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Astrocytes: Understanding Their Development and Reactivity Using Novel Tools

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ABSTRACT OF THE DISSERTATION

Astrocytes: Understanding Their Development and Using Novel Tools to Assess their Immune Reactivity

by

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In the healthy brain, astrocytes function as key homeostatic players that support neuronal signaling, communicate with the blood brain barrier to maintain its selective permeability, and participate in the glymphatic system to ensure proper waste removal. Astrocytes were originally named *nervenkitt*, or neuronal glue and were considered to be homogenous and physiologically inactive cells, providing little more than structural support. Recently we have begun to understand the dynamic and diverse roles these cells occupy. As the importance of astrocytes has come into focus, so has the need to understand the mechanisms that drive their development. Here, we review current understanding of the developmental processes that give rise to the mature astrocyte and produce diverse astrocyte populations. We will also look at the commonalities that developing astrocytes share with another process, astrocyte reactivity.

In response to immune challenges, astrocytes become reprogramed into reactive astrocytes in a process known as reactive gliosis. Reactive astrocytes are commonly

identified by upregulation of intermediate filaments and hypertrophy. The mechanisms underlying reactivity and the functions that it serves are yet to be fully understood. Currently, there are a lack of tools which allow us to specifically target astrocytes after they become reactive. Studies characterizing reactivity have revealed several genes expressed in astrocytes only after the brain is immune challenged. Using this knowledge, we developed a transgenic mouse that expresses Cre recombinase under the promoter of one of the identified markers, *Lcn2* (*Lcn2CreErt2*). The *Lcn2CreErt2* mouse was validated across a variety of disease models including systemic inflammation and parasitic infection. We used this mouse to drive the permanent expression of a fluorescent marker, which allowed us to observe changes in reactive gene expression during and after the resolution of inflammation. In order to address potential off target effects of *Lcn2CreErt2*, we developed viruses that drive expression of Cre dependent genes under the GFAP promoter. We were also able to use the *Lcn2CreErt2* mouse to isolate labeled cells through flow cytometry. The experiments performed represent only a fraction of the possibilities for the ways the *Lcn2CreErt2* mouse will enhance our understanding of reactive astrocytes.

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Abbreviations:

Glial Fibrillary Acidic Protein (GFAP)

Central Nervous System (CNS)

Perisynaptic Astrocyte Process (PAP)

Aquaporin 4 (AQP4)

Cerebral Spinal Fluid (CSF)

Lipopolysaccharide (LPS)

Intraperitoneal (IP)

Toll-like Receptor 4 (TLR4)

Alzheimer's Disease (AD)

Huntington's Disease (HD)

Parkinson's Disease (HD)

Neuronal Stem Cell (NSC)

Glutamate Aspartate Transporter (GLAST)

Aldehyde Dehydrogenase 1 Family Member L1 (ALDH1L1)

Notch Intracellular Domain (NICD)

Suppressor of Hairless (RPB-J)

Hairy and Enhancer of Split (HES)

Basic Helix Loop Helix (bHLH)

SRY-related HMG-box9 (Sox9)

Nuclear Factor 1 (NF1)

DNA methyl transferase 1 (DMNT1)

Signal Transducer and Activator of Transcription (STAT)

Janus Kinase (JAK)

Interleukin-6 (IL-6)

Cardiotrophin-1 (CT-1)

Ciliary Neurotrophic Factor Receptor (CNTFR)

Secreted Protein-Acidic and Rich in Cysteine (SPARC)

Thrombospondin (TSP)

Blood Brain Barrier (BBB)

Stem Cell Leukemia (Scl)

Reactive Astrocyte (RA)

Lipocalin2 (Lcn2)

Adeno-Associated Virus (AAV)

Immunohistochemistry (IHC)

Fluorescent *in situ* Hybridization (FISH)

Fluorescent Activated Cell Sorting (FACS)

Chapter 1: Astrocytes in Health and Disease

Abstract

Astrocytes are glial cells which serve critical homeostatic roles in the healthy brain. They exhibit a highly ramified morphology, which connects them to all other cell types in the central nervous system. Their networked structure allows them to support synaptic function, transport waste and nutrients into and out of the brain, influence cerebral blood flow, and perform many other tasks. Astrocytes are also a critical part of the brains immune system, responding to insults through a process known as reactive gliosis. Reactive gliosis produces reactive astrocytes, but much remains unknown regarding how this process contributes to different disease contexts. Here we review some of the functions of astrocytes in both the healthy and diseased brain.

Introduction

Astrocytes

Astrocytes, what a concept. Astrocytes are the brains most abundant cell, and are characterized by a starshaped morphology from which their name originates. Astrocytes were first identified with other glia by Rudolf Virchow in the 1850's as part of the *nervenkitt,* or the glue that holds the nervous system together (Virchow, 1858). Cajal then distinguished from other glial populations in the 1890's when he developed a gold chloride-sublimate technique which labeled the glial fibrillary acidic protein (GFAP) (y Cajal, 1913). Despite the early identification of astrocytes in the field of neuroscience, until recently they remained relatively understudied. This was due in part to the hypothesis that their primary function was to act as an inert stabilizer for the nervous

system, which was supported by their relatively tame electrophysiology when compared to the neuronal action potential (Sofroniew & Vinters, 2010). As new molecular and cellular techniques have developed, we have come to understand astrocytes as dynamic contributors in nervous system functioning. Advancements in histology and imaging have revealed the intimate interactions between astrocyte processes and neuronal synapses, given rise to the tripartite synaptic theory (Farhy-tselnicker & Allen, 2018). The application of these advancements has revealed an astrocyte morphology far more expansive and complex than was ever considered, showing off processes that make connections with many other neuronal cell types to perform a variety of critical functions (Yu et al., 2020). Beyond our enhanced ability to visualize these cells, looking beyond the action potential revealed astrocytes participate in dynamic calcium signaling that can influence blood brain barrier permeability and thus, transport of important signals, nutrients and waste into and out of the central nervous system (CNS) (Engelhardt & Sorokin, 2009; Straub et al., 2006).

In addition to the numerous vital roles that astrocytes play in the healthy brain, they are also a critical part of its innate immune response. The brain is isolated from the periphery by complex and dynamic system known as the blood brain barrier, which exhibits a selective permeability to ensure that the proper extracellular environment for neuronal signaling can be maintained (Engelhardt & Sorokin, 2009). A consequence of this selective permeability is that the immune system that circulated through the bloodstream and lymphatic system does not have immediate access to the CNS. As a result, the CNS must mount its own immune response when challenged by reacting with

its native inhabitants (Ransohoff & Brown, 2012). The primary cells identified as CNS immune responders are microglia and astrocytes, which change to address perturbations through a process known as reactive gliosis (Burda & Sofroniew, 2014). After undergoing astrogliosis, astrocytes change into reactive astrocytes. The functions of reactive astrocytes long remained elusive, and until recently they were largely considered to be detrimental to CNS recovery. In the following sections, we will review the functions of astrocytes in the healthy brain, as well as the recent work that has highlighted the critical importance of reactive astrocytes and all the questions about these cells that remain unanswered.

Astrocytes in the Healthy Brain

Astrocytes are broadly grouped into two major categories, protoplasmic astrocytes of the grey matter and fibrous astrocytes of the white matter (Miller & Raff, 1984). Protoplasmic astrocytes exhibit processes radiating out from a cell body in a globular fashion which establish unique nonoverlapping domains. The processes of fibrous astrocytes are long and unsurprisingly, fiber like, and may exhibit a strong bipolar bias (Tabata, 2015). The morphology and the connections made by their complex ramifications give insight into the functions that these astrocytes serve. Astrocyte branches terminate in several major structural connections, including gap junctions with adjacent astrocytes, and endfeet that contact blood vessels (Matyash & Kettenmann, 2010). Fibrous astrocytes have also been demonstrated to make contacts with nodes of Ranvier, whereas protoplasmic astrocytes envelope synapses through extension of

astrocyte synaptic processes (Sofroniew & Vinters, 2010). These connections establish astrocytes as major utility players in ensuring the proper function of the CNS. In the following sections, we detail the major roles astrocytes play through the lens of their morphological interactions.

The astrocyte perisynaptic process (PAP) are fine structures that envelop both presynaptic boutons and postsynaptic dendrites. Study of PAPs was previously limited as the fine structures can reach sizes smaller than resolvable with light microscopy. Not all synapses are infiltrated by PAPs, and coverage varies between brain regions (Wolff, 1970). When first identified, PAPs were theorized to be inert structures serving as structural support for synapses (Kettenmann & Verkhratsky, 2008). This theory is in part true, as astrocyte processes present on their membrane protein complexes which interact with both the pre and post synaptic cell in order to ensure proper organization (Allen & Eroglu, 2017). While our knowledge of how PAPs form physical contacts with synapses is still developing, Ephrin mediated mechanisms have been described in the hippocampus, where astrocyte ephrin A3 interreacts with the ephrin 4a receptor in neurons (Murai et al., 2003). Infiltration of a PAP into a synapse influences the transition from immature to mature synapses, and communication between astrocytes and microglia drives synaptic removal and degradation during pruning (Chung et al., 2013; Farhy-tselnicker & Allen, 2018; Stogsdill et al., 2017).

While PAPs are important for synaptic stability, they are equally critical for synaptic signaling. Neurons signal through the release of neurotransmitters into the synaptic cleft after arrival of an action potential into the presynaptic terminal (Bean,

2007). This process is highly energy intensive, and creates robust changes in both ion and neurotransmitter concentrations in the synaptic cleft, which must be rapidly buffered in order for the next signal to appear unique. The PAP synaptic membrane contains many specialized proteins which drive this buffering process to ensure the proper synaptic environment is maintained. Ion channels like Kir4.1 assist in the removal of potassium released during action potentials so that the proper electrochemical gradient is maintained (Djukic et al., 2007; Kuffler, 1967). Glutamate transporters such as GLAST/Slc1a3 and GLT-1 ensure that glutamate is rapidly removed from the synaptic cleft so that future signal does not appear as noise (C. M. Anderson & Swanson, 2000; David et al., 2016; Tanaka et al., 1997). The complex functionality of these processes has led to the modern theory of a tripartite synapse consisting of a PAP along-side the pre and post synaptic neuronal projections.

The contribution of astrocytes to neuronal signaling does not end at the synapse. Neurotransmitters removed from the synaptic cleft must be efficiently recycled to provide neurons with the necessary materials for continued signaling. Astrocytes are key contributors towards the metabolism of glutamate in the CNS (Schousboe et al., 2014). After entering the astrocyte, glutamate will either be broken down into glutamine by glutamine synthetase (GS), or released back to the extrasynaptic area where it may have both pre-and post-synaptic effects (Rose et al., 2013). Glutamine will then be transported back into neurons via the SLC1 family of transporters, where it can be converted back to glutamate or into the inhibitory transmitter GABA (Walls et al., 2014).

The uptake of potassium and waste products at the synapses dictates that astrocytes must be highly efficient at buffering. Astrocytes have several specialized connections that help accomplish this task. Endfeet connect astrocytes to the vasculature that permeates the CNS (Engelhardt & Sorokin, 2009). These foot-shaped structures have a high concentration of potassium channels, as well as the water channel aquaporin 4 (AQP4) (Jessen et al., 2015). Rapid transport of potassium out of astrocytes into the bloodstream is coupled to water transport through AQP4, which maintains proper intracellular ionic concentrations (Papadopoulos & Verkman, 2013). Endfeet also allow astrocytes to drive vascular constriction and dilatation (MacVicar & Newman, 2015). Spikes in intracellular calcium that invade endfeet drive the production of arachidonic acid (Bindocci et al., 2017; Mulligan & MacVicar, 2004; Straub et al., 2006). Arachidonic acid is metabolized differently depending on nitrous oxide levels, resulting in either vasodilation or vasocontraction (Metea & Newman, 2006). Thus, astrocytes can directly influence the flow of nutrients and oxygen through their communication with blood vessels.

Astrocytes are connected to one another through gap junctions which are composed of several classes of connexins (Orthmann-murphy et al., 2008). Astrocyte gap junction networks allow for rapid buffering of ions and small molecules, as well as the propagation of calcium waves between connected astrocytes (Bazargani & Attwell, 2016). The passage of small molecules is not only important for buffering, it also allows for the passage of glucose metabolites, a process that has been shown to be modulated in

an activity dependent manner (Giaume et al., 1997). This allows astrocytes to ensure the proper nutrition is supplied to neurons, whose signaling is highly energy intensive.

Energy intensive neuronal signaling also produces a large amount of waste, which would quickly become toxic if not properly disposed of. The lymphatic system does not penetrate the CNS in order to assist in this waste removal. As such astrocytes participate in the removal of soluble waste proteins and metabolic products from the CNS by acting in the glymphatic system, a sewage network formed of connected glial cells (Plog & Nedergaard, 2018). Linked astrocytes help drive the ventricular flow of cerebral spinal fluid (CSF), a primary pathway for waste removal, by allowing it to enter into the parenchyma through AQP4 (Jessen et al., 2015). The passage of CSF through the glymphatic network is enhanced during sleep, pointing to a potential mechanism for why sleep is required (Mendelsohn & Larrick, 2013). This glial network is also critical to distributing essential proteins and nutrients, and its disruption is linked to a number of diseases, including Alzheimer (Reeves et al., 2020; Taoka et al., 2017).

Astrocyte Reactivity

Astrocyte functions are critical to the healthy CNS. These functions allow the maintenance of homeostasis in the brain, creating the proper environment for neuronal signaling and ensuring the buffering and removal of neuronal waste products. When the brain is challenged by an insult or disease, astrocytes undergo a dramatic transformation known as reactive gliosis, turning into reactive astrocytes (Escartin et al., 2021). Originally, reactive astrocytes were identified by their hypertrophy and upregulated

expression of glial fibrillary acid protein GFAP (Andriezen, 1893; Eng et al., 1971). More recently, the process of gliosis has been given many definitions, which were expertly summarized by the Sofroniew lab through the following four characteristics: (i) reactive astrogliosis is a spectrum of molecular, cellular, and functional changes in astrocytes that occur in response to all forms of CNS perturbations; (ii) more severe perturbations will result in more severe changes undergone by the reactive astrocyte; (iii) the changes in reactive astrogliosis are context dependent, and regulated by both inter- and intra- cellular pathways; (iv) the changes undergone by reactive astrocytes lead to both loss and gain of function (Sofroniew, 2015a). This broad characterization highlights a major open question in the field of reactive astrocytes- is there a universal reactive response or is reactivity finely tuned towards individual diseases and insults (Escartin et al., 2021)?

Previously, astrocytes were viewed as a homogenous population and reactivity was viewed as a hindrance to recovery (M. a Anderson et al., 2014; Rio Hortega et al., 1927). As modern techniques have allowed us to observe reactivity in more context and greater detail, we have begun to uncover the vast differences in reactive responses.

As described above, the severity of the gliosis depends on the severity of the immune challenge. This observation was one of the first indications that gliosis occurs on a spectrum and may be a varied response. Minor injuries such as contusions result in minor gliosis, marked by upregulation of GFAP but no proliferation or loss of astrocytic domain (Sofroniew, 2009). In more severe gliosis, astrocytes can become hypertrophic as their major processes increase in size, and they can start to proliferate, migrate and

polarize in order to form a glial scar around areas of severe damage (Sofroniew & Vinters, 2010). Whether there are common pathways that drive each of these levels of response also remains to be determined. For example, the JAK/STAT3 pathway has been identified in several reactive models as being required to induce GFAP upregulation, but not all disease contexts may produce this form of reactivity (Escartin et al., 2021). As the response ranges so greatly, it is critical that we learn more about the functions of reactive astrocytes in every part of the reactivity spectrum.

The provided characterization of gliosis also mentions both gain and loss of function. This aspect is critical to another major question regarding reactive astrocytes- are they helpful or are they harmful? As astrocytes play such critical roles for maintaining normal brain function, the conversion to a reactive astrocyte may inhibit their ability to perform these roles. Severe reactivity can result in loss of fine astrocyte structures, resulting in a simplified morphology with major processes enhanced (Schiweck et al., 2018). As reviewed above, these structures connect astrocytes to other cells and allow for synaptic maintenance, buffering of ions, waste removal, and much more. Furthermore, once an astrocyte becomes reactive, little is known about its ability to resume its healthy CNS roles (Escartin et al., 2021). These questions remain unanswered and create the need for new tools which will allow us to study reactivity throughout the course of the response.

As the more modern approach to gliosis is to look at how astrocyte reactive to an individual disease or insult, it is critical that we study gliosis on a disease-by-disease basis. Here we will detail several disease models which have advanced our understanding

of reactivity, including inflammation through exposure to lipopolysaccharide (LPS), parasitic infection from *toxoplasma gondii*, traumatic brain injuries, epilepsy, and neurodegenerative disease. In each of these models, we will highlight what is currently understood about mechanism that induces reactivity, what changes reactive cells undergo, and studies that have highlighted how the reactive response impacts the progression of the perturbation.

LPS induced inflammation

LPS, also known as endotoxin, is found on the capsule of graham negative bacteria (Nava Catorce & Gevorkian, 2016). The purified form triggers a systemic but transitory inflammatory response when injected intraperitoneally (IP). Astrocytes react strongly in following IP LPS, and the mechanism for their reaction has recently been described. In the CNS, the inflammatory stimulant is recognized by toll-like receptor 4 (TLR4), whose expression is limited to microglia (Lehnardt et al., 2002). The activation of TLR4 triggers a proinflammatory cascade, leading microglia to the release the cytokines Il1α, TNFα, and C1q. Astrocytes respond to these cytokines by activation of the JAK/STAT and NFκB pathways, which upregulate expression of reactive genes such as GFAP (Liddelow et al., 2017). This form of reactivity appears to be transient, as reactive markers such as lipocalin-2 and serpina3a return to a basal level within 72 hours (Zamanian et al., 2012). It remains possible that permanent functional and morphological changes occur within astrocytes after responding to LPS, however the timeline of response must be detailed further at the level of an individual cell in order to determine this possibility.

LPS models have been used in testing whether the contributions of reactive astrocytes are helpful or harmful after gliosis. Interestingly, LPS induced reactive astrocytes appear to release neurotoxic factors, as evidenced by cell culture experiments where conditioned media from LPS treated astrocytes was found to drive neuronal death (Liddelow et al., 2017). These experiments have given rise to the hypothesis that LPS induced gliosis results in a neurotoxic reactive phenotype. The functionality of this phenotype remains unclear, as does the released factor that acts as a neurotoxin. It is possible that the astrocytes are responding to what they perceive as a sceptic environment. Detection of LPS in the brain would signal that bacteria had intruded the CNS as observed in severe conditions like meningitis. Unlike meningitis, the LPS signal is extremely transient, with a half-life between 2 and 4 minutes (Yao et al., 2016). Therefore, the strength of the observed astrocyte reaction which leads to the release of toxins is likely in response a perceived threat much greater than the reality. This distinction between what LPS represents and the limited duration of its action raises some questions about its functionality as a model for inducing reactivity. However, LPS remains one of the best characterized and highly used models, leading to the discovery of critical morphological and genetic changes that have been observed in many disease models.

Brain injury and glial scar formation

Injuries that result in structural damage to the CNS and breakage of the blood brain barrier elicit some of the most severe reactive phenotypes in astrocytes (Escartin et al., 2019; Sofroniew, 2015b). Injury models have led to observations of astrocytes

polarizing, proliferating, and migrating in order to form a glial scar (Rio Hortega et al., 1927; Sofroniew, 2009). Glial scars form around injury sites where the blood brain barrier has broken down allowing peripheral signals to enter the CNS. The mechanisms behind glial scar formation and its functionality have been investigated in models of spinal cord and brain injury. While astrocytes have been shown to divide during scar formation, the extent of this proliferation is currently debated, with some studies showing the scar is primarily formed by newly born astrocytes and others finding only minimal astrocyte divisions (Ferrer-Acosta et al., 2017; Wanner et al., 2013). While proliferation remains an active topic of discussion, astrocyte reactivity is generally agreed as critical for the recruitment of other glia and fibromeningeal cells leading which leads to collagen deposition and restructuring of the extracellular matrix around the injury site (Adams & Gallo, 2018). While scars may or may not be primarily comprised of newly divided astrocytes, blocking of astrocytic JAK/STAT3 signaling still strongly inhibits scar formation, indicating that the reactive response is still critical for scar formation (Katsouri et al., 2020; Pekny et al., 1999; Sofroniew, 2009).

As the CNS requires a highly regulated environment in order to ensure proper neuronal signaling, it would appear that scar formation is a critical step towards reestablishing homeostatic balance isolating the damaged region. Despite the obvious protection provided by scar formation, the glial scar had previously been labeled as a net negative towards CNS recovery (Yang et al., 2020). Early studies proposed that scar formation blocked the ability of neurons to regenerate and reform connections, pointing towards a number of signals released in glial scars that inhibit neuronal growth

(Brambilla et al., 2005; Clemente & Windle, 1954; Sugar & Gerard, 1940). More recently, studies observing the progression of spinal cord injury in the absence of reactive astrocytes found that scar formation actually limited the spread of injury, and that the chemical milieu released from scars actually contains a mixture of signals that both promote and inhibit neuronal regeneration (M. A. Anderson et al., 2016; Okada et al., 2006). Thus, recent evidence suggests that scar formation is actually an important step towards limiting the injury spread, but the presence of the glial scar may later inhibit recovery. Tools that would allow us to target astrocytes at specific time points during scar formation will help us further elucidate their roles.

Epilepsy

Epilepsy is a common neurological disorder characterized by unpredictable seizures that result from pathological excitability in the nervous system (Coulter, Douglas, Steinhauser, 2015). As described above, astrocyte performs numerous essential processes to maintain the normal functioning of excitatory synapses. The synaptic functions of astrocytes support the theory that these cells may play an active role in the pathology of the disease, making them an interesting target for future therapies. Furthermore, as reactivity can result in loss of normal astrocytic function, epileptic gliosis is a particularly interesting case for studying the benefits and costs of this process.

Astrocytes respond to enhanced neuronal activity by increasing their expression of Kir4.1, and increase the density of this channel at the synapse by shuttling them from the endfeet (Steinhauser et al., 2016). This allows for more rapid uptake of released potassium, but reduces the ability of the astrocyte to dispose of it. Inability to efficiently

buffer potassium can lead to astrocytic swelling, which contributes to the pathological excitability by reducing extracellular space and potentially promoting astrocytic glutamate release (Lauderdale et al., 2015; Malarkey & Parpura, 2008; Wetherington et al., 2008).

Potassium buffering may be further inhibited by breakdown of astrocyte gap junction connections. Several animal models have shown that breakdown of astrocyte connectivity can contribute to the epileptic environment, although results have been conflicting as to whether the effect is pro or antiepileptic (Bedner et al., 2015; Giaume, Koulakoff, Roux, Holcman, & Rouach, 2010; Seifert, Carmignoto, & Steinhäuser, 2010; Rouch 2017 unpublished). Finally, the inflammatory response triggered during seizures can lead to the breakdown of the blood brain barrier. This leads to leakage of albumin into the parenchyma, where it is cleared by astrocytes, a process that impedes their spatial buffering abilities by downregulating Kir channel activity (Seifert et al., 2010).

Epileptic gliosis drives changes in gene expression which may further exacerbate pathology. Seizures have been shown to dysregulate expression of glutamine synthetase and GLT-1 which disrupts proper glutamate cycling. This can lead to enhanced astrocytic glutamate release as external concentrations build, as well as a reduction in glutamate uptake (Hubbard et al., 2016; Ortinski, Dong, Mungenast, Yue, & Takano, 2010; Sun et al., 2016). The result of this disruption is slower glutamate clearance, which directly promotes a hyperexcitable environment. Reactive astrocytes are also key regulators of the neuroinflammatory response, which promotes a pro-epileptic environment in several ways (Colombo & Farina, 2016; Cordiglieri & Farina, 2010). For example, they release

proinflammatory cytokines which promote the breakdown of the blood-brain barrier, reducing extracellular space and taxing normal astrocytic function (Argaw et al., 2012; Cordiglieri & Farina, 2010; Sofroniew, 2009). Finally, selective induction of astrogliosis leads to deficiencies in GABA signaling and neural inhibition, and has even been shown to cause seizures in some models (Ortinski, Dong, Mungenast, Yue, Takano, et al., 2010; Robel et al., 2015). Selective inhibition of astrogliosis has been shown to have the opposite effect, leading to a reduction in epileptic severity (Wang et al., 2017). While these studies highlight an important role for astrocytes in epileptic pathology, the underlying mechanisms of reactivity that are harmful or helpful remain to be fully explored.

Parasitic infection and toxoplasma gondii

Toxoplasma gondii is a protozoan parasite that is currently estimated to infect 30 percent of the world's population (Hofhuis et al., 2011). Infections are detected in the brain by the presence of intracellular cysts. The majority of these infections appear to be asymptomatic, as the growth of cysts is inhibited by the patient's immune system. In immune-compromised patients, such as those with HIV/AIDS, *T. Gondii* cysts can begin to replicate unchecked leading to severe consequences (Luft et al., 1993).

The interaction between astrocytes and *T. Gondii* is a subject of continued research. Mouse models of infection have led to the discovery of reactive phenotypes in astrocytes which may contribute to the prevention of cyst growth after the infection has been established. Reactive markers such as GFAP and C3 are upregulated in astrocytes during *T. gondii* infection, and expression of astrocytic GLT-1 is disrupted (David et al.,

2016; Jin et al., 2019). Furthermore, prevention of reactivity leads to an enhanced pathology following infection. Astrocyte specific removal of gp130, a receptor for inflammatory cytokine signals such as interlukin6, leads to enhanced spread of inflammatory lesions and eventual death from *toxoplasma* encephalitis (Drögemüller et al., 2008). Furthermore, STAT1 and TGF-β signaling in astrocytes is required for inhibition of parasitic growth (Drögemüller et al., 2008; Hidano et al., 2016). Future studies should look towards the changes in astrocyte expression at different time points during infection, as well as changes that might allow for cysts to reenter the cell cycle following immune challenges.

Neurodegenerative diseases

Neurodegenerative disease is an umbrella term for a number of debilitating disorders, such Huntington's (HD), Parkinson's (PD) and Alzheimer's disease (AD), which are marked by the progressive loss of neurons (Dugger & Dickson, 2017). Research into these illnesses has historically focused on the neuronal detriments, but new discoveries have implicated changes in glia as a critical component of disease pathology (Li et al., 2019). Reactive astrocytes have long been identified in postmortem tissue of patients with these disorders, but the function of this reactivity remains an active source of debate (Ben Haim et al., 2015; Palpagama et al., 2019; Yun et al., 2018). Here, we will focus on some of the recent advances made towards understanding how astrocytes contribute to AD.

Alzheimer's disease is the most common form of dementia, which is characterized in the CNS by the build-up of amyloid plaques and tau neurofibrillary

tangles (Breijyeh & Karaman, 2020). Astrocytes in the healthy brain play a critical role in beta-amyloid clearance, and this function has been shown to be disrupted in AD patients (Plog & Nedergaard, 2018). AD triggers a strong inflammatory response, where astrocytes polarize towards beta-amyloid plaques and upregulate GFAP, a phenotype that is recapitulated in mouse models that drive plaque accumulation (Duyckaerts et al., 2008; Pike et al., 1995). Furthermore, astrogliosis has been shown to occur prior to neuronal loss and plaque deposition, and the severity of the reactive response increases as the pathology worsens (Jo, Yarishkin, Hwang, Chun, Park, Woo, Bae, Kim, Lee, & Chun, 2014; Simpson et al., 2010; Tarkowski et al., 2003).

In recent years, conflicting evidence has emerged regarding whether astrogliosis is beneficial or detrimental to AD progression and severity. Reactive astrocytes increase parenchymal GABA levels, leading to enhanced neuronal suppression and memory deficits (Ben Haim et al., 2015; Jo, Yarishkin, Hwang, Chun, Park, Woo, Bae, Kim, Lee, Chun, et al., 2014). Furthermore, blocking microglial induced astrocyte reactivity in two mouse models of Alzheimer's resulted in increased neuronal survival (Yun et al., 2018). In support of a protective role of reactive astrocytes, when proliferating astrocytes were genetically ablated, animals suffered increased plaque load and more severe behavioral deficits (Katsouri et al., 2020). While the consensus on how reactive astrocytes contribute to pathology is still developing, these studies clearly demonstrate that AD is at least in part a non-neural autonomous disease strongly impacted by the reactive response.

Investigating Astrocytes

The review of literature provided here demonstrates astrocytes as a fertile ground for future research. Our recent appreciation for the functionality of astrocytes outside of their original role as neuronal glue has led to many discoveries regarding the how the nervous system functions as an integrated unit. Still, the field of astrocyte studies contains many unanswered questions. Astrocytes serve as a nexus for communication between all other CNS cell types, but the mechanisms in development that allow astrocytes to form their network require further investigation. Recent discoveries have also identified astrocytes as an immensely heterogenous population, and the origins and mechanistic consequences of this heterogeneity must be addressed. In order to ask the appropriate questions regarding these phenomena, we provide a detailed review of our current understanding of astrocyte development as the first aim of this thesis.

The field of astrocyte development is rapidly evolving as we come to discovery new populations of astrocytes and their functional niche. In chapter 2, we review the current understanding of astrocyte development as it pertains to the early specification of glia following neurogenesis, the acquisition of their mature functions, and the mechanisms that allow diverse populations of astrocytes to arise. The goal of this review is not only to highlight the recent discoveries in the field of astrocyte development, but also point towards the abundant opportunities for progress that will help us understand the how the nervous system functions as a whole.

The study of astrocyte reactivity has also advanced greatly in recent years. Old views of a homogenous astrocyte response to any nervous system perturbation have been

challenged, leading to the need to study gliosis in a disease-by-disease case. Furthermore, the reactive response is likely changing throughout the course of disease progression. These discoveries necessitate the availability of tools that will allow us to study reactive astrocytes across different disease models with greater specificity and temporal control. The second aim of this thesis was to expand the toolkit available to address these issues. In Chapter 3, we describe the development of a tool that will allow us to answer many of the outstanding questions in the field.

Gliosis occurs as a universal response to CNS immune challenges, but the extent of this response context dependent, and the role it plays needs to be investigated in a disease specific manner with high temporal specificity. In order to accomplish this, we developed the *Lcn2CreERT2* mouse, which expresses a Cre-ERT2 under the promoter of *Lcn2*. *Lcn2* is strongly upregulated in astrocytes across a number of disease models, making it a strong candidate for targeting reactivity on a trans-disease basis. Using this promoter to express a tamoxifen inducible Cre gives us the ability to drive recombination in an inflammation specific manner without affecting astrocytes that have not undergone gliosis. We demonstrate the ability of this tool to label astrocytes across a number of disease models, develop combinatorial approaches to increase the specificity of *Lcn2CreERT2*, and provide the first proof that reactive astrocytes remain alive in the CNS following resolution of inflammation. The *Lcn2CreERT2*mouse will open many experimental doorways for the future study of reactive astrocytes.

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Chapter 2: Astrocyte development: Origins of Mature Functions and Diversity

Abstract:

Astrocytes are star shaped cells that tile the central nervous system. As astrocytes do not share the same dramatic physiological activity as neurons, they were once considered to be quiescent members of the CNS which contributed little more than structural support. More recently, astrocytes have been shown to be dynamic contributors to brain physiology, whose functions are critical to maintaining homeostasis, supporting synaptic signaling, clearing waste, and even participating in the CNS's innate immune response. Studies of astrocytes have also revealed immense morphological and functional diversity in what was previously considered to be a relatively homogenous population. As the importance of astrocytes has emerged, focus has turned to understanding how an astrocyte acquires its critical functions during development. Furthermore, studying the development of astrocytes has provided a window into their heterogeneity and the mechanisms that allow different populations of astrocytes to arise. While research in the last 20 years has provided a strong foundation for understanding the fundamentals of glial development, uncovering the means by which diversity and functional complexity arise promises to be fruitful for years to come.

Introduction

Astrocytes are the most abundant population of glial cells in the mammalian brain, comprising up to 50 percent of the cell population in the human central nervous system (CNS). Despite their extreme abundance, our understanding of astrocyte functionality has

lagged behind that of other CNS cell types. Astrocytes were first identified over a century ago and were long considered to be quiescent cells providing little more than structural support (Virchow, 1858). More recently, we have come to understand astrocytes as an active and diverse population whose functionality is critical for the maintenance and defense of the CNS. The functions of astrocytes in the mature CNS include participation in neuronal signaling through the tripartite synapse, direct communication to a broad network of other astrocytes through gap junction coupling, transport of nutrients and removal of waste through connections with the blood brain barrier, and maintenance of the parenchymal environment through buffering of molecules and ions (Engelhardt & Sorokin, 2009; Farhy-tselnicker & Allen, 2018; James I. Nagy & Rash, 2000; Sofroniew & Vinters, 2010). Astrocyte functions are further tuned to the suiting the needs of their local environment, a recent discovery that has led to major questions about how astrocytes might be further subclassified (Batiuk et al., 2020; John Lin et al., 2017; S. J. Miller et al., 2019). In this review, we discuss our current understanding of early astrocyte development and the mechanisms that drive astrocyte maturation. We also summarize new findings about astrocyte heterogeneity, and examine how that diversity arises during development. In the conclusion, we briefly explore the common pathways shared between development and astrocyte reactivity and how this shapes directions for future astrocyte studies.

Astrocyte development - general considerations

Neural development in the CNS follows a series of programed steps which can be broadly broken up into the processes of specification, determination, differentiation, and

maturation (A. V. Molofsky & Deneen, 2015). Specification begins during cell division: neural cells born from neuronal stem cells (NSC) in the germinal layers of the ventricular zone are specified for a particular lineage through asymmetric division, as well as external and internal cues (Tropepe et al., 2001). Next, specified cells migrate away from the ventricular zone as they exit the cell cycle. During and after the completion of migration, cells become determined and begin to differentiate into their specified type through the expression of fate-specific determinants (Corbin et al., 2008). Finally, they acquire the adult features and functionality through the process of maturation (Chaboub & Deneen, 2013). While these stages have been classified extensively for the development of neurons and oligodendrocytes, whether or not astrocytes undergo the same stepwise processes is still being investigated.

The study of astrocyte lineage has previously been held back by a lack of stage specific markers, as well as a lack of understanding of the heterogeneity between astrocyte populations. Early studies looking to examine astrocyte development relied on differential expression of intermediate filaments such as vimentin, glial fibrillary acidic protein (GFAP), and nestin to look at early, intermediate and late stages (CHU et al., 2001; Hirano & Goldman, 1988; Kálmán & Ajtai, 2001). Each of these markers are flawed for the purposes of studying astrogenesis, as they are either expressed in other cell types at concurrent times during development, or fail to label all developing astrocyte populations (Holst et al., 2019; A. V. Molofsky & Deneen, 2015). More recently, the discovery of astrocyte markers that provide greater specificity, such as Glutamate Aspartate Transporter (GLAST) and Aldehyde Dehydrogenase 1 Family Member L1

(ALDH1L1), has allowed for more advanced studies of this lineage. However, these markers may also fail to identify all astrocyte subpopulations at different developmental stages (Perego et al., 2000; Regan et al., 2007).

The diverse means by which adult populations of astrocytes arise further complicates whether all astrocytes undergo the same stages of developmental progression. Astrocytes are produced from radial glia, subventricular zone progenitors, NG2 glia, and from local proliferation (Reviewed in W. P. Ge & Jia, 2016). The local production of astrocytes is a unique feature among cells derived from the neuroepithelium, the result of a second phase of post migratory proliferation (W.-P. Ge et al., 2012). This second proliferative phase has been shown to generate the majority of mature astrocytes, increasing the population 6 to 8 fold in the rodent CNS (W.-P. Ge et al., 2012). Each environment that these populations of astrocytes are born into is radically different making it exceedingly unlikely that their developmental progressions are identical. In the following sections, we will cover the current understanding of the processes which govern early astrocyte development and maturation.

Part 1 - Early Astrocyte Development- Specification, Migration, Proliferation, and Differentiation.

The development of the CNS begins with the early specification of neuronal precursors from a population of NSCs located in the ventricular zone (Kohwi & Doe, 2013). Following this early stage of neurogenesis, a switch to gliogenesis occurs in ventricular NSC which gives rise to populations of astrocyte and oligodendrocyte

precursors (Gallo & Deneen, 2014). As neurogenesis precedes gliogenesis, early astrocyte development is largely determined by the preservation of pluripotency in the neuroepithelium as populations of these cells are selected to become neurons. After being prevented from acquiring a neuronal fate, glial cells are specified through instructive and permissive signals. Thus, the gliogenic switch is characterized by two major processes: inhibition of neurogenesis through permissive signals, and activation of gliogenesis through instructive signals.

Several pathways are critical to the inhibition of neurogenesis in developing NSC as well as astrocytic specification. Among the most well characterized is the Notch signaling pathway (Gaiano et al., 2000). Gain- and loss-of-function experiments demonstrated that disruption of notch signaling leads to major impairments in glial development (Furukawa et al., 2000; Gaiano et al., 2000; Taylor et al., 2007). Early specified neurons suppress neuronal fate in the surrounding neuroepithelium through the expression of delta/jagged ligands which activate Notch receptors in neighboring NSC (Namihira et al., 2009b). This leads to cleavage of the notch intracellular domain (NICD), which translocates to the nucleus and through interactions with the DNA binding protein suppressor of hairless (RPB-J) leads to the expression of downstream targets such as the Hairy and Enhancer of Split (HES) family of genes (Reviewed in Bray, 2016). Release and activation of NICD leads to large scale remodeling of chromatin structures, which inhibits neuronal gene expression while opening up astrocytic genes allowing them to be transcribed (Sanosaka et al., 2017).

As mentioned above, Notch mediation of gliogenesis requires downstream expression of HES proteins, basic helix-loop-helix (bHLH) transcription factors which function as transcriptional repressors (Borggrefe & Oswald, 2009; Castro et al., 2006; Nieto et al., 2001). NICD fails to suppresses neurogenesis in the absence of Hes1 and Hes5, which function to repress the expression of neuronal specific genes, such as Mash1 and Neurogenin2 (Castro et al., 2006; Furukawa et al., 2000; Kageyama et al., 2008). While preventing the expression of neuronal genes allows for gliogenesis, Notch signaling does not directly induce the expression of glial genes (Deneen et al., 2006). Therefore, early in development Notch signaling is considered to be permissive rather than instructive for astrocyte development.

Another important cue in early glial specification is the SRY-related HMG-box 9 (Sox9) high mobility group transcription factor. Early knockout studies showed that in the absence of Sox9 the period of neurogenesis is extended (Stolt et al., 2003). Sox9 also plays a critical role in maintaining stemness during development and in adult populations (Cheng et al., 2009; Stolt et al., 2003). Sox9 functions along with and downstream of notch signaling to suppress neurogenesis, but it's absence does not prevent the onset of gliogenesis, indicating this signal is again permissive and not instructive (Janssens et al., 2009; Vong et al., 2015).

While Notch and Sox9 serve as permissive cues for gliogenesis, they both regulate the expression and activity of the nuclear factor I (NFI) family of transcription factors (Janssens et al., 2009; Namihira et al., 2009b). NFIA in particular has been identified as an instructive factor for the specification of astrocytes, and its expression

serves as one of the earliest indicators of glial identity (Deneen et al., 2006). While NFIA is expressed in both astrocyte and oligodendrocyte precursors at the onset of glial specification, it becomes restricted to astrocytes later and is both necessary and sufficient for astrogenesis (Deneen et al., 2006). The NFIA promoter region features a binding site for RPB-J making it a downstream Notch target (Janssens et al., 2009). Sox9 acts both upstream and in concert with NIFA, both inducing its transcription and physically associating with it to drive the expression of gliogenic genes (Kang et al., 2012).

In conjunction with SOX9, NFIA has been shown to drive the development of functional human astrocytes from pluripotent stem cells, indicating its instructive nature in this process extends across species (Tchieu et al., 2019). Much remains to be discovered about the precise mechanisms by which NFIs regulate the astrocyte specific gene expression of a number of downstream targets (Harris et al., 2015; Kang et al., 2012). The Sox9-NFIA complex drives the expression of *ACPD1, Mmd2* and *Zcchc24*, a combination of regulatory and metabolic proteins which are able to restore gliogenesis in the absence of Sox9 or NFIA (Kang et al., 2012). The zinc finger- and BTB domain-containing protein Zbtb20 was recently shown as another downstream effector of NFIA/Sox9, promoting cortical astrogliogenesis through the suppression of neurogenic genes (Nagao et al., 2016). Furthermore, NFIA drives the dissociation of DNA methyl transferase 1 (DMT1) from astrocytic genes such as *GFAP* and various other targets of Signal Transducer and Activator of Transcription 3 (STAT3), a critical transcription factor for astrocyte differentiation, although the mechanism by which this occurs remains unresolved (Namihira et al., 2009a; Takouda et al., 2017).

Epigenetic modifications to chromatin structure represent a final permissive step in early astrocyte development. The stepwise appearance of neurons and glia from NSC is accompanied by two successive waves of demethylation (Sanosaka et al., 2017). This might underlie why methylation of glial cells remains remarkable low compared to neurons, resembling a fetal methylome (Lister et al., 2013; Sanosaka et al., 2017). The large-scale demethylation during the gliogenic switch is accompanied by an increase in available binding sites for instructive transcription factors, and an increase in methylation based silencing of neuronal genes. In addition to Notch/Sox9/NFIA dependent inhibition of DNMT1, bone morphogenic protein-2 drives epigenetic changes synergistically with leukemia inhibitory factor (LIF) through prompting histone-acetylation (Nakashima et al., 1999). This occurs by driving the expression of Smad1, which physically associates with STAT3 through the transcriptional coactivator p300, forming a complex with histone-acetylation capabilities that further increases astrocytic gene availability (Nakashima et al., 1999; Sanosaka et al., 2009).

Advancement in cell culturing techniques has also provided insight into important pathways both early and late in astrogenesis. NSC cultures can be readily differentiated into neurons, astrocytes, or oligodendrocytes depending on the molecular milieu of the culture media (Mantle & Lee, 2018; Sher et al., 2008; Xiong et al., 2014). Mouse NSC cultured in the presence of bovine serum and leukemia inhibitory factor readily differentiate into astrocytes (Conti et al., 2005; Pe et al., 2018). Astrocytes cultured in this manner were sequenced throughout their developmental process revealing stage specific transcriptional changes which readily distinguished astrocytes as they progressed

from unspecified NSC to differentiated and mature cells. For example, GFAP expression was upregulated in astrocytes at the start of differentiation, whereas aquaporin 4 did not appear until later stages, confirming this model recreates patterns of expression seen in vivo (J. D. Cahoy et al., 2008). This culture model was thus used to examine transcription factors which drive the expression changes at each stage in development. NFIA and ATF3 were identified as transcription factors critical for the differentiation of early astrocytes, whereas STAT3, Runx2 and Prdm9 drove astrogenesis at later stages (Pe et al., 2018). Driving the overexpression of these factors in vivo was also able to promote astrocyte fate at the cost of neuronal differentiation, further supporting their case as key instructors of astrocyte development (Pe et al., 2018).

Following specification, astrocyte precursors migrate away from the germinal layers in which they were born and begin to differentiate. During this time period, astrocytes precursors replicate during two distinct phases of proliferation. The first phase occurs as the progenitors divide asymmetrically in the ventricular zone. The second phase happens following migration to the region they will occupy until maturation. This biphasic pattern of proliferation occurs cortically, in the retina and in the spinal cord (W. P. Ge & Jia, 2016; SANDERCOE et al., 1999; Tien et al., 2012). The RAF/MEK/ERK pathway has been shown to be critical to astrocyte proliferation, with the RAF isoform BRAF being both necessary and sufficient for normal astrocyte proliferation in the spinal cord (Tien et al., 2012). Prolactin activation of JAK/STAT signaling has also been shown to induce astrocyte proliferation both in culture and embryonic astrocytes (DeVito et al., 1992; Mangoura et al., 2000).

Migration of astrocytic precursors occurs following division in the ventricular zone. Early fate mapping studies showed that immature astrocytes remain tethered to their germinal site of origin while migrating on the same radial glial tracks used by neurons (Goldman et al., 1997; Jacobsen & Miller, 2003). This radial migration only accounts for the 40 percent of the astrocyte population that is specified early in development. Later, the radial glial processes disappear, however astrocytes are still proliferating at this stage, suggesting a different means for migration may exist. The extent to which astrocytes born outside of the ventricular zone undergo a second migratory phase to their terminal location, or are simply moved as a result of displacement from mitosis, remains to be investigated (Schiweck et al., 2018). Furthermore, different varieties of astrocytes show differential preferences for direction of migration. Protoplasmic astrocytes prefer to migrate radially, whereas fibrous white matter astrocytes show a preference for longitudinal migration along axon tracks (Jacobsen & Miller, 2003).

Much remains to be known about the repulsive and attractive cues which are responsible for astrocytes finding their terminal location. Early studies showed glial precursors respond to similar migratory cues as neurons, as is seen in the developing optic nerve where netrin-1 and semaphorin 3a serve as repulsive cues for heterogenous glial populations (Sugimoto et al., 2001; Tsai & Miller, 2002). In addition to long-range cues, short-range signals such as ephrins likely play a critical role during this process. For example, targeted deletion of *EphA4* leads to disorganization of the astrocytes in the olfactory bulb (Todd et al., 2017). In vitro studies looking at mechanisms driving

astrocyte migration have also identified additional factors which may be critical during this developmental event, although these findings remain to be validated in vivo (Etienne-Manneville, 2006; A. V. Molofsky & Deneen, 2015; Osmani et al., 2006). As we come to learn more about the diverse subpopulations of astrocytes and the unique domains that they occupy, it will be of great interest to understand the cues that allow each population to find its domain.

Astrocytes begin to differentiate as they migrate to their terminal positions. The process of astrocyte differentiation is marked by the expression of several markers, including S100b, ALDH1L1, aquaporin4, and GFAP (Chaboub & Deneen, 2013). While none of these markers label all astrocyte populations, they have provided windows into the regulation of astrocyte differentiation. Among the most well characterized pathways for astrocyte differentiation is Janus Kinase/Signal Transducer and Activator of Transcription 3 (JAK/Stat3). JAK/STAT3 signaling is activated by extracellular members of the interleukin (IL)-6 family of cytokines, which bind to IL receptors leading to homodimerization and the activation of JAK through autophosphorylation (Rawlings, 2004). JAK/STAT3 signaling during differentiation can be induced through cardiotrophin-1 (CT-1) induced activation of the ciliary neurotrophic factor receptor (CNTFR) as well as IL-6 activation of glycoprotein-130 (Bonni et al., 1997; Sriram et al., 2004; Wang et al., 2014). The signals activating these receptors are released from new born neurons and endothelial cells (Imura et al., 2008; Namihira et al., 2009b). JAK then phosphorylates STAT, leading to its homodimerization and translocation to the nucleus. Upon entering the nucleus, STAT3 has been shown to directly bind to the newly

available promoter regions of astrocytic genes and enhance their expression (He et al., 2005). Genes under the control of STAT3 include some of the most commonly studied markers of astrocytic differentiation, including *GFAP* and *S100B* (Freeman, 2010).

The importance of microRNAs in astrocyte development has only begun to be explored. Several studies have broadly addressed the importance of these regulatory elements by knocking out Dicer, an enzyme critical to miRNA maturation. Removal of Dicer in the murine spinal cord disrupts gliogenesis without affecting early motor neuron development, and leads to disorganized, hyperproliferative Aldh1-1 positive astrocytes in cerebellum (Howng et al., 2015; Zheng et al., 2010). Stat3 signaling leads to the expression of miR-31, which inhibits stem cell renewal through the suppression of Lin28, thus promoting terminal astrocyte differentiation (Meares et al., 2018). MiRNA-124 has also been identified as a key suppressor of glial fate early in development: the removal of this microRNA promotes early astrocyte differentiation (Neo et al., 2014). The precise epigenetic mechanisms that drive early astrocyte development will continue to be a fruitful area of study as we come to understand more about how these regulatory elements can impact cell fate.

Part 2: Astrocyte maturation:

Astrocytes in the mature CNS have remarkable morphological complexity. Astrocytes exhibit extensive ramification which allows them to contact and communicate with other astrocytes, neurons, other glial cells, and endothelial cells (Schiweck et al., 2018). Processes extending from astrocytes can be broadly categorized based on the

location in which they terminate. Astrocytic perisynaptic processes (PAPs) form connections with neurons through synapse enveloping (Allen & Eroglu, 2017). Astrocytes connect and communicate with the blood brain barrier (BBB) through astrocytic endfeet (Engelhardt & Sorokin, 2009). Finally, other major astrocytic processes terminate in gap junction connections with other astrocytes and myelinating oligodendrocytes, or by enveloping the soma of other neuronal cell types (Khakh & Deneen, 2019; Molina-Gonzalez & Miron, 2019). These remarkable complex features form largely during early postnatal development, a process that has been characterized extensively in rats (Bushong et al., 2004; Schiweck et al., 2018). As the extension of astrocyte processes results in connections to numerous cell types, astrocytic responses to extrinsic signals are of particular importance during maturation. In the following sections, we will detail our current understanding about how each of these types of processes is formed.

Astrocytic perisynaptic processes:

Neurons in the mature nervous system communicate with each other through synaptic connections, which allow for a presynaptic neuron to pass information to a postsynaptic neuron by the release of neurotransmitters or the direct transfer of ionic currents (Bean, 2007). Our understanding of the role of astrocytes in neuronal signaling has greatly blossomed in recent years. Previously, astrocytes were seen as structural support surrounding synapses to enhance stability. Now, we understand that astrocytes play a direct role in both the formation of inhibitory and excitatory synapses and are important for synaptic pruning and maturation, and function of the mature synapse (Farhy-

tselnicker & Allen, 2018). Despite neurons being the first cells to differentiate in nervous system development, the majority of synaptic development occurs only after the onset of astrogenesis (Huttenlocher, 1999). Furthermore, the majority of synaptic connections are enveloped by an astrocytic process, forming the tripartite synapse. Astrocytic Perisynaptic processes (PAPs) allow a single astrocyte to contact over one hundred thousand synapses in the mouse cortex, while in humans this number is estimated to be as large as 2 million (Oberheim et al., 2009). Incredibly, some of this complex branching ability appears to be innate, as human astrocyte grafts transplanted into a mouse brain are capable of forming enhanced numbers of PAPs relative to their murine counterparts, resulting in greater synaptic plasticity and learning capabilities (Han et al., 2013). PAPs serve a number of critical functions at the synapse. These include the uptake of neurotransmitters, buffering of ions, as well as structural and trophic support though the release of factors like ATP (Farhy-tselnicker & Allen, 2018). The following section will cover recent studies which have illuminated our understanding of how astrocytes drive synapse formation as well as the instructional cues which lead to astrocyte process development.

The formation of the mature tripartite synapse comes from communication between astrocytes and their environment (Freeman, 2010; Schiweck et al., 2018). Astrocytes secrete synaptic factors, and receive instruction to express and translocate PAP proteins from neurons and microglia. At a later stage, they also instruct the pruning of nonfunctional synapses through, either by acting directly on synaptic terminals or by

promoting microglial engulfment (Freeman, 2010; Montaner et al., 2018; Risher & Eroglu, 2012; Vainchtein et al., 2018).

Astrocytic PAP contains a number of proteins that are critical for synaptic functioning. These include ion and water channels, neurotransmitter transporters and receptors, and cell adhesion molecules (Durkee & Araque, 2019; Farhy-tselnicker & Allen, 2018; Panatier & Robitaille, 2016; Risher & Eroglu, 2012). The ion and water channels allow for buffering of released ions to maintain concentration gradients critical for neuronal signaling (Amiry-Moghaddam et al., 2003; G. Seifert et al., 2009). The neurotransmitter transporters and receptors aid in the rapid removal of transmitters from the synaptic cleft, as well as transmitter recycling (Perego et al., 2000; Rose et al., 2013). Finally, a variety of cell adhesion molecules provide structural integrity and mediate astrocyte synapse contact (Murai & Pasquale, 2011; Stogsdill et al., 2017). In addition to these synapse supportive functions, astrocytes possess the unique capability to process and recycle waste metabolites created by neurons which divert the majority of their energy to maintaining osmotic balance (Weber & Barros, 2015). Regulation of PAPs remains dynamic throughout maturity and plays a critical role in neuronal plasticity (Chung et al., 2015). The dynamic nature of PAPs is now described by the term astrocyte plasticity, and the mechanisms governing this plasticity in maturity mirror PAP development. It is now widely understood that neuronal activity influences astrocyte process motility, and the presence of astrocytic PAPs remains extremely dynamic even after maturation (Chai et al., 2017; Freeman, 2010).

Astrocytes secrete a number of synaptic factors which are both instructive and permissive for synapse development. The signals released by astrocytes that drive synaptic formation can broadly classified as structural cues that establish basic synapse formation, and functional cues which drive synapses to be physiologically active (Allen & Eroglu, 2017). Among the structural cues that drive the formation of glutamatergic synapses are the thrombospondins and hevin (Christopherson et al., 2005; Kucukdereli et al., 2011). Thrombospondin was the first prosynaptic astrocytic secreted factors identified which was shown to be critical for synapse formation both during development and plasticity (Christopherson et al., 2005; Risher & Eroglu, 2012; Stenina-Adognravi, 2014). Hevin is expressed by astrocytes in the superior colliculus, and is sufficient to drive the formation of immature synapses in rat retinal ganglion cells (Kucukdereli et al., 2011). This activity of hevin is antagonized by another astrocyte secreted protein, Secreted Protein-Acidic and Rich in Cysteine (SPARC), which negatively regulates synapse formation while promoting synapse maturation (Eroglu, 2009; Kucukdereli et al., 2011).

While the synapses induced by thrombospondin and hevin are postsynaptically silent, a second category of astrocyte signals are utilized to promote physiologically active synapses (Christopherson et al., 2005; Farhy-tselnicker & Allen, 2018). For example, glypicans 4 and 6 were identified as sufficient to induce functional synapses in purified retinal ganglion cells by driving the accumulation of GluA1 AMPA receptors at the synaptic surface (Allen et al., 2012). It is now understood that glypican 4 drives mature AMPA accumulation by releasing the AMPA receptor clustering factor pentraxin 1 (Farhy-Tselnicker et al., 2017). The switch from immature to mature AMPA receptors

can also be driven by astrocyte secreted chordin-like1 (Blanco-Suarez et al., 2018). Other astrocytic synaptic factors secreted during development may promote both synapse maturation and removal (Baldwin & Eroglu, 2017). These factors include TNF-α, BDNF, TGF-β, and the aforementioned SPARC (Beattie et al., 2002; Blanco-Suarez et al., 2018; Bosworth & Allen, 2017; Gómez-Casati et al., 2010). Furthermore, astrocyte lipid metabolism provides essential cholesterol and phospholipids for neurons which are critical for synaptic maturation in the hippocampus (van Deijk et al., 2017).

Beyond influencing the growth of neuronal synaptic components through secreted ligands, astrocytes directly participate in synapse development through contact mediated mechanisms. Astrocyte PAPs can stabilize by forming physical connections to the synaptic regions they envelop, which helps synaptic target selection (Allen & Eroglu, 2017). Physical connections through EPH3A can be found in developing PAPs, whereas neurons express its receptor (Murai & Pasquale, 2011). More recently, astrocytic neuroligins were shown to be critical to the formation and morphology of the tripartite synapse (Stogsdill et al., 2017). Still, much remains to be discovered regarding how astrocytes find synaptic targets within their domain.

Studies of astrocytic signaling during synapse development have also revealed extensive astrocytic diversity in terms of the trophic support they provide to the synapse. Secreted factors appear to vary to suit the distinct circuits forming in the astrocytes' environment (Bosworth & Allen, 2017). In support of this idea, astrocyte-conditioned media from different regions of the CNS confer different synaptic potential to cultured neurons, due to varying concentrations of previously mentioned secreted factors (Buosi et

al., 2018). Thrombospondin (TSP) signaling further highlights this aspect of astrocyte diversity, as different TSP proteins are expressed by astrocytes in a region dependent manner (Benner et al., 2013; Eroglu, 2009). These studies suggest that astrocytes are critical to the tuning which allows for neuronal circuit establishment and refinement.

One of the most critical functions PAPs must perform is the uptake of neurotransmitters (Perego et al., 2000; Weber & Barros, 2015). In this regard, the majority of studies have focused on astrocyte uptake of excitatory glutamate, as glutamatergic synapses are more frequently tripartite. Astrocytes uptake glutamate through high affinity transporters GLAST and GLT-1. The expression of these proteins is upregulated in a progressive fashion postnatally, a period of massive synaptogenesis (John D Cahoy et al., 2008; Zhang et al., 2016a). The expression of GLT-1 is regulated in part by endothelial cells through a Notch dependent mechanism, providing another example of reusing signaling pathways at different stages of development. (Lee et al., 2018). PAPs can also express metabotropic glutamate receptors (mGluR) which allow astrocytes to directly respond to glutamate release (Sun et al., 2013). Astrocytic mGluR-5 is expressed prior to the first major wave of synaptogenesis, suggesting that it may be important for astrocyte detection of neuronal activity and thus play a role in forming the initial connection between PAPs and neurons (Petrelli & Bezzi, 2018). As we come to understand how astrocytes are tuned to the individual needs of the synaptic unit they are supporting, the processes by which this specialization is acquired will need to be further examined.

Astrocytic Endfeet and the Blood Brain Barrier:

Astrocytic endfeet cover 99.7 percent of capillary space in the mammalian CNS (Mathiisen et al., 2010). Endfeet perform a number of essential functions to maintain homeostasis in the CNS. The connections between astrocytes and the vasculature are critical for the transport of nutrients into the CNS, waste out of the CNS, and control blood brain barrier permeability (Plog & Nedergaard, 2018; Schiweck et al., 2018; Weber & Barros, 2015). Furthermore, astrocytes serve as intermediaries in neurovascular coupling, where neuronal signaling drives changes in cerebral blood flow to ensure highly active areas receive additional resources (Abbott et al., 2006). Astrocytes allow this process to occur by releasing signals in response to neuronal activity that drives vasodilation or constriction (Metea & Newman, 2006).

Astrocytic endfeet are easily labeled through expression of GFAP, and blood vessel dilatation is increased in GFAP deficient mice (Oberheim et al., 2009; Pekny et al., 1999). Other prominent proteins expressed in endfeet include extracellular matrix proteins such as laminin which help establish connections to the vasculature, and functional proteins such as aquaporin4 (AQP4) and Kir4.1 (Amiry-Moghaddam et al., 2003; Kalsi et al., 2004; Yao et al., 2014). The expression of laminin peaks early in the astrocyte endfoot at P7, whereas Kir 4.1 and AQP4 increase in expression as the animal reaches adulthood indicating a long period of maturation (Lunde et al., 2015). The maturation of the endfeet and blood brain barrier appear to occur in tandem. Following the expression of AQP4, astrocytes upregulate GlialCAM and MCL1, two transmembrane proteins in the complex that connect endfeet to the BBB (Gilbert et al.,

2019). This occurs simultaneously with endothelial expression Claudin-5 and P-gP, although a causal link between endfeet and BBB maturation remains unknown (Gilbert et al., 2019). During development, endothelial cells release signals such nitric oxide, bone morphogenic protein, and leukemia inhibitory factor which strongly drive astrocyte differentiation (Covacu et al., 2006; Imura et al., 2008; Lee et al., 2018; Mi et al., 2001). Astrocytic endfeet in turn contribute to the formation of the blood brain barrier by secreting the growth factors bFGF, GDNF and TFGβ which drive the formation of tight junctions (Abbott et al., 2006; Chaboub & Deneen, 2013; Hayashi et al., 1997; Sobue et al., 1999). Still, much remains to be discovered regarding the exact mechanisms endfeet use to find their targets in the vasculature.

Astrocytic Gap Junctions:

Astrocytic ramification increases dramatically during postnatal development as PAPs are formed and envelop synapses. During this time period, around the second postnatal week in mice, astrocytes establish their nonoverlapping domains through a process known as tiling. Tiling is mediated through contact inhibition although its significance and the specific mechanisms that allow overlapping branches to be pruned are questions that still need to be investigated (Distler et al., 1991; Freeman, 2010). Astrocytes form gap junction connections to other astrocytes in adjacent tiles, as well as oligodendrocytes within their domain (Molina-Gonzalez & Miron, 2019). Gap junction networks allow for the rapid diffusion of neuronally released waste products and ions, making astrocyte networks extremely effective at buffering (Gerald Seifert et al., 2010).

Gap junction connections are composed of connexin proteins, 4 of which are heterogeneously expressed by astrocytes (Bachoo et al., 2004; Mansour et al., 2013). Importantly, astrocyte connection expression profiles are uniquely tuned to serve the physiological needs of their region, one of the earliest discoveries to indicate the importance of astrocyte heterogeneity (Bachoo et al., 2004). The formation of astrocyte gap junction networks has been characterized in the mouse hippocampus, where it increases dramatically in the first 3 postnatal weeks leading to significantly reduced membrane resistance (Schools et al., 2006). The timeline of gap junction coupling corresponds to postnatal peaks in hippocampal synaptic development (O'Kusky et al., 2000). Beyond ensuring astrocyte domain sovereignty, contact between astrocytes may also play a role in driving the ramification process that results in mature astrocyte morphology (Li et al., 2019). Astrocyte gap junction networks have been shown to exhibit region specificity as demonstrated in the barrel cortex and hippocampus (Anders et al., 2014; Houades et al., 2008). Much remains to be explored about the extent to which astrocyte networks partition other brain regions, and how these partitions are formed, however their existence points towards an additional level of complexity in terms of connected neuronal circuits.

Part 3: Astrocyte diversity and its developmental origins

In the last thirty years our understanding of astrocyte heterogeneity has increased dramatically. We now know that astrocytes display a remarkable level of diversity, both between regions and within them. The earliest notions of astrocyte diversity stem from

Ramón y Cajal, who revealed numerous morphological categories of astrocytes through Golgi staining (y Cajal, 1913). A next step taken to categorize different astrocyte populations was the classification of astrocytes into two major groups: protoplasmic astrocytes and fibrous astrocytes (R. H. Miller & Raff, 1984b). Protoplasmic astrocytes exist in the grey matter and are noted for their remarkable bushy morphology marked by diverse functional branching. Each protoplasmic astrocyte envelops synapses with perisynaptic processes, extends endfeet to connect with blood vessels, and connects to other astrocytes with gap junctions (Bushong et al., 2004). Fibrous astrocytes have not been studied as extensively. These astrocytes exist in the white matter, have relatively straight processes that connect with nodes of Ranvier, and stain strongly and broadly for GFAP, an intermediate filament that in protoplasmic astrocytes is restricted to the endfeet (R. H. Miller & Raff, 1984a; Rungger-Brändle et al., 1993). Despite the morphological and functional differences between these two classes of astrocytes, distinguishing their developmental origins has remained elusive. Protoplasmic astrocytes are have been shown to arise earlier in development, and may largely be the product of subventricular glial progenitors following the gliogenic switch (Gressens et al., 1992; R. H. Miller & Raff, 1984a). Removal of proliferative cells from E17 to E18 exclusively reduces protoplasmic astrocytes, whereas the majority of fibrous astrocytes can be eliminated with postnatal knockout of Olig -2 (Cai et al., 2007; Gressens et al., 1992). Furthermore, linage tracing studies have shown that fibrous and protoplasmic astrocytes arise almost exclusively from distinct progenitor populations, but the identity of these progenitors remains to be discovered (García-Marqués & López-Mascaraque, 2013; Tabata, 2015).

Classification of astrocytes into protoplasmic and fibrous populations represents only the tip of the astrocyte diversity iceberg (Bachoo et al., 2004; Batiuk et al., 2020; Bayraktar et al., 2018, 2020; Chai et al., 2017; John Lin et al., 2017; Zhang et al., 2016b). As our understanding of astrocyte diversity has evolved, we now know that populations of astrocytes display remarkable heterogeneity both between and within regions of the brain. Different regions of the brains can sometimes be delineated by their local astrocyte morphology, density, functional properties and proliferation rate (Emsley & Macklis, 2006). Molecular markers which were once considered common among astrocytes, such as connexin-30, GLT-1, s100β, and GFAP, are now known to be differentially expressed across different brain regions (Bachoo et al., 2004; J I Nagy et al., 1999). As a result of our greater understanding of regional astrocyte differences, a number of additional astrocyte subpopulations have emerged. Müller glia of the retina are characterized by their integration into a columnar unit with photoreceptors and neurons, and a specifically tuned to maintaining the retinal environment required for photo-information processing (Farmer & Murai, 2017; Reichenbach & Bringmann, 2013). In the cerebellum, two subpopulations of astrocytes have already been extensively characterized. Bergmann glia are integrated into the Purkinje cell layer, where they show polarization and express high levels of AMPA receptors to assist in motor coordination (Saab et al., 2012). Velate astrocytes are in the granule cell layer and more strongly express AQP-4 (Papadopoulos & Verkman, 2013). Furthermore, region specific subsets of astrocytes that are hormone responsive, tuned to circadian rhythms, or even pH sensitive have been identified (Reviewed in Farmer & Murai, 2017).

The importance of region specificity in astrocytes has come to light in recent years. In a 2017 study, Morel *et al.* identified distinct patterns of astrocytic gene expression through ribosomal purification which closely follow the dorsal ventral axis. Furthermore, astrocytes from a given region were found to selectively promote synaptic activity and neurite outgrowth from region matched neurons, while failing to do so for neurons of other regions (Morel et al., 2017). A recent detailed examination of astrocytes in the hippocampus and striatum revealed numerous physiological and morphological differences which distinguished these populations (Chai et al., 2017). For example, spontaneous calcium signaling was observed at a much higher frequency in hippocampal astrocytes, and the gap junction blocker carbenoxolone dramatically reduced coupling in striatal astrocytes compared to those in the hippocampus. These studies clearly demonstrate that close examination will reveal populations of astrocytes that are finely tuned to their specific environment.

Beyond the differences between astrocytes across different regions of the CNS, astrocytes within a given region also exhibit remarkable molecular, morphological and functional heterogeneity. Cortical astrocytes have emerged as a bountiful frontier for studying intraregional diversity. Astrocytes display remarkable differences across the cortical layers. Layer 1 astrocytes closely resemble fibrous astrocytes, exhibiting limited branching and strong expression of GFAP (Colombo & Reisin, 2004). Astrocytes in layer 6 also exhibit fibrous characteristics, and are frequently associated with larger cortical blood vessels (Sosunov et al., 2014). Protoplasmic astrocytes exist in layers 2-5, and have recently been shown to exhibit lamination in a manner distinct from the neuronal layers

(Bayraktar et al., 2020). By using a high throughput method for imaging and analysis, combined with fluorescent *in situ* hybridization techniques that allow for labeling multiple genes simultaneously with distinct fluorescent probes (RNAscope), Bayraktar *et al.* were able to identify three distinct layers of protoplasmic astrocytes established in early postnatal development and maintained through adulthood. Layers of astrocytes were distinguished through differential expression of *Chrld1, Scel, Eogt, Spry1, Paqr6* and *Il33,* and differential expression of these markers not only identifies astrocyte layers, but is also seen across differing regions of the cortex (Bayraktar et al., 2020). The expression of different molecular markers indicates an abundance of astrocyte subpopulations, but does not detail why having diverse subpopulations is functionally important. Thus, it is critical to ask whether these populations are in fact functionally heterogenous.

Several recent studies have addressed this question across different regions of the CNS. In the spinal cord, Molofsky et al. showed that astrocytes express regional markers which are required for proper postnatal circuit refinement. They identified *Sema3a* as a signal distinct to ventral astrocytes, which specifically promotes correct α-motor neuron orientation and survival (A. V Molofsky et al., 2014). A study published by the Deneen lab in 2017 used advanced cell sorting techniques to identify five distinct subpopulations of astrocytes across the cortex, thalamus, and brainstem based on the expression of CD51, CD63, and CD71. These populations exhibited differing developmental properties, such as proliferation rate, time of appearance and migratory potential. Furthermore, these populations showed differential support of synaptogenesis, with

certain populations expressing much higher levels of presynaptic proteins (John Lin et al., 2017). Batiuk et al. also identified five distinct astrocyte subpopulations across the cortex and hippocampus through the use of single cell sequencing. Interestingly, these subpopulations existed within spatially distinct domains, and displayed functional differences in calcium signaling (Batiuk et al., 2020). Some of the functional specification of astrocytes appears to require continued neuronal input, as was recently shown by Farmer et al. in 2016. In the cerebellum, Bergmann glia and velate astrocytes are segregated into the Purkinje and granule cell layers respectively, and have distinct expression patterns of important functional proteins such as AMPA receptors an aquaporin-4 (Papadopoulos & Verkman, 2013; Saab et al., 2012). The diversification of these populations is driven by neuronal expression of sonic hedgehog (Shh), as removal of *Shh* signal transducer *Smo* causes Bergman glia to adopt an expression profile similar to that of the velate astrocyte (Farmer et al., 2016). While much remains to be discovered about the functions of individual populations, and the level to which each population remains functionally adaptable, it has become clear that astrocyte subpopulations exist and are tuned to serve their local environment.

The origins of astrocyte heterogeneity may be traced back to their progenitors. Astrocytes arise from multiple progenitor types in distinct regions and at different time points in development (Clavreul et al., 2019; Hewett, 2009). In addition to radial glial cells, astrocytes are also born in the neonatal subventricular zone, a population distinguished from radial glia by the expression of distal-less homeobox-2 (Marshall & Goldman, 2002). Furthermore, a second population of SVZ cells, distinguished by the

expression of the chondroitin sulfate proteoglycan NG2, also give rise to gray matter astrocytes in the spinal chord (Zhu et al., 2008). Some studies have also suggested that a population of OPCs may differentiate into astrocytes, however the contribution of this population in vivo appears to be minimal at most (Tabata, 2015). As mentioned above, different subpopulations of cortical astrocytes, such as fibrous and protoplasmic, have been shown to arise from distinct progenitor pools, however it remains unknown if distinct progenitors exist for subpopulations within a given region (García-Marqués & López-Mascaraque, 2013).

Beyond the diverse progenitor pool, astrocyte heterogeneity is also driven by regional patterning. Regional patterning is a consequence of the distribution of morphogen gradients that drive the combinatorial expression of transcription factors (Briscoe & Small, 2015). This process is critical to ensuring proper organization of distinct regions within the CNS (Sansom & Livesey, 2009). As astrocytes were long considered a homogenous population, the question of how astrocytes achieve proper regional patterning has only recently begun to be explored. Many of the signals used to organize neuronal populations in development may play a similar role in astrocytic patterning. Morphogen gradients help establish axes in the developing neural tube while setting up the boundaries of unique progenitor domains (Rowitch & Kriegstein, 2010). These boundaries are maintained during the neuroglial switch, thus the progenitors from which astrocytes arise are already positionally distinct. Regional patterning ensures proper astrocyte development from the earliest stages of specification. Prior to macroglial specification, *Stem cell leukemia* (*Scl*) drives developing NPCs towards astrogenesis

specifically in the P2 domain of the developing spinal cord (Muroyama et al., 2005). In the spinal cord white matter, three positionally distinct classes of astrocytes can be identified by combinatorial expression of *reelin* and *slit1.* The specification of these classes is dependent on Pax6 and Nkx1, a homeodomain code also used to pattern interneurons in the region (Hochstim et al., 2008). Proper specification of cortical neuronal layers was also shown to be important for the organization of the recently discovered astrocytic lamina. In *reelin* mouse mutants, which display inversion of their cortical layers, deep layer astrocytes were observed throughout the cortical depths (Bayraktar et al., 2020). These results suggest that astrocyte heterogeneity is established early during or soon after specification, and maintained into maturation.

Conclusion

Astrocytes are now appreciated as dynamic contributors to both the healthy nervous system, as well as its innate response to disease. The development of astrocytes is of particular interest because of the similarities this process shares with astrocyte reactivity, or the mechanisms by which astrocytes react to nervous system damage or disease. The process of astrocyte reactivity involves heterotrophy which parallels the robust growth observed during development, and pathways important for growth such as JAK/STAT, NFIA, and Notch signaling are critical in both of these contexts (Haim et al., 2015; Laug et al., 2019; Qian et al., 2019). Understanding more about the unique origins of astrocyte populations could be critical to our understanding not only of how the brain

is properly formed, but also the roles of these different subpopulations in the reactive astrocyte disease response.

Astrocytes are now known to be a widely heterogeneous population that serves a wide variety of functions critical for the nervous system. As we come to understand more about the diversity within astrocyte populations, the questions of how this diversity arises during development, and how it is maintained into maturity must be addressed. Classical pathways in astrocyte development play different roles in different populations of astrocytes, and the function of these pathways change during different developmental windows. Astrocytes may also reuse a number of the same external signals used by neurons during development to guide their patterning, migration and maturation. Our rapidly expanding knowledge of the diversity within astrocytes necessitates that we examine whether pathways previously assumed to be canonical are truly utilized in the same manner across different populations. Uncovering the precise mechanisms that allow for the nervous system to be properly assembled is an important step in understanding how astrocytes contribute to both the healthy and diseased brain.

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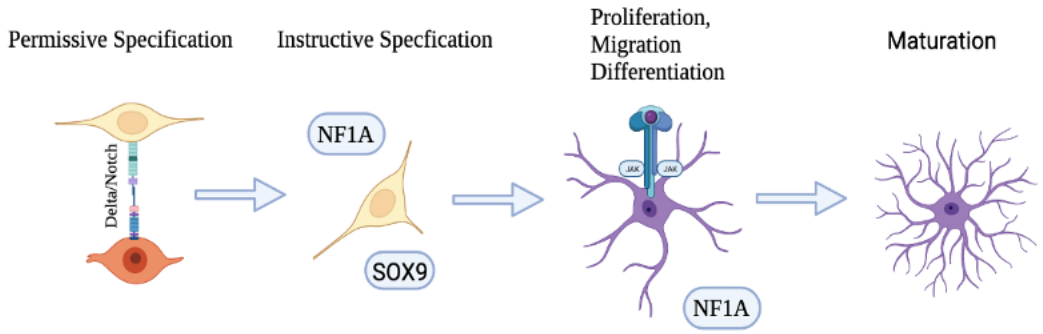

Figure 2.1: Early Astrocyte Development. Early development of the central nervous sytem begins with early specification of neurons in the ventricular zone born from a population of neuronal stem cells (NSC). These neural precursors then suppress neuronal fate in adjacent cells of the neuroepithelium through the delta-notch signaling pathway. Following early neurogenesis, a gliogenic switch occurs which is marked by the specification of glial progenitors from NSCs. Activation of notch signaling represents a permissive step for gliogenesis, however it is not sufficient signal to instruct progenitors toward a particular glial fate. The specification of astrocytes requires the presence of nuclear factor IA (NFIA), a signal that has been shown to be sufficient and instructive for this process. NFIA works in conjunction with SOX9 and notch signaling in order to drive the transcriptional programing which mark early astrocyte specification. Following specification in the sub ventricular zone, astrocyte precursors begin the process of migration and differentiation, where they translocate to the region they will occupy in the mature CNS, and begin to express specialized astrocyte markers. These steps in astrocyte development are heavily dependent on JAK/STAT signaling. NF1A once again plays an instructive role in these final steps. Upon arrival at their destination in the CNS parenchyma, some astrocyte precursors undergo an additional round of cell division before exiting the cell cycle and beginning the process of maturation, where ramification of astrocytes increases as they extend processes to interreact with other cells.

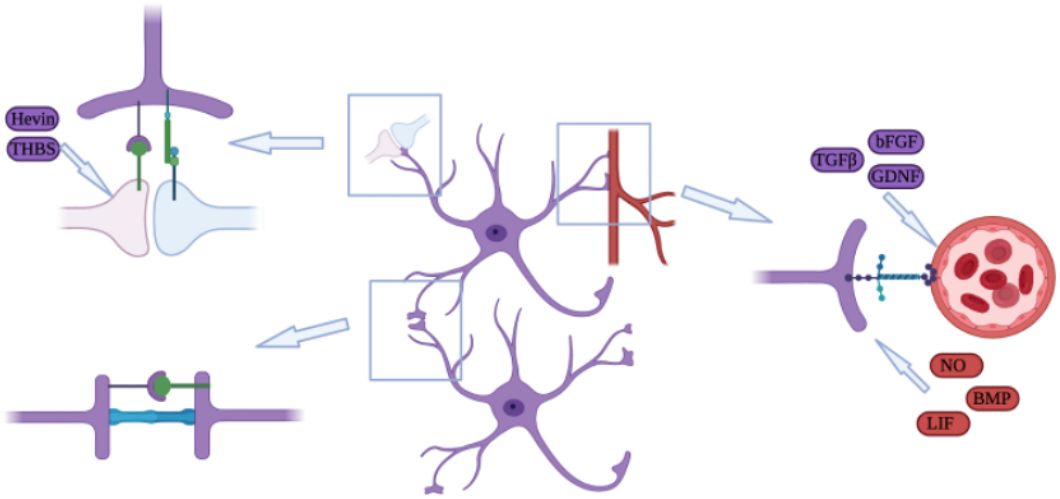

Figure 2.2: Astrocyte maturation and the acquisition of morphological connections. Astrocyte maturation is marked by the development of a complex branched morphology which results in connections with other cells in the CNS. The major connections that form during this developmental period include perisynaptic processes which envelop synapses, astrocyte endfeet which terminate at the blood brain barrier, and gap junctions with other astrocytes. The formation of these connections is the result of both intrinsic pathways and communication with their target cells. The formation of the perisynaptic process (top left) requires contact between astrocytes and both pre and postsynaptic cells which is mediated by neuroligin/neurexin and ephrin signals. Astrocytes then release a number of signals such as thrombospondins and hevin which drive both the formation and maturation of the synapse. Astrocytes connect to other astrocytes through gap junctions formed by connexin proteins (bottom left). Ephrin-mediated contact inhibition also helps astrocytes establish unique domains among the parenchyma. Astrocytes connect to the vasculature through endfeet through extracellular matrix proteins such as laminin. Blood vessels and astrocytes then release a number of factors to drive the maturation of the other. For example GDNF from astrocytes helps induce formation of blood vessel tight junctions, and BMP from endothelial cells helps drives expression of astrocytic proteins such as GLT-1.

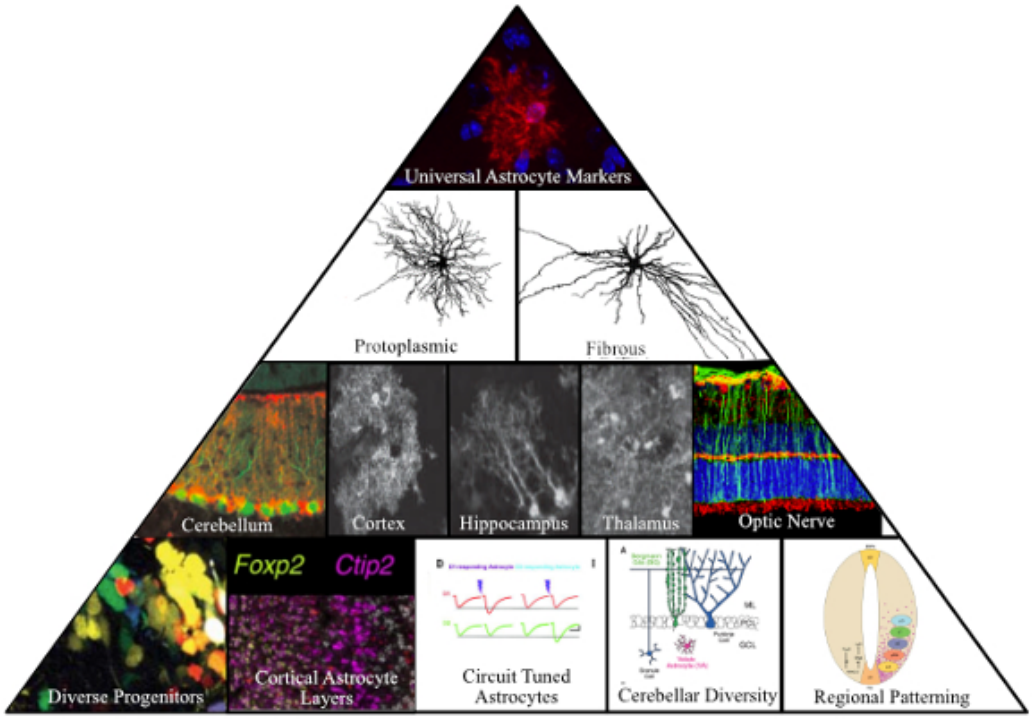

Figure 3: The evolution of our understanding astrocyte heterogeneity. The levels of the pyramid here represent how our understanding of astrocyte diversity has grown. The top level represents astrocytes as they were previously considered, a homogenous population that shares universal makers such as aldh1. In level two, we see the early classification of astrocytes into two broad subtypes, fibrous and protoplasmic (Flectcher, 2007). In level three, we see inter-regional morphological diversity that was made apparent by new IHC markers and intracellular stains (Emsley & Macklis, 2006; Hippert et al., 2015; Lippman Bell et al., 2010). Level four describes where modern research is currently focused regarding astrocyte diversity. Recent work has highlighted the developmental origins of diversity and intra-regional heterogeneity in both form and function (Bayraktar et al., 2020; Farmer et al., 2016; García-Marqués & López-Mascaraque, 2013; Martín et al., 2015; Rowitch, 2004).

Chapter 3: *Lcn2CreERT2*: A Novel Tool for Assessing Reactive Astrocytes

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Abstract

Reactive astrocytes (RAs) respond to all of the immune challenges the brain faces. Despite being identified over 50 years ago, much remains to be understood about their function in different disease and inflammatory contexts. Our ability to study RAs has been hindered by a lack of tools which allow us to specifically target them. *Lipocalin-2* (*Lcn2*) was recently identified as a gene that is upregulated in RAs, but not expressed in the healthy brain. Using this knowledge, we developed an *Lcn2CreERT2* mouse, which expresses a tamoxifen-dependent Cre recombinase under the *Lcn2* promoter. Using this tool, we were able to identify reactive astrocytes following inflammation caused by a variety of insults. The biggest advantage of this model is that it can be used to permanently label RAs, which allowed us to perform longitudinal studies of astrocyte gene expression during and after the resolution of inflammation. These studies confirmed RAs remain in the CNS once reactive markers have returned to basal levels. In addition, the *Lcn2CreERT2* mouse was used to isolate reactive cells through flow cytometry.

These experiments represent only a fraction of the possible experiments made available by the *Lcn2CreERT2* mouse.

Introduction

Astrocytes are the most abundant cell in the central nervous system (CNS) where they serve as critical regulators of homeostasis (Sofroniew & Vinters, 2010). In a healthy CNS, astrocytes function by providing synaptic support, transporting nutrients and waste, and maintaining the blood brain barrier (BBB) (Engelhardt & Sorokin, 2009; Jessen et al., 2015; Panatier & Robitaille, 2016). The BBB isolates the CNS from the circulating immune system of the periphery, so the brain has evolved its own innate immune system where local glia are converted into immune responders (Escartin et al., 2021). This process is known as reactive gliosis, where microglia and astrocytes become reactive following inflammation. Reactive astrocytes are universal to any CNS inflammatory response, seen in all manner of perturbations such penetrating brain injuries, epilepsy and Alzheimer's disease (Binder & Steinhauser, 2006; Katsouri et al., 2020; Laird et al., 2008). Reactivity in astrocytes has historically been identified by the hypertrophy of its major processes and upregulation of GFAP (Anderson et al., 2014; Eng et al., 1971). In response to severe damage, astrocytes can migrate and polarize to form a glial scar, or even reenter the cell cycle and proliferate (Ferrer-Acosta et al., 2017; Wanner et al., 2013). Despite the prevalence of this response, many questions remain regarding the function of reactive astrocytes (RAs). Some evidence suggests that reactivity in astrocytes is harmful to CNS repair and recovery, whereas more recent data suggest that

preventing reactivity actually increases the damage caused by a brain injury (Katsouri et al., 2020; Liddelow, Guttenplan, Clarke, Bennett, Bohlen, Schirmer, Bennett, Munch, et al., 2017; Okada et al., 2006; Shinozaki et al., 2017; Yun et al., 2018). Little is known about the differences in reactive responses across different disease models, with studies suggesting that there may be great variability in astrocyte gene expression in a disease dependent manner (Park et al., 2021; Yun et al., 2018; Zamanian et al., 2012). Furthermore, within a given disease astrocyte reactivity may vary both regionally and over time, with some astrocytes remaining completely unreactive (Sofroniew, 2020; Wheeler et al., 2020; Zamanian et al., 2012). While molecular markers associated with reactivity appear to return to baseline levels a month after the removal of an acute inflammatory stimulus, it remains to be elucidated whether that is due to programmed cell death of the RAs or changes in gene expression in the reactive cells (Zamanian et al., 2012). Following the fate of individual cells that become reactive requires the use of tools that specifically label astrocytes post gliosis

These outstanding questions regarding reactivity demand that we develop tools which will allow us to specifically target reactive astrocytes and observe their behavior, physiology, and gene expression over time. Previous tools used to study reactive astrocytes have the downsides of expressing also in healthy astrocytes and not being able to target astrocytes at specific time points (Cahoy et al., 2008; Robel et al., 2009; Sofroniew, 2009).The recent identification of several genes that are upregulated only in reactive astrocytes has allowed us to address these pitfalls. *Lipocalin-2* (*Lcn2*), an iron chelator expressed abundantly in the peripheral immune system but importantly

expressed at negligible levels in the healthy brain, was identified as being upregulated strongly in astrocytes in mouse models of inflammation and stroke (Zamanian et al., 2012). Using this knowledge, we developed a *Lcn2CreERT2* mouse that expresses a tamoxifen-dependent Cre recombinase (Cre-ERT2) under the *Lcn2* promoter.

Using the *Lcn2CreERT2* mouse, we were able to examine reactive astrocytes across a variety of disease models and even isolate labeled cells from LC animals to be used for single cell sequencing. More importantly, this new mouse line can be used to permanently label reactive astrocytes soon after a brain insult, and follow them over time. This allows for longitudinal studies of reactive astrocyte morphology, physiology and gene expression after the onset of a particular neuropathology. Using this type of approach, we were able to show astrocytes that were labeled at the height of reactivity remain in the CNS after acute inflammation is resolved, while their expression of reactive markers returns to a basal level. Overall, our experiments revealed that the majority of RAs remain alive after the resolution of inflammation, but their gene expression and morphology appear to go back to baseline. Our data also highlights the great potential of the *Lcn2CreERT2* mouse to advance our understanding of astrocyte reactivity.

Results

Validation of Lcn2 as a reactive marker and design of the Lcn2CreERT2 mouse

Lcn2 expression has been previously shown to be upregulated hundreds of folds in mouse models of stroke and systemic inflammation (Liddelow, Guttenplan, Clarke, Bennett, Bohlen, Schirmer, Bennett, Münch, et al., 2017, Zamanian et al, 2012). To

confirm that *Lcn2* is strongly upregulated in astrocytes following an inflammatory insult, swiss webster mice were injected with 5 mg/kg LPS or equivalent saline IP and collected after 24 hours. Collected forebrains were analyzed for expression of *Lcn2* along with the astrocyte marker *SLC1a3* and the endothelial cell marker *PECAM1* by means of fluorescent *in situ* hybridization (FISH) using RNAscope kit from ACD bio (Figure 3.1A). While *Lcn2* expression in saline treated brains was negligible, it was notably upregulated in LPS treated mice over 266 fold. In the paper that identified *Lcn2* upregulation in reactive astrocytes, potential expression in endothelial cells was also noted (Zamanian et al., 2012). Our results supported this, as upregulation was primarily observed in astrocytes (*SLC1a3*+) and endothelial (*PECAM1*+) cells. After validating the strong upregulation of *Lcn2* only in LPS treated animals, we decided to target the *Lcn2* locus by homologous recombination to design an *Lcn2CreERT2* mouse. To generate this mouse, a CreERT2 transgene followed by a P2A signal was targeted downstream of the endogenous *Lcn2* promoter and start codon, but upstream of the rest of the first coding exon (Figure 3.1B). Under this design, it is expected that the transgene will drive the expression of a tamoxifen inducible Cre in cells where *Lcn2* is expressed, without affecting endogenous *Lcn2* expression.

Validation of the Lcn2CreERT2 mouse during systemic inflammation

To begin to characterize our newly developed mouse line, the *Lcn2CreERT2* mouse was crossed into the Cre-reporter line *Ai9*. This cross generates mice that express the red fluorescent tdTomato reporter in a Cre-dependent manner. Using the progeny from this cross, we tested the expression of *Lcn2CreERT2* following a low dose LPS

treatment protocol. In this protocol, mice were given LPS dissolved in saline 5 consecutive days in order to induce sustained inflammation, and treated with tamoxifen dissolved in corn oil in order to allow expressed Cre to be active (Nava Catorce & Gevorkian, 2016). To ensure that any expression was dependent on treatment of both LPS and tamoxifen, control animals were given saline and tamoxifen, saline and oil, or LPS and oil. Following the last day of treatment, animals were collected and their brains stained for expression of tdTomato. These stains revealed that tdTomato was strongly upregulated in animals treated with both LPS and tamoxifen, however its expression was negligible in all other treatment groups (Figure 3.2A-B). This result suggests that *Lcn2CreERT2* dependent recombination occurs in an inflammation and tamoxifen dependent manner. We observed the most robust expression of our Cre dependent reporter in the thalamus, and so decided to focus future experiments on this region.

Next, we wanted to explore which cell types were expressing our Cre dependent reporter. *Lcn2CreERT2*;*Ai9* brains from animals that received LPS and tamoxifen were stained for the neuronal marker NeuN, the astrocyte marker GFAP, and the endothelial/microglial marker tomato lectin (Figure 3.2 C-D). No tdTomato expressing cells were found to be NeuN positive, where as 51 ± 2.2% of tdTomato cells were copositive for lectin and 45 ± 1.8% of tdTomato cells were copositive for GFAP. The morphology of the lectin positive cells suggested that they were most likely endothelial cells, whereas the GFAP positive cells appeared to be astrocytes. To further confirm the identity of these cells we performed RNAscope staining, looking at expression of *SLC1a3* and *Pecam1* in the brains of the LPS and tamoxifen treated *Lcn2CreERT2* ;*Ai9*

mice (Figure 3.2 E-F). Using this technique, 47 ± 1.3% of identified tdTomato positive cells were shown to be copositive for *SLC1a3*, whereas 51 ± 1.3% were copositive for *Pecam1*. Taken together, these results suggest that *Lcn2CreERT2* is expressed in the CNS only after an inflammatory insult and when tamoxifen is present, and that its expression is found primarily in astrocytes and endothelial cells.

Expression of Lcn2CreERT2 in other disease models

To determine if the *Lcn2CreERT2* mouse would function as a good tool for studying reactive astrocytes in different inflammatory contexts, we next wanted to examine the expression of *Lcn2CreERT2* in other disease models. While several models were briefly examined (Figure 3.7), here we highlight two in particular: Parasitic infection by *Toxoplasma gondii*, and direct CNS exposure to LPS through striatal LPS injections.

In order to test whether *Lcn2CreERT2* mice will be useful in studying chronic inflammation, *Lcn2CreERT2*;*Ai9* mice were infected with 20 *toxoplasma gondii* cysts via IP injection or given a sham injection of saline. *T. gondi* infection provides a model of chronic inflammation that is human disease-relevant, as one third of the world's population is currently estimated to be infected (Hofhuis et al., 2011). Three weeks following the injection with *T. gondi,* animals were given tamoxifen 3 times a week for 4 weeks and collected after the last treatment. Expression of tdTomato was primarily restricted to infected animals while being negligible in the sham controls (Figure 3.3A). Of the tdTomato positive cells, zero were found to be NeuN positive, 64 ± 3.4% were copositive for GFAP and 37 ± 1% were copositive for tomato lectin (Figure 3.3 B-C).

This suggests robust upregulation of *Lcn2CreERT2* following long term *T. gondi* infection. Importantly, this also revealed that *Lcn2CreERT2* is not induced by long term treatment of tamoxifen alone.

We also looked at directly exposing the CNS to LPS via striatal injections. We reasoned that this could serve as a robust model of encephalitis. This model was adapted from Hunter *et al*. who showed that striatal LPS injections could induce dramatic inflammation, followed by dopaminergic neuronal loss in the substantia nigra (Hunter et al., 2009). *Lcn2CreERT2*;*Ai9* animals were given tamoxifen for three days. On the second day, four LPS or saline injections were made into the striatum. One week after the injections, brains were collected and stained for tdTomato. Expression of tdTomato could be found broadly and abundantly in the CNS of LPS injected animals, whereas expression was much lower in the saline injected animals and restricted to the injection site (Figure 3.3D-E). Taken as a whole, these results suggest that there are multiple disease models for which the *Lcn2CreERT2* mouse can be used to examine reactivity.

Combinatorial approaches with the Lcn2CreERT2 mouse

While *Lcn2CreERT2* expression appears only after an immune challenge, IHC and FISH revealed non-astrocyte cells, in particular endothelial cells, also were also Cre positive. In order to address this, we designed several adeno-associated viruses (AAV) that express Cre dependent genes under the GFAP promoter (Table 3.1). There are several benefits to this combinatorial approach. First, the use of the GFAP promoter to drive our Cre dependent genes ensures expression of our recombinase target only in cells that are expressing both the astrocyte specific GFAP and *Lcn2CreERT2*. Second, we can

target specific brain regions by injecting our designed AAVs stereotactically, or we can drive broader expression of the gene by performing intraventricular injections in neonatal pups (Stoica et al., 2013). Finally, because AAVs can be cloned into with relative ease, we are able to customize the Cre-dependent expression of any gene of interest that we would want expressed.

In order to validate this approach, *Lcn2CreERT2* animals were injected into the right thalamus with AAV-*GFAP-lsl-GCAMP7* which drives the expression of the calcium indicator GCAMP7 only in cells expressing GFAP that are also positive for Cre expression (Helassa et al., 2016). Three weeks following injection of the virus, mice were gavaged with tamoxifen for 3 consecutive days, and given 5 mg/kg LPS or equivalent saline on the second day of tamoxifen. Three days after the final tamoxifen treatment, animals were collected and their brains were stained for GFP. Expression of GFP was much stronger in LPS treated animals, although there was some expression in both saline and LPS treated animals around the injection site (Figure 3.4C). This result suggests that the combinatorial approach will be a powerful and adaptable method for using the *Lcn2CreERT2* mice to study reactive astrocytes.

Flow sorting of labeled cells using the Lcn2CreERT2 mouse

The ability of the *Lcn2CreERT2* mouse to label reactive cells with a fluorescent maker opens the door to fluorescent-activated cell sorting (FACS) through flow cytometry, which allows us to isolate permanently labeled cells and characterize them. As proof of this concept, *Lcn2CreERT2*;*Ai9* animals underwent low dose LPS. 24 hours after the final LPS treatment, animals were sacrificed and their brains were prepared for cell

sorting. Isolated cells were stained for DAPI in order to create a live/dead gate. The prepared cells were FACS sorted and tdTomato positive, DAPI negative cells were collected, ranging from 1.5 to 3% of the total cells ran (Figure 3.5). Overall, more than14000 cells were isolated and prepared for sequencing. These results show the potential of the *Lcn2CreERT2* mouse to allow us to isolate and sequence reactive astrocytes in different disease contexts.

Reactive astrocytes remain in the CNS following resolution of inflammation

The fate of reactive astrocytes after inflammation is resolved has been a major unanswered question in the field (Escartin et al., 2021). While reactive markers return to basal levels, whether this is a result of RAs dying and being replaced, or downregulation of reactive markers within RAs cannot be determined without a tool that permanently labels astrocytes at the time of gliosis. In order to address this, we performed low dose LPS treatment on *Lcn2CreERT2*;*Ai9* animals, labelled them during the early stages of inflammation by tamoxifen gavage, and collected them at two separate time points: 24 hours after the final LPS treatment when *Lcn2* should still be upregulated, and 4 weeks after the final LPS treatment when reactive markers have returned to their basal level (Zamanian et al., 2012). While expression of tdTomato remained negligible in saline treated animals, it was robustly expressed in animals collected at both 24 hours and 1 month (Figure 3.6 A-B). Furthermore, expression at one month was not significantly different compared to 24 hours (Figure 3.6 B, $t(4)=0.32$, $p=0.38$). These results indicate that cells that had expressed *Lcn2CreERT2* remain in the CNS long after inflammation should have resolved.

Next, we wanted to investigate whether the remaining cells maintained their expression of reactive markers, or returned to a basal level. We preformed FISH on low dose LPS animals collected at 24 hours and 4 weeks after the final treatment, as well as saline treated control animals looking at the overall expression of *GFAP* and *Lcn2* (Figure 3.6 C-D). A one way ANOVA confirmed differences in fluorescence between the groups for both markers (*Lcn2:* $F(2,35)=27.98325$, $p<0.00001$, *GFAP*: $F(2,34)=9.26055$, $p=0.000616$). Tukeys HSD was then performed as post-hoc analysis to look for differences between the individual groups. In support of the findings made by Zamanian et al., 2012, *Lcn2* was upregulated in animals collected 24 hours after LPS treatment when compared to saline treated animals and animals collected at 1 month post LPS ($p=0.00000$ and $p=0.00000$ respectively). Furthermore, there was no significant difference in *Lcn2* expression between animals treated with saline, and animals treated with LPS collected 1 month after treatment ($p=0.99989$, Figure 3.6 D). Similarly, *GFAP* was significantly upregulated in LPS treated animals collected at 24 hours relative to other groups ($p=0.00145$, $p=0.00293$) with no difference between saline animals and animals collected 1 month after LPS ($p=0.96454$). These results confirm that upregulated reactive markers return to a basal level.

The overall changes in these reactive markers between these time points suggested that cells that had previously been reactive return to basal levels of expression. To confirm this, we identified cells in LPS treated animals collected at both 24 hours and 1 month that were expressing tdTomato, indicating that they had at one-point upregulated *Lcn2*. We looked at both *Lcn2* and *GFAP* expression in these cells from animals collected

at both time points. *Lcn2* fluoresce was much higher in cells from animals collected at the 24 hour time point (Figure 3.6 E-F, $t(29)=2.90272$, $p=0.002612$). Similarly, *GFAP* was also upregulated in tdTomato positive cells collected at 24 hours relative to those collected at 1 month (Figure 3.6 G-H, $t(29)=4.91487$, $p<0.00001$, Figure 3.6E-H). These results confirm that reactive markers are transiently upregulated in astrocytes following inflammation. Furthermore, we can confirm that astrocytes which upregulate reactive markers following inflammation survive in the brain while no longer strongly expressing reactive markers after inflammation is resolved.

Discussion

Reactive astrocytes are a universal response of the brain to any immune challenge, including infection, trauma, and even neurodegenerative disorders like Alzheimer's (Sofroniew, 2020). While astrocyte reactivity has been observed in many contexts, we still understand very little about how this response functions in each of them (Escartin et al., 2021). An astrocyte reacting to a beta amyloid plaque may upregulate GFAP in the same way one does when it's activated by a penetrating brain injury, but questions remain about how similar these responses really are, and these questions can only be answered by examining reactivity in a context dependent manner in great detail. In order to do so, we need tools which allow us to target reactive astrocytes specifically. As demonstrated here, the *Lcn2CreERT2* mouse represents an important first step in the development of these tools.

Lcn2CreERT2 shows no significant basal expression in the CNS, and is strongly upregulated in a number of different disease contexts. This is a key aspect of *Lcn2CreERT2*, as previous tools to study reactive astrocytes have relied on markers such as *GFAP* or *Aldh1l1*, which are already expressed in the healthy CNS (Brambilla et al., 2005; Cahoy et al., 2008; Robel et al., 2009; Sofroniew, 2009; Srinivasan et al., 2016). Astrocytes play a critical role in brain homeostasis, and any tool that effects astrocytes before they become reactive has the potential downside of impacting those serving their regulatory functions. Furthermore, after most insults there is a mix of reactive and healthy astrocytes present in the brain, so even when using tools for which timing can be controlled (e.g. CreERT2 lines), both types of astrocytes will be targeted under those pan-astrocytic promoters. The *Lcn2CreERT2* line takes advantage of the minimal expression of *Lcn2* in the healthy nervous system to ensure that Cre is only expressed after an immune insult and in reactive cells. This guarantees that Cre expression in astrocytes will only be turned on in a reactive setting.

When developing the *Lcn2CreERT2*;*Ai9* mouse, we decided to employ a CreERT2, a tamoxifen-dependent Cre recombinase (Figure 3.1B). Cre recombinases function by acting on loxp sites, where they can make cuts, invert, or translocate DNA sequences depending on loxp orientation and location (Nagy, 2000). A number of tools have already been developed which make use of Cre to drive recombinatorial gene expression, meaning the experiments performed with the *Lcn2CreERT2* mouse are highly customizable (Van Duyne, 2015). The mutant estrogen receptor ERT2 acts as a nuclear export signal until it is bound to tamoxifen, in which case it translocates Cre to the

nucleus allowing it to act on loxp sites (Feil et al., 1997). Having the *Lcn2CreERT2* mouse express a Cre-ERT2 allows us to control the timing of when our Cre is active. This approach opens up a number of experimental possibilities. Firstly, we can prevent Cre activity outside of our experimental window. This is an important step towards preventing off target recombination in other *Lcn2* expressing cells, such as the peripheral immune system. Secondly, we can examine different windows of astrocyte response during the course of a disease. For example, in the kainic acid model of epilepsy, after the induction of *status epilepticus* there is a week-long silent period before the development of progressive seizures (Coulter, Douglas, Steinhauser, 2015). By changing the timing of tamoxifen treatment, the *Lcn2CreERT2* tool would allow us to examine the differences in reactive astrocytes induced by the initial seizures compared to those that develop only at the chronic stage of the disease.

As astrocyte reactivity has been observed almost universally in response to CNS injuries and disease, the ideal tool for studying these cells should be expressed in reactive astrocytes regardless of the disease model. In addition to not being expressed prior to the induction of reactivity, here we demonstrate *Lcn2CreERT2* expression in several models of inflammation. *Lcn2CreERT2* is upregulated following IP LPS, *Toxoplasma gondii* infection, and intrastriatal LPS (Figures 3.2, 3.3). We also examined several other disease models that are not detailed in this paper, including kainic acid models of epilepsy, the 5XFAD mouse model of Alzheimer's, penetrating brain injuries, and developmental gliomas (Figure 3.7). While these models require further investigation, astrocytic expression of *Lcn2CreERT2* was identified in each of them. Future studies should

continue to expand the scope of disease investigated with the *Lcn2CreERT2* mouse, which will allow us to determine differences in how astrocytes are responding.

The ability of the *Lcn2CreERT2*mouse to permanently label reactive astrocytes with a fluorescent marker opens up a number of experimental possibilities. Here we employ this technique to great effect for several experiments. First, we were able to isolate labeled cells for sequencing using FACS. Isolation of reactive astrocytes will allow us to perform single cell sequencing in the future, and look in detail at changes in gene expression across different disease contexts. Secondly, we were able to answer a long-standing question in the field by performing longitudinal studies on RAs after inflammation. Using this approach, we observed that previously reactive astrocytes remain in the CNS after the resolution of inflammation, and demonstrated that the expression of reactive markers reaches a basal level in these cells.

The observation that activated cells remain in the CNS opens the door to several exciting experiments. Reactive astrocytes undergo a number of functional changes which may impact their normal homeostatic functions (Sofroniew, 2015). It is currently unknown if a reactive astrocyte is able to return to its normal role after inflammation is resolved, a question that the *Lcn2CreERT2* mouse will allow us to examine in the future. Fluorescent labeling also creates the possibility of observing changes to reactive astrocytes in real time through the use of two-photon microscopy, allowing us to look at some controversial aspects of reactivity such as migration, proliferation, and changes in astrocyte connectivity.

The *Lcn2CreERT2* has two limitations when it comes to the study of reactive astrocytes. Although Cre was expressed in a number of different disease models, the expression of *Lcn2CreERT2* vastly under-represents the total number of reactive astrocytes compared to upregulation of other reactive markers such as GFAP. Thus, we are unable to use the *Lcn2CreERT2*;*Ai9* mouse for experiments that would remove all reactive astrocytes to observe disease progression in their absence. The second limitation is the expression of *Lcn2CreERT2* in non-astrocytic cells. *Lcn2CreERT2* seems to be strongly expressed in endothelial cells following LPS, which was expected from analyzing endogenous Lcn2 expression. In order to address this, we employed a combinatorial approach which uses AAVs to drive the expression of Cre dependent genes under the GFAP promoter. The use of this approach allowed us to prevent LPS induced endothelial expression of the Cre dependent gene, in this case the calcium indicator GCAMP7. As we were able to observe robust expression of GCAMP7 in astrocytes using this approach, we hope to study changes in astrocyte calcium signaling in the near future.

In summary, the *Lcn2CreERT2* mouse is a powerful new tool for studying reactive astrocytes which opens up a broad range of experimental possibilities. Future studies will apply this tool to sequence reactive astrocytes in different disease contexts, observe changes in their physiology and connectivity, determine the progression of reactivity in chronic illness, and much more. The *Lcn2CreERT2* mouse can be used in combination with genetic tools that ensure expression is restricted to astrocytes, and allow for targeting of specific regions during specific timepoints. The deep pool of conditional KO mice and Cre-dependent tool that can be readily delivered through viral

approaches allows experiments with the *Lcn2CreERT2* mouse to be highly customizable. In all, the *Lcn2CreERT2* mouse represents a critical first step towards understanding astrocyte reactivity.

Materials and Methods

Animals

Wild-type swiss webster mice were used for histology to examine LCN-2 expression in the naive and inflamed brain. *Lcn2CreERT2*animals were maintained in the swiss webster background and crossed into the Ai9 tdtomato reporter line available from JAX for experiments. Cre negative controls were taken from *Lcn2CreERT2* negative littermates, while saline controls were performed on Cre positive littermates. All procedures occurred when animals were 4-6 months of age. All animal procedures detailed in the following sections were preformed according to the University of California, Riverside's Institutional Animal Care and Use Committee (IACUC) guidelines.

Generation of Lcn2CreERT2 Mice

The mouse line carrying a CreErt2 between the *Lcn2* promoter and the 5' untranslated region (UTR) of the *Lcn2* gene was generated using the vector pBS-PGK-DTA-pa, modified by Bac recombineering (Liu et al., 2003). The *Lcn2-CreErt2*^{neo} allele was engineered to encode for an Ert2 tagged Cre recombinase upstream of the 5' UTR and an FTR-flanked *neomycin* cassette. Germline transmission of the *Lcn2-CreErt2*^{neo} allele was verified by southern blot and a PCR genotyping strategy. In order to eliminate the *neomycin* cassette, *Lcn2-CreErt2*$^{neo/+}$ animals were crossed to the deleter mice carrying

ACT-FlpE, which expresses FlpE recombinase under the ACTB promoter. Expression of FlpE generates the *Lcn2CreERT2* allele by removal of the *neomycin* gene while leaving a single FRT site in the genomic DNA. All breeding at the chimera stage were done into the C57/B16J mouse strain and then were backcrossed into the swiss webster background. PCR genotyping of the *Lcn2CreERT2* mouse was done using the following primers: *Lcn2CreERT2* Forward: 5'-GGC AGT CCA GAT CTG AGC TGC-3' and *Lcn2CreERT2* Reverse: 5'-TGC ATC GAC CGG TAA TGC AGG-3'.

Treatments and Surgeries

Low dose LPS

Lcn2CreERT2 animals crossed into the Ai9 tdTomato reporter line were separated into 4 treatment groups; Oil and Saline, Oil and LPS, Tamoxifen and Saline, and Tamoxifen and LPS. Animals in the two tamoxifen treated groups were given an oral gavage of 150 ug/g of tamoxifen (Sigma Aldrich) dissolved in corn oil while animals in the two oil groups received an equivalent dose of corn oil. Tamoxifen/oil treatments occurred every 24 hours for 6 consecutive days. 24 hours after the first tamoxifen or oil treatment, animals in the two LPS treatment groups received intraperitoneal (IP) injections of 1.5 mg/kg lipopolysaccharide (Sigma Aldrich) dissolved in sterile 1x phosphate buffered saline (PBS) while animals in the two saline treated groups received an equivalent dose of sterile 1x PBS. LPS/saline treatments occurred every 24 hours for 5 consecutive days. Animals were collected for tissue processing 24 hours to 4 weeks following the final treatment.

Toxoplasma gondii injections

The Me49 strain of *T. Gondii* was prepared from chronically infected CBA/ca mice and *Lcn2CreERT2* mice crossed into the Ai9 tdtomato reporter were infected intraperitoneally with 20 cysts or given sham IP injections. 3 weeks following the initial infection date, mice were given 150 ug/g tamoxifen dissolved in corn oil. Tamoxifen treatments continued 3 times a week for 4 weeks. 24 hours after the final tamoxifen treatment, animals were collected for tissue processing.

Intrastriatal LPS

Lcn2CreERT2 animals crossed into the *Ai9* tdTomato reporter line were given 150 ug/g tamoxifen dissolved in corn oil every 24 hours for 3 consecutive days. Striatal LPS was injected as described in Hunter *et al*. 2009. Briefly, on the day of the second tamoxifen treatment animals were stereotactically injected with 1 µl of sterile saline or 1µl of 7.5 mg/ml LPS in four locations in the striatum using the following coordinates from Bregma: a/p +1.18 mm, m/l +/– 1.5 mm, and d/v –3.5 mm as well as a/p –0.34 mm, m/l +/– 2.5 mm, and d/v –3.2 mm. Injections were performed at 0.5 µl/minute and the needle was kept in place for 5 minutes prior to withdrawing. Animals were kept on a heating pad and received sterile saline subcutaneously until they became hydrated and free moving. 7 days after the injections, animals were collected for tissue processing.

Stereotactic injections

Stereotactic injections were performed as previously described on *Lcn2CreERT2* animals. Mice were put under isoflurane anesthesia for the duration of the procedure. Injections of AAV-GFAP-lsl-GCAMP7 (1.3×10^{13}) were performed targeting the right thalamus (coordinates: A/P -1.70 mm, M/L -1.00 mm, D/V -3.00 mm). Mice were

injected with 250 nl using a Hamilton Neuros 32 gage syringe at 75 nl/min. Three weeks after injection, animals were treated with tamoxifen and LPS as described above.

Flow Cytometry

To isolate astrocytes, mouse brains were perfused with sterile 1x PBS and placed in 1% RPMI. Perfused brains were transferred to a chilled petri dish and diced with razor blades before being treated with 4 ml of 0.25% tripsin. Brains in tripsin were moved using a P1000 pipet into 50 ml conical falcon tubes and digested at 37°C for 30 minutes. During digestion, the mixture was inverted every 5 minutes. Tripsin digestion was stopped with the addition of 30 ml of 20% RPMI. The mixture was then centrifuged at 1000 rpm for 5 minutes at 4°C, the supernatant was decanted and the pellet was resuspended in 1% RPMI to a total volume of 7 ml. The suspension was transferred to a conical tube with 3 ml of 100% Percoll and gently mixed through pipetting up and down. Following mixing, 1 ml of 70% Percoll was underlaid at the bottom of the tube. Tubes were centrifuged at 2500 RPM for 20 minutes at 4°C, creating a top myelin later, and a layer containing glial cells at the surface of the underlaid 70% Percoll. Layers above the formed glial layer were aspirated and the glial layer and remaining Percoll were diluted in 1% RPMI before pelleting the cells through 10 minute centrifugation at 2000 RPM for 10 minutes at 4°C. The supernatant was aspirated and the cells were resuspended in FACS buffer for cell sorting. DAPI was added to the FACS buffer to create a live dead gate. The samples were analyzed using the MoFLo Astrios EQ Cell Sorter and data was analyzed using FlowJo 10.1.

Fluorescent in situ Hybridization

Fluorescent *in situ* hybridization was performed on mouse tissue using the RNAScope LS Multiplex Assay (Advanced Cell Diagnostics, ACD). In this assay, the target RNAs are hybridized to single stranded DNA "z-probes" composed of a complementary approximately 20 nucleotide sequence to the RNA of interest, a spacer sequence, and a 14 nucleotide tail region. 28 nucleotide -reamplifier olgios bind to the tail region of z-probe pairs bound to adjacent sequences in the RNA, which are then bound to amplifiers that are labeled with horse radish peroxidase (HRP) enzyme molecules. Tyramide conjugated fluorophores are added, leading to HRP enzymatic conversion of tyramide into a highly oxidized intermediate which covalently binds to proteins near the HRP label thus depositing a large number of detectable fluorophores. Multiple RNA targets can be labeled with the use of detection probes in different channels with distinct tail sequences which allow for the generation of unique amplification trees for each target. Each target/probe is then sequentially developed using tyramide signal amplification by using channel specific HRP labels and tyramide conjugated dyes. At the end of signal development, some sections were selected for immunohistochemistry as described below.

Immunofluorescence

Mice were perfused and extracted brains were fixed with 4% paraformaldehyde for 2 hours to overnight at 4°C, rinsed, and sunk in 30% sucrose overnight prior to being frozen in Optimum Cutting Temperature media for cryopreservation (Tissue-Tek). Coronal sections (20 μm) were obtained on a Leica CM3050 cryostat. Sections that were

immediately used for immunohistochemistry were next blocked in 10% goat serum and 0.1% Triton-X100 for 1 hr at room temperature. This step was skipped for sections that had previously used for FISH, but all following steps were the same. Sections were then incubated overnight at 4°C with primary antibodies. Following incubation, sections were washed with PBS and incubated with secondary antibodies and DAPI. Sections were washed in PBS and mounted using vectorshield hard-set fluorescence mounting medium (Vector Laboratories). Confocal fluorescence images were taken using a Leica SPE II microscope. Primary antibodies used in this study include rabbit anti-DSRed (Takara Bio, 1:500), rat anti-GFAP (Invitrogen 1:1000), chicken anti foxo3 (EnCor 1:1000), and chicken anti-GFP (AVES, 1:1000). Tomato-lectin (Vector Laboratories, 1:75), was combined with primary antibodies as described in (Robertson et al., 2015; Villacampa et al., 2013).

Image Analysis

All images were analyzed using FIJI (Schindelin *et al*., 2012). Cells expressing tdTomato were counted by thresholding to create a binary image, the watershed function was used and followed by the analyze particles function to count cells with a size between 20-2000 pixels^2. Colocalization of IHC markers was performed by manual counting. Cell expressing tdTomato were identified and their area was turned into outlines. Cells showing expression of markers labeled in the green or far red channel within the outlined areas were then manually counted. Total expression of RNAs identified through FISH was performed by analyzing total fluorescent area in selected ROIs. ROIs were drawn around the thalamus. Background fluorescence was subtracted

with the subtract background function. The measure function was then used to look at total fluorescent area in the drawn ROI. For measuring expression of reactive markers within tdTomato positive astrocytes, the tdTomato positive cells were outlined and the outline was turned into an ROI. The fluorescent channel corresponding to the RNA was then made binary, watershed, and particles were analyzed within the ROI using the analyze particles function. All counts are reported with ± standard error.

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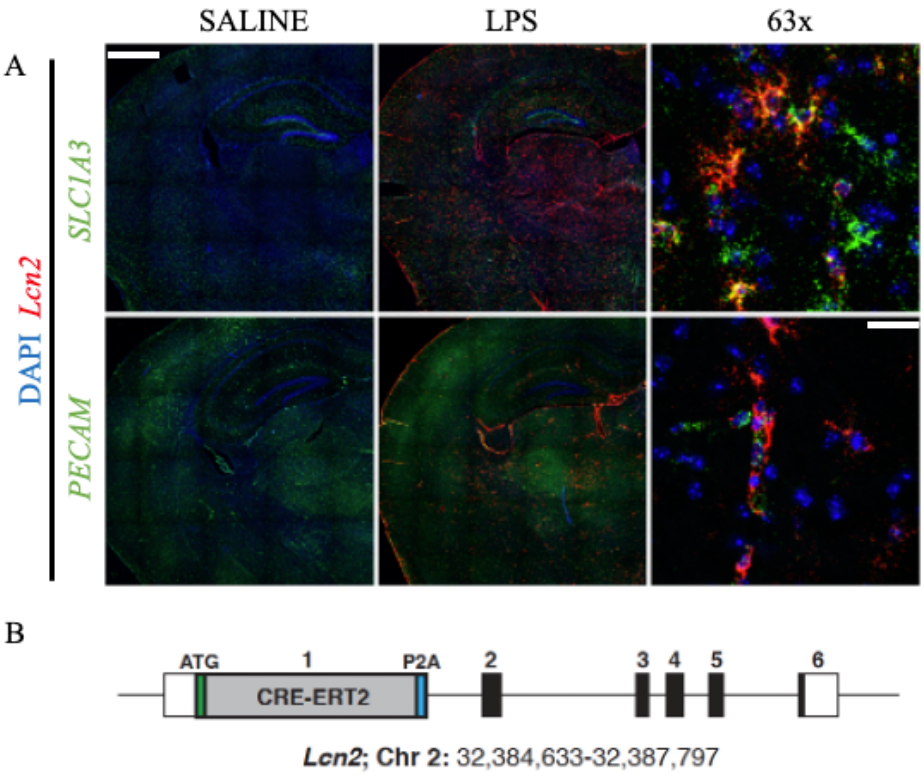

Figure 3.1: Validation of LCN-2 and Design of the Lcn2CreErt2 mouse. (A) Swiss webster animals were given IP injections of LPS or saline and collected after 24 hours. Prepared tissue was then used for fluorescent *in situ* hybridization to detect the expression of *Lcn2*, and the astrocytic marker *SLC1A3* (top) or the endothelial cell marker *PECAM1* (bottom). Expression of *Lcn2* (red) was observed only in LPS treated animals, in both *PECAM1* and *SLC1A3* positive cells. Scale bars: Top left: 250 µm. Bottom right: 15 µm. (B) Design of the *Lcn2CreErt2* transgene. CreErt2 followed by a P2A signal was targeted downstream of the *Lcn2* promoter and start codon, but upstream of the remainder of the first coding exon.

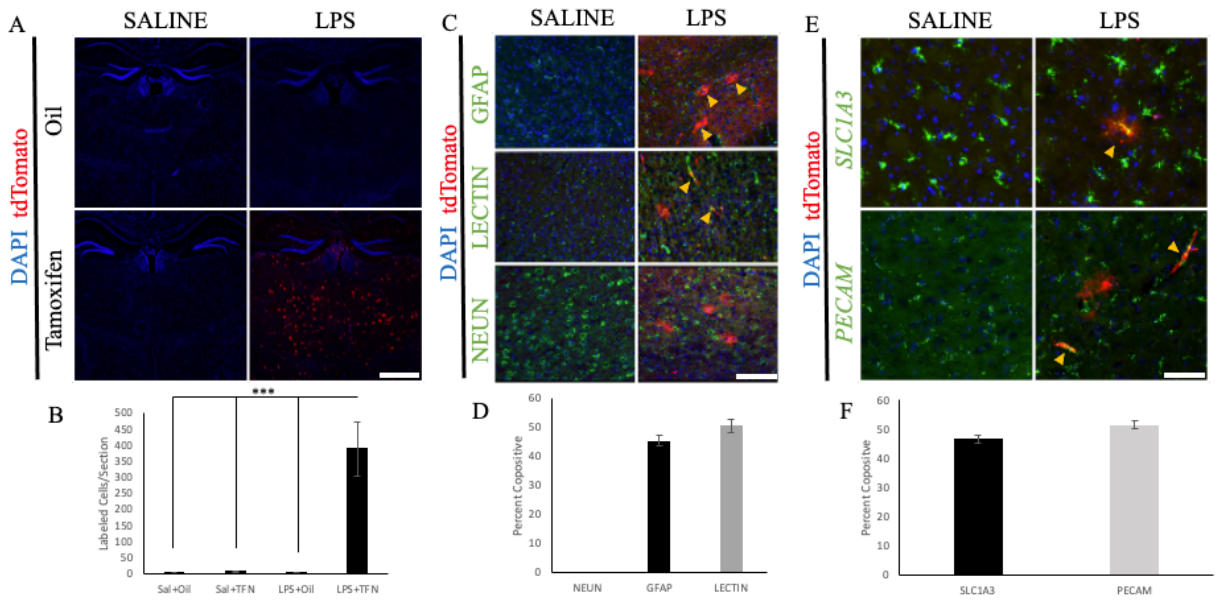

Figure 3.2: Expression of Lcn2CreErt2 following repeated low dose LPS. (A) *Lcn2CreErt2* mice were crossed with the *Ai9* reporter line. Mice from this cross were given one of the following four treatments for 5 consecutive days: Saline and corn oil, saline and tamoxifen, LPS and corn oil, LPS and tamoxifen. Saline and LPS dissolved in saline were given through IP injection, where as corn oil and tamoxifen dissolved in corn oil were given through oral gavage. 24 hours after the last treatment, animals were collected and brains were immunostained for tdTomato and counter stained with DAPI. (B) Quantification of (A), total number of red cells/ section focusing on thalamic regions. Significant differences marked by *, as revealed by one way ANOVA and Tukey's pos-hoc test. (C) Immunostaining for cell type markers on tissue collected from animals treated with Saline (left) or LPS (right) as described in (A). Sections were stained for tdTomato as well then the astrocytic marker GFAP (top), the endothelial and microglial marker lectin (middle), and the neuronal marker NeuN. Sections were counter stained with DAPI. Yellow arrows indicate copositive cells for tdTomato and the panel maker. (D) Quantification of copositive cells from (C). (E) FISH performed on tissue collected from LPS and saline treated animals to look at expression of astrocytic *SLC1a3* (top) and endothelial *PECAM1* (bottom). Following FISH, sections were immunostained for tdTomato and counterstained with DAPI. (F) Quantification of cells copositive for tdTomato and RNA markers described in (E). Scale bars: (A)-250 µm. (C)-50 µm. (E)-25 µm. All quantifications are shown +/- standard error.

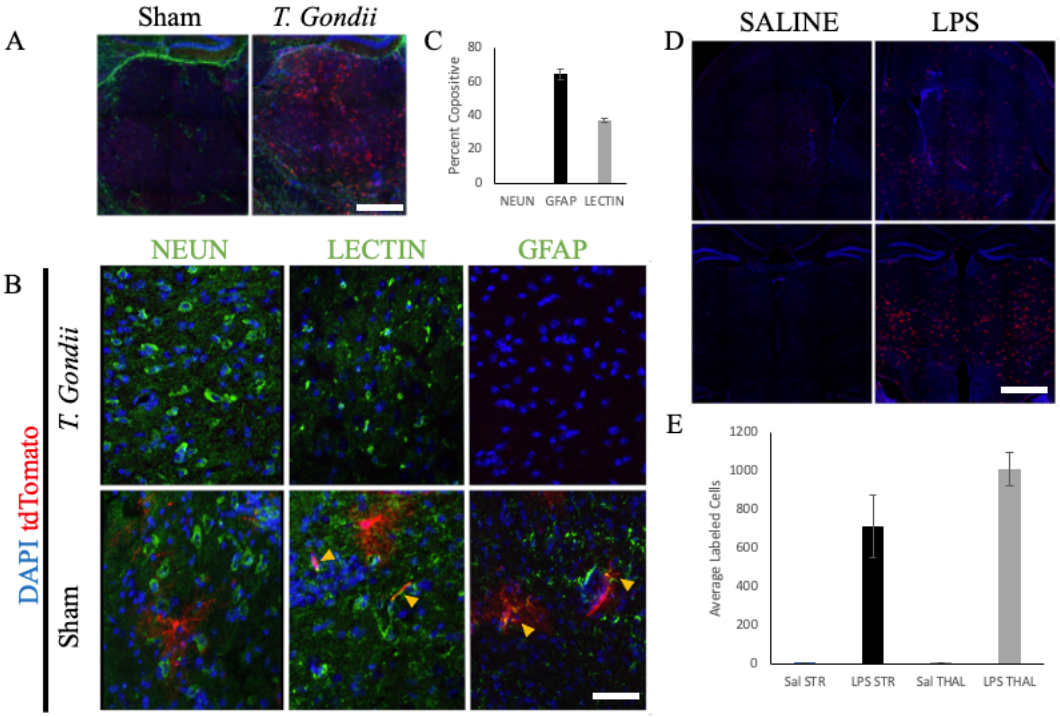

Figure 3.3: Expression of Lcn2CreErt2 in models of infection and striatal LPS. (A) *Lcn2CreErt2* mice were crossed with the *Ai9* reporter line, and mice from this cross were given IP injections of 20 *toxoplasma* cysts or a sham solution. 3 weeks after injection, mice were gavaged with tamoxifen 3 times a week for 4 weeks. Tissue was then collected and immunostained for tdTomato. (B-C) Tissue from animals described in (A) was immunostained for tdTomato and the neuronal marker NeuN (left), the endothelial and microglial marker lectin (middle), and the astrocytic marker GFAP (right). Copositive cells indicated by yellow arrows, quantified in (C). (D-E) Animals from the *Lcn2CreErt2* x *Ai9* cross were gavaged for 3 days with tamoxifen. On the second day, 4 striatal injections of LPS dissolved in saline or sterile saline were performed using a stereotactic frame. One week after injection, animals were collected and tissue was immunostained for tdTomato and counterstained with DAPI. (E) Quantification of total number of labeled cells in the striatum and thalamic regions. Scale bars: (A,D)- 250 μm. (B)- 50 μm. All error bars +/- SE.

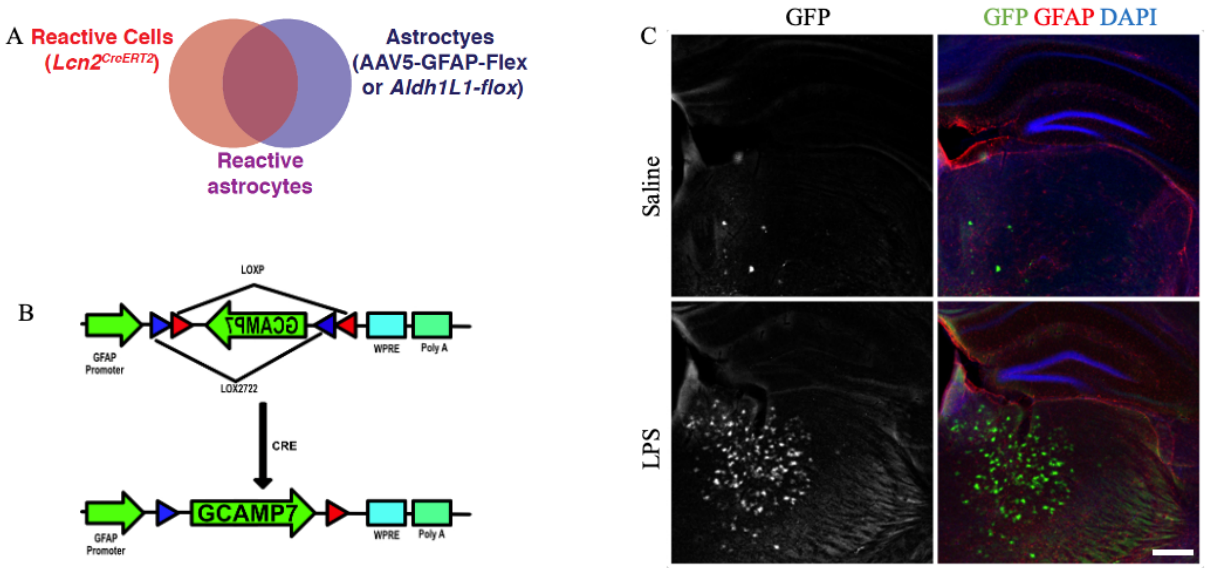

Figure 3.4: Combinatorial approaches for targeting reactive astrocytes. (A): Representative schematic of how reactive astrocytes can be specifically target via using the *Lcn2CreErt2* mouse in combination with tools that drive expression of Cre dependent genes under astrocyte specific promoter. (B) Example of combinatorial approach. Here, a virus has been used to deliver a transgene which expresses the calcium indicator GCAMP7 under the *GFAP* promoter in a Cre dependent manner. Cre acts on flanking LOXP and LOX2722 sites, inverting the GCAMP7 gene into a sense direction from which it can be transcribed. (C) Validation of combinatorial approach. *Lcn2CreErt2* animals were injected in the thalamus using a stereotactic frame with an AAV5-GFAP-Flex-GCAMP7 as described in (B). Three weeks following injection, animals were given tamoxifen and saline or LPS dissolved in saline as previously described. Tissue was collected and immunostained for GFAP, as well as GFP to reveal the expression of the GCAMP7 indicator, and counterstained with DAPI. Scale bar: 250 μm

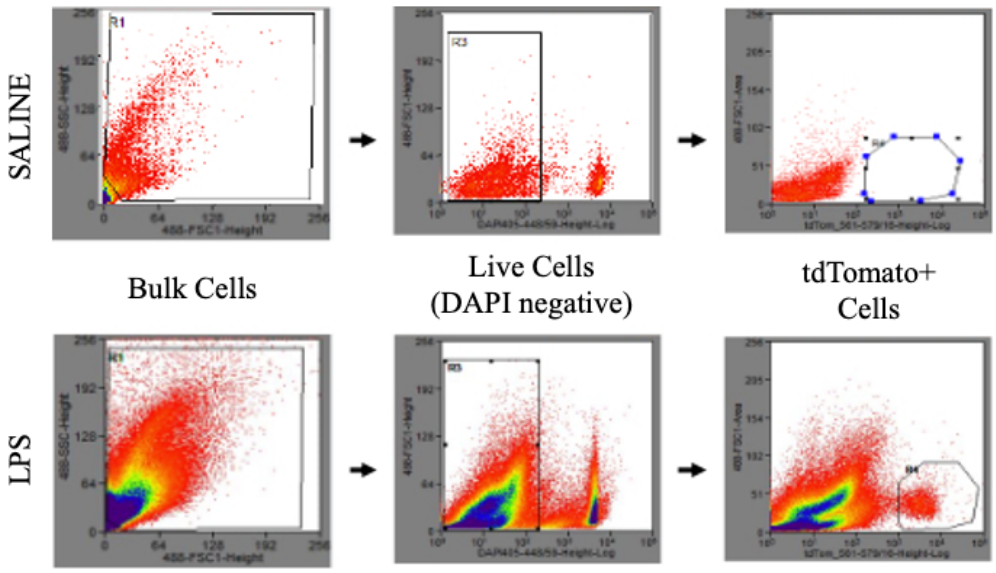

Figure 3.5: Isolation of reactive cells from the Lcn2CreErt2 mouse. *LcnCreErt2* x *Ai9* mice were treated with tamoxifen and saline or LPS dissolved in saline as described. Collected brains were prepared for FACS sorting through tissue dissociation, followed Percol gradients and filtration to size select for glia. Prepared cells were stained with DAPI in order to identify live vs dead cells. The work flow for FACS sorting tdTomato positive cells is shown here. First bulk cells were identified from the sample on the basis of forward versus side scatter. From the bulk cell population, the DAPI negative population was identified to isolate live cells. From the DAPI negative population, we then selected cells that were positive for tdTomato, only identified in LPS treated animals.

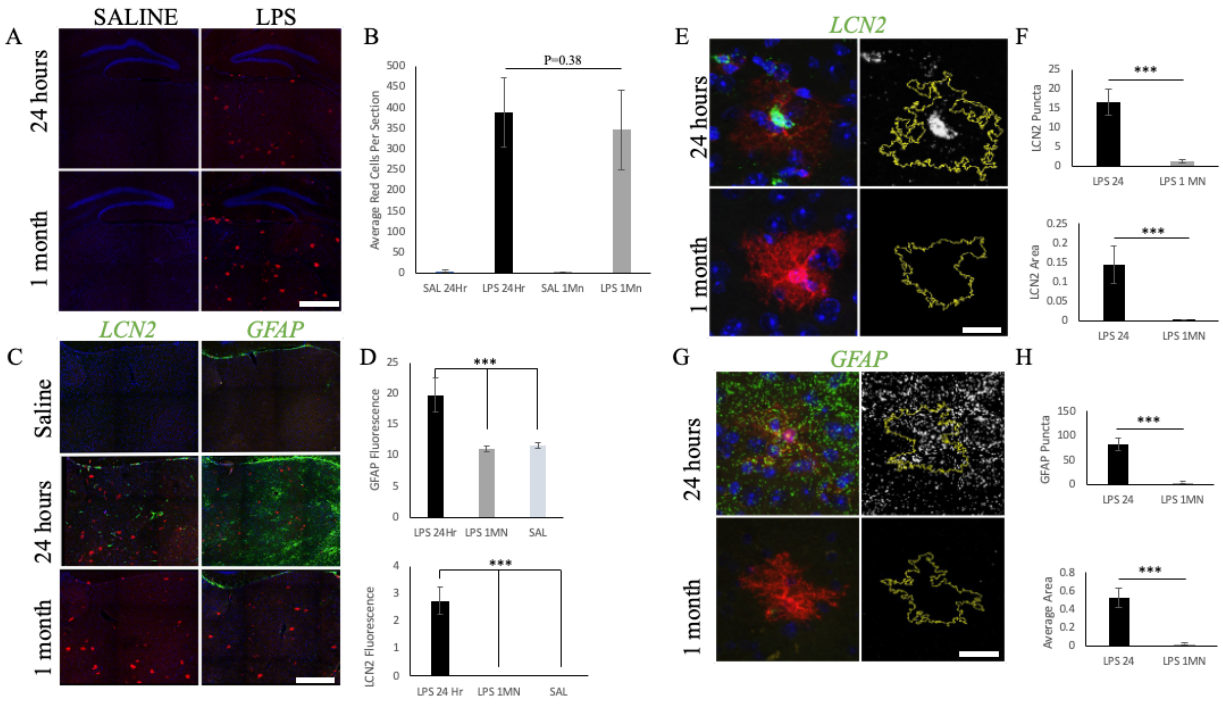

Figure 3.6: Reactive astrocytes remain in the brain after resolution of inflammation. (A-B): *Lcn2CreErt2* x *Ai9* animals were given tamoxifen and saline or LPS dissolved in saline as previously described. Animals were then collected at either 24 hours or 1 month after the last treatment and brain sections were immunostained for tdTomato and counterstained with DAPI. tdTomato positive cells in the thalamic region were then counted, shown in (B). (C-H): Tissue collected from the experiments described in (A) was used for FISH to examine expression of the reactive markers *GFAP* and *Lcn2*, followed by immunostaining of Tdtomato and counterstaining with DAPI. First, we looked at total expression of fluorescent markers used to label *GFAP* and *Lcn2* mRNA (C-D). Total fluorescent area was quantified for *GFAP* (D-top) and *Lcn2* (D-bottom) and differences were confirmed first by one way ANOVA followed by Tukeys HSD post-hoc. (E-H) Analysis of reactive markers within tdTomato positive cells. For each animal 10 tdTomato positive cells were identified and outlined (E,G- right), and expression of the reactive markers *Lcn2* (E-F) and *GFAP* (G-H) was quantified by looking at both number of fluorescent puncta (F,H-top) and total fluorescent area (F,H-bottom). Paired t-tests were performed to confirm any significant differences. All error bars are standard error. Significant differences are indicated by *. Scale bars: (A,C)- 250 μm, (E,G)-50 μm

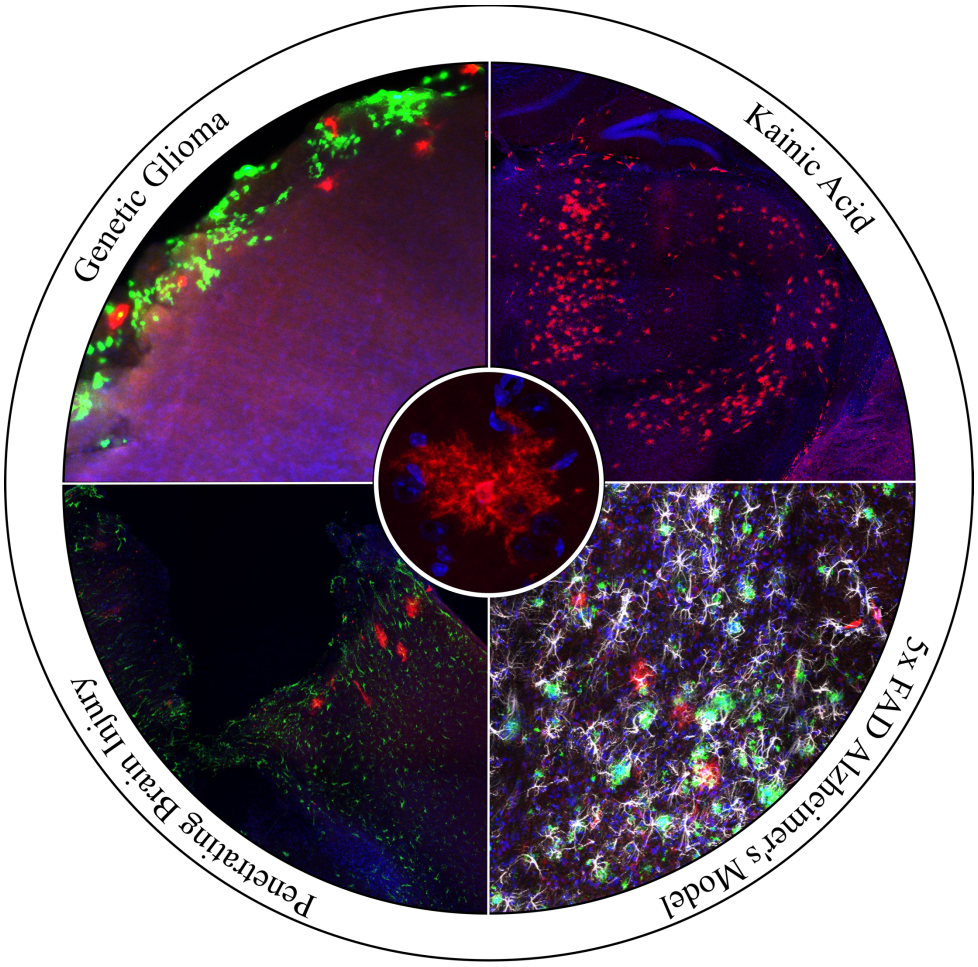

Figure 3.7: Lcn2CreErt2 expression in other disease models. Summary of other disease models briefly examined but not fully characterized. Animals in the *Lcn2CreErt2* x *Ai9* background were used for all studies. Genetic gliomas (top left) were induced through *in utero* electroporation of RAS oncogenes in combination with a GFP promoter. Mice were given tamoxifen at P7-10 and collected at P14, and tissue was immunostained for GFP, tdTomato and counterstained with DAPI. Kainic acid (top left) was stereotactically injected into the thalamus on the second of three days of tamoxifen treatments. 48 hours after the injection, tissue was collected, immunostained for tdTomato and counterstained with DAPI. Penetrating brain injury (bottom left) was created by using a blunted 20 gage needle to stab into the left temporal lobe. Injuries occurred on the second of three days of tamoxifen treatment, and 48 hours after the last treatment animals were collected and brains were immunostained for GFAP (green), tdTomato, and counterstained with DAPI. A mouse model of Alzheimer's, 5xFAD (bottom right), which expresses 5 mutations to familial Alzheimer's disease genes leading accumulation of beta amyloid plaques. *Lcn2CreErt2* x *Ai9* animals were crossed into this background and given tamoxifen 3 times a week from 4-6 months old. Brains were then collected and immunostained for beta amyloid (green), GFAP (white), and tdTomato.

Virus	Effect
AAV5-GFAP-lsl-GFP	Express fluorescent GFP reporter in GFAP positive cells that express Cre
AAV5-GFAP-lsl-mCherry	Express fluorescent mCherry reporter in GFAP positive cells that express Cre
AAV5-GFAP-lsl-GCAMP7	Express calcium indicator GCAMP7 in GFAP positive cells that express Cre
AAV5-GFAP-ll-SOCS9-P2A-mCherry-ll	Express the JAK/STAT inhibitor SOCS9 and fluorescent mCherry reporter in GFAP positive cells that express Cre

Table 3.1: Available viruses for combinatorial approaches. This table contains the viruses we have available to use in combination with the *Lcn2CreErt2* mouse. AAV5 was selected as the infection agent as it has previously been identified as more strongly expressed in astrocyte populations (Haery et al 2019). The available viruses allow for specific labeling of Cre positive astrocytes with a fluorescent reporter and offer options for studying astrocyte biology, such as changes in calcium signaling and inhibition of JAK/STAT, an important pathway identified in a number of gliosis models.