from a *Aldh1l1*-GFP mouse brain that is stained for Tcf4. From these images, we were able to confirm that Tcf4 staining did indeed colocalize with a subset of GFP labeled astrocytes (white arrows indicating examples of this colocalization). We also found expression of Tcf4 in oligodendrocytes when costaining WT mice for Tcf4 and myelin oligodendrocyte glycoprotein (MOG), a protein that is uniquely expressed on the surface of oligodendrocytes (*data not shown*). While other studies have also gone on to show that Tcf4 is expressed in astrocytes, neurons, and oligodendrocytes, but not microglia [7,9,39,48], to this date there is still no work done on understanding the role of Tcf4 in astrocytes.

3.2c: Astrocyte-specific Tcf4 KO in mice causes seizures and impacts animal lifespan

Since we now hypothesize that Tcf4 may regulate essential genes in astrocytes for brain function and development, we generated heterozygous and homozygous *Tcf4* knock-out mice in astrocytes. This was done by crossing mice containing a floxed conditional allele of *Tcf4* (*Tcf4^{fl/fl}*) [105] with mice constitutively expressing Cre recombinase under control of the *Aldh1l1* promoter (*Aldh1l1-Cre*) that is only expressed in astrocytes in the brain (**Figure 3.3A**) [106]. This breeding pair gave us both homozygous and heterozygous deletions of *Tcf4* in mouse astrocytes. For controls, we used littermate mice with floxed alleles that did not express cre (*Tcf4^{fl/+}* and *Tcf4^{fl/fl}*). Interestingly, when breeding the mice for future experiments that aimed to explore the function of *Tcf4* in astrocytes, we noticed that both *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice developed seizure activity, whereas seizures in control mice were never observed (**Figure 3.3B**). The observed seizures occurred in both sexes starting around three months of age and later, and are classified as tonic-clonic seizures with post-seizure hyperactivity. While we have only observed handling induced seizures, we will later discuss our future directions in which we aim to continuously monitor mice to characterize seizure type and occurrence in depth. Additionally, we observed significant premature death in these mice (**Figure 3.3C**). Since it is known that strong seizures can cause death

by hypoxia [107], this opens the possibility that these seizures could be contributing to the premature death that is noted in *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice. These observations show that our novel mouse model recapitulates the seizures that are reported with PTHS, which has not been reported in any of the other mouse models of PTHS currently. This raises the possibility that PTHS seizures reflect a dysfunction of astrocytes, rather than other cell types like neurons. However, further validation is needed to ensure that there is no leaky expression of Cre.

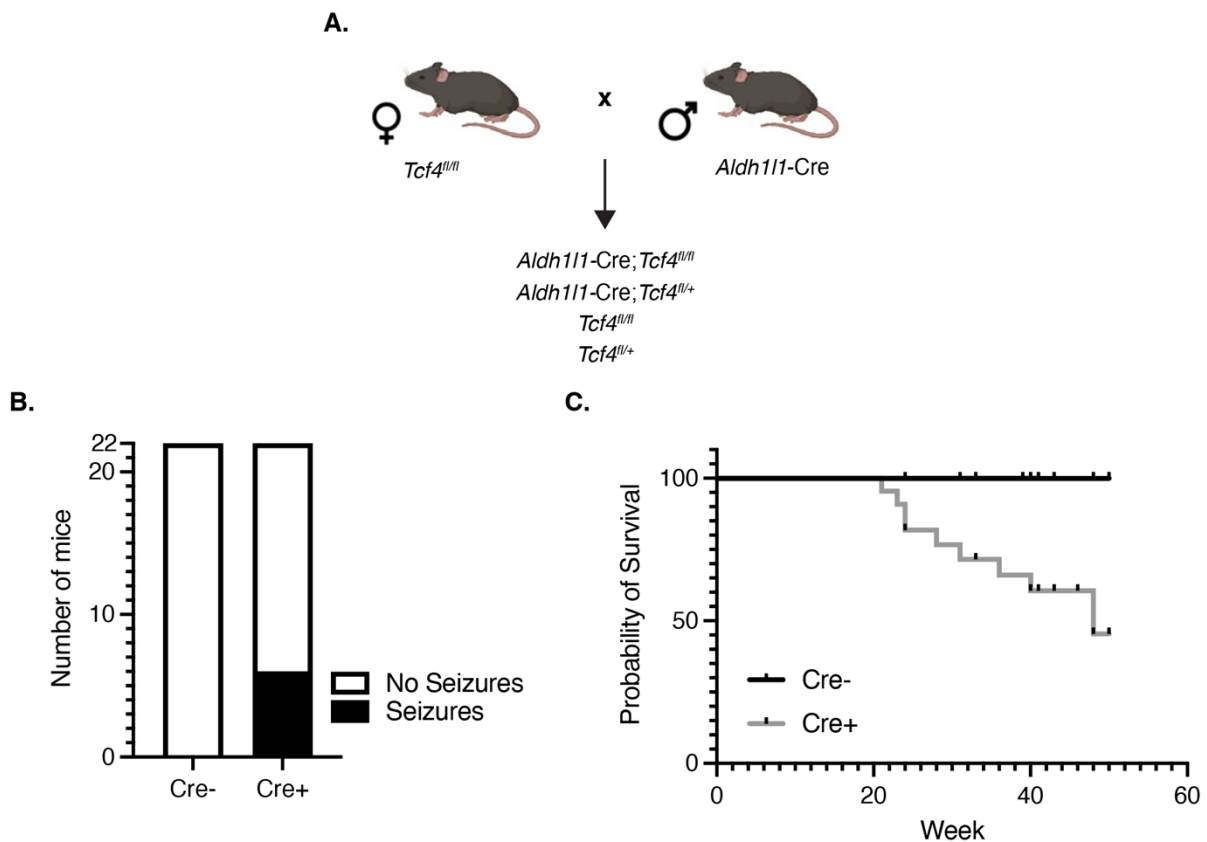


Figure 3.3. *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice exhibit seizures and significantly shortened life spans.

A) Schematic of breeding strategy. *Tcf4^{fl/fl}* female mice were crossed with *Aldh1l1-Cre* male mice for *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice and *Tcf4^{fl/+}* and *Tcf4^{fl/fl}* controls. **B)** Plot of cohort indicating number of mice with observed seizures. There were no seizures in control mice (Cre-), whereas 6 Cre-expressing mice were seen having tonic-clonic seizures. $p=0.0089^{**}$ (Fisher's exact test). **C)** Survival of Cre+ ($n=22$) and Cre- (control; $n=22$) mice was tracked over the course of a year. Chi square=10.66, $df=1$, $p=0.0011^{**}$ (Mantel-Cox test). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

The observation that an astrocyte-specific knock-out of *Tcf4* causes seizures is quite intriguing, and has changed the trajectory we originally thought this project would take. Since our lab is not equipped with the tools to study seizures, we have started a collaboration with the laboratory of Dr. Jeanne Paz at the Gladstone Institute. We have

spent the past several months rederiving our mouse lines at their facilities. Once a sufficient cohort is generated, they will use chronic electrocorticography (ECoG) with synchronized video recordings in freely behaving mice, starting at the age of two months in order to be able to capture the pre-symptomatic phase and define the precise onset of seizures. Through their work, we will be able to identify when the mice start having seizures, the types of seizures that they are having (e.g. tonic-clonic or absence, which might not all be observable by eye), and also identify the brain regions involved in the seizures.

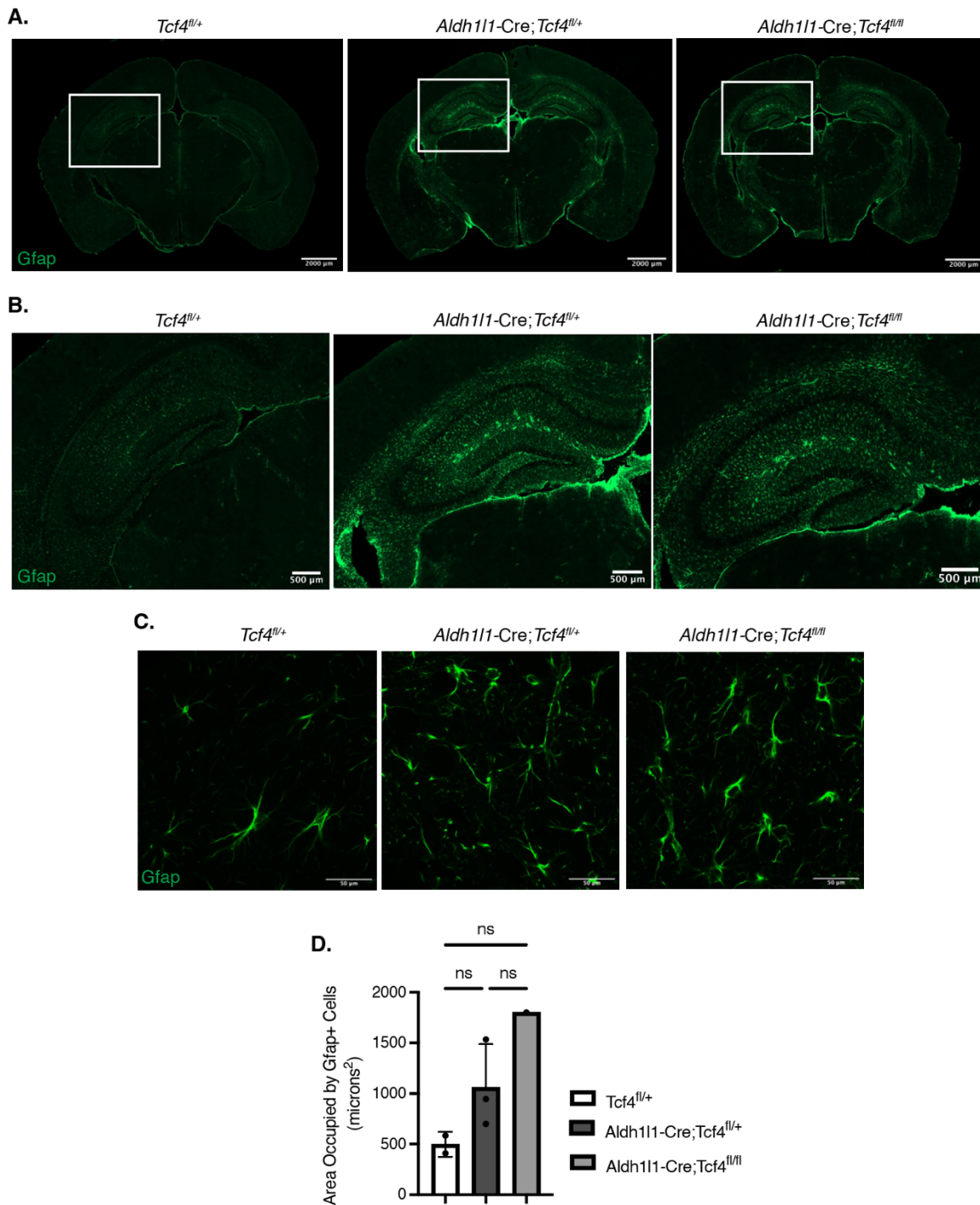


Figure 3.4. Gfap expression trends towards increasing in hippocampal sections of *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* compared to controls.

A) Representative epifluorescence image of coronal section containing hippocampus, from *Tcf4^{fl/+}* (left), *Aldh1l1-Cre;Tcf4^{fl/+}* (center) and *Aldh1l1-Cre;Tcf4^{fl/fl}* (right) mice. White box indicates hippocampal structure, which is shown in higher magnification in B. Gfap (green) shows extent of astrogliosis. Scale bar is 2000µm. **B)** Hippocampus of *Tcf4^{fl/+}*, *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice at higher magnification from A. Gfap (green) showing extent of astrogliosis. Hippocampus structure of *Aldh1l1-Cre;Tcf4^{fl/fl}* mouse is malformed. Scale bar is 500µm. **C)** High resolution confocal microscopy images of hippocampal astrocytes from *Tcf4^{fl/+}*, *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice. **D)** Quantification of area occupied by astrocytes. Data is shown as an average of area occupied by Gfap+ cells and error bars indicates S.D. n.s. indicates not significant.

3.2d: Astrocyte-specific *Tcf4* KO in mice causes reactive astrogliosis and changes hippocampal anatomy

We hypothesize that due to the seizures observed in *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice, we will also find reactive astrogliosis, as it is one of the most common features of epilepsy and is observed across different animal models [50–53]. To reveal this, we have begun to perform immunofluorescence imaging analyses on the brains of the astrocyte-specific *Tcf4* knock-out mice and their littermate controls. A defining feature of reactive astrocytes is their overexpression of intermediate filament proteins like *Gfap* and vimentin. For this reason, they are often used as markers to assess for reactive astrogliosis. For this initial experiment, we chose to use *Gfap* as the marker to assess whether or not the *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice have reactive astrogliosis, as other mouse models of epilepsy do.

Figure 3.4A shows expression of *Gfap* in coronal sections of *Tcf4^{fl/+}* (control), *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* containing the hippocampus. The expression of *Gfap* is visibly much higher in the hetero- and homozygous astrocytic *Tcf4* knock-outs. In **Figure 3.4B**, enlarged images of the hippocampus from the above coronal section further demonstrates the extent of the reactive astrogliosis observed in the hippocampus. Additionally, the *Aldh1l1-Cre;Tcf4^{fl/fl}* mice seem to have malformed hippocampal layer, as the overall structure of the hippocampus is altered compared to the control and even *Aldh1l1-Cre;Tcf4^{fl/+}* mice. Higher magnification images of cells in the hippocampus where obtained by confocal microscopy (**Figure 3.4C**) in order to quantify the extent to which *Gfap* expression increases in the *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}*. These images also illustrate the extent of astrocyte hypertrophy, which is characterized by an increase in the thickness and number of cellular processes. The area occupied by *Gfap*+ astrocytes from these images is calculated and graphed in **Figure 3.4D**. Proliferation is not as common in astrocytes during gliosis, therefore we are calculating the hypertrophy of astrocytes as seen in the changes of cell area occupied. While the data is currently not significant, there is a clear trend showing an increase in *Gfap*+ area, with the full homozygous knock-out have the highest amount of area occupied. This is due to the small sample size, which we are actively working on increasing as we breed more mice.

3.3: Discussion and Future Directions

We first demonstrate that *Tcf4* can regulate the inflammatory response of microglia. Interestingly, depending on the proinflammatory factor and stimulus that we examined, we found different effects of *Tcf4* on microglia response. *Tcf4*^{KD2} cells, which had about a 50% knock-down deficiency, showed that at the 1 hour time point, Poly(I:C)-induced responses of SIM-A9 cells was dampened, as indicated by the decreased expression of *iNOS* and *TNF-α*. This suggests that in this situation, *Tcf4* could be acting as an activator of specific proinflammatory transcription. However, these effects were not observed in *Tcf4*^{KD2} cells stimulated by LPS, as the knock-down did not seem to impact the response at 1 hour. Since LPS and Poly(I:C) and LPS have differing signaling mechanisms, these data suggest a signal-dependent role for *Tcf4* in microglia. More work is necessary to further explore this, such as sequencing the knock-down and overexpression SIM-A9 after treating with LPS or Poly(I:C). This would give us a broader sense of how many other inflammatory factors are impacted by the changes in *Tcf4* levels, as opposed to our current data set that is limited to *iNOS* and *TNF-α* measurements. Interestingly, recent work by Shariq and colleagues demonstrated that adult hippocampal neural progenitor cells have an inflammatory potential that is suppressed by *Tcf4* [108]. It is known that *Tcf4* can act as either a transcriptional activator or repressor, depending on the circumstances. Their study used a mouse model with an inducible deletion of *Tcf4* in *nestin*-expressing progenitor cells in the adult brain, therefore the changes in inflammatory modulation could be due to differences in cell types, as we were using mouse microglia cells. Alternatively, we only looked at the expression levels of two proinflammatory factors, therefore the response of other inflammatory factors could be different.

When continuing our work on microglial *Tcf4 in vivo*, we found that murine microglia do not express *Tcf4* at levels that are detectable by immunohistochemistry. Instead, we noticed very high expression in neurons, as was expected given previous literature. We also noticed *Tcf4* labeling other cells that were not neurons, and found a particularly high expression of *Tcf4* in astrocytes. In 2020, Kim *et al.* published a study that shows the cell type-specific distribution of *Tcf4* [39]. The team generated a mouse model in which a GFP reporter is under the control of the endogenous *Tcf4* promoter, thus allowing visualization of all *Tcf4* expression in the brain at a cellular level. Their mouse model definitively showed that *Tcf4* is expressed in neurons, astrocytes, and oligodendrocytes, but not microglia, as we also observed. This was consistent across all major brain regions, including the hippocampus, cortex, striatum and cerebellum. Such studies on cell specificity have pushed other researchers to examine *Tcf4* in other cell types. There is considerable focus now on *Tcf4* and OLs, as several papers have now identified *Tcf4* to be important for OL maturation and myelination [7,48]. However, no work has been done on *Tcf4* and astrocytes, despite clear evidence for high expression of this transcription factor.

Epilepsy is a problem in 40-60% of PTHS patients, with an onset during early school age [14,109–112]. Both absence and tonic-clonic seizures have been described [14,109–

^{112]}. While deletion of *Tcf4* in neurons and oligodendrocytes have recapitulated the autistic behaviors and ID of PTHS, no current mouse models of PTHS have reported seizure activity. Remarkably, our *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice went on develop seizures. We observed about one-third of our knock-out mice to have strong, tonic-clonic seizures. However, we expect this number to be higher for the following reasons. For one, many of our knock-out mice had premature death. Since seizures were often observed later in life (three months of age and on), it is possible that these mice died before we noticed their seizures. Additionally, we only handle the mice once a week, therefore it is a short window of time in which we are observing the mice. In order to fully capture the number of mice that have seizures, they would have to be constantly watched. Lastly, absence seizures are also reported in PTHS patients, which we would not be able to detect without monitoring of brain activity, as they are hard to spot since they lack the classic convulsions that is normally associated with seizures. For these reasons, our collaboration with Dr. Jeanne Paz will be key for determining the nature of the seizures in our *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice. ECoG with video recording will begin at around two months of age with these mice in order to detect when these seizures begin to occur. From here, recording will be performed for 48 hours over the course of several months. These data will determine the brain regions involved and the type(s) of seizures that are occurring.

Given that reactive astrogliosis is observed in human patients and in nearly every mouse model of epilepsy ^[50–53], we have begun to test for this by staining with anti-Gfap antibody. Thus far, we have only been able to stain several adult mice from our first cohort that are post-symptomatic. However, even in this small sample size, we observe clear tendency of increasing in Gfap expression in *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* compared to controls across all major brain regions, including the hippocampus, prefrontal cortex, striatum, nucleus accumbens, and substantia nigra. We are also currently breeding mice to test for reactive gliosis before the onset of seizures. There is currently much interest in whether reactive astrogliosis is a pathology that occurs before or after onset of seizures. It has been traditionally thought that astrogliosis is a consequence of epileptic activity, as astrocytes are direct sensors of changes in the brain milieu, such as hyperexcitability. However, there is growing evidence that direct changes in astrocytes are in fact the cause of seizures. Other astrocytes-specific manipulations have been shown to cause astrogliosis and seizures in mice, such as β 1-integrin and Gfap ^[55,56,59]. To further test this, we will use both pre- and post-symptomatic mice for RNA-sequencing. By detecting DEGs, we can better understand the mechanism behind seizure development in *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice. Past work in neuron-specific deletions of *Tcf4* in mice have identified potassium and sodium ion channels that are transcriptionally regulated by *Tcf4* and are critical for maintaining neuron excitability ^[5]. Therefore, we expect to see similar mechanisms of action in astrocytes. Other models of epilepsy have reported many other mechanisms by which astrocytes contribute to brain hyperexcitability, such as water transport (via aquaporins), ion homeostasis, glucose metabolism, gliotransmission, and glutamate reuptake and recycling. Through our RNA-sequencing results, we aim to identify if any of these

mechanisms are also contributing to the seizure activity that is observed in our *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice.

3.4: Methods and Materials

3.4a: *In vivo* experiments

Animals

To generate mice lacking *Tcf4* in astrocytes only, we purchased *Tcf4^{tm1a(EUCOMM)Wtsi}* mice were purchased from the EMMA mouse repository^[105]. These mice contained a FRT flanked LacZ/neomycin sequence that needed to first be bred out using a Flp-deleter mouse purchased from Taconic in order to generate *Tcf4* conditional ready mice. *Tcf4^{fl/fl}* females from these mice were bred with mice constitutively express Cre recombinase under control of the *Aldh1l1* promoter Tg(*Aldh1l1-Cre*), purchased from the Jackson Laboratory^[106]. Exon 4 from the *Tcf4* gene was floxed and targeted for deletion. Experimental controls were littermates with *Tcf4^{fl/fl}* or *Tcf4^{fl/+}* genotype, thus negative for Cre but of comparable genetic background (C57BL/6J, Jax #000664). We confirm the genotype of mice by PCR using primer sequences listed in Table 3.1. All experimental animals were virgins. Both male and female mice were used in this study, bred and housed in the specific pathogen- free (SPF) animal facility of the Li Ka Shing building at University of California, Berkeley. All animals were weaned around three weeks postnatally, housed in cages of 2-5 same-sex mice, and maintained on a 12-hour/12-hour light/dark cycle (on 7:00am; off 7:00pm) with *ad libitum* access to food and water. During the COVID-19 pandemic, the facility light cycle was changed to a 14-hour/10-hour light/dark cycle (on 6:00am; off 8:00pm). All animal care and procedures were approved by the University of California, Berkeley Institutional Animal Care and Use Committee and Office of Laboratory Animal Care.

Immunofluorescence (IHC) of fixed tissue

Adult mice (aged 6-8 months) were anesthetized and transcardially perfused with phosphate buffer solution (PBS) and 4% PFA in 0.1M PBS. Brains were harvested and post-fixed in 4% PFA overnight, followed by transfer into 30% sucrose (in PBS) before freezing in Optimal Cutting Temperature mounting medium (OCT; Tissue Tek). Sections were obtained coronally at 30 μ m using a cryostat (CM1950; Leica Biosystems) directly onto Superfrost slides (ThermoFisher). Sections were blocked by incubating in PBST (0.1% Triton-X-100 in PBS) containing 5% normal donkey serum, and then incubated with primary antibodies (Table 3.1) for 48h at 4°C. After washing, brain sections were incubated with corresponding secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 594 (Table 3.1) overnight at 4°C. Sections were then stained with iamidino-2-phenylindole (DAPI; 1:10000) to label nuclei and mounted in fluoromount-G mounting media (SouthernBiotech). Images of wholes sections were acquired using Zeiss Axio ScanZ1 slide scanner using a 20x objective lens and Zen Blue 3.1 software (Carl Zeiss; UC Berkeley Molecular Imaging Center). High magnification images were obtained with

confocal fluorescence imaging using the Zeiss laser scanning microscope 710 AxioObserver with a 40x objective lens and Zen 2021 software (Carl Zeiss; UC Berkeley Molecular Imaging Center). Image analysis and quantification was performed using FIJI (National Institutes of Health). All cells were cultured in incubator at 37°C and 5% CO₂.

3.4b: In vitro experiments

Cell Culture

SIM-A9 (murine microglial cell line, kindly provided by Dr. Nagamoto-Combs) [113] cells were maintained with DMEM/F12 (Corning) supplemented with 10% fetal bovine serum (FBS; Hyclone GE Healthcare Life Sciences), 5% horse serum (HS; Hyclone GE Healthcare Life Sciences) and 1% penicillin/streptomycin (pen/strep; Gibco) (complete medium). HEK 293T cells were cultured in DMEM (Corning) supplemented with 10% FBS and 1% pen/strep.

shRNA-mediated knockdown of Tcf4 in murine SIM-A9 microglial cells

pGIPZ lentiviral shRNA targeting *Tcf4* was purchased from Dharmacon. Sequences are listed in Table 3.1. Lentivirus was packaged via co-transfection of each pGIPZ shRNA plasmid with pCMV-VSV-G (Addgene plasmid #8454) and pCMV-dR8.2 (Addgene plasmid #8455) into HEK 293T cells using Lipofectamine 3000 reagent (Life Technologies) according to the manufacturer's instructions. Viral supernatant was harvested after 48h and added to SIM-A9 cells. 24h post-transduction, cells were changed into complete medium. After 48 hours, GFP+ cells were bulk sorted via FACS (Aria Fusion, BD Biosciences; UC Berkeley Cancer Research Laboratory), expanded, and subculture for LPS and Poly(I:C) stimulation experiments. Percent knockdown of *Tcf4* was determined via RT-qPCR.

Immune Stimulation

For all immune stimulation time course experiments, *Tcf4*^{KD2} and *Tcf4*^{OE1} SIM-A9 cells were seeded in 24-well plates at a density of 10⁵ cells/well, in triplicate, and stimulated with lipopolysaccharide (LPS; 0.1 µg/ml; Sigma-Aldrich) or Poly(I:C) (final concentration of 25 µM; Sigma Aldrich) for 0, 1 or 6 hours. No stimulation controls received an equivalent volume of sterile 1x DPBS (Invitrogen). Following completion of experiment, cells were lysed and stored at -80°C for RNA isolations.

RNA extraction and RT-qPCR

Total RNA was isolated from SIM-A9 cells using the quick-RNA Miniprep kit (Zymo Research). Primers were designed using Primer-BLAST and are listed in Table 3.1. Complementary DNA was reverse transcribed using SuperScript III (Invitrogen) and qPCR was performed using SYBR green master mix (Roche) on a Quant Studio6 qPCR machine (ThermoFisher). All data collected by QuantStudio Real Time PCR Software v1.3 were normalized to housekeeping gene hypoxanthine phosphoribosyltransferase (*Hprt*) using the delta-delta Ct method. Biological and technical replicates were indicated in the figure legends. Primers used in this assay were designed using Primer-BLAST and are listed in Table 3.1.

3.4c: Statistical Analysis

Whenever possible, quantification and analyses were masked to treatment or genotype. Statistical analysis was performed using GraphPad Prism 9 software. A one-way ANOVA with Dunnett's multiple comparison test was used to compare the means of three or more group (mRNA expression of shRNA KD). Two-way ANOVAs with Tukey's multiple comparisons were used to compare differences between groups for experiments with two independent variables (mRNA expression of proinflammatory factors and Gfap quantification). The Fisher's exact test was used to analyze Figure 3.3B. *P* values were corrected for multiple comparisons. Survival curve was analyzed with chi square and Mantel-Cox test. **<0.01. Data are represented as mean ± SEM, unless otherwise indicated. Significance was set in the figure panels as: **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

3.4d: Table 3.1

RT-qPCR Primers	
<i>Hprt</i>	F: TCAGTCAACGGGGGACATAAA R: GGGGCTGTACTGCTTAACCAG
<i>TNF-α</i>	F: CCCTCACACTCAGATCATCTTCT R: CCCTCACACTCAGATCATCTTCT
<i>iNOS</i>	F: GTTCTCAGCCCAACAATACAAGA R: GTGGACGGGTTCGATGTCAC
Genotyping Primers	
<i>Aldh1l1-Cre</i>	F: CCTGTCCCCTTGACAGTAG R: CGGTTATTCAACTTGCACCA
<i>Tcf4</i> WT	F: CCGATGACAGTGATGATGGT R: AAGTTAAGCTGAAGTAAATACCCACA
<i>Tcf4</i> floxed	F: CCGATGACAGTGATGATGGT R: TCGTGGTATCGTTATGCGCC
<i>Tcf4</i>	https://www.scbt.com/scbt/product/itf-2-antibody-c-1
Gfap	https://www.thermofisher.com/antibody/product/GFAP-Antibody-clone-2-2B10-Monoclonal/13-0300
Alexa Fluor® 488	https://www.thermofisher.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11006
Alexa Fluor® 594	https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32744

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Chapter 4: Closing Remarks

4.1: Summary of Findings

In chapter 2, we identified changes in copper levels within microglia and astrocytes when presented with specific inflammatory stimuli using a copper probe, CD649. Copper was seen in puncta within astrocytes at rest, which then disappeared when treated with TNF- α and IL-1 α . In contrast, microglia had moderate levels of copper throughout the cell body when at rest, which then significantly increased when stimulated with LPS or ODN. This provides direct evidence that copper levels change within glial cells during inflammation. Deciding to focus on microglia, we next found that depleting intracellular copper levels dampened the inflammatory response to LPS. Finally, we've successfully replicated the LPS-induced DA neuron death in mice in order to study how copper levels are impacted in a real, pathological inflammatory disease with collaborator in Dr. Christopher Chang's lab.

For chapter 3, after finding a potentially activating role of *Tcf4* in microglial inflammation, we were unable to detect levels of *Tcf4* in microglia *in vivo*. Instead, we found that *Tcf4* was expressed in astrocytes, and generated an homo- and heterozygous astrocyte-specific *Tcf4* knock-out mouse model to elucidate its function in this specific cell-type. Surprisingly, we found that astrocytic deficiency of *Tcf4* in mice caused a decreased life span, anatomical changes in brain structures, and induced seizure activity. We are currently waiting upon our collaborators in Dr. Jeanne Paz's lab at the Gladstone Institute to help characterize the nature of these seizures.

4.2: Future Directions

Currently, both chapters are works-in-progress, as we are awaiting on data from our collaborators to complete each project for publication. However, our current data opens many future directions on its own. In Chapter 2, we decided to focus our work on microglia, dropping our interesting finding on copper puncta in astrocytes during inflammation. This makes us wonder why copper behaves differently in distinct cell types when examining the same function (in this case, neuroinflammation). Further experiments on astrocytes and copper levels are needed to probe this question, such as assessing inflammatory levels of astrocytes when presented with decreased copper pools using TTM or genetic knock-downs of *Ctr1*. To support our experiments on inflammation in response to decreased copper levels, we also hope to repeat the experiments in SIM-A9 cells with increased copper levels. For example, Cu^{II}(atsm) (or diacetyl*bis*(*N*(4)-methylthiosemicarbazonato)-Cu^{II}) is a complex that delivers copper intracellularly and is used to increase chemically increase copper levels ^[1,2]. As a genetic approach, we can also knock-down copper exporter *Atp7a*, which will prevent copper from leaving the cell and increase intracellular copper. To support our hypothesis, we would expect to see that

increasing intracellular copper exacerbates microglial inflammatory responses. Next, we hope to use these genetic knockdown cells targeting *Ctr1* and *Atp7a* for RNA-sequencing experiments. Our current data is limited to only the few proinflammatory factors that we are examining, therefore it would be intriguing to see whether copper similarly impacts the expression of other proinflammatory factors as well. RNA-sequencing would provide use with a more holistic perspective of how copper is impacting microglial inflammation. Finally, to fully show that microglia copper level contribute to neuroinflammation, and therefore progression of DA neuron death, we hope to do stereotaxic injections of LPS or PBS in the SN of mice that are heterozygous for *Ctr1* in microglia, specifically. If decreased copper levels truly dampen the inflammatory response of microglia, then mice with less *Ctr1* in microglia will show less DA neuron death in response to LPS, slowing the progression of PD pathology. This experiment would be critical to supporting treatment trials of PD with methods that decrease brain copper levels. Lastly, we hope that this work not only inspires new therapeutic treatment development for PD, but also potentially influences public health policies. This work provides direct evidence that copper impacts neuroinflammation. Thus, it is critical to implement save work environments to minimize exposure to toxins, which includes heavy metals like copper.

When we first began our *Tcf4* project, relatively little was known about cell specific expression levels of the transcription factor. In addition to our own imaging experiments, many neuroanatomical studies have now been done on *Tcf4* to gain better spatiotemporal and cell type-specific expression patterns; all these studies distinctly show *Tcf4* is present in astrocytes [3–5]. To study the function of *Tcf4* in astrocytes, we generated the first astrocyte-specific *Tcf4* knock-out mouse model that is also the first PTHS-associated mouse to present with seizures. Our immediate future directions are completing the characterization of seizures in these mice with our collaborators in Dr. Jeanne Paz's lab. Additionally, we are excited to see what transcriptional changes are occurring in these astrocytes using RNA-sequencing, as we hypothesize that *Tcf4* could be transcriptionally controlling the expression of proteins that are critical for astrocytic maintenance of neural excitability. Current drug treatments for seizures mainly target neurons, as that's where most of the research lies, despite being ineffective for many types of epilepsy. Results from this work will find innovative mechanisms for epilepsy by targeting astrocyte function.

Aside from extensively exploring how *Tcf4* causes seizures in our *Aldh1l1-Cre;Tcf4^{fl/+}* and *Aldh1l1-Cre;Tcf4^{fl/fl}* mice, we suspect that there are many other pathological phenotypes in these mice as well. However, given the strong seizures and shortened life span that these mice have, this is outside the scope of our project goals. Other mouse models of PTHS targeting neurons and oligodendrocytes have reported behavior, brain structure, and neuron function abnormalities [3,5–16]. We hope that future work in the *Tcf4* field continues to explore these questions in our astrocyte model as well. For example, due to the structural abnormalities that we see in *Aldh1l1-Cre;Tcf4^{fl/fl}* mice, we would expect to observe behavior changes that are reminiscent of PTHS patients, such as poor sociability, vocalizations, and impaired learning and memory. Further, these experiments need to be replicated in human cellular models before any true drug treatments are established, as other NDCs have been modeled in human organoids of the brain [17].

Animal research has its limitations; this is especially true with astrocyte research, as human astrocytes are incredibly more complex and expansive ^[18].

4.3: Conclusion

Innovative projects are shifting our neuron-centric view of the central nervous system towards a holistic perspective wherein glia and neurons cooperate to form and maintain a healthy brain throughout an organisms life. Given that both astrocytes and microglia are emerging choreographers of neural circuit formation and surveyors of brain homeostasis, it is necessary to reevaluate brain diseases from a glial perspective. Broadly, our work continues to contribute to this body of knowledge, illustrating that the pathogenesis underlying neurological diseases is attributable to the dysfunction of microglia and astrocytes. Currently, treatment for most neurological diseases—including neurodegenerative diseases and ASDs—occurs solely by moderating symptoms and consequently are still incurable despite years of research. By approaching diseases from a glial perspective, we can potentially identify new molecular targets in these cells that will guide future research on discovering novel therapeutic treatments.

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