A NOVEL OPTOGENETIC TOOL TO IMAGE CALCIUM IN THE TRIPARTITE SYNAPSE DURING EPILEPTOGENESIS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

In the past few decades, a revolution in our understanding of brain function has occurred based on demonstrations that astrocytes play critical roles in synaptic physiology. These findings led to the concept of the "tripartite synapse," which redefines the synapse to be comprised of not only the pre- and postsynaptic neuronal elements, but also the astrocyte processes that interact with them. Likewise, temporal lobe epilepsy (TLE) is a seizure disorder that affects the structure and function of networks of both neurons and astrocytes. One of the hallmark findings in TLE is the profound change in astrocyte structure and gene expression, a process called astrogliosis, throughout the brain regions involved in seizure generation. Using a well-established rat model of TLE, our lab recently demonstrated that astrocytes begin to express kainate receptor subunits during the development of TLE, and this increased expression persists throughout chronic epilepsy, suggesting that this pathway may play a role in the development of hyperexcitable circuits. However, the functional consequences of changes in reactive astrocytes and their impact on tripartite synapse function are not known.

To facilitate imaging experiments at the tripartite synapse, I developed a novel genetic tool that uses a fluorescent reporter system to label astrocytes with tdTomato and neurons with Cerulean. This plasmid also includes the genetically encoded calcium-indicating protein, Lck-GCaMP6f, enabling the monitoring of calcium transients in the fine processes of all transfected cells. I expressed this novel tool in the rat brain with in

utero electroporation and characterized expression throughout development using immunohistochemistry for markers of astrocytes and neurons. I demonstrated the utility of this tool to investigate functional subcellular Ca^{2+} signals in both astrocytes and neurons. This tool was used in experiments that showed that Ca^{2+} signaling is altered during the development of temporal lobe epilepsy in the rat kainic acid-induced model of status epilepticus. Spontaneous Ca^{2+} events in the processes of reactive astrocytes exhibited longer interevent intervals and longer duration of events compared to astrocytes from healthy tissue. I also found that increased protein expression of kainate receptors translated to functional expression of kainate receptors on a subset of reactive astrocytes following kainic acid-induced SE, suggesting a pathway that may be involved in pathological neuron-glial signaling contributing to the development of epilepsy.

Taken together, these results indicate that alterations in Ca^{2+} signaling in astrocytes during the development of TLE may have important consequences on function at the tripartite synapse. This dissertation lays the groundwork for future studies in the tripartite synapse and points to a novel pathway that may be involved in pathological neuron-astrocyte signaling that contributes to hyperexcitability during epileptogenesis.

This dissertation is dedicated to my mom, Geri Gibbons, for her unconditional love and support.

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CHAPTER 1

CONTRIBUTIONS OF ASTROCYTES TO EPILEPTOGENESIS FOLLOWING STATUS EPILEPTICUS: OPPORTUNITY FOR PREVENTIVE THERAPY?

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Abstract

Status epilepticus (SE) is a life threatening condition that often precedes the development of epilepsy. Traditional treatments for epilepsy have been focused on targeting neuronal mechanisms contributing to hyperexcitability, however, approximately 30% of patients with epilepsy do not respond to existing neurocentric pharmacotherapies. A growing body of evidence has demonstrated that profound changes in the morphology and function of astrocytes accompany SE and persist in epilepsy. Astrocytes are increasingly recognized for their diverse roles in modulating neuronal activity, and understanding the changes in astrocytes following SE could provide important clues about the mechanisms underlying seizure generation and termination. By understanding the contributions of astrocytes to the network changes underlying epileptogenesis and the development of epilepsy, we will gain a greater appreciation of the contributions of astrocytes to dynamic circuit changes, which will enable us to develop more successful therapies to prevent and treat epilepsy. This review summarizes changes in astrocytes following SE in animal models of SE and human temporal lobe epilepsy and addresses the functional consequences of those changes that may provide clues to the process of epileptogenesis.

Status Epilepticus Can Result in Temporal Lobe Epilepsy

Status epilepticus (SE) is a medical emergency with a poor prognosis. It is defined as continuous seizure activity lasting greater than 10 minutes or two or more sequential seizures without full recovery between seizures (Lowenstein et al., 1999). While the risk of death following SE has declined somewhat in the industrialized world, older patients experiencing prolonged SE are still at high risk of death. Furthermore, patients who

survive a prolonged bout of SE often experience cognitive decline and develop epilepsy (Engel, 1996). Patients with temporal lobe epilepsy (TLE) often first present with SE. TLE is a common seizure disorder characterized by complex partial seizures that can secondarily generalize and are often pharmacoresistant to currently available anti-seizure drugs (ASDs). Because SE can result in difficult to treat forms of epilepsy such as TLE, it is the focus of much research.

Administration of chemoconvulsant agents and electrical stimulation have been used in rodents to induce SE that is then followed by the progressive development of epilepsy. Two of the most well studied rodent models of SE are the systemic kainic acid (KA) and pilocarpine (PILO) models. Following injection of either of these two agents, animals begin a bout of SE that can last hours (Curia et al., 2008; Lehmkuhle et al., 2009). While mortality is lower for the KA model, survival can be enhanced in the PILO model by treatment with a variety of anticonvulsant agents after about an hour of SE. After a latent period of several days to weeks following SE, animals develop recurrent seizures that are limbic in origin. Animals treated with a repeated low-dose paradigm of KA have been found to develop spontaneous convulsant seizures on average 18 days following SE (Williams et al., 2009), while animals treated with pilocarpine begin to exhibit spontaneous seizures earlier, with some reported seizures occurring within five days following SE (Raol et al., 2006). Likewise, electrical stimulation of the perforant pathway can induce sustained SE that is followed by the development of recurrent seizures. Depending on the stimulus paradigms used to evoke SE in these models, spontaneous seizures can begin within days (Bumanglag and Sloviter, 2008; Norwood et al., 2010). In all of these models, considerable neuronal cell death is observed throughout

the brain and astrocytes begin to exhibit signs of reactive gliosis soon after evoking SE (do Nascimento et al., 2012; Glushakova et al., 2012; Lauritzen et al., 2012a). Animal models of SE-induced epilepsy provide an unparalleled opportunity to unravel the complex changes that occur in the CNS following SE and during the process of epileptogenesis. In particular, they help identify disease-modifying therapies that can prevent the development of epilepsy following SE or stop the progression of seizure activity once epilepsy is established. While much is known about the complex changes that occur in neurons following SE in these animal models, researchers are now investigating the changes that occur in astrocytes following SE. Astrocytes post SE may acquire functional changes that contribute to, or even prevent, network excitability. By understanding these functional changes in astrocytes, it may be possible to identify novel therapeutic targets or strategies for the prevention of epilepsy. In this review, we will focus our attention on recent findings of the effect of SE or related CNS insults on astrocyte function in limbic and cortical regions known to be associated with the initiation of seizure activity and the development of epilepsy.

Astrocytes Become Reactive Following SE and This Persists in Epilepsy

Since their discovery over 100 years ago, glial cells have traditionally been considered only as passive support cells of the nervous system (for reviews on the history of glial research, see Garcia-Marin et al., 2007; Somjen, 1988; Verkhratsky, 2006). However, recent application of cellular imaging, physiology, and genetics to astrocytes has shown that they are active participants at the neuronal synapse; their diverse roles are

entwined with the function of neurons. The structural and functional relationship between astrocytes and the neuronal synapse has led to the use of the term "tripartite synapse" to reflect the important contribution of astrocytes in influencing synaptic transmission (Halassa et al., 2007). The processes of astrocytes are in close proximity to neuronal membranes and express a host of receptors, transporters, and ion channels that enable astrocytes to "listen" to the chemical conversation at the synapse. In return, they are able to "respond" and modify the tone of synaptic transmission by regulating the availability of neurotransmitters and ions in the synaptic cleft, such as glutamate and potassium.

As a consequence of SE and subsequent inflammation, astrocytes change their morphology and protein expression in a process termed reactive astrogliosis. A number of molecules and signaling pathways are known to trigger reactive astrogliosis (for a comprehensive review, see (Sofroniew, 2009)) and many of those receptors/pathways are activated as a consequence of SE. Molecules released from dying neurons, such as reactive oxygen species, ATP and glutamate, are all known to induce astrogliosis. In addition, inflammatory cytokines such as TNF-α and Il1-β are also known triggers and these cytokines can be released by microglia and neurons following SE. Interestingly, cytokines are also released by reactive astrocytes, which can result in a feed-forward system that enhances gliosis (Vezzani et al., 2011). Furthermore, the JAK-STAT pathway is known to be elevated as a consequence of SE and this pathway, when stimulated, can also contribute to the observed changes in astrocytes (Lund et al., 2008; Sofroniew, 2009).

While reactive astrogliosis is one of the most common pathological lesions in epilepsy and other brain insults, it continues to be poorly understood. Dramatic changes

in the expression of neurotransmitter receptors, voltage gated ion channels, inflammatory cytokines, and other proteins, have been identified in astrocytes in both animal models and human TLE. However, the time course and the functional significance of such diverse changes that accompany astrogliosis is not clear and it is currently unknown to what extent "reactive astrocytes" either contribute to, or prevent the ultimate development of TLE. Indeed, some components of reactive astrogliosis may be critical compensatory mechanisms following injury, resulting in the dampening of excitability, while other changes, e.g. decreased expression of aquaporin, may in fact contribute to epileptogenesis and/or seizure generation (Lee et al., 2012). Finally, it is possible that some alterations in structure and/or function of astrocytes may even be unrelated to epileptogenesis. We contend that the study of the role of reactive astrogliosis in epileptogenesis is in its infancy and therefore, before disease modifying therapies can be identified for the treatment and/or prevention of SE-induced epilepsy, a mechanistic understanding of structure and function of reactive astrocytes in the disease process must be attained.

Properties of Reactive Gliosis Following SE

Following SE, reactive astrocytes become hypertrophic, increase expression of intermediate filament proteins (for example, GFAP), and develop longer and thicker processes (Binder and Steinhauser, 2006) (Figure 1). Although the functional consequence of changes in domain organization (see below) and hypertrophied processes with increased expression for GFAP are not exactly clear (Fedele et al., 2005; Gouder et al., 2004), some of the changes in expression of other proteins could have direct

consequences on the regulatory mechanisms of gene transcription in reactive astrocytes (Ridet et al., 1997). The term “reactive astrocyte” is rather ambiguous because reactive astrogliosis is not an all-or-none response (Sofroniew, 2009). There can be significant diversity within a reactive astrocyte population (Kálmán, 2003; Ridet et al., 1997). Mild to moderate gliosis, found early on in SE models, typically does not induce the proliferation of new astrocytes (Fernaud-Espinosa et al., 1993; Sofroniew, 2009). There are, however, some studies that have addressed the proliferative ability of astrocytes in the CA fields of the hippocampus following KA-induced SE or lesions (Murabe et al., 1982; Niquet et al., 1994). These studies show that while astrocytes can in fact proliferate after insult, the proliferation accounts for a very small number of the new cells in the area, suggesting that in models of SE, reactive astrocytes are comprised mainly of the resident astrocytes present before the insult.

Two of the hallmarks of reactive astrocytes are the increase in expression of intermediate filament proteins (for example, GFAP) in response to injury (Ridet et al., 1997) and hypertrophy of the main cytoskeletal branches. During this process, however, the fine spongiform processes of astrocytes are not immunoreactive for intermediate filaments. GFAP immunolabeling, therefore, does not detect changes in the spatial domains (i.e., cell volume) of reactive astrocytes in the neuropil. Changes in domain organization in reactive astrocytes are important to consider, as they may be indicative of astrocytes that are no longer controlling or modulating the same group of synapses and these changes could indicate pathological astrocyte-astrocyte or astrocyte-neuron relationships.

Two independent groups investigated whether domain organization is preserved

in reactive astrocytes. Wilhelmsson et al. (2006) dye filled astrocytes in the hippocampus following disruption of the perforant path from the entorhinal cortex. In this model, astrocytes increased the expression of GFAP and were reactive (presumably due to the axonal degeneration of the perforant path) but did not change their domain volume and did not interdigitate with neighboring astrocytes; thus, reactive astrocytes in this model maintained their spatial domains. In contrast, Oberheim et al. (2008) diolistically labeled astrocytes in the hippocampus of three mouse models of epilepsy: the ferrous chloride model of post traumatic epilepsy, KA-induced SE model, and the SWXL-4 mouse, a genetic model of epilepsy. Hypertrophied astrocytes (observed with GFAP) in these models of epilepsy had increased domain volumes and almost a complete loss of domain organization (observed with diolistic labeling). These changes were observed 1 week post injection of ferrous chloride, and 6 months after, during a time of chronic seizure activity. Interestingly, in ferrous chloride injected animals whose seizures were reduced with the anti-epileptic drug valproate, astrocytes were less hypertrophied, had domain volumes similar to controls, and did not lose their domain organization by interdigitating extensively with neighboring astrocytes. Therefore, changes in domain organization paralleled the severity of seizure activity.

The results from these studies on the changes in spatial domains of reactive astrocytes suggest that a loss of domain organization may be specific to astrocytes in epileptic tissue. In support of this notion, Oberheim et al. (2008) noted that reactive astrocytes in a mouse model of Alzheimer's disease did not lose their domain organization, even though the astrocytes in this model were reactive (i.e., hypertrophied processes and an increase in GFAP expression). The research highlights two salient

points. The first point is that reactive astrocytes, as determined by increased immunoreactivity to GFAP and hypertrophied processes, may not be homogeneous. Astrocytes may respond differently according to the type of cerebral insult, even though they may appear identical by immunohistochemistry (i.e., reactive, as revealed by GFAP). Indeed, recent genomic analysis of reactive astrocytes revealed a different pattern of gene expression depending on the type of insult (Zamanian et al., 2012). The CNS insults compared in this study were ischemia and LPS injection. Therefore, while it is not clear what patterns of gene expression would be observed following SE, it is possible that an entirely different gene expression pattern would be observed. Second, reactive astrocytes that have lost their domain organization in epileptic tissue may confer unique functional properties that are specific to this neurological disorder. Based on the differences in structural changes, therefore, reactive astrocytes in various models of neuronal injury may have different functional properties. This is particularly important to note because reactive astrocytes are often considered a homogenous population, although the data increasingly indicates that the term reactive gliosis encompasses a very wide range of features with important functional consequences to studying pathologies. An additional point to consider is that the work described above was performed in rodents. However, there exists a dramatic heterogeneity of human astrocytes. It is not currently known how the individual types of human astrocytes differentially respond to either SE or prolonged TLE (Oberheim et al., 2009).

Potassium Buffering Is Not Altered Shortly After SE

Astrocytes play a critical role in maintaining neuronal homeostasis by buffering extracellular potassium (K+) through the highly permeable, inwardly rectifying potassium channel KIR4.1 (Higashi et al., 2001; Poopalasundaram et al., 2000). Neurons extrude K+ with each action potential repolarization (Dichter et al., 1972) and during periods of high activity, K+ concentration in the extracellular space can be rapidly elevated (Nicholson and Sykova, 1998). Thus, the tight regulation of [K+]o via astrocytic uptake is critical for maintaining neuronal homeostasis. Given the importance of KIR channels in spatial buffering and the role of potassium in neuronal function, it is no surprise that its role has been heavily investigated in epilepsy. For instance, conditional knock-out of the KIR 4.1 channel in mice results in behavioral ataxia, seizures, and early lethality is observed resulting from deficient K+ uptake in astrocytes (Djukic et al., 2007). Likewise, in the sclerotic hippocampus of patients with TLE, the KIR channel blocker Ba^{2+} was shown to be ineffective in increasing extracellular potassium levels measured by potassium selective microelectrodes in response to stimulus as was expected, suggesting dysfunction in KIR channels (Kivi et al., 2000). This finding was also observed in some brain slices obtained from epileptic rats that had received pilocarpine to induce SE (Gabriel et al., 1998). These results were corroborated using patch-clamp electrophysiology in acute hippocampal slices obtained from surgical specimens of patients with TLE, which showed reduced inward rectifying potassium conductances in reactive astrocytes from this tissue (Hinterkeuser et al., 2000). Together these findings support the altered ability of astrocytes to effectively buffer potassium in epileptic tissue. However, until recently, changes in KIR and potassium uptake have not

been investigated in reactive astrocytes during the latent period in an animal model of SE-induced MTLE. Studies from our laboratory (Takahashi et al. 2010) have demonstrated that potassium conductances were not altered in astrocytes during the latent period one-two weeks following SE in the KA-treated rat. This finding suggests that soon after SE and prior to the development of epilepsy, astrocyte potassium buffering capability remains intact. Further investigation is necessary to determine how and if KIR function contributes to epileptogenesis. Indeed, therapeutic strategies which result in the continued expression and function of specific KIR channels in astrocytes might prove to be useful in delaying the onset of epilepsy following SE.

Gap Junctions Between Astrocytes Increase Following SE

Astrocytes are by far the most extensively gap junction coupled cells in the central nervous system and are critical in maintaining homeostatic balance of ions and molecules (for reviews, see Kielian, 2008; Nagy and Rash, 2000; Theis et al., 2005). A complete gap junction is formed by the direct contact of two opposing hemichannels, or connexons, on two different cells. Each connexon consists of a hexameric structure consisting of six connexin (Cx) proteins with a hydrophilic pore at its center. This pore allows transfer of ions, second messengers, metabolites, and other small molecules (Nakase and Naus, 2004), allowing not only electrical coupling between cells but also chemical and metabolic coupling. Astrocytes express predominately Cx43 and Cx30 and form a metabolically coupled syncytium in CNS tissue (Rash et al., 2001). Our laboratory recently showed that the number of coupled astrocytes in brain slices was significantly increased in KA-treated rats early in the process of epileptogenesis, and consistent with

that observation, CX43 expression was greatly elevated (Takahashi et al., 2010). Interestingly, in brain slices obtained from normal rats, epileptiform burst-like activity has been shown to enhance coupling between astrocytes in a rapid activity dependent manner (Rouach et al., 2008). The extent of coupling in astrocytes is thought to underlie spatial buffering and the ability to redistribute potassium and glutamate (Theis et al., 2005). Gap junction coupling has also been implicated in spreading depression, a pathophysiological phenomenon in the CNS characterized by a combined reaction of neurons and glia in the form of a propagating wave of neuronal depolarization followed by neuronal inactivation. Interestingly, a conditional knock out of the main gap junction protein in astrocytes, connexin (Cx) 43 results in an animal with decreased intracellular coupling among astrocytes and enhanced velocity in the wave of spreading depression (Theis et al., 2003), suggesting that the redistribution of ions and intracellular molecules in astrocytes is critical for minimizing spreading depression and hyperexcitability. Therefore, in animals experiencing SE, an increase in gap junctionally-coupled astrocytes could be viewed as compensatory, with an enhanced capacity for buffering both glutamate and potassium.

The data concerning the expression of Cx43 and Cx30 in other models of chronic epilepsy and in human resected tissue are conflicting. In the kindled rat and tetanus toxin-induced model of epilepsy, no change in mRNA of Cx43 was observed four weeks following injury (Elisevich et al., 1997a). Likewise, no change in mRNA or protein expression of Cx43 or Cx30 was detected in kindled rats and in the kainate-treated rat four weeks following injury (Sohl et al., 2000). However, in human resected tissue from patients with intractable epilepsy, Cx43 mRNA and protein are generally shown to be

increased (Collignon et al., 2006; Fonseca et al., 2002; Naus et al., 1991) except for (Elisevich et al., 1997b). There is evidence of increased gap junction coupling by measuring calcium signaling induced by glutamate exposure using fluorescence recovery after photo bleaching (FRAP) assays in surgically resected tissue (Lee et al., 1995). These findings suggest that gap junction coupling, along with increased Cx43 and Cx30 expression, might be a common feature in human epilepsy, something not always recapitulated in animal models of chronic epilepsy. Some of the discrepancies between studies could be due to the dynamic nature of the connexin protein. Connexins have a rapid turnover rate of ~1 hour and the antigens that have been used to label Cx43 in the past were difficult to detect and were sensitive to fixation (Hossain et al., 1994; Laird et al., 1991).

Glutamate Transporter Function Is Enhanced Following SE

Unlike other neurotransmitters that can be enzymatically degraded after being released into the synaptic cleft, glutamate must be actively removed by Na+-dependent glutamate transporters. Fast efficient clearance is essential for high signal-to-noise ratios in neurotransmission and for the prevention of excitotoxicity (for review see Danbolt, 2001; Marcaggi and Attwell, 2004; Tzingounis and Wadiche, 2007). The main glutamate transporters, GLAST and GLT-1 are predominately expressed in astrocytes (Lehre et al., 1995), and are responsible for >90% of the total glutamate uptake (Tanaka et al., 1997). The alterations that occur to this system, as well as the closely linked processes governing glutamate and glutamine cycling during epilepsy are complex and the literature is often conflicting in regards to changes in glutamate transporter expression

following SE.

In animal models, as would be expected, knock down of GLT-1 expression in the mouse leads to neuronal loss and seizure activity that is caused by the build-up of extracellular glutamate (Tanaka et al., 1997). Similarly, eight weeks following stereotaxically injected KA into the amygdala of rats, spontaneous motor seizure activity was observed and hippocampal tissue homogenates showed reduced expression of GLT-1 and GLAST (Ueda et al., 2001). In human tissue from patients with TLE, immunohistochemistry consistently demonstrates reductions in GLT-1 and GLAST expression (Mathern et al., 1999; Proper et al., 2002; Sarac et al., 2009). However, similar studies in human tissue using Western Blotting on hippocampal homogenates do not show a change in GLT-1 or GLAST expression (Bjornsen et al., 2007; Tessler et al., 1999). However, the changes in transporter protein expression described above do not provide a direct assessment of the changes in transporter dependent glutamate uptake in epilepsy. A more quantitative measurement of transporter function, and hence glutamate clearance, in epileptic tissue is to directly record synaptically evoked glutamate transporter dependent currents in reactive hippocampal astrocytes.

In a recent study, we recorded from astrocytes in hippocampal brain slices obtained from control animals and those that had been subjected to KA-induced SE. We demonstrated that there is no effect of SE on the amplitude of glutamate transport currents, nor any concomitant change in the expression of GLT-1 expressed in astrocytes within 1-2 weeks after SE. However, we did determine that there is a significant decrease in the decay kinetics of glutamate transport currents recorded in reactive astrocytes, suggesting that following SE, astrocytes have an enhanced capacity to more quickly take

up extracellular glutamate (Takahashi et al., 2010). Previous work by other investigators demonstrated that the time course of glutamate transport currents is not affected by changes in stimulus strength, the probability of release of glutamate, or the diffusion kinetics of glutamate. Instead, the rate of decay of the current is likely due to a variety of mechanisms, including the number and type of glutamate transporters present at the synapse and the extracellular volume (Diamond, 2005; Diamond and Jahr, 2000). Therefore, there are several hypotheses that could explain the faster kinetics of the glutamate transport currents in reactive astrocytes. For example, it has recently been demonstrated that the GLT-1 transporter is rapidly trafficked to activated synapses. (Benediktsson et al., 2012). Therefore, it is possible that following the intense neural activity that occurs during SE, existing transporters may be trafficked to activated synapses. It is also known that several astrocyte signaling pathways can regulate glutamate transporter trafficking (Robinson, 2002) and while we did not detect a change in GLT-1 expression in our studies, it is possible that trafficking of existing transporters could contribute to the changes we observed. Alternatively, decreased extracellular volume contributes to the observed decrease in glutamate transporter current decay kinetics observed during development (Thomas et al., 2011) and thus may also play a role in epilepsy, where the extracellular space is reduced and known to contribute to hyperexcitability (Hochman, 2012). Finally, the rapid buffering of glutamate observed following SE may also be facilitated by the increase in gap junctions between astrocytes (Takahashi et al., 2010). Indeed, recent work in animals with Cx43 and Cx30 deleted from astrocytes, and consequently no gap junctions between astrocytes, demonstrated that the decay time constant of the glutamate transport current was significantly slowed,

consistent with the notion that extensive networks are essential for proper buffering of glutamate (Pannasch et al., 2011). Thus, the maintenance of extensive networks of coupled astrocytes could very well be viewed as an essential mechanism for dampening excitability in epileptic networks.

Glutamate Receptor Expression

Astrocyte function depends in part on cell surface receptor expression. Astrocytes in juvenile animals and in cell culture have been shown to express nearly every transmitter receptor as their neuronal counterparts, including metabotropic and ionotropic glutamate receptors; for review see (D'Antoni et al., 2008; Kimelberg, 1995; Lalo et al., 2010). In brain slices obtained from juvenile animals, the functional expression of a-amino-3-hdroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Jabs et al., 1994; Schroder et al., 2002; Seifert et al., 2004; Seifert et al., 1997) and N-methyl-Daspartate receptors (NMDA) (Serrano et al., 2008; Steinhauser et al., 1994) have been described. Expression for group I mGluRs is increased in reactive astrocytes 1 week after SE (Aronica et al., 2000), in human MTLE (Notenboom et al., 2006), and in reactive astrocytes following neuronal injury (Ferraguti et al., 2001; Ulas et al., 2000), however, the electrically passive, GFAP-expressing, protoplasmic astrocyte of the adult hippocampus does not express ionotropic glutamate receptors under basal conditions. Interestingly, kainate receptors (KARs) and NMDA receptors are expressed in reactive astrocytes following ischemia in adult animals (Gottlieb and Matute, 1997; Krebs et al., 2003). Furthermore, two recent reports have demonstrated that the protoplasmic GFAP+ astrocyte of the cortex acquires electrophysiological responses to kainate application

following ischemic insult or within an Alzheimer's disease model, again suggesting possible KAR expression in response to injury (Peters et al., 2009; Wang et al., 2008). Interestingly, preliminary immunohistochemical studies from our laboratory suggests that following SE, astrocytes begin to express KAR subunits (Vargas et al., 2006). The modulating role of KARs and their unique ability to couple to G-proteins makes this family of glutamate receptor an important component of CNS function. It is currently unknown what functional role KARs expressed in astrocytes following KAinduced SE play in the disease process of epileptogenesis. However, it is enticing to hypothesize that increased calcium signaling and subsequent gliotransmission mediated in part by the pathological expression of ionotropic KARs and increased expression of mGluRs in reactive astrocytes could cause the synchronization of neurons thus contributing to hyperexcitability.

Blood-Brain Barrier Disruption Following SE

Astrocytes form close associations with the endothelial cells of capillaries and help maintain proper function of the blood brain barrier (BBB). Disruption of the BBB is often observed in epileptic brain regions, but has generally been thought to result from seizure activity, rather than contribute to the generation of seizures. A body of clinical and experimental evidence now suggests that opening of the BBB triggers a chain of events that may contribute to the processes underlying epileptogenesis. Van Vliet and coworkers have demonstrated that pronounced levels of BBB leakage occur during the acute and latent phases following SE in humans and rats (van Vliet et al., 2007). In addition, following BBB disruption, uptake of the serum protein albumin by astrocytes

through TGF-□ receptors has been associated with reactive gliosis (Ivens, Kaufer et al. 2007). Studies in cultured astrocytes from rat brains show that albumin induces calcium signaling (Nadal et al., 1995) and that TGF-B receptor activation causes a down regulation of KIR channels (Perillan et al., 2002), suggesting that albumin can directly modulate astrocyte functions and enhance excitability. Together these data suggest that disruptions of the blood brain barrier may directly contribute to processes underlying epileptogenesis through astrocyte-mediated mechanisms (Ivens et al., 2007).

It is also important to note that changes in BBB permeability following SE and other insults may also have significant consequences for delivery of pharmacotherapies and drug-resistant epilepsies (Loscher and Potschka, 2005). For instance, increased expression of multidrug-resistance-associated proteins (MDPs) in endothelial cells of blood vessels in the brain following seizures has been implicated as a possible mechanism in drug-resistant epilepsies. In chronic epilepsy, increased levels of MDPs are thought to facilitate the efflux of antiepileptic drugs such as carbamazepine and valproate, thereby preventing the accumulation of therapeutic levels of antiepileptic drugs in brain tissue (Loscher, 2007). One study demonstrated that increased expression of MDPs in reactive astrocytes and blood vessels in the hippocampus occur immediately following electrically evoked status epilepticus (SE) in the rat, indicating that changes in MDP expression may occur earlier than initially thought (van Vliet et al., 2005). Furthermore, increased expression of MRPs during the acute and chronic phase was also accompanied by decreased levels of an administered ASD, phenytoin (PHT). These results suggest that the development of pharmacoresistant epilepsy via the increased expression of MRPs may be initiated after a single bout of SE and should be

taken into account when considering potential therapeutic interventions aimed at disrupting the process of epileptogenesis.

Additionally, reductions in MCT1, a key BBB carrier molecule for monocarboxylates and amino acids, have been observed on the endothelial cell membrane of microvessels in the hippocampus of human patients with TLE (Lauritzen et al., 2011) as well as two rodent models of SE (Lauritzen et al., 2012b). Reduced levels of MCT1 in the epileptogenic hippocampus may impair the uptake of monocarboxylates resulting in decreased concentrations of blood-derived fuels, such as ketone bodies, which could contribute to enhanced seizure susceptibility. Intriguingly, the transport of drugs such as valproic acid across the blood brain barrier is also facilitated by MCT1. Thus, concentrations of anti-seizure drugs in the MCT1-deficient epileptogenic hippocampus may be severely reduced, representing a possible explanation for pharmacoresistance in TLE (Lauritzen et al., 2012b).

Brain inflammation occurs as a consequence of seizures and is intimately linked to changes in BBB permeability. Reactive astrocytes have been identified as key players in the mechanisms involved in CNS inflammation (Aronica et al., 2012). Indeed, mRNA of inflammatory mediators are induced within 30 minutes of SE onset (De Simoni et al., 2000) and upregulation of astrocyte genes related to immune and inflammatory responses persists for up to 1 week following SE (Gorter et al., 2006). It is now recognized that a myriad of immune responses, many of which are mediated by astrocytes, can occur as a consequence of seizure activity and may contribute to the development of epilepsy. A more complete discussion of these topics are included in this special issue (see Carson and Fabene's reviews).

Increased Expression of Adenosine Kinase Following SE

In addition to the list of the changes in astrocytes following SE, alterations in the astrocyte-mediated adenosine/adenosine kinase system have shown intriguing potential as a target in the prevention and treatment of epilepsy. For over 40 years, adenosine has been recognized as an endogenous anticonvulsant. Adenosine levels are elevated in patients following seizures, suggesting that adenosine is released during a seizure and may mediate seizure termination (Boison, 2005). Astrocytes regulate adenosine levels via the production of the enzyme adenosine kinase (ADK) and during epilepsy, reactive astrocytes produce increased levels of ADK. Studies from transgenic animals that have increased expression of ADK in the brain show that ADK overexpression leads thedevelopment of spontaneous seizures and mortality following KA-induced SE, presumably due to a reduction in adenosine (Li et al., 2008). Furthermore, SE may cause an imbalance of adenosine/ADK that leads to epileptogenesis. Gouder et al. (2004) demonstrated at ADK is increased in reactive astrocytes around 1 week post-SE, prior to the development of spontaneous seizures. Intriguingly, reduction of ADK in the brain can prevent the development of spontaneous seizures following KA-induced SE and prevents cell death, indicating that ADK reduction confers a neuroprotective effect following status (Li et al., 2008). Recently Theofilas et al. demonstrated that an antisense viral approach to modulate astrocytic ADK expression was able to trigger or prevent seizures in mice, providing the first line of evidence that gene therapy may be successfully be applied to this system in order to control epilepsy (Theofilas et al., 2011).

Changes in Astrocyte Calcium Signaling After SE

While astrocytes do not exhibit prominent electrical excitability as do neurons, they are able to dynamically regulate calcium using internal stores (Charles et al., 1991; Cornell-Bell et al., 1990; Finkbeiner, 1992). Calcium transients in astrocytes occur in response to the activation of g-protein coupled receptors (e.g. mGluRs) and are thought to modulate the release of a number of gliotransmitters that could influence synaptic function (Aguado et al., 2002; Araque et al., 1998; Fellin et al., 2004; Halassa and Haydon, 2010; Parpura and Haydon, 2000; Pasti et al., 1997; Shigetomi et al., 2008). Consistent with effective synaptic modulation, astrocytic calcium transients have been shown to correlate with structured neuronal network activity (Aguado et al., 2002; Kuga et al., 2011; Winship et al., 2007). These observations naturally lead to the question of potential changes in astrocytic calcium dynamics during and following SE and how any changes might affect neural network function. Astrocyte calcium dynamics and SE have been studied in only a couple of papers to date (Ding et al., 2007) (Smeal et al., In press). Ding et al. used two-photon microscopy to study cortical astrocytic calcium dynamics in vivo using a pilocarpine model of SE (Ding et al., 2007). They found that astrocytic calcium activity increased during and after SE. Interfering with the increase in calcium activity in astrocytes that followed SE was neuroprotective, suggesting that glutamate release from astrocytes was contributing to excitotoxic cell death following SE (Ding et al., 2007).

Recent work from our laboratory using two-photon microscopy and a fast scanning technique showed that calcium waves mediated by the astrocytic syncytium in the CA3 region of the hippocampus can be very fast and widespread *in vitro* (Smeal et

al., In press). This fast wave activity was much reduced in brain slices prepared from juvenile rats that experienced SE induced with kainic acid. Therefore, based on these two studies, calcium dynamics in reactive astrocytes following SE appear to be altered. The functional consequences of these changes in the context of epileptogenesis however, remains to be determined.

One of the primary difficulties in studying astrocyte calcium dynamics related to SE is the inability to bulk load calcium indicating dyes in brain slices obtained from the older animals that are used in the models of SE. The labeling of cells *in vitro* using the acetoxymethyl (AM) ester calcium indicator dyes becomes much more difficult as the animals age past a few weeks (Peterlin et al., 2000; Reeves et al., 2011). Transfection of protein calcium indicators using viruses is a potential solution, but has the drawback of causing astrocytes to become reactive independent of the insult that causes the SE (Ortinski et al., 2010). In order to bypass these loading problems, we have recently used the in utero electroporation technique (Chen and LoTurco, 2012; Gee et al., 2011; LoTurco et al., 2009) to introduce genetic calcium indicators into animals that are subsequently used in the standard SE animal models. Using plasmids that contain modifications to introduce the genetic calcium indicator (GCaMP-3) into the genome and subsequently target it to the cell membrane (Shigetomi et al., 2011) has resulted in well-labeled astrocytes in a number of brain regions including the hippocampus (Figure 2). Calcium transients can be detected even in the fine processes (Figure 2). Future experiments will study both the potential changes of calcium activity within the fine structure of individual astrocytes and in the calcium dynamics of large networks of astrocytes following SE and during the process of epileptogenesis.

Disease Modifying Therapies Targeting Astrocytes

The vast majority of anti-seizure (ASD) drugs target ion channels and receptors that directly modulate neuronal function (Bialer and White, 2010; White et al., 2007). While this approach can confer seizure control to a majority of patients with epilepsy, it is estimated that between 25-35% of patients do not have their seizures adequately controlled with existing ASDs and the side effects of these drugs also negatively impact the quality of life of patients with epilepsy. Thus, novel ASDs that target inflammation and as a consequence, reactive astrocytes, may provide viable approaches to seizure control. Indeed, there is currently a clinical trial investigating the ability of VX-765, a caspase-1 inhibitor that prevents the production of IL-1B, to reduce seizures in patients with refractory partial seizures. This is the first clinical trial designed to target inflammatory processes in patients with epilepsy and a positive outcome will set the stage for novel therapeutic approaches for the treatment of existing seizure disorders.

There is currently no strategy to prevent the development of epilepsy in patients at risk following a CNS insult such as SE. However, initial work with VX-765 in rodents suggested that this compound may possess disease modifying properties that could alter the development of epilepsy (Ravizza et al., 2008). Furthermore, the increase in experiments that study the function of astrocytes following SE has opened the door for the development of novel compounds or approaches for targeting astrocyte function in an effort to interfere with the process of epileptogenesis. In addition to the VX-765 studies already discussed, the manipulation of the adenosine kinase system as described in this issue by Boison and colleagues may prove to be an important new therapeutic direction. Likewise, as more information is gleaned about the role of reactive astrocytes in the

progression of epilepsy, the more opportunities we will have for interfering with the process of epileptogenesis.

Conclusions

SE results in rapid structural and functional changes to not only neurons, but astrocytes as well. Numerous studies performed in resected epileptic tissue and in chronic models of epilepsy have noted many changes in astrocyte function that might contribute to seizure activity. However, less is known about early changes in function in astrocytes following SE and to what extent astrocytes prevent or contribute to the development of epilepsy following SE. Ideally, understanding the wide array of astrocyte functions will give us clues as to how to exploit those processes that dampen excitability, such as glutamate uptake, gap junctions, and potassium buffering, and how to minimize those processes that contribute to network excitability, such as decreased expression of aquaporins and secretion of cytokines and gliotransmitters. The development of new tools for wide scale imaging of networks of neurons and astrocytes with genetically encoded calcium indicators will undoubtedly contribute to our quest for disease modification and prevention of acquired epilepsy.

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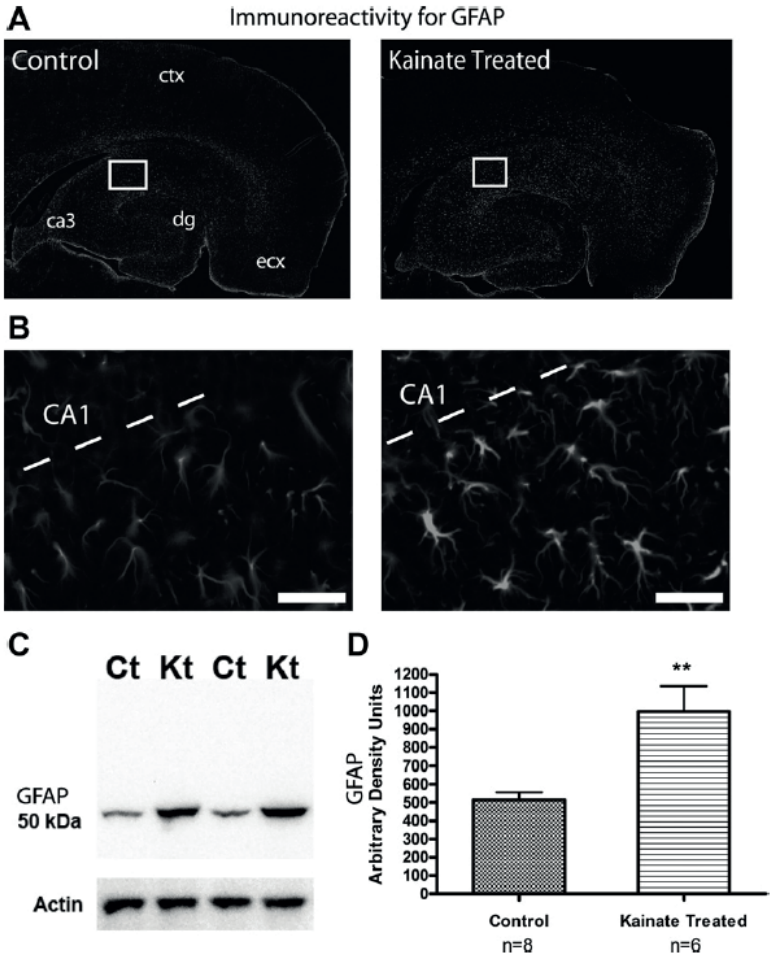

Figure 1.1. Reactive astrocytes are present in the hippocampus 1 week following KA-induced SE.

(A) Immunoreactivity for GFAP is observed throughout the hippocampus and cortex in horizontal brain sections from both control and KA-treated rats. dg = dentate gyrus, ecx = medial entorhinal cortex. (B) Close up of CA1 and stratum radiatum from the area indicated by the white box in (A). Sections from KA-treated rats (right) show characteristics typical of reactive astrocytes including a hypertrophied morphology and an increase in the immunoreactivity for GFAP. Scale bar, 50 lm. (C) Western Blot of crude hippocampal membrane fractions from control (Ct) and kainate-treated (Kt) animals probed with an antibody directed against GFAP. Actin served as a lane loading control. (D) Densitometry values of immunoreactivity for GFAP from kainate-treated samples (n = 6) were found to be significantly increased compared to control (n = 8) samples (⁄⁄$p < 0.0001$, Student's t test). Figure reprinted from (Takahashi et al., 2010) with permission from Elsevier.

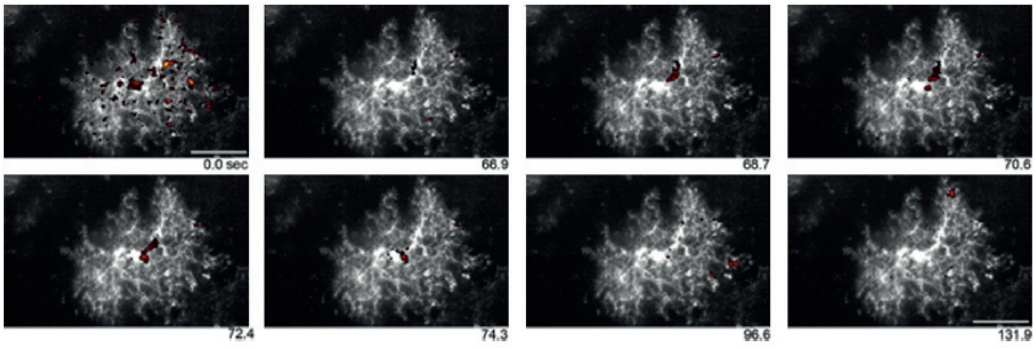

Figure 1.2. Ca^{2+} transients recorded at high resolution in the soma and throughout the processes in an astrocyte expressing Lck-GCaMP3 are observed in a series of images extracted from a movie using a standard x-y raster.
Changes in fluorescence as a consequence of increased Ca^{2+} are highlighted in pseudo-color and superimposed on the mean image of the movie. At time 0.0 s, there is activity throughout the astrocyte. Time 66.9 to 74.3 s shows a slow wave moving from the main processes to the soma. The final two panels demonstrate isolated Ca^{2+} transients in the astrocytic processes. Scale bar = 100 μM.

CHAPTER 2

CHALLENGES FOR STUDYING THE TRIPARTITE SYNAPSE

As the previous chapter discussed, astrocytes, the main cell type responsible for the maintenance of brain homeostasis, become reactive and undergo widespread changes after status epilepticus (SE). The functional significance of changes that accompany astrogliosis during the development of temporal lobe epilepsy (TLE) is still very unclear, and it is currently unknown to what extent reactive astrocytes might contribute to, or prevent, seizure generation in TLE. Understanding the role of astrocytes during the process of epileptogenesis is key to providing a better grasp on the changes that take place during the development of epilepsy and for designing new therapies for treating individuals at risk for developing acquired epilepsy. The overall goal of this dissertation was to understand the functional alterations in astrocytes during TLE and I focused on investigating how Ca^{2+} signaling might be altered in reactive astrocytes in the kainic acid-induced status epilepticus model of human mesial temporal lobe epilepsy.

Although astrocytes express numerous ligand and voltage gated ion channels, receptors, and transporters, they do not respond to stimulation with significant changes in membrane potential (Takahashi, Vargas, & Wilcox, 2010). As a result, the electrophysiological methods that are commonly used to monitor neuronal activity are not particularly useful for studying astrocyte activity. Astrocytes do, however, exhibit

dynamic changes in intracellular calcium that respond to neurotransmitters and evoked neuronal activity. Measuring Ca^{2+} dynamics in astrocytes and linking these events to neural activity has been a substantial challenge for the field and has required the development of new methods to monitor activity in physiologically-meaningful contexts (Bazargani & Attwell, 2016; Nedergaard & Verkhratsky, 2012). Most early investigations of astrocyte Ca^{2+} activity were based on bulk-loaded membrane-permeable chemical Ca^{2+} indicators. Fluo4-AM and Oregon Green BAPTA1-AM, the most popular chemical indicators used to image Ca^{2+} activity in astrocytes, allow imaging the somatic region and the larger proximal processes of astrocytes but do not diffuse into the very thin processes that closely appose neuronal structures. Therefore, it is likely that many early investigations of astrocyte Ca^{2+} signaling missed important signals in the fine processes. Furthermore, synthetic dyes are particularly difficult to load into adult or pathological tissue, limiting their use exclusively to young, healthy tissue.

The recent availability of genetically encoded calcium indicating proteins (GECI's) has provided the opportunity to monitor Ca^{2+} activity in cells from healthy and diseased tissue. Several variants of the GFP-based Ca^{2+} sensor GCaMP have been used to obtain important information about Ca^{2+} states in both neurons and astrocytes (Chen et al., 2013; Li, Agulhon, Schmidt, Oheim, & Ropert, 2013; Rose, Goltstein, Portugues, & Griesbeck, 2014). Furthermore, the inclusion of a membrane-tethering domain, Lck, increases the level of GCaMP expression near the plasma membrane, enabling the monitoring of Ca^{2+} signals in previously inaccessible fine processes (Gee et al., 2015; Haustein et al., 2014; Shigetomi, Bushong, et al., 2013; Shigetomi, Kracun, & Khakh, 2010; Shigetomi, Patel, & Khakh, 2016). Monitoring astrocyte activity using GECI's, it

has become clear that astrocytes exhibit dynamic increases in intracellular Ca^{2+} throughout their entire cell bodies, but especially in the fine processes. Distinct types of astrocytic Ca^{2+} signals have been described that involve the release of Ca^{2+} from internal stores as well as the activation of Ca^{2+}-permeable membrane channels (Petravicz, Fiacco, & McCarthy, 2008; Rungta et al., 2016; Shigetomi, Jackson-Weaver, Huckstepp, O'Dell, & Khakh, 2013).

The most distinguishing feature of astrocytes in the hippocampus is their highly complex, bushy morphology. This complex morphology enables astrocytes to penetrate all areas of the neuropil and maintain intimate associations with the axons and dendrites that comprise synapses. The concept of the "tripartite synapse" was coined in order to describe the role of the astrocyte processes as morphological and functional partners of the synapse (Araque, Parpura, Sanzgiri, & Haydon, 1999; Perea, Navarrete, & Araque, 2009). This term proposes that the synapse should take into account the role of the astrocyte, as well as the pre- and postsynaptic neuronal elements.

The close proximity and complex interactions between astrocytes and neurons have made it clear that a complete understanding of brain circuit function relies on the ability to monitor and differentiate activity in both cell types. However, many questions remain about the functional relationships between astrocytes and neurons at synapses, how different astrocyte subtypes encode information in Ca^{2+} signals, and how Ca^{2+} signaling might be altered in brain disorders such as epilepsy.

The following chapter in this dissertation describes the development and characterization of a unique genetic tool to differentiate and color code astrocytes and neurons during Ca^{2+} imaging experiments. Furthermore, the development of this plasmid

lays the groundwork for experiments, discussed in Chapter 4, examining alterations in calcium signaling in reactive astrocytes during the development of epilepsy in the rat kainic acid-induced model of TLE.

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CHAPTER 3

A NOVEL PLASMID FOR GENETICALLY DIFFERENTIATING NEURONS AND ASTROCYTES DURING CALCIUM IMAGING EXPERIMENTS

Abstract

Elucidating the relationship between astrocytes and neurons is a fundamental challenge to understanding how the myriad of astrocyte subtypes contributes to normal synapse function and neurological disorders. Most genetic approaches for studying astrocyte-neuron interactions rely on selective targeting of proteins to either astrocytes or neurons. Currently, no single tool exists capable of differentiating astrocytes and neurons in real time. To address this gap in research tools, we developed a novel transposable transgene, the Neuron/Astrocyte Specified Transgene by in utero electroporation (*NASTIE)*. The *NASTIE* transgene is composed of a strong promoter driving expression of the genetically encoded calcium indicator protein, Lck-GCaMP6f, and a red fluorophore, tdTomato; the tdTomato coding region is flanked by *loxP* sites. Downstream of tdTomato is the blue fluorescent protein, Cerulean. The transgene also carries a Synapsin I promoter driving Cre expression. When cells destined to populate the rat hippocampus are transfected using in utero electroporation, only cells that express Synapsin I undergo Cre-mediated excision of the tdTomato coding region and express

Cerulean instead; any cell that does not normally express Synapsin I will continue to express tdTomato. We evaluated *NASTIE* transfected rat hippocampi using 2-photon imaging and immunohistochemistry and show the utility of this tool in capturing high-resolution images of the close morphological associations of astrocytes and neurons. Next, we recorded subcellular calcium activity in both astrocytes and neurons, demonstrating the use of *NASTIE* to investigate functional aspects of calcium signaling in transfected cells. We have created a novel tool that allows for distinguishing the fine processes of astrocytes from dendritic processes of neurons and for monitoring changes in calcium activity in astrocytes and neurons in the rat brain. The modular design of the transgene makes it possible to swap in different promoters or indicators for customized application of the tool. Thus, this versatile genetic tool could be used to elucidate the relationships between different combinations of cells of interest.

Introduction

In the last few decades, the roles of astrocytes as active partners in neural circuit function have rapidly expanded to include the ability to integrate neuronal signals (Di Castro et al., 2011; Murphy-Royal, Dupuis, Groc, & Oliet, 2017; Wang et al., 2006), regulate neuronal activity(Letellier et al., 2016; Newman, 2003), participate in neurotransmission (Brancaccio, Patton, Chesham, Maywood, & Hastings, 2017; Harada, Kamiya, & Tsuboi, 2015), and regulate neural plasticity (Murphy-Royal et al., 2015; Poskanzer & Yuste, 2016). Astrocytes maintain intimate and dynamic associations with neurons, both morphologically and functionally (Haber, Zhou, & Murai, 2006; Medvedev et al., 2014; Perez-Alvarez, Navarrete, Covelo, Martin, & Araque, 2014; Theodosis,

Poulain, & Oliet, 2008; Ventura & Harris, 1999). A typical protoplasmic astrocyte in the hippocampus exhibits an extremely complex, bushy, sponge-like network of processes (Bushong, Martone, & Ellisman, 2004; Bushong, Martone, Jones, & Ellisman, 2002). Astrocytes communicate through ultrathin distal processes that contact and sometimes enwrap synapses (Halassa, Fellin, Takano, Dong, & Haydon, 2007). Functionally, this places astrocyte processes in a position to detect, integrate, and shape synaptic activity. Practically, it makes investigation of these interactions highly challenging, as visual confirmation of real-time activity between the two cells, in an experimental model with only one cell labeled, can be almost impossible. The increasing recognition of the role of astrocytes in synaptic transmission in the last few decades has led to the concept of the tripartite synapse, which reflects the critical role that astrocytes play in synapse function (Araque, Parpura, Sanzgiri, & Haydon, 1999). However, the study of astrocyte-neuronal networks has been hampered by a lack of tools that facilitate the selective labeling of both of these cells. Many unresolved questions remain about the anatomical and functional relationships of astrocytes and neurons in the healthy brain, as well in neurological and psychiatric disease. Thus, a tool capable of selectively distinguishing astrocytes and neurons would be a great benefit to the field.

Another reason a tool has been difficult to design is the fact that astrocytes are electrically-passive, rendering standard electrophysiological techniques ineffective (Kimelberg, 2010). Using a genetically encoded calcium indicator (GECI), like the GCaMP family of proteins which fluoresce in the presence of calcium, allows us to use intracellular Ca^{2+}, a physiologically integral second messenger important to astrocyte physiology (Bazargani & Attwell, 2016; Rusakov, 2015), to monitor the activity and

intercellular signaling between neurons and astrocytes with subcellular resolution in physiologically relevant preparations (Shigetomi, Patel, & Khakh, 2016). GECIs, optimized for studying calcium activity in astrocytes, have been particularly useful to elucidate the activity of astrocytes in several biological contexts, and have revealed the complexity and relevance of the dynamic range of fluctuations of calcium, particularly in the fine processes of astrocytes (Shigetomi, Kracun, & Khakh, 2010; Shigetomi et al., 2016; Srinivasan et al., 2016). Previous studies have employed two strategies for expressing GECIs to study astrocyte-neuronal networks brain: 1) use of targeted transgenic mouse lines with cell-type specific promoters in combination with conditional, Cre-Lox or tetO-tTA, transgenes, delivered by viral infection or by crossing to other transgenic mouse lines, to target expression specifically to astrocyte or neurons (Gee et al., 2014; Srinivasan et al., 2016; Szokol et al., 2015; Tang et al., 2015); or 2) use of viral infection or electroporation to transfect all cells of a brain region with a transgene, carrying a strong ubiquitous promoter, and relying on posthoc identification, through morphological characteristics or immunohistochemistry, in order to identify cell type (Gee et al., 2015; O'Donnell, Jackson, & Robinson, 2016).

Given that it is not feasible to generate transgenic lines for many of the long established neurological disease models in rats, and the insurmountable challenges of differentiating processes of astrocytes and neurons using posthoc identification techniques, we sought to develop a single tool that can assay physiologically relevant signaling in astrocytes and neurons in real-time. This tool, *Neuron/Astrocyte Specified Transgene by in utero Electroporation* (*NASTIE*), was designed to differentially label neurons and astrocytes and allows for the real-time monitoring of calcium transients in

both cell types. *NASTIE* utilizes Cre/lox recombination to differentially express fluorescent reporter proteins and reliably labels neurons with Cerulean and astrocytes with tdTomato in the hippocampus. The simultaneous labeling of both cell types enables high magnification imaging of closely associated astrocyte and neuronal structures. The construct can be used to investigate calcium signaling, in astrocytes and neurons, in primary neuronal cell culture, organotypic slice cultures, acute brain slices, and in vivo with newly developed head-mounted cameras. We designed this tool to be modular, such that it is straightforward to substitute new GECIs, as technology advances, or by switching the promoter sequences, for another cell-specific promoter, researchers can modify the specific interacting cell populations they wish to study. This tool offers a new strategy for researchers interested in combining high-resolution visualization and identification of astrocytes and neurons combined with the ability to image calcium activity in networks of cells in the brain, or any other region of the body, where cells are closely enmeshed.

Methods

Plasmid construction

The Lck-GCaMP6f gene was moved into a plasmid harboring inverted terminal repeats recognized by *piggyBac* transposases, pPB.GFP.neo, using Xba1 (5') and Not1 (3') restriction sites. Ires-Lox-tdTomato was cloned from a fragment obtained from Ben Xu (Capecchi lab, unpublished) into HindIII and AscI sites. A polylinker containing SpeI and PacI was inserted into pPB.GCaMP6-IRES-lox-tdTomato. A *loxP* site was added to the 5' end of the Cerulean (Addgene# 15214) coding sequence and an SV40 polyadenylation sequence was added immediately after the stop codon and it was cloned

via PCR into PacI and SpeI sites in the polylinker immediately downstream of tdTomato. The rat Synapsin I promoter (Gift from Megan Williams, Univ. of Utah, Addgene #27232) was cloned before the Cre gene into pBluescript SK+ vector at the NotI site using Gibson Assembly Cloning (NEB E5510S). For Gibson assembly, primers were designed using the Gibson primer design application on the NEB website. The Synapsin I-cre fragment was cloned into pPB. GCaMP6.IRES.lox.tdTomato.lox.Cerulean to complete the *NASTIE* construct (Figure 3.1).

SH-SY5Y cultures

To test expression of tdTomato, expression and functionality of GCAMP6f, and synapsin specific expression of Cre and subsequent recombination on and expression of Cerulean, we transfected the SH-SY5Y cell line. This cell line was previously shown to express Synapsin from the endogenous promoter as well as the P2Y ATP receptor subclass. We transfect $1x10^6$ cells with ~1 μg of the *NASTIE* transgene and ~300 ng of a plasmid that expressed the *piggyBac* transposase source using the Amaxa IIb electroporator. Cells were scored for tdTomato expression, Cerulean expression, basal levels of GFP fluorescence in the absence of an induced response, as well as documenting through live-time lapse imaging, using a Zeiss Axiovert 200M and a Hamammatsu ORCA R^2, the dynamic increase in GFP fluorescence intensity, indicating a clear calcium transient in the transfected cell, following the addition of ATP to the media.

Primary neuronal cultures

Primary cultures of cortical neurons were prepared from embryonic (E-18) CF-1 mice (Charles River, Wilmington, MA), as described previously (Otto, Kimball, & Wilcox, 2002). Embryos (Day 18) were removed from anesthetized CF-1 mice and the brains were quickly removed from the embryos. Dissected cortical hemispheres were gently chopped into small pieces and then incubated 2 min in 15 mL DMEM with 0.25% trypsin. Two mL of heat inactivated horse serum (Invitrogen) was then added and this mixture was centrifuged 2 min at 1800 RPM. The supernatant was removed and the brains triturated and then spun again for 2 min at 1800 RPM. Cells were resuspended in 5 mL of Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 10% fetal calf serum, 3% glucose, and 2% L-glutamine and strained through a 70 um cell strainer (BD Falcon). Cells were plated at a low density of 100,000 cells/ml on poly-L-lysine (Sigma) coated coverslips placed in 12 well plates. Cultures were maintained in a humidified incubator at 37°C and 5% CO2. When cells were about 70% confluent, the cultures were treated with Ara-C (Sigma) 10- 14 μL of a 1 μM stock, usually between 5 to 6 days after plating.

Cells were transfected 24 hours after Ara-C treatment with lipofectamine 2000 (Thermofisher). Each coverslip with cells was placed into 1 well of a 12 well plate in Neuronal Incubation media (NIM). The *NASTIE* plasmid (1 μg) in 50 μL of h-DMEM (with 1X GlutaMax (Gibco) buffered with 10 mM HEPES (Gibco)) was mixed with 3 μL of lipofectamine diluted in 50 μL of h-DMEM and incubated at room temperature for 15 min. 100 μL of this solution was then added to the well with the coverslip and incubated 1 hour at 37C without CO_2. The coverslip was then placed back into the well containing

the original conditioned DMEM. Cells were imaged 24 to 48 hours later (Shepherd et al., 2006).

In utero electroporation

Transfection of the embryonic rat brain was accomplished using an established in utero electroporation protocol (Gee et al., 2015). In utero electroporation was performed on embryonic day (E) 14.5-15.0. The final concentration of DNA plasmid solution was 4 μg/μl final (2 μg/μl of *NASTIE* and 2 μg/μl of transposase source). In order to target the developing hippocampus, the paddles were rotated across the top of the head with the positive electrode placed opposite the injected side, as previously described (Gee et al., 2015; Navarro-Quiroga, Chittajallu, Gallo, & Haydar, 2007). Animals were weaned from the mother on postnatal day (P) 21 and males and females housed separately until used for experiments.

Preparation of acute brain slices

Brain slices were prepared using established techniques (Takahashi, Vargas, & Wilcox, 2010). Briefly, electroporated rats were deeply anesthetized using isoflurane and decapitated. Brains were rapidly dissected and placed in ice-cold (4 °C) oxygenated sucrose Ringer's solution (95% O_2/5% CO_2) containing (in mM): 200 sucrose, 26 $NaHCO_3$, 10 D-glucose, 3 KCl, 3 $MgSO_4$, 1 $CaCl_2$, and 1.4 NaH_2PO_4. Brains were then cut down the midline and glued dorsal side down to the mounting disk of a Vibratome tissue slicer (Vibratome, St. Louis, MO). Horizontal brain slices (400 μm) containing both the medial entorhinal cortex and hippocampus were cut in oxygenated sucrose

Ringer's solution. Following sectioning, slices were transferred to a holding chamber filled with oxygenated artificial cerebrospinal fluid (ACSF; room temperature) containing (in mM): 126 NaCl, 26 $NaHCO_2$, 10 D-glucose, 3 KCl, 2 $MgSO_2$, 2 CaCl and 1.4 NaH_2PO_4 for at least 1 hour. The normal Ringer's solution was continuously bubbled with 95% O_2/5% CO_2, the pH was adjusted to between 7.35 and 7.40 with HCl or NaOH, and the osmolarity was adjusted to between 300 and 305 mOsm with double distilled water or 10X Ringers.

Two-photon imaging

During imaging, slices were placed in a heated (31°C ± 1) immersion style recording chamber (Warner Instruments, Hamden, CT, USA) mounted on a microscope stage and perfused with oxygenated ACSF. Two-photon imaging was performed using a Bruker 2-photon microscope assembled around a mode-locked laser source emitting 140 fs pulses at an 80 MHz repetition rate with a wavelength adjustable from 690-1040 nm (Spectra Physics). Laser emission wavelengths of 940-950 nm were used to excite GCaMP (T. W. Chen et al., 2013; Tian et al., 2009), 1020 nm to excite tdTomato, and 860 nm to excite Cerulean (Drobizhev, Makarov, Tillo, Hughes, & Rebane, 2011). Full field of view images were acquired with an x-y raster scan. We used 20x 0.95 NA objective (Olympus, Tokyo, Japan). Emitted photons were bandpass filtered (Semrock, Rochester, NY, USA) at (peak/bandwidth): 525/50 nm (GCaMP), 593/46 nm (tdTomato) and collected by a wide band (300-650 nm) and low noise photomultiplier tube (H7360-01; Hamamatsu, Hamamatsu City, Japan). To avoid the "dead zone" on the surface of brain slices, only cells in focal planes at least 30 μm beneath the surface of the slice were

monitored.

Pharmacological activation of neurons

To activate neurons in acute brain slice, 500 μM of kainate, a glutamate receptor agonist was focally applied to cells. Glass electrodes were pulled from borosilicate glass capillaries (Schott #8250 glass, World Precision Instruments, Sarasota, FL) to ~2 μm using a micropipette electrode puller (P-97, Sutter Instruments, Novato, CA). The pipette was placed ~100 μm or less away from a cell of interest. Receptor agonists were delivered by pressure application (200-500ms, ~20 psi) using a picospritzer. Ca^{2+} activity was monitored in a single optical plane and measured by plotting the intensity of a region of interest (ROI) over time, after the intensity of the background ROI had been subtracted (Jia, Rochefort, Chen, & Konnerth, 2011).

Immunohistochemistry and quantification

On P9, P35, P60, electroporated rats were deeply anesthetized with sodium pentobarbital (25mg/kg, i.p.) and transcardially perfused with ice cold PBS followed by 4% paraformaldehyde diluted with PBS. Brains were removed and postfixed for 24 hours in a solution containing 20% sucrose and PBS. Frozen coronal sections were cut at 35 μm on a cryostat and mounted onto glass slides (Superfrost Plus Micro Slide, VWR). Sections were incubated for 48 hours with mouse anti-NeuN (1:100; Millipore MAB377) and rabbit anti-GFAP (1:2000; Millipore AB5804) diluted in Cyto-Q Immuno Diluent and Block (Innovex Biosciences). The primary antibody was localized with Alexa Fluor 647 goat anti-mouse and anti-rabbit IgGs (respectively) (1:200; Invitrogen A21235,

A21245) for 2 hours at room temperature. Following antibody labeling, slides were mounted with ProLong Gold Antifade (P36930 Invitrogen, Carlsbad, CA, USA). All imaging was performed with a Leica TCS SP5 confocal microscope equipped with a 20x objective. For quantification of colocalization of tdTomato/Cerulean and GFAP/NeuN, 512 x 512 pixel images were taken of the CA1, CA3, and DG regions of the hippocampus. Images were acquired in 2μm z stacks. Quantification of cells displaying colocalization of tdTomato/Cerluean with Alexa Fluor 488 was manually performed by a trained experimenter.

Western Blot

Wildtype rats on P9, P20, P35, and P60 were deeply anesthetized with pentobarbital, decapitated, and the hippocampi dissected. Protein expression of Synapsin I was quantified by Western Blot analysis. Briefly, hippocampi were rapidly isolated, frozen immediately on 2-methylbutane on dry ice, and stored at -80°C until further processing. The hippocampi were homogenized in 10 µl of lysis buffer (25mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Igepal CA-630, 5% glycerol, protease inhibitors (cocktail tablet, Roche), and 1mM sodium orthovanadate) per mg of tissue and the supernatant was collected. Total protein concentration was measured by BCA protein assay (Pierce) and 10 µg of total protein was subjected to electrophoresis using polyacrylamide gel (4-12% Bis-Tris gel, NuPAGE™, Invitrogen) under denaturing conditions. The proteins were transferred to a PVDF membrane, and Synapsin I and Actin were detected by chemiluminescence (NEL105001EA, PerkinElmer) using rabbit polyclonal antibodies against Synapsin I (1:25000; Invitrogen 51-5200) and actin

(1:250,000; Sigma A2103) followed by HRP-conjugated secondary antibody (1:3000; Invitrogen 65-6120). Densitometric analysis of protein levels was performed using ImageJ software (NIH).

Spontaneous calcium event selection and data analysis

In order to minimize the effect of region of interest (ROI) selection bias on our results, ROI selection was accomplished using GECIQuant scripts from the Khahk laboratory (http://www.physiology.ucla.edu/Labs/khakh/astrocytetools.htm) (Srinivasan et al., 2015). Automatic time-series event detection was accomplished with custom-written MATLAB (MathWorks, Natick, MA, USA) scripts. $\Delta F/F_0$ time-series plots were generated for each ROI by averaging F values of each pixel at each time point and using the median fluorescence of all image frames as the baseline fluorescence (F_0). The $\Delta F/F_0$ trace for each selected ROI was filtered with an order-3 one-dimensional median filter. The noise floor was defined as the median of the lower 30th percentile of fluorescence intensity values. The event threshold was set to three standard deviations above the noise floor with a $\Delta F/F_0$ of at least 30%. Using this threshold, event peaks were found with the findpeaks command in MATLAB. Events were eliminated if the time points adjacent to the event peak were not large enough (i.e., did not have a $\Delta F/F_0$ of at least 60% of the event threshold). Event durations were calculated as the time between the troughs before and after each event peak. The troughs before and after each peak were detected automatically and were defined as, respectively, the last or first data points before or after the peak that had a value less than three standard deviations above the noise floor.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). We used Student's t-test to compare means of normally distributed data. The Kolmogorov-Smirnov test (KS-test) was used to compare cumulative distributions. Western Blot data were compared using ANOVA with Tukey's Multiple Comparison test. Statistics are presented as mean ± SEM unless otherwise noted. A significance level of $p < 0.05$ was used.

Results

NASTIE, a Lck-GCaMP6f harboring plasmid for differentially labeling astrocytes and neurons following *IUE*

Genetically encoded calcium indicators offer a number of advantages over organic calcium indicators for studying calcium activity in the brain (Akerboom et al., 2013). GCaMP6f is the superior GECI, as it has faster kinetics and higher signal to noise than its predecessors (Tian et al., 2009). Furthermore, the plasma membrane targeting Lck-GCaMP is ideal for studying astrocyte calcium signaling as it reliably reports calcium signals in near membrane regions and fine processes (Shigetomi et al., 2010). A transgenic mouse line expressing Lck-GCaMP6f in astrocytes exists (Srinivasan et al., 2016). However, rat models are sometimes preferable to mouse models. In particular, rat models of Parkinson's disease and the development of epilepsy following status epilepticus, closely mimic particular aspects of human conditions and are critical tools for translational neuroscience research (Duty & Jenner, 2011; Gibbons, Smeal, Takahashi, Vargas, & Wilcox, 2013). Viral vectors are often used to deliver GCaMP to rat brains,

but the need for invasive injections near the imaging site and low carrying capacity of viral vectors (as discussed in (Gee et al., 2015)) renders this approach suboptimal in some settings. As an alternative, in utero electroporation (*IUE*) is a proven method for transfecting cells in the rat brain without strict limitations on transgene size or invasive injections associated with viruses. *IUE* has been used to transfect populations of both astrocytes and neurons in the brain and can be combined with *piggyBac* transposon systems to ensure stable integration in to the genome of the transfected cell. Integration facilitates transmission of the transgene to the entire lineage of transfected progenitor and stable expression of genes throughout the lifespan of the cells in the animal (F. Chen & LoTurco, 2012; Gee et al., 2015). However, baseline fluorescence from GCAMP protein is dim due to low concentrations of resting calcium in cells. To address this, we previously developed a tool kit of plasmids for *IUE* where we coexpressed tdTomato, a bright red fluorescent protein, with GCaMP under the control of the same promoter to enable detection of electroporated cells (Gee et al., 2015). Because transfection by electroporation is not specific to any one cell type, fluorescent reporters can also aid in determining the identity of the transfected cells by delineating a cell's morphology, since the reporter proteins will freely diffuse throughout the entire cytoplasm of cells, including the fine processes. However, we found that a single fluorophore was insufficient because it can be difficult to distinguish between astrocyte and neuronal processes labeled with the same fluorophore. In light of this, we designed *NASTIE* to conditionally change expression from tdTomato to Cerulean in cells that express the neuronal protein Synapsin Ia.

The *NASTIE* transgene is a *piggyBac* transposable element, flanked on both sides

with inverted terminal repeats (iTR) (Figure 3.1). The *piggyBac* transposase recognizes these iTRs and excises *NASTIE* from the plasmid and inserts it into a chromosome in the transfected cell. *NASTIE* also harbors GCaMP6f, which is targeted to the membrane with the Lck tag, and is driven by the strong ubiquitous promoter cytomegalovirus early enhancer/chicken beta actin (CAG) (Niwa, Yamamura, & Miyazaki, 1991). Expression of tdTomato (Shaner et al., 2004), is also under the control of the CAG promoter via an internal ribosomal entry site (IRES). IRES is a bicistronic sequence that allows for simultaneous expression of two proteins individually but from the same RNA transcript. The tdTomato gene is flanked by lox^{2272} sites (Suzuki, Inui, & Yukawa, 2007). The transgene also contains the gene for the blue fluorescent protein, Cerulean, downstream of tdTomato (Shaner, Steinbach, & Tsien, 2005). The pan neuronal specific rat promoter, Synapsin I (Hoesche, Sauerwald, Veh, Krippl, & Kilimann, 1993) drives expression of Cre, so that Synapsin I expressing cells transfected with *NASTIE* will also express Cre and Cre-mediated recombination will excise the tdTomato coding region, placing the Cerulean gene directly downstream of the IRES. Thus, in cells that express Synapsin I, Cre-mediated recombination results in the removal of tdTomato and the expression of Cerulean.

The entire *NASTIE* transgene is 12,426 base pairs and is flanked by terminal repeat sequences recognized by the *piggyBac* transposase. We used *IUE* to cotransfect the *NASTIE* transposon and a second plasmid that encoded the transposase, into progenitor cells in the rat hippocampus. We tested both wildtype *piggyBac* transposase (pbase) and hyperactive *piggyBac* transposase and observed nearly two-fold increase in the number of cells labeled by hyperactive *piggyBac* transposase (Figure 3.2). Therefore,

we opted to use the hyperactive *piggyBac* transposase when using *IUE* to express *NASTIE*.

Expression of *NASTIE* in cell culture and rat hippocampus demonstrates intact transgenes

We initially tested the *NASTIE* transgene by transfecting the Synapsin I-expressing human neuroblastoma cell line SH-SY5Y (Figure 3.3a-c). We observed expression of tdTomato and Cerulean in cells, indicating that tdTomato expression and Synapsin induced Cre-mediated recombination and subsequent Cerulean expression occurred as designed. We also observed a population of cells that demonstrated both tdTomato and Cerulean fluorescence, likely due to perdurance of the tdTomato protein and nascent expression of Cerulean, following Cre-lox recombination. These data provided initial confirmation of functional expression of tdTomato, Cre, and Cerulean genes from the *NASTIE* transgene. We also validated the functionality of the GECI, GCaMP6f, by transfecting primary neuronal cultures with *NASTIE*. Primary neuronal cultures express Synapsin and display high frequency spontaneous recurrent synchronized bursting behavior. The average magnitude of spontaneous calcium transients in primary cultured neurons was 258.34% $\Delta F/F_0 \pm 24.3$ and the frequency (number of events in 100 seconds) was 15.3 ±1.2 (n = 30 ROIs in 6 cells) (Figure 3.3d). These data validate the expression and function of the GCaMP6f gene in cells that underwent synapsin-dependent Cre-mediated recombination to express Cerulean.

NASTIE can be transfected in the rat brain using *IUE* to label astrocytes and neurons. High resolution 2 photon imaging in acute brain slices prepared from *NASTIE*

rats revealed labeling of two predominant cell types, marked by bright expression of tdTomato and Cerulean throughout their cellular compartments and fine processes (Figure 3.4). TdTomato positive cells were most frequently observed in the stratum radiatum (SR) of the hippocampus and displayed unambiguous morphological features of astrocytes, marked by a relatively small soma surrounded by heavily branched, fine processes. Cells labeled with Cerulean were most frequently observed in the cell body layer of the hippocampus and demonstrated characteristic morphological features of pyramidal neurons, including triangular shaped cell bodies and a large proximal dendrite projecting into the SR.

Developmental time course of *NASTIE* expression in hippocampal cells

We also validated the types of cells expressing Cerulean and tdTomato using immunohistochemistry in *NASTIE* transfected rat brains harvested from P9, P35, and P60 animals. To identify astrocytes, we used an antibody against the astrocyte marker, glial fibrillary acidic protein (GFAP) (Figure 3.5a). To positively identify neurons, we used an antibody against the neuronal antigen NeuN (Figure 3.5b). Colocalization of GFAP and NeuN with tdTomato and Cerulean-positive cells was quantified at each time point. Colocalization analysis of Cerulean-positive cells with NeuN revealed that the majority of cells that expressed Cerulean were also positive for NeuN staining (P9 = 88.1% ± 4.7; P35 = 79.13% ± 1.9; P60 = 71.26% ± 4.42; n =4-5; Figure 3.5e). Interestingly, a subset of Cerulean-positive cells at P35 (9.01% ± 2.06; n = 4; Figure 3.5e), P35 (21.25% ± 1.88; n = 4; Figure 3.5e) and P60 (25.50% ± 2.45; n = 4; Figure 3.5e) were also found to

coexpress with GFAP (Figure 3.5d), suggesting that Cre-dependent recombination of tdTomato had occurred in a subset of astrocytes, allowing for expression of Cerulean.

Colocalization of tdTomato with transfected cells showed a complex pattern during development. At the earliest time point observed, P9, less than half of tdTomato-positive cells colocalized with the astrocyte marker GFAP (44.9 ± 1.20; n= 4; Figure 3.5d). The remaining tdTomato cells at this time-point colocalized with NeuN (52.05% ± 6.37; n = 4; Figure 3.5d), suggesting that the transgene had not yet undergone Cre-dependent recombination in neurons or the Cerulean protein had not yet accumulated to the threshold level necessary for visualization. However, at the P35 and P60 time-points, approximately 90% tdTomato-positive cells colocalized with GFAP. These data suggest that tdTomato expression is a reliable marker of astrocyte cell identity. Taken together, the data indicate that the *NASTIE* transgene preferentially labels neurons with Cerulean and astrocytes with tdTomato in mature (P35 and P60) rats, making it a useful tool for the identification of astrocytes, neurons, and their processes in the hippocampus.

Synapsin promoter activity of *NASTIE* corresponds to the developmental expression of endogenous synapsin I in the rat hippocampus

We chose the rat Synapsin I promoter to drive Cre-mediated recombination due to well-reported specificity for achieving pan neuronal expression throughout the brain (Hoesche et al., 1993). We observed many tdTomato-positive cells in the stratum pyramidale layer of the hippocampus that showed clear morphological features of pyramidal neurons at the P9 time point (Figure 3.6a). We quantified total numbers of

tdTomato-positive cells during development and found that approximately 70.1% ± 5.054 (n = 4 rats) cells labeled were positive for tdTomato at P9, compared to 33.0 % ± 7.110 at P35 (n = 4 rats) and 29.11% ± 5.300 (n = 4 rats) at P60 (Figure 3.6a), indicating that the majority of Cre-mediated recombination events occur between the ages P9 and P35.

Synapsin I is a developmentally regulated gene whose product, the Synapsin I protein, is tightly linked with synaptogenesis and maturation of synapses during the first postnatal weeks (Cesca, Baldelli, Valtorta, & Benfenati, 2010; Hoesche et al., 1993). We examined total postnatal Synapsin I protein levels in the wildtype rat hippocampus at several postnatal time-points by immunoblot using an antibody against Synapsin I. At P9, Synapsin I levels were low (1.554 ± 0.06 Optical Density (OD), approximately 70% of adult levels, Figure 3.6e) and rapidly reached adult levels by P20 (2.19 ± .1202 OD; p = 0.0033), then remained stable throughout the P35 (2.102 OD ± 0.1247) and P60 (2.206 OD ± 0.1266) time points. These results are consistent with studies that examined Synapsin I expression in the rat cortex (Pinto, Jones, & Murphy, 2013) and mouse hippocampus, as well as the rat Synapsin I promoter expression in mouse brain (Hoesche et al., 1993) during development. Taken together, these data indicate that Cre-mediated recombination of tdTomato and subsequent expression of Cerulean in *NASTIE* is linked to the developmental profile of Synapsin I expression. Furthermore, it indicates that developmental regulation of Synapsin I during early life has important consequences on transgene labeling and must be taken into account when using *NASTIE* during early postnatal development.

Monitoring spontaneous and evoked calcium dynamics in *NASTIE* labeled hippocampal astrocytes and neurons

We next determined whether *NASTIE* would drive GECI expression sufficient for calcium signaling studies. Astrocytes play a prominent role in the modulation of synapses and synaptic transmission but experimental approaches for studying their activity have been very limited until recently. Genetically encoded calcium indicators such as GCaMP6 have proven to be useful for imaging studies of astrocyte activity. Astrocytes, in vivo and in brain slices, display frequent spontaneous astrocyte calcium events, particularly in the fine processes. We recorded astrocyte calcium signals in acute brain slices of the hippocampus prepared from *NASTIE* transfected rats. Astrocytes were identified by their characteristic morphology and the expression of tdTomato throughout in cell bodies and processes (Figure 3.7a, b). We observed large amplitude spontaneous localized calcium transients throughout astrocyte processes but very infrequent somatic events, as previously reported by our group and others (Gee et al., 2015). Frequent calcium transients were also observed in small compartments in astrocyte microdomains (Figure 3.7c).

Next, we validated the GCaMP6f response in individual neurons. Neurons in acute brain slices show little spontaneous activity, so we investigated direct activation of neurons by focal application of kainate to acute brain slices prepared from *NASTIE* rats. Fig. 3.7l-p shows an example of the change in GCaMP fluorescence observed upon application of a 20 ms puff of 500 μM kainate. Upon application of the agonist, a robust increase in calcium was observed (Fig 3.7q). In seven neurons, there was an average increase in fluorescence 258.34 $\Delta F/F_0 \pm 24.3$ in response to application of kainate (7

brain slices; from 3 animals). Cerulean fluorescence remained stable throughout the recordings with minimal photobleaching.

Spontaneous calcium activity differs between tdTomato and Cerulean positive astrocytes

Our data indicated a subpopulation of *NASTIE* astrocytes in the mature rat expressed Cerulean, indicating they had undergone Synapsin I-dependent Cre-mediated recombination. We were surprised by this observation, as numerous studies have used the Synapsin I promoter in order to achieve strong panneuronal selective expression in the brain[27]. However, astrocytes within the brain display a range of morphologies, markers, and functional specializations (Oberheim, Goldman, & Nedergaard, 2012; Y. Zhang & Barres, 2010), and our results suggest that *NASTIE* detected a novel population of astrocytes that express Synapsin I at some point during development. To further investigate these potentially distinct populations of tdTomato-positive and Cerulean-positive astrocytes, we evaluated spontaneous calcium transients in astrocyte processes.

Cerulean-positive cells that strongly resembled astrocytes were also observed in acute brain slices prepared for 2-photon imaging from mature *NASTIE* rats (Figure 3.8a-f). These Cerulean-positive astrocytes exhibited typical features of astrocytes, and were morphologically distinct from Cerulean-positive neurons (arrow in Fig. 3.8f). Frequent spontaneous calcium events were detected in both tdTomato-positive astrocytes and Cerulean-positive astrocytes, throughout the entire astrocyte syncytium. There were no differences detected in interevent interval (IEI), indicative of event frequency (Figure 3.8g). The average amplitude ($\Delta F/F_0$) of events was similar between tdTomato-positive

astrocytes (66.86 $\Delta F/F_0$ ± 46.82 SD; a total of 3760 events in 557 ROIs from 10 cells, 7 slices, 6 rats) and Cerulean-positive astrocytes (55.89 $\Delta F/F_0$ ± 29.16 SD; a total of 3123 events in 543 ROIs from 9 cells, 7 slices, 6 animals) but the distribution of events showed separation in the ocurrence of larger $\Delta F/F_0$ events, indicating that Cerulean-positive astrocytes were more likely to have events that were larger in amplitude (Figure 3.8h; p = $1.0330e^{-17}$, KS-test). Event duration of tdTomato-positive (15.05 seconds ± 13.012 SD; 3760 events in 557 ROIs from 12 cells, 8 slices, 6 rats) and Cerulean-positive (14.83 seconds ± 12.9 SD; 3123 events in 543 ROIs; 11 cells, 9 slices, 6 rats) astrocytes was also measured (Figure 3.8i). Comparison of the distribution of event duration between the two groups indicated that there was no separation in the traces or significant differences (Figure 3.8i; p = .015, KS-test).

Discussion

The goal of this study was to develop a novel tool, *NASTIE*, capable of distinguishing between astrocytes and neurons, while allowing us to monitor cell-cell interactions using GCaMP to study calcium signaling in both cell types. Significant advances in tools in the last decade, specifically the development and subsequent improvements upon GECIs, have provided new opportunities for understanding both neuron and astrocyte contributions to brain circuitry. The close proximity and complex interactions between these two cell types have made it clear that a complete understanding of brain circuit function relies on the ability to monitor and differentiate activity in both cell types. The *NASTIE* transgene was designed to allow for the monitoring of calcium transients in the fine processes of all transfected cells with Lck-

GCaMP6f, while also providing a fluorescent reporter system for labeling astrocytes with tdTomato and neurons with Cerulean.

The *NASTIE* transgene is 12,426 base pairs and is flanked by terminal repeat sequences recognized by the *piggyBac* transposase. The hyperactive *piggyBac* transposase was beneficial in achieving substantially higher integration of *NASTIE* in the rat hippocampus than wildtype *piggyBac* transposase. The large transgene underscores the utility of *IUE*, when compared to viral vehicles, to deliver increasingly sophisticated genetic tools such as *NASTIE*, as the relatively limited carrying capacity of most viral vectors disqualifies viral delivery methods for plasmids of this size with complex strong promoters that rely on introns (Hirsch, Agbandje-McKenna, & Samulski, 2010). Furthermore, the modular design of *NASTIE* makes it highly customizable. By swapping GCaMP6f for any of the growing number of number of optogenetic sensors, such as opsins (F. Zhang et al., 2010), iGluSnFR (Marvin et al., 2013), or rCaMP (Wu et al., 2014), to name a few, *NASTIE* could be used to monitor a variety of cellular functions. In addition, by replacing either the CAG or Synapsin I promoter, any two specific populations of cells could be targeted in order to genetically distinguish cells during experiments in real time.

The *NASTIE* transgene was designed to specifically label neurons with Cerulean and astrocytes with tdTomato, because the established dogma held that only neurons express the protein Synapsin. In these experiments, *NASTIE* identified a subpopulation, of up to 25% in mature rats, of cells that expressed Cerulean, labeled positive for the astrocyte-specific marker GFAP, and also displayed many morphological and functional characteristics of astrocytes. We were somewhat surprised by this observation since

Synapsin I has been reported to be a neuron-specific protein (Huttner, Schiebler, Greengard, & De Camilli, 1983), although expression has been shown in the testes (Harno, Cottrell, & White, 2013). Furthermore, expression of the rat Synapsin I promoter with viruses was reported to be restricted to neuronal cells (Dittgen et al., 2004; Jackson, Dayton, Deverman, & Klein, 2016). Cerulean-positive astrocytes might be explained by a number of possible scenarios such as a) "leaky" expression of the Cerulean gene, (i.e., expression of Cerulean occurred in cells that did not undergo recombination), b) or by nonspecific expression of the Cre gene (i.e., expression not under the control of the Synapsin I promoter), or c) expression of Synapsin in a subset of astrocytes. It is unlikely that "leaky" Cerulean expression is responsible for Cerulean+ astrocytes, because in a control experiment in MEF cell lines that were transfected with either the complete *NASTIE* transgene or a the immediate predecessor plasmid, lacking Synapsin-Cre, only tdTomato gene expression occurred until Cre was provided exogenously. In addition, we believe that nonspecific Cre expression is unlikely to account for Cerulean+ astrocytes, given that the temporal pattern of Cerulean expression corresponds to the developmental regulation of Synapsin I expression in the hippocampus, with more astrocytes expressing Cerulean at the later developmental time points. Furthermore, chromosomal integration site-mediated missexpression of Cerulean is unlikely because Cerulean expressing astrocytes were seen in multiple independent electroporated rats. It is extremely unlikely that the random integration of *NASTIE* in to the chromosome identified multiple sites in the genome that yield the same misexpression pattern. Indeed, the presence of Cerulean+ astrocytes suggests that this population reflects a genetically distinct population of astrocytes that expresses Synapsin I at some point during their lineage and therefore may

have a distinct function associated with this expression pattern.

Cellular heterogeneity of astrocytes is a topic that has received little direct attention until very recently. There is a growing recognition of an expansive heterogeneity among astrocytes, including the existence of specific astrocyte subpopulations with differential molecular and functional properties (Farmer et al., 2016; Lin et al., 2017). That these subpopulations are determined, in part by neuronal-astrocyte signaling during development, suggests that a modular plasmid such as *NASTIE,* would be beneficial for this line of study. The study of diverse astrocyte subpopulations represents a potential new way of thinking about brain circuit function and with it, will come the need for new tools. Spontaneous calcium-induced fluorescent transients are a prevalent feature of astrocytes and are proposed to be an important factor in intracellular signaling. While the role of spontaneous astrocytic calcium activity is not well understood, it seems likely to serve as a mechanism of intracellular signaling. While some small difference was seen, our initial analysis did not identify spontaneous transients as a distinguishing factor between tdTomato-positive and Cerulean-positive astrocytes. However, investigation of spontaneous calcium activity with GECIs has illuminated the diversity of signals exhibited within territories of single astrocytes. Therefore, while we are currently exploring, in greater depth, the study of spontaneous calcium activity in astrocytic-neuronal networks, the data gathered here clearly demonstrate the kind of information that can be gathered using *NASTIE* to label multiple cell populations in the brain.

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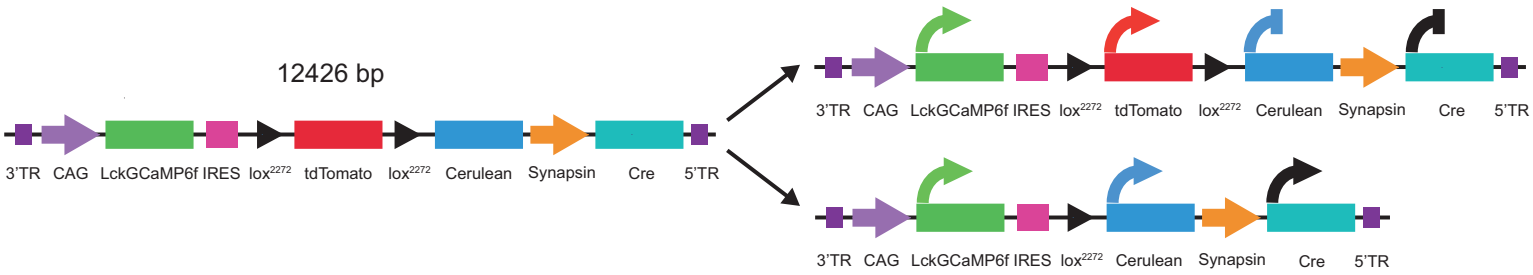


Figure 3.1. A schematic diagram of the Neuron Astrocyte Specified Transgene with in utero Electroporation, *NASTIE*, transgene and Cre-recombinant product.
This 12,462 base pair transposable transgene enables Cre-mediated recombination through rat synapsin I promoter driven Cre that restricts expression to cells expressing Synapsin, *NASTIE* differentiates Synapsin I expressing cells from other transfected cells, through the expression of Cerulean. Cerulean is expressed as a consequence of the Cre-mediated recombination of tdTomato. All transfected cells express the calcium indicator, GCAMP6f. The *NASTIE* transgene is flanked by two *piggyBac* terminal inverted repeats (TIR) that are recognized by the *piggyBac* transposase (encoded on a second cotransfected plasmid, not shown) that mediates the insertion of the transgene into a chromosome.

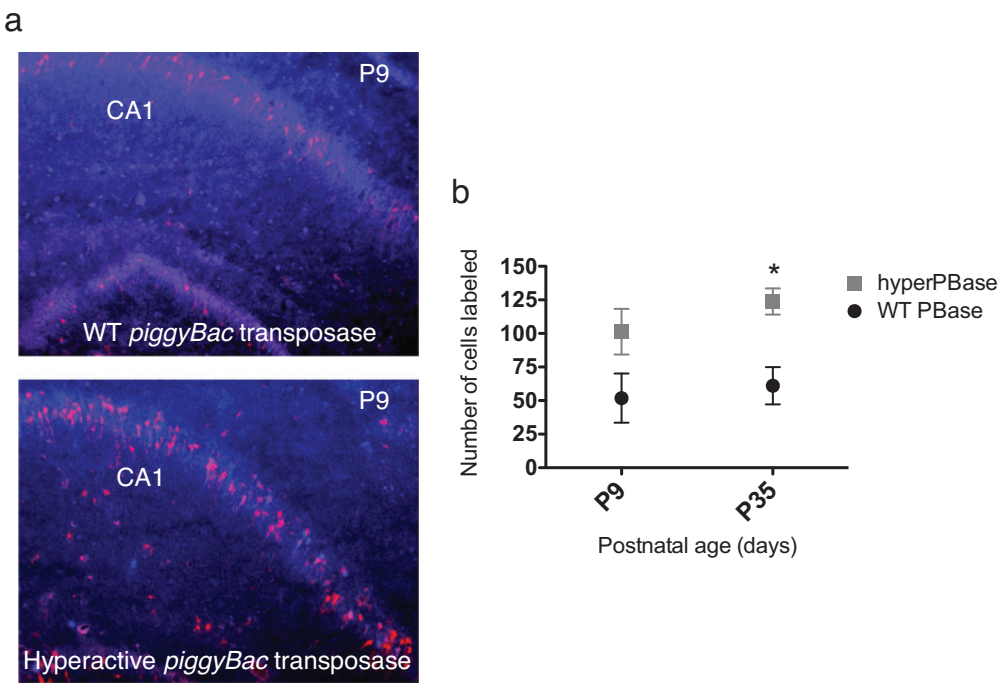


Figure 3.2. Comparison of *NASTIE* expression with hyperactive and wildtype *piggyBac* transposase.
NASTIE was colectroporated with the same concentration of either wildtype transposase (WT PBase) or hyperactive transposase (hyperPBase)**. (a)** Example 20x images of transfected rat hippocampus demonstrates higher number of cells expressed either tdTomato or Cerulean at P9 with WT compared to Hyperactive transposase. (**b**) Quantification of number of cells labeled with hyperactivePBase (grey, square) vs WT pbase (black, circle) demonstrates a two-fold increase in the overall number of cells expressing either Cerulean or tdTomato fluorophores with 4PB. Statistical analysis: unpaired t-test.

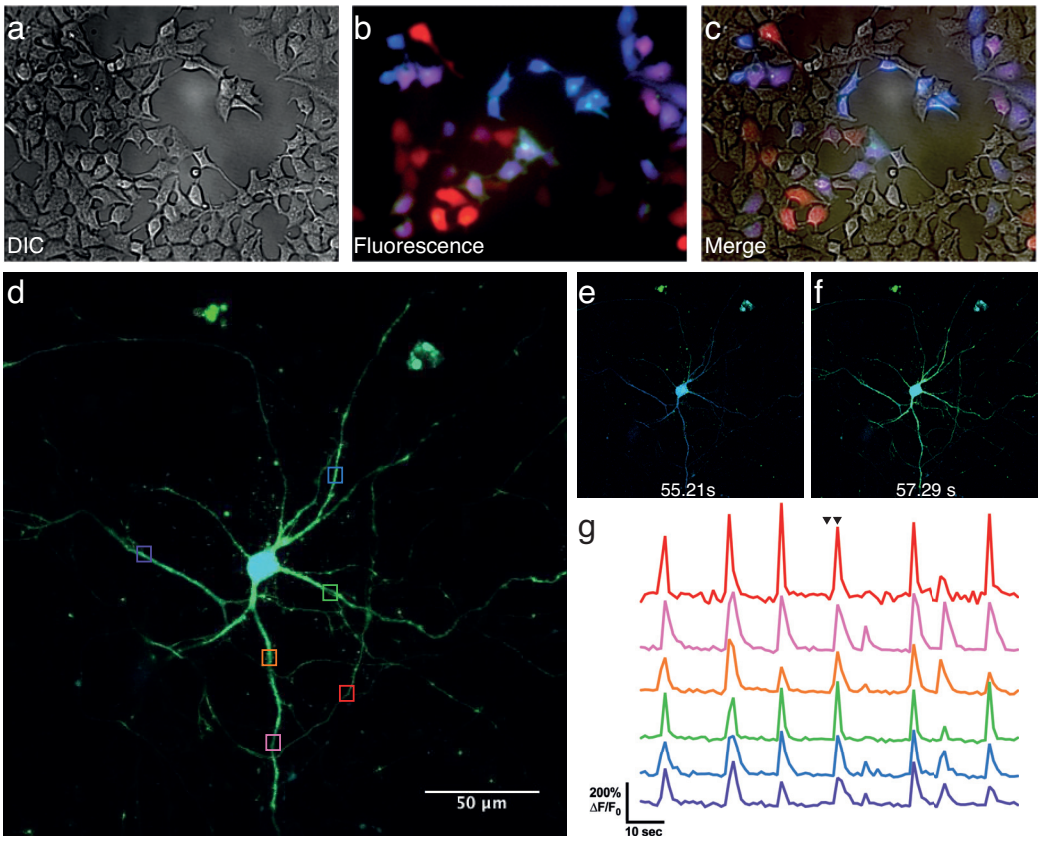


Figure 3.3. Validation of *NASTIE* transgene expression and function in cell culture. A human neuroblastoma cell line, SH-SY5Y (**a-c**) and primary dissociated neuronal cultures (**d-f**) were used to validate expression of *NASTIE* transgenes. Transfected SH-SY5Y cells demonstrate functional expression of TdTomato, Cerulean, and GCaMP genes. Fluorescence is contained in the boundaries of cells. Primary neurons transfected with *NASTIE* exhibited GFP and Cerulean (**d**) fluorescence and exhibited spontaneous epileptiform bursting behavior. (**e-f**). Representative individual traces of GCaMP6f fluorescence changes ($\Delta F/F_0$) of bursting behavior (**g**). The color-coding of the traces corresponds to square ROIs shown in **(d)**.

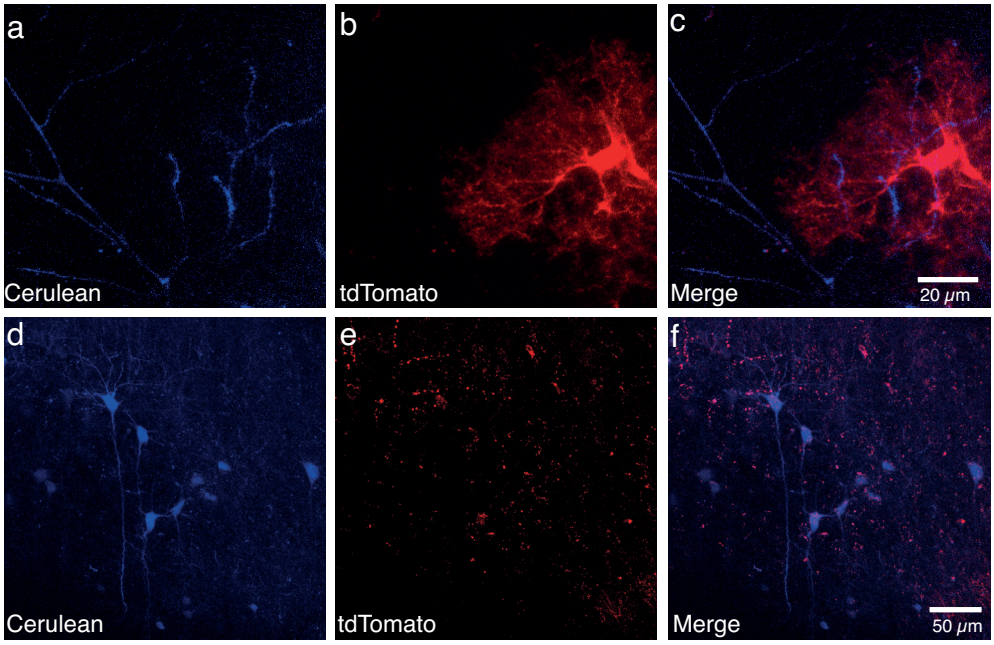


Figure 3.4. Close apposition of astrocyte and neuron processes in acute brain slices. High magnification 2Photon images of *NASTIE* labeled neuronal processes (blue) and astrocyte (red) in the stratum radiatum of hippocampus in acute brain slice. (**a-f)** *NASTIE* labeled neuronal cell bodies (blue) and astrocyte processes (red) in the CA1 region of hippocampus.

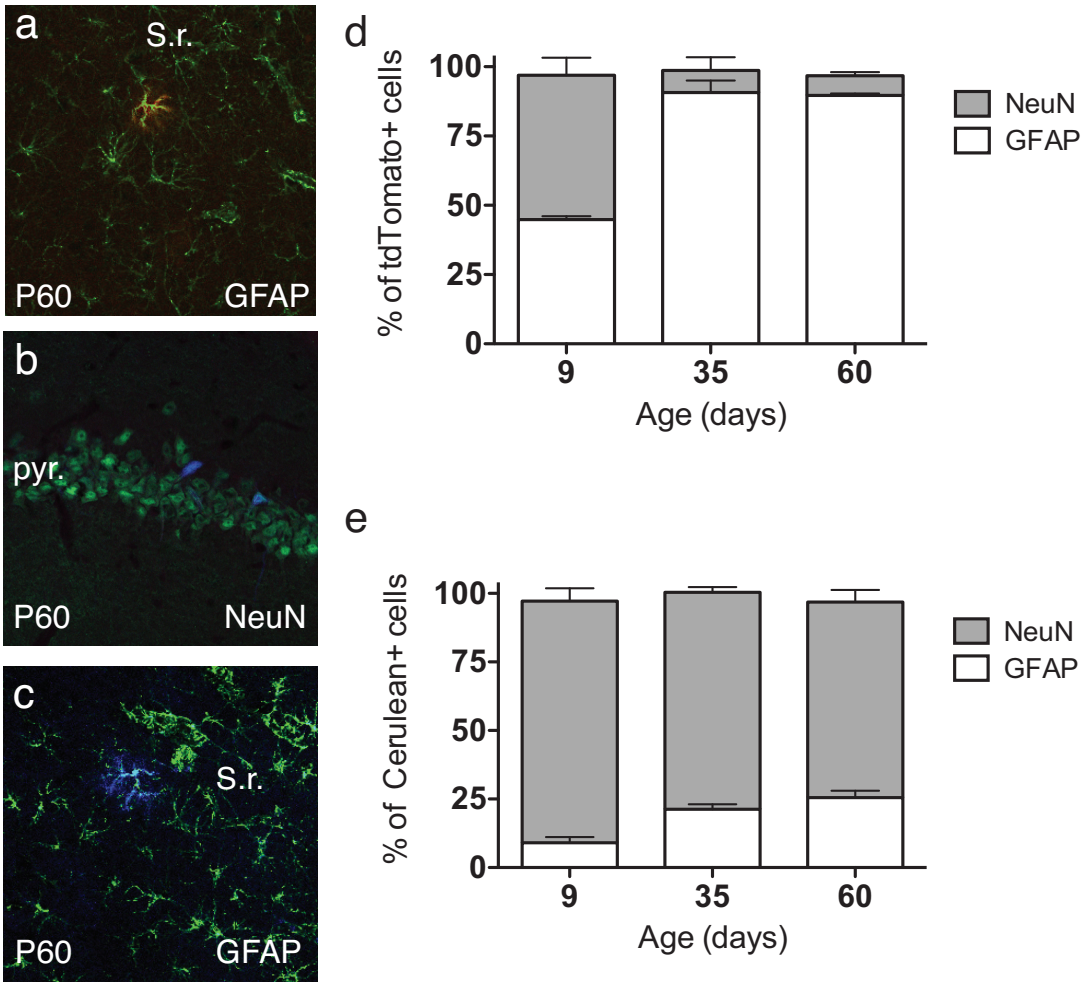


Figure 3.5. Characterization of cell-types labeled by *NASTIE* throughout development.
Representative images of (**a**) GFAP and tdTomato positive cells; (**b**) Cerulean and NeuN positive; (**c**) and Cerulean and GFAP positive cells in P60 animals. Quantification of tdTomato- and Cerulean-positive cells that colabeled with NeuN (neuronal marker) and GFAP (astrocyte marker). The total percentage of tdTomato or Cerulean cells that labeled with either GFAP or NeuN was calculated (n = 4-5 brains)(**d** and **e**).

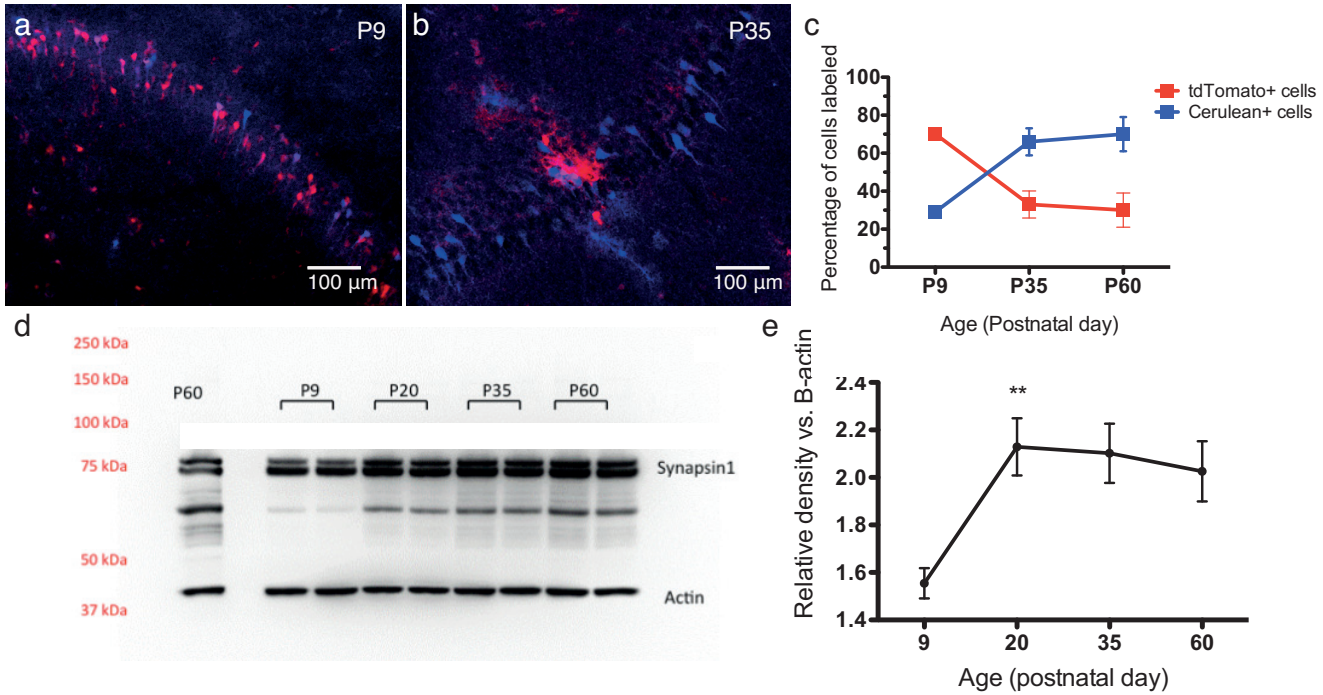
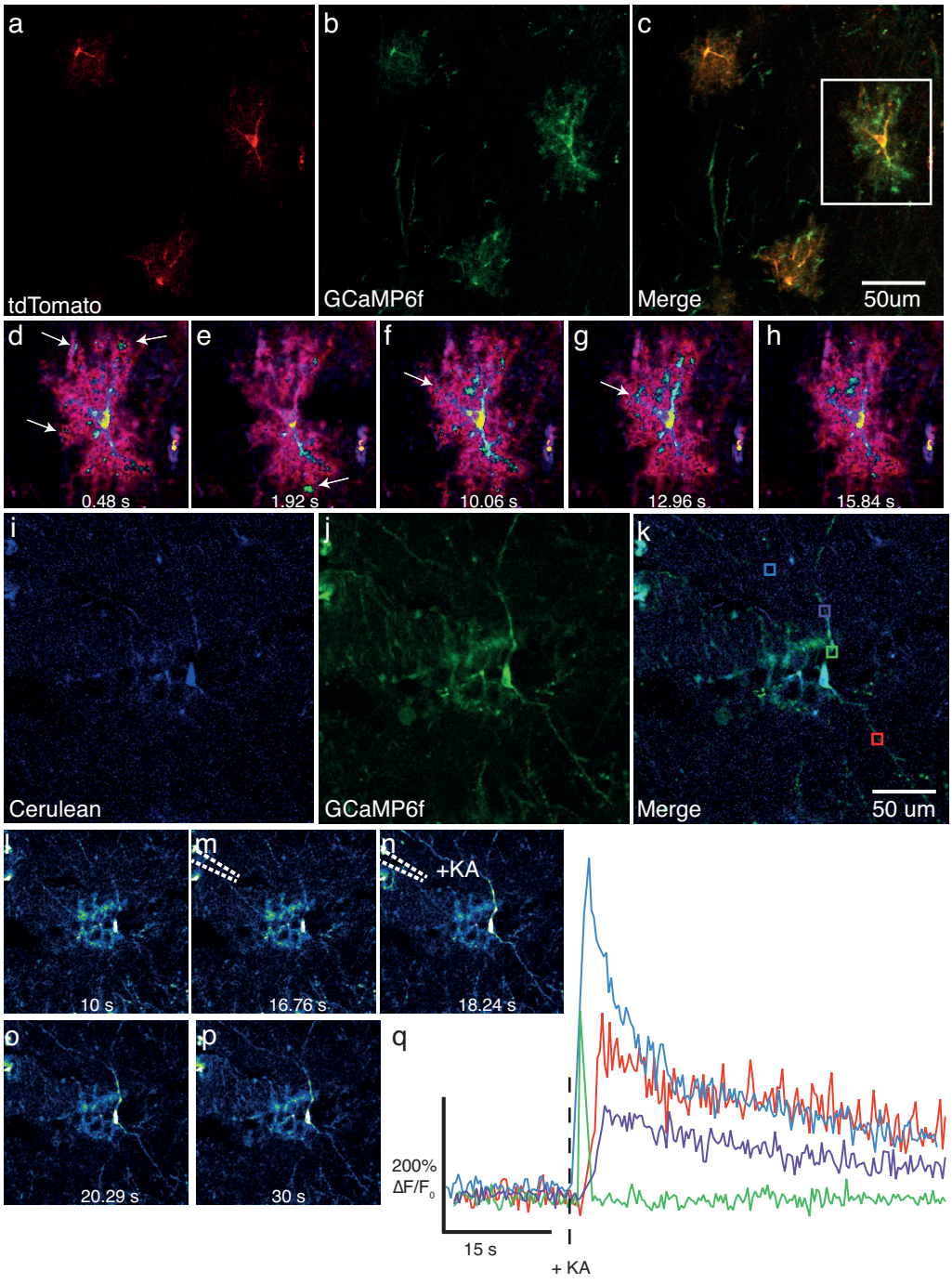


Figure 3.6. Cre-mediated recombination of *NASTIE* mimics endogenous Synapsin I protein expression.
(a-b) Representative images of the CA1 region from postnatal age P9 and P35 rat hippocampi transfected with *NASTIE*. (**c**) Quantification of labeled cells demonstrates that at P9, the majority of *NASTIE* transfected cells are tdTomato-positive. By P35, however, the majority of cells are Cerulean. *** = p < .001 (**d**) Western Blot of endogenous Synapsin I expression in P9, P20, P35, and P60 WT rat hippocampus. (**e**) Quantification of the relative density of bands compared to control (B-actin) over time demonstrates that synapsin levels are initially low and rise rapidly between P9-P20 to reach adult levels. ** = p <. 01

Figure 3.7. Spontaneous and evoked calcium transients are observed in *NASTIE* astrocytes and neurons in acute brain slices.
Mean calcium activity projection image of an astrocyte expressing tdTomato (**a**), Lck-GCaMP6f (**b**), and overlay (**c**) in an acute brain slice. (**d-h)**, frames from a movie demonstrating spontaneous calcium transients throughout the astrocyte, including activity in distinct microdomains, as indicated arrows. Example mean calcium activity projection of a neuron expressing Cerulean **(i**), GCaMP **(j)**, and overlay (**k).** (**l-p)** Time series of intracellular neuronal calcium increases induced by focal application of kainate (500 uM) from a glass pipette (white dotted line) in an acute brain slice prepared from *NASTIE* rat. The pseudocolor scale displays changes in relative Lck-GCaMP6f emission. (**q**) Representative individual traces of GCaMP6f fluorescence changes ($\Delta F/F_0$) in response to KA administration. The color-coding of the traces corresponds to square ROIs shown in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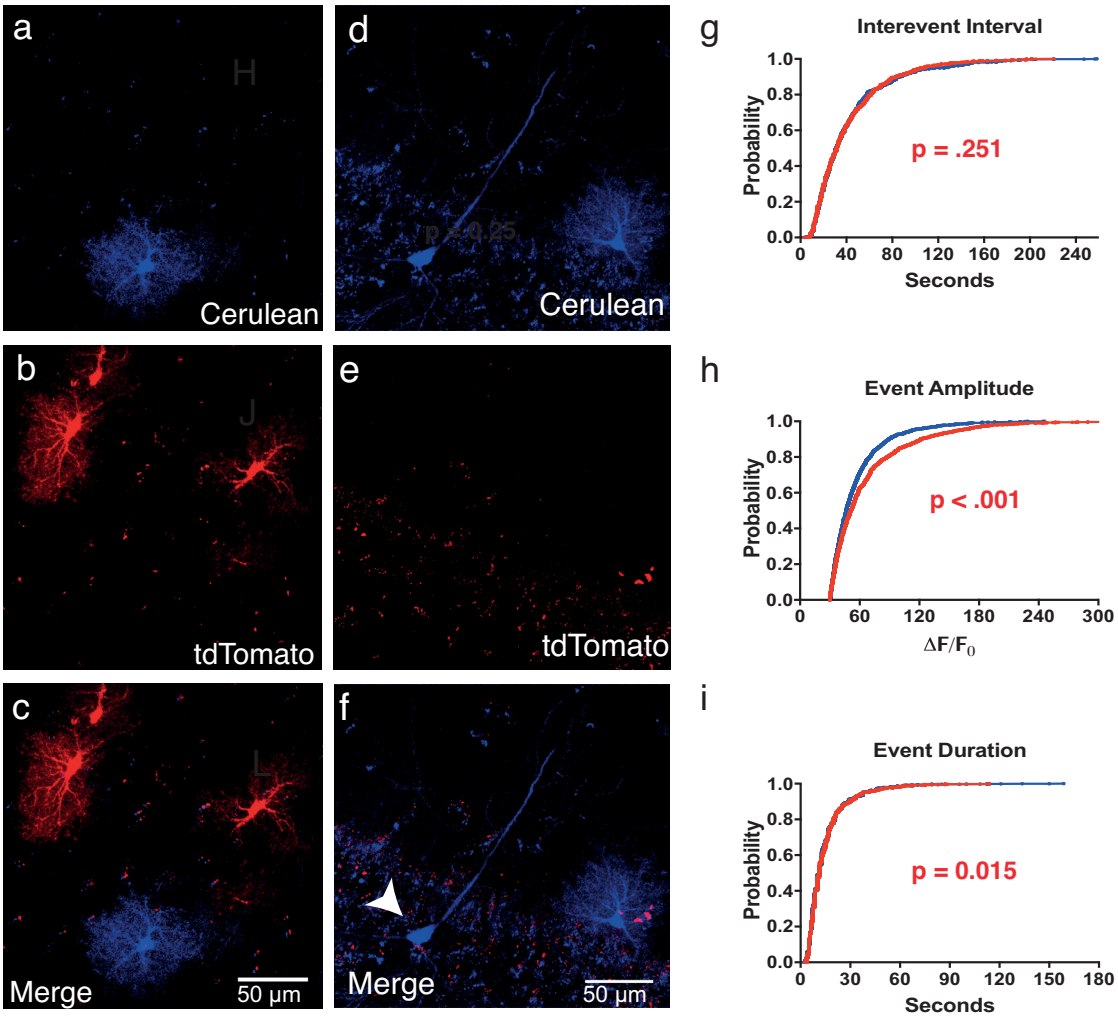

Figure 3.8. Spontaneous astrocyte calcium activity varies between tdTomato-positive and Cerulean-positive astrocytes.
Example images of *NASTIE* transfected Cerulean-positive and tdTomato-positive astrocytes in brain slices prepared from mature rats show complete expression of either Cerulean or tdTomato . Panels **a-f** show example images of Cerulean-positive astrocyte and neuron from a mature *NASTIE* rat. Cumulative histograms displaying spontaneous event amplitude (**g**), Interevent Interval (**h**), Amplitude (**i**), Event Duration are shown. TdTomato-positive astrocyte frequencies are shown in red and Cerulean-positive astrocyte frequencies are in blue. The calculated p-value from a Kolmogorov-Smirnov test is shown in the plot.

CHAPTER 4

CALCIUM SIGNALING IN REACTIVE ASTROCYTES IN A MODEL OF TEMPORAL LOBE EPILEPSY

Abstract

The importance of astrocytes in almost all aspects of brain physiology is now widely recognized. The processes of astrocytes are closely apposed to neuronal synapses, and astrocytes can modulate neuronal activity by regulating extracellular potassium and glutamate. Furthermore, astrocytes may directly affect the excitability of neurons through the release of signaling molecules such as glutamate and ATP. Temporal lobe epilepsy is a disorder that has historically been understood through a "neurocentric" lens with a focus on how neural circuit dysfunction leads to hyperexcitability and seizures. Reactive astrocytes in epileptic and epileptogenic tissue also show dramatic changes in morphology and protein expression, but their functional properties remain poorly understood.

In the kainic acid-induced status epilepticus model of temporal lobe epilepsy, astrocytes in the hippocampus become reactive shortly after status epilepticus and before the onset of spontaneous seizures, during a window referred to as the latent period. Recently, our laboratory found that reactive astrocytes during the latent period begin to express kainate receptor subunits and this expression persists through the development of

chronic epilepsy. The function of kainate receptors on reactive astrocytes, however, is currently unknown. The experiments in this chapter were designed to answer two questions: 1. What are the properties of spontaneous calcium signals in reactive astrocytes during the latent period? 2. Are the kainate receptors that are expressed on reactive astrocytes functional? To answer these questions, the genetically encoded calcium indicator, Lck-GCaMP6f, was expressed in the rat brain. Reactive astrocytes imaged in acute brain slices obtained from rats experiencing status epileptics (SE) showed longer interevent intervals and duration of spontaneous calcium events compared to saline-treated controls. In addition, astrocytes exhibited a bimodal response to application of the kainate receptor agonist, (RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid (ATPA), suggesting that there are two populations of reactive astrocytes, those that express functional kainate receptors and those that do not. Taken together, these results identify changes that occur in calcium signaling in reactive astrocytes during the process of epileptogenesis and may thus provide new targets for disease modifying therapies.

Introduction

Astrocytes occupy a strategic position in the brain where they can act as an intermediary between neurons, blood vessels, and the cerebral spinal fluid (CSF). This location is ideal for sensing and responding to overall changes in brain state. Much consideration has focused on the role of intracellular Ca^{2+} signaling in the physiology and function of astrocytes in a variety of contexts throughout the brain. Work over the past two decades has led to a revolution in the way that astrocytes are understood in the

context of brain physiology. In light of this, it is perhaps unsurprising that recent studies have also begun to elucidate their involvement in the etiology in a host of neurological disorders, including temporal lobe epilepsy (TLE) (Coulter & Steinhauser, 2015; Steinhauser & Boison, 2012; Wetherington, Serrano, & Dingledine, 2008).

Astrocytes become reactive in response to CNS injury

In response to injury, healthy astrocytes undergo a multistage, defensive reaction and transform into a reactive state, commonly referred to as reactive astrogliosis (Burda & Sofroniew, 2014). Astrocyte reactivity involves a host of morphological, transcriptional, and functional changes. Of these changes, the most commonly recognized characteristic of reactive astrocytes is high levels of expression of the intermediate filament Glial Fibrillary Acidic Protein (GFAP) (Clarke, Shetty, Bradley, & Turner, 1994). Increases in GFAP expression, and in the number and length of GFAP-positive processes, is a characteristic trait of reactive astrocytes, and as such, immunohistochemical detection of increased expression of GFAP is used as standard and reliable marker of reactive astrocytes throughout the CNS and spinal cord (Faulkner et al., 2004; Oberheim, Goldman, & Nedergaard, 2012; Zamanian et al., 2012).

The extent of reactive astrogliosis is often graded, depending on the type and severity of the insult, and can range in intensity from a mild, to severe phenotype called a glial scar (Burda & Sofroniew, 2014; Sofroniew & Vinters, 2010). Astrocytes express many transmembrane receptors and can detect and respond to signals in the extracellular space that reflect changes in overall brain homeostasis. Reactive gliosis is regulated by a large array of extracellular signals, which are largely dependent on the type of triggering

CNS insults. The molecular signals causing reactive astrogliosis can be traced back to all cell types found in the CNS, including neurons, microglia, oligodendrocytes, pericytes, endothelial cells, and other astrocytes (Burda & Sofroniew, 2014). Many different types of intercellular signaling molecules are able to trigger reactive astrogliosis, including growth factors and cytokines, immune mediators such as lipopolysaccharide and other Toll-like receptor ligands, neurotransmitters, purines such as ATP, reactive oxygen species (ROS), hypoxia and glucose deprivation, proteins associated with neurodegeneration such as β-amyloid, molecules associated with metabolic toxicity such as NH_4^+, and regulators of cell proliferation (Ben Haim, Carrillo-de Sauvage, Ceyzeriat, & Escartin, 2015; Sofroniew, 2009; Sofroniew & Vinters, 2010).

These extracellular signals act on numerous receptors at the astrocyte membrane to trigger a host of intracellular signaling cascades causing large-scale transcriptional changes (Ben Haim et al., 2015). These signaling cascades seem to converge on the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway (Ceyzeriat, Abjean, Carrillo-de Sauvage, Ben Haim, & Escartin, 2016; Pekny & Pekna, 2014). Pharmacological inhibition of the JAK/STAT pathway in a mouse model of Parkinson's disease decreases GFAP expression, suggesting that the JAK/STAT3 pathway is required to induce astrocyte reactivity. Furthermore, other cascades including the Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells (NF-κB) pathway, the calcineurin (CN) pathways, and the Mitogen-Activated Protein Kinase (MAPK) pathway (Ben Haim et al., 2015) are involved in regulating and maintaining astrocytes in a state of reactivity. Furthermore, many additional levels of complexity potentially exist due to cross talk between these intracellular signaling pathways, as well

as posttranscriptional regulation in gene expresses due to changes in microRNAs (Ben Haim et al., 2015; Iyer et al., 2012).

The causal relationship and contributions of reactive astrocytes to disease progression is unclear (Sofroniew, 2005). The effects of reactive gliosis can be viewed as beneficial or detrimental, depending on experimental procedure, disease model, and extent or stage of astrogliosis (Ben Haim et al., 2015). Two recent key studies provide compelling evidence that reactive astrocytes cause hyperexcitability in brain networks. The first study used high titers of adeno-associated virus (AAV) to induce astrogliosis throughout the hippocampus (Ortinski et al., 2010). This led to downregulation of the astrocytic enzyme glutamine synthetase, disrupting hippocampal inhibition, and leading to widespread neuronal hyperexcitability in acute brain slices. In addition, Robel et al. (2015) developed a mouse model of reactive astrogliosis by chronically deleting β1-integrin in GFAP-expressing cells (Robel et al., 2015). β1-integrin knockout mice had spontaneous, recurrent, generalized tonic–clonic seizures in vivo and the astrocytes showed a number of impairments, including alterations in potassium and glutamate buffering. Taken together, these two studies provide evidence to suggest that reactive astrogliosis leads to hyperexcitability and seizures.

Human mesial temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most common form of medically intractable epilepsy and is defined by seizures that originate from the temporal lobe. A common pathological finding in patients with TLE is the presence of hippocampal sclerosis. It is found in approximately 50%-75% of temporal lobe resections and is characterized by

neuronal cell loss in the CA1 and CA3 cell fields, dentate gyrus, and entorhinal cortex, tissue shrinkage, and marked reactive gliosis throughout the hippocampus (Al Sufiani & Ang, 2012; Janigro, 2008; Thom et al., 2009). In a study of surgically resected sclerotic hippocampal tissue from TLE patients, GFAP immunoreactivity and inflammatory mediators (COX-2, TGF-β, NFkB) were present in astrocytes, suggesting widespread changes in reactive astrocytes in the sclerotic hippocampus (Das et al., 2012).

A long-standing and fundamental question in epilepsy research is whether damage causes hippocampal sclerosis, and this in turn is responsible for seizures, or whether there is a preexisting pathology, or underlying genetic factors, which predisposes to seizures and later on hippocampal sclerosis. The data available suggest that among TLE patients undergoing surgical resection of the temporal lobe for uncontrolled seizures, precipitating injuries early in life are highly associated with hippocampal sclerosis. Precipitating injuries include febrile seizures, head trauma, birth injuries or *status epilepticus* (defined as a seizure causing loss of consciousness lasting longer than 30 minutes) (Mathern, Babb, Vickrey, Melendez, & Pretorius, 1995). Thus, a common clinical manifestation of TLE in humans is a precipitating injury that is followed by a variable latent period, and eventually the development of spontaneous motor seizures.

The current treatment for epilepsy is based on the suppression of symptoms (i.e., seizures) by antiseizure drugs. There are currently no therapies to modify the disease process in patients with a known increased risk of epilepsy due to a history of precipitating injury (Terrone, Pauletti, Pascente, & Vezzani, 2016). The term *epileptogenesis* encompasses the cascade of molecular and cellular events that take place causing a healthy brain to develops spontaneous seizures (Curia et al., 2014;

Pitkanen et al., 2007). The development of treatments to prevent epileptogenesis is a major objective in epilepsy research. However, exploring these processes in human tissues is limited. Often, human tissue that is available for study comes from surgical resection of hippocampal tissue, and thus reflects an advanced stage of epilepsy as well as years of exposure to antiseizure drugs. Animal models of TLE, discussed in the next section, provide the opportunity for careful study of this period.

Animal models of TLE

In order to better understand the pathophysiology of TLE, and to carefully study the mechanisms of epileptogenesis, several in vivo animal models that present features similar to human TLE have been developed. There are several TLE models available, and all recapitulate the clinical manifestations of TLE in humans, including a precipitating injury that is followed by a latent period with the subsequent development of spontaneous motor seizures. The systemic low-dose kainic acid model of TLE in rat uses the systemic administration of the glutamate receptor agonist kainic acid (KA) to induce status epilepticus (SE), defined as a period of seizure activity lasting for at least 30 minutes during which full consciousness does not recover (Hellier, Patrylo, Buckmaster, & Dudek, 1998; Williams et al., 2009). Following SE, there are no clinical signs of seizure activity, but significant alterations in neuronal and glial cell structure and function can occur. Axon sprouting, structural changes in pre- and postsynaptic receptors, changes in voltage-gated ion channels, alterations of homeostatic mechanisms, and neuronal degeneration have all been associated with epileptogenesis (Dudek & Staley, 2012). The duration of the latent period typically spans between 10 and 30 days after the initial SE in

rats, but can last as long as several months (Williams et al., 2009). Approximately 96% animals that have SE in the low-dose systemic KA model will go on to develop spontaneous recurrent seizures (Thomson, Modi, Glauser, Rausch, & Steve White, 2017).

As mentioned previously, in human TLE and in the KA-treated rat model of TLE, reactive astrocytes are observed throughout the hippocampus. Data acquired from the KA model of TLE demonstrate that following KA-induced SE, there are dramatic increases in GFAP expression 7 days following SE and the increased expression of GFAP persists in astrocytes throughout the development of chronic epilepsy (8 weeks) (Figure 1.1A). In addition, reactive astrocytes following SE exhibit numerous changes in potassium buffering, and gene expression, including transmembrane receptor expression. These changes are summarized in Chapter 1. In the next section, we will discuss an aspect of astrocyte biology, Ca^{2+} signaling, that has only recently begun to be explored in TLE.

Astrocyte signaling

Astrocytes, while not electrically excitable, show a type of chemical excitation in the form of increases in intracellular calcium (Agulhon et al., 2008; Bazargani & Attwell, 2016). Astrocytes express numerous G-coupled protein receptors (GPCRs) receptors coupled to second messenger systems, and have been shown to mobilize calcium from intracellular stores in response to several neurotransmitters, such as glutamate, ATP, GABA, adenosine, and norepinephrine (Agulhon et al., 2008; Bekar, He, & Nedergaard, 2008). The most widely accepted mechanism for astrocytic Ca^{2+} increases is via the canonical phospholipase C (PLC)/inositol (1,4,5)-trisphosphate (IP3) pathway. Upon activation of GPCR, PLC hydrolyzes the membrane lipid phosphatidylinositol 4,5-

bisphosphate to generate diacylglycerol (DAG) and IP_3, leading to IP_3 receptor (IP_3R) activation and Ca^{2+} release from the endoplasmic reticulum (ER) (Fiacco & McCarthy, 2006). Calcium elevations can be increased throughout the entire cell including the soma, or be localized to a few processes or astrocyte endfeet, or small, physiologically restricted volumes dubbed microdomains (Mulligan & MacVicar, 2004; Volterra, Liaudet, & Savtchouk, 2014). A recent study showed that including norepinephrine, ATP, or the mGluR-agonist in the extracellular solution caused unique patterns of microdomain activity, suggesting that microdomains are physiologically distinct, contain different receptors, and can experience different modes of Ca^{2+} increases (Agarwal et al., 2017).

Furthermore, astrocytes in the hippocampus, cortex, and thalamus have been shown to exhibit intrinsic oscillations independent of neuronal activity (Aguado, Espinosa-Parrilla, Carmona, & Soriano, 2002; Nett, Oloff, & McCarthy, 2002; Parri & Crunelli, 2003; Rungta et al., 2016). When neuronal action-potential-mediated and spontaneous synaptic vesicle release is blocked by both TTX and bafilomycin, astrocytes still exhibit transient increases in intracellular calcium, particularly in the fine distal processes (Aguado et al., 2002; Nett et al., 2002; Parri & Crunelli, 2003). Subsequent experiments demonstrated that the spontaneous Ca^{2+} dynamics observed in astrocytes in vivo are similar to those described in acute slice preparations (Hirase, Qian, Bartho, & Buzsaki, 2004; Nimmerjahn, Kirchhoff, Kerr, & Helmchen, 2004; Wang et al., 2006). When the IP_3R2 gene is deleted, spontaneous calcium transients are reduced in the soma but are still present in the fine processes (Haustein et al., 2014). This suggests that many signaling pathways are involved in generating spontaneous transients. To this end, a recent report showed that spontaneous Ca^{2+} signals in the fine processes depend on

transmembrane Ca^{2+}influx. When Ca^{2+} is removed from the external solution, Ca^{2+} transients were reversibly eliminated, suggesting some unidentified Ca^{2+} influx pathway is responsible for transients in fine processes (Rungta et al., 2016).

The observation of intracellular Ca^{2+} activity has led many to wonder whether pathological neuron-astrocyte signaling could promote abnormal neuronal synchronization in epilepsy (Carmignoto & Haydon, 2012; Wetherington et al., 2008). However, calcium dynamics in reactive astrocytes in a model of TLE have only been investigated in one study to date (Ding et al., 2007). This study was performed using the bulk-loaded calcium indicator Fluor4. Fluor4 and other synthetic dyes do not diffuse throughout the entire astrocytes, and tend to be restricted to the soma and major branches, making it difficult to accurately record calcium fluctuations throughout the entire astrocyte, including the fine processes (Reeves, Shigetomi, & Khakh, 2011). Since the publication of Ding et al. (2007), the development of genetically encoded Ca^{2+} indicating proteins (GECIs) has enabled improved monitoring of astrocyte Ca^{2+} dynamics throughout astrocyte fine processes (Gee et al., 2015; Gee et al., 2014; Shigetomi, Kracun, & Khakh, 2010; Shigetomi, Patel, & Khakh, 2016). A recent study in a mouse model of Huntington's disease, GECIs uncovered dramatic changes in spontaneous and stimulation evoked Ca^{2+} dynamics in striatal astrocytes (Jiang, Diaz-Castro, Looger, & Khakh, 2016). Changes in the frequency, duration, and amplitude of spontaneous astrocyte activity were observed, and these changes proceeded the appearance of astrogliosis in this model, prompting the authors to hypothesize that disrupted astrocyte engagement within the striatal circuit may drive Huntington's disease progression. To date, no study has utilized GECIs to investigate changes in spontaneous astrocytes signals

during the latent period during TLE. Therefore, we sought to explore astrocyte signaling in the hippocampal circuit in saline and KA-treated rats during the latent period.

The standard method for delivering GECIs to the rat brain has been through viral vectors, but this approach has a number of drawbacks (Gee et al., 2015). Viruses require invasive injections, often times near the location and time of imaging. Furthermore, adeno-associated virus, while extremely useful for delivering transgenes and relative non-immunogenic, has a relatively small carrying capacity. To address this, we used in utero electroporation (*IUE*) to express a plasmid containing the newest GECI, Lck-GCaMP6f, which was optimized for imaging in astrocyte fine processes by the inclusion of the membrane-tethering tag, Lck (Shigetomi et al., 2010). The details of the construction and validation of the *NASTIE* plasmid are discussed in Chapter 3. We monitored intrinsic calcium activity in astrocyte processes in the hippocampus of adult rats that had previously undergone *IUE* and were 6-12 days postSE and compared them to saline treated age-matched controls.

Reactive astrocytes in the latent period of epilepsy express kainate receptors

Reactive astrocytes in TLE demonstrate profound changes in protein expression, including expression of cell surface receptors. Several reports indicate an increased expression of Type 1 and Type 2 metabotropic glutamate receptors (mGluRs) in astrocytes in both patients and animal models of TLE (Aronica et al., 2000; Ferraguti, Corti, Valerio, Mion, & Xuereb, 2001; Notenboom et al., 2006; Umpierre et al., 2016). To date, only one study has investigated the functional consequences of increased

expression of mGluRs following SE (Ding et al., 2007). Intriguingly, this study found that mGluR activation played a role in altered Ca^{2+} activity in astrocytes, and that blocking Ca^{2+} release was neuroprotective, suggesting that altered Ca^{2+} dynamics of astrocytes could contribute to the development of TLE.

Interestingly, data from our laboratory utilizing immunohistochemistry and colocalization analysis as well as Western Blotting on glial enriched tissue fractions in control and KA-treated animals revealed a dramatic increase in the expression of KAR subunits 1 week following KA-induced SE that, for some subunits, persisted 8 weeks following SE, at a time when chronic epilepsy has developed (Vargas, Takahashi, Thomson, & Wilcox, 2013). Kainate receptors are a class of ionotropic glutamate receptor comprised of "low-affinity" GluK1, 2, and 3 and "high affinity" GluK4 and GluK5 subunits. The GluK1, 2, and 3 subunits can form functional homomeric KARs, while the GluK4 andGluK5 subunits cannot. However, GluK4 and GluK5 can assemble with GluK1, 2, or 3 to form functional heteromeric receptors (Lerma, Paternain, Rodriguez-Moreno, & Lopez-Garcia, 2001). In addition to their classical ionotropic function, KARs can also couple to G-proteins, thus bestowing a metabotropic signaling mechanism onto these receptors (Lauri et al., 2003; Rozas, Paternain, & Lerma, 2003; Ruiz, Sachidhanandam, Utvik, Coussen, & Mulle, 2005). It is anticipated that kainate receptor activation will trigger Ca^{2+} transients in astrocytes, regardless of signaling mechanism, from the latent groups while having no effect in nonreactive astrocytes from control tissue.

The functional significance of KAR expression in reactive astrocytes is unknown. The novel expression of KARs on reactive astrocytes could represent a previously

uncharacterized mechanism that mediates pathological neuron-glia signaling through activation of ionotropic and/or metabotropic KARs expressed during epileptogenesis. Therefore, as a first step towards determining if KARs play a role in epileptogenesis, the present study tests the hypothesis that KARs on reactive astrocytes are functional and that activation of KARs results in intracellular calcium transients.

Methods

Animals

Male and Female Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were used in this study. Animals were housed in individual cages in a temperature controlled room (70-74ºF) with a 12h light/12h dark cycle, and had ad libitum access to water for the entire study. All animal procedures were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the University of Utah Institutional Animal Care and Use Committee.

In utero electroporation

Transfection of the embryonic rat brain was accomplished via *IUE*. After induction of a deep anesthetic state with ketamine/xylazine (80/20 mixture, i.p.), the abdomen of a pregnant rat was shaved and swabbed with betadine. A laparotomy was performed and embryos (gestational day 13.5–14.5) within the uterine horn were exposed and gently placed on a sterile and irrigated gauze pad. Because the embryos at this age stretch the uterus, they can be clearly visualized with only the aid of a bright fiber optic lamp. A glass capillary tube was pulled (Sutter Instrument, Novato, CA, USA) to a fine

point by high heat and filled with a mixture of fast green (1%; Sigma-Aldrich, St. Louis, MO, USA) and DNA plasmid solution (4 μg/μl final). The DNA plasmid solution consisted of 2 μg/μl of *NASTIE* and 2 μg/μl of transposase source (Figure 4.1). The pipette tip was inserted through the uterus into one of the lateral ventricles of the embryo brain and a small volume (~2–5 μl) of the mixture was pressure injected by applying positive pressure through a mouth pipette. Successful injection was confirmed visually by observing fast green filling of the ventricle. For electroporation, a pair of sterilized gold copper alloy plate electrodes (1 × 0.5 cm) were positioned across the head of the embryo on the uterus exterior, and three very brief (1 ms) voltage pulses (65 V) were discharged across the electrodes. In order to specifically target the developing hippocampus and cortex, the paddles were rotated across the top of the head with the positive electrode placed opposite the injected side, as previously described (Gee et al., 2015; Navarro-Quiroga, Chittajallu, Gallo, & Haydar, 2007; Pacary et al., 2012). The embryos within the uterus were returned to the body cavity and the incision closed with sterile Ethicon silk suture 4.0 (Ethicon, Somerville, NJ, USA). Animals were weaned from the mother on P21 and males and females were housed separately until used for experiments.

EEG electrode implantation

2-month-old (~220–280 g) male and female rats that had previously undergone *IUE* were implanted with EEG electrodes as follows. After anesthetization with a mixture (20:80) of ketamine/xylazine, animals were placed in a stereotaxic apparatus. A bipolar (with ground) electrode (MS333-3- B, Plastics One, Roanoke, VA) was used for surface recordings (Thomson & White, 2014). Two holes were drilled on the right side of the

midline, and one lead was placed into each of the craniotomies to provide differential recordings. A third lead was placed in a third craniotomy left of the midline to be used as the ground electrode. Three additional holes were drilled in the surrounding space and screws were fashioned. The electrodes were fixed in place with dental cement, and the skin was sutured around the skull.

Video EEG recording and monitoring of latent period

Rats were permitted 14 days to recover from surgery before being placed into a video-EEG (vEEG) suite, and monitored 24/7 using vEEG for the duration of the experimental trial. Animals were tethered to a rotating commutator (Plastics One, Roanoke, VA) which in turn was connected to an EEG100C amplifier (BioPac Systems, Goleta, CA), which filtered signal below 1 Hz and above 100 Hz at a sampling rate of 500 Hertz. EEG was streamed from a BioPac MP150 Recording system (BioPac Systems Inc., Goleta, CA), and video was captured by a pair of DVP 7020BE capture cards (Advantech, Milpitas, CA). Video and EEG data were synchronized and written to disk using custom software (Thomson & White, 2014). Video was stored in MPEG4 format, and EEG was stored in a custom file format. Spectrograms representing changes in electrographic power (1–100 Hz, 90 s window, 45 s step) during KA injections were plotted across a 24 hour period that included 1 hour of baseline activity used to normalize EEG power spectra. Postprocessing was performed in MATLAB (MathWorks, Natick, MA), using the Chronux Toolbox (http://chronux.org/).

This study was concerned with changes in astrocytes following SE during the latent period, prior to the development of recurrent spontaneous seizures, so rats were

monitored continually for spontaneous seizures and eliminated from the study if spontaneous seizures were observed. Video EEG monitoring was performed 24 hours per day until rats were sacrificed for the imaging portion of the study. Video EEG files were reviewed for seizures by a reviewer blinded to the treatment paradigm. Two rats, 12 and 13 days after SE, displayed seizure activity after the initial KA-induced SE activity and were thus eliminated from the study (Table 4.1).

Induction of status epilepticus

After a 24 hour acclimation period in the video-EEG chambers, SE was induced in rats 150 – 180 g using the repeated low-dose kainic acid (KA) protocol as described previously (Hellier et al., 1998; Smith, Adams, Saunders, White, & Wilcox, 2007). KA (Tocris Bioscience, Bristol) was dissolved for 1 hour in sterile isotonic saline prior to first injection. Rats received an initial 10 mg/kg injection of KA, followed by a 5 mg/kg injection an hour later. Animals continued to receive 5 mg/kg injections every 30 minutes. Rats were continuously monitored for electrographic and convulsive motor seizures. For controls, saline (0.9%) was substituted for KA. Injections ceased following the onset of the first Racine stage 4 or 5 seizure, defined as forelimb clonus with rearing (stage 4), possibly escalating into loss of postural control (stage 5)(Racine (1972)). To be included in the study, rats had to exhibit a minimum of 3 stage 4/5 seizures over a 3-hour observation period. If an animal was nearing its endpoint, half-doses (2.5 mg/kg) were given to avoid excessive toxicity and mortality. All animals were observed for behavioral seizures and EEG data was captured with synchronized video recording. SE self-terminated by 24 hours after the first KA injection. Approximately 6 hours after the first

injection, rats were given an injection of 0.9% saline (1 ml, subcutaneous) and access to -Pedialyte, a sweetened beverage fortified with electrolytes, to prevent dehydration. Subcutaneous fluids and access to Pedialyte (Abbott Laboratories, purchased at a local grocery store) were continued for 2–3 days if the animals appeared dehydrated. Rats were allowed access to food and water *ad libitum* while being housed individually in a temperature- and light-controlled (12 h light/dark cycle) environment until experimentation.

Acute brain slice preparation

Rats previously administered KA, as well as vehicle treated control rats, were monitored using video EEG as described above to ensure the absence of spontaneous seizures and confirm that the rodents were still in the latent period of TLE disease progression. Acute brain slices were prepared from rats 6-13 days following KA-induced SE, or from saline-injected controls, in a manner similar to previously described methods (Otto, Yang, Frankel, White, & Wilcox, 2006; West, Dalpe-Charron, & Wilcox, 2007). Rats were deeply anesthetized with isofluorane and brains were rapidly dissected and placed in ice-cold (4 ºC) oxygenated sucrose Ringer's solution (95% O_2/5% CO_2) containing (in mM): 200 sucrose, 26 $NaHCO_3$, 10 D-glucose, 3 KCl, 3 $MgSO_4$, 1 $CaCl_2$, and 1.4 NaH_2PO_4. The pH was maintained at 7.4 by saturation with 95% O_2-5% CO_2. Brains were then trimmed, cut down the midline, and one hemisphere was glued dorsal side down to the mounting disk of a Vibratome tissue slicer (Vibratome, St. Louis, MO). Coronal brain slices (400 μm) containing both the medial entorhinal cortex and hippocampus were cut in oxygenated sucrose Ringer's solution (4 ºC). Following

sectioning, slices were transferred to a holding chamber filled with oxygenated artificial cerebrospinal fluid (ACSF; room temperature) containing in mM: 126 NaCl, 26 $NaHCO_2$, 10 D-glucose, 3 KCl, 2 $MgSO_2$, 2 CaCl and 1.4 NaH_2PO_4 and containing 10 μM TTX for at least 1 hour.

2-photon imaging

Before imaging, slices were transferred to a heated submersion recording chamber (Warner Instruments, Hamden, CT, USA) and perfused with oxygenated ACSF containing 10 μM TTX at a rate of ~1.0 ml/min and a temperature between 29° and 30° C. Slices were evaluated for tdTomato-transfected astrocytes in the hippocampus using epifluorescence. Two-photon imaging was performed using a Prairie Technologies Ultima Multi-Photon Microscopy System (Bruker, Billerica, MA, USA) built around a SpectraPhysics Mai Tai laser source with a wavelength adjustable from 690-1040 nm (SpectraPhysics, Newport CA). GCaMP6f and tdTomato imaging was done with a laser emission wavelength of 950 nm (Akerboom et al., 2012; Drobizhev, Makarov, Tillo, Hughes, & Rebane, 2011). Laser scanning was accomplished using X-Y galvanometer-mounted mirrors. Emitted photons were bandpass filtered at 490-560 for GCaMP6f and 570-620 nm for tdTomato. Full field of view images (512 x 512) were acquired with an X-Y raster scan at depths spanning 20-100 μm below the edge of the slice to avoid the "dead zone" resulting from brain slice preparation. A water immersion 20 × 0.95 NA objective (Olympus, Tokyo, Japan) was used. PrairieView 5.2-5.3 software was used for imaging acquisition. Table 4.1 lists all controlled imaging parameters.

Pharmacological activation of receptors

Synaptic transmission was suppressed, in order to reduce neuronal sources of glutamate, by including 1 μM TTX in the ACSF solution to block voltage gated sodium channels. 500 μM each of ATPA and ATP were dissolved in ACSF and an Alexa Fluor Dye 568 was included in the solution. A glass electrode was pulled from borosilicate glass capillaries (Schott #8250 glass, World Precision Instruments, Sarasota, FL) to ~10 μM using a micropipette electrode puller (P-97, Sutter Instruments, Novato, CA). The pipette was placed ~100 microns or less away from a cell of interest using a micromanipulator (Sensaplex, Gaithersburg, MD). Receptor agonists and ACSF were delivered locally by pressure application (200-500ms, ~20 psi) using picospritzer (III, Parker Instrumentation, Huntsville, AL) and PrairieView 5.2-5.3 Software (Bruker, Billerica, MA, USA) while simultaneously imaging Lck-GCaMP6f activity with the laser tuned to 950 nm. Movies of calcium activity were captured in a single focal plane.

Image analysis

Image analysis was accomplished using ImageJ along with Fiji: an open source platform for biological image processing and custom-written Matlab software that was developed by Roy Smeal and Tony Umpierre in the Wilcox laboratory. Example traces of Ca^{2+} activation in figures were generated by plotting the intensity of a region of interest (ROI) over time, after the intensity of the background ROI (F_0) had been subtracted. The procedure for determining changes in the area of fluorescence in response to application of pharmacological agonists was as follows. First, .xml files containing all frames and metadata from movies were opened in Fiji using the BioFormats plugin. All images in the

GCaMP and tdTomato channels were filtered using the Hybrid 3D Median Filter plugin. Images from the GCaMP channel were opened in a custom written Matlab program in order to remove background fluorescence and reduce noise. To visualize the puff radius, we relied on visualization of diffusion of an Alexa Fluor 568 dye that was included in the pipette solution. A maximum projection of the frames containing puff diffusion was generated. This image was then subtracted from a single frame image just prior to the puff and the magic wand function in ImageJ was used to outline the puff area. The puff area was selected and added to the Region of Interest Manager. The "Clear Outside" function was used so that cells outside of the puff area were not included in subsequent analysis to determine changes in area of fluorescence. To determine the area of fluorescence of each cell before and after the puff, maximum intensity projection images of GCaMP activity were generated from 10 sequential frames that occurred directly before and after the puff was triggered. The maximum intensity projection images were thresholded using the IJ_IsoData algorithm in Image J. The area of fluorescence activation on thresholded images was measured using the "Analyze Particles" function within ImageJ. The change in fluorescence area was calculated as follows: PostPuffArea/PrePuffArea – 1 to give the change in area of fluorescence before and after the puff.

Region of interest selection and spontaneous event detection

In order to minimize the effect of region of interest (ROI) selection bias on our analysis of spontaneous calcium events, ROI selection was accomplished using GECIQuant from the Khahk laboratory (Srinivasan et al., 2015). Each cell had 40-60 ROI

(Table 4.2). Automatic time-series event detection was accomplished with custom-written MATLAB (MathWorks, Natick, MA, USA) scripts developed by Marsa Taheri in the White Laboratory. $\Delta F/F_0$ time-series plots were generated for each ROI by averaging F values of each pixel at each time point and using the median fluorescence of all image frames as the baseline fluorescence (F_0). The $\Delta F/F_0$ trace for each selected ROI was filtered with an order-3 one-dimensional median filter. The noise floor was defined as the median of the lower 30th percentile of fluorescence intensity values. The event threshold was set to three standard deviations above the noise floor with a $\Delta F/F_0$ of at least 30%. Using this threshold, event peaks were found with the findpeaks command in MATLAB. Events were eliminated if the time points adjacent to the event peak were not large enough (i.e., did not have a $\Delta F/F_0$ of at least 60% of the event threshold). Event durations were calculated as the time between the troughs before and after each event peak. The troughs before and after each peak were detected automatically and were defined as, respectively, the last or first data points before or after the peak that had a value less than three standard deviations above the noise floor.

Statistics

Statistical tests were performed with Graph Pad Prism 9 (GraphPad Software, La Jolla, CA, USA) or MATLAB Statistics Tool Box. Data are presented as mean ± SEM. For each set of data to be compared, we determined within GraphPad I whether the data were normally distributed or not. If they were normally distributed, we used parametric tests; otherwise, we used nonparametric tests. Paired and unpaired Student's two-tailed t tests (as appropriate) and two-tailed Mann-Whitney tests were used for most statistical

analyses with significance declared at $p < 0.05$, but stated in each case with a precise p value. Fisher's exact test was used to compare two groups with a binomial outcome. Cumulative frequency distributions were compared using Komologrov-Smirnoff (KS) test. When the p value was less than 0.0001, it is stated as $p < 0.0001$ to save space. The numbers of ROI's, cells, slices, and/or rats are defined throughout on a case-by-case basis depending on the particular experiment.

Results

EEG verification of latent period

To study the functional changes in reactive astrocytes occurring after SE and before the onset of spontaneous seizures acute, SE was induced in rats that had undergone in utero electroporation of the *NASTIE* plasmid using a low-dose KA injection protocol (Hellier et al., 1998; Smith et al., 2007; Williams et al., 2009). In the low-dose kainate model, the latent period is a period of low seizure probability immediately following the insult, which is characterized by a gradual increase in seizure probability that ends in the first spontaneous recurrent seizure. To verify that no seizures occurred following the initial SE, animals were monitored with continuous 24/7 video EEG during kainate injections and until sacrifice for imaging experiments. A trained individual blinded to treatment condition evaluated the vEEG for each animal. The entire study timeline is defined in Figure 4.2a. Seizures were defined by the occurrence of spikes or sharp wave (amplitude & >2-fold background) that lasted ~10s (Williams et al., 2009). In addition, electrographic seizures included an increase in EEG amplitude and a decrease in spike frequency as the seizure progressed. Electrographic seizures were considered

seizures if they occurred with a behavioral correlate such as freezing, head bobbing, face twitching, forearm clonus, tonic clonic seizures, etc. Two KA treated animals exhibited spontaneous recurrent seizures 12 and 13 days after initial SE, perhaps not unexpectedly, as these time points were at late end of the time frame typically thought as the latent period in this model, where the probability of seizure occurrence is indeed very high (Williams et al., 2009). These seizures were a behavioral Racine 4 and 5 and were accompanied by the appropriate electrographic traces. These rats were removed from the study (Table 4.3). EEG traces of kainite-treated, but not saline-treated, rats sometimes displayed interictal spiking, defined as transient electrographic events 3 x the baseline signal amplitude.

Figure 4.2b shows an example control EEG spectrogram covering a 24 hour period including 1 hour of baseline at approximately hour -1 to 0. Saline injections begin at approximately hour 0. The spectrogram is similar before and after the injections with sporadic small increases in power in the 0-10 Hz range across the entire period. An example of an EEG spectrum for a kainate-injected animal is shown in Figure 4.2c. After KA injections, there are larger increases in power in the range of 0-25 Hz indicating the occurrence of seizures. An example representative seizure approximately 6 hours after the initial injection is shown in the insert. Of the 23 animals that were implanted with EEG electrodes and subjected to KA treatment, 7 (30%) did not have successful transfections in the hippocampus, 2 were removed from the study due to spontaneous seizures before sacrifice, 1 lost their EEG implant, and data from 1 rat was removed from the analysis due to z-drift during imaging experiments.

Astrocytes in hippocampus of KA-treated rats exhibit thicker processes

One of the hallmarks of the brain following SE is the dramatic increase in expression of GFAP in astrocytes. Following SE, increased GFAP expression in astrocytes is observed throughout the entire hippocampus including in the CA1 stratum radiatum (Figure 1.1). In this study, astrocytes in the stratum radiatum of the hippocampus in acute brain slices prepared from saline and kainate treated rats were identified by tdTomato fluorescence. Individual astrocytes varied considerably in shape and appearance, but exhibited bushy morphology with many fine processes protruding from the main cellular processes. Maximum projections of tdTomato revealed that reactive astrocytes from kainate-treated rats exhibited thicker main processes and a higher density of fine astrocyte processes than astrocytes from saline-treated (Figure 4.3). Although we did not quantify the morphological changes seen here, out observations are similar to those from a recent study were intracellular dye-loading was used to visualize the spatial domains in reactive astrocytes in the dentate gyrus following electrical lesioning of the entorhinal cortex. Quantification of the number of processes confirmed that reactive astrocytes had increases the number of primary processes leaving the soma, and increases in the number of processes 15 μm from the soma, but there were no changes in in the overall volume accessed by astrocytes (Wilhelmsson et al., 2006).

Astrocyte have longer, but less frequent spontaneous calcium events following SE

Astrocyte processes maintain morphologically close relationships with neuronal synapses. Astrocytes can detect release of neurotransmitters through calcium-dependent

signaling mechanisms but also show spontaneous, or intrinsic calcium elevations in the absence of synaptic transmission.

In the past, synthetic calcium dyes such as Fluo-4 have been used to probe SE-induced changes in calcium dynamics in astrocytes (Ding et al., 2007). However, these dyes diffuse only through the soma and major branches of astrocyte following bulk loading, missing important activity in the fine processes. To bypass this technical challenge, we employed *IUE* in embryonic rats to express the GECI, Lck-GCaMP6f. Because Lck-GCaMP6f is targeted to the membrane, we were able visualize calcium activity in fine processes. Rats that had undergone *IUE* were subsequently treated with either low-dose injections of KA or saline. Movies of spontaneous astrocyte activity were collected in the presence of 1 μM TTX to block synaptic transmission.

Nonreactive astrocytes from healthy (control) rats demonstrated frequent spontaneous calcium transients throughout the entire astrocyte syncytium, in agreement with many other studies (Figure 4.4a) (Gee et al., 2015; Shigetomi et al., 2010; Volterra et al., 2014). Most of the spontaneous calcium activity events did not propagate to the entire cell but were spatially restricted to small regions within the astrocytes.

There were differences detected in the interevent interval (IEI) following SE and compared to slices prepared from control animals (Figure 4.4b). Reactive astrocytes had longer intervals between events (30.57 ± 22.99 (SD) seconds; n = 3819, from 13 cells, 11 slices, 5 rats) than control astrocytes (42.78 ± 33.29 (SD) seconds; n = 3813 events, from 15 cells, 12 slices, 6 rats; $p < .0001$; Mann Whitney), indicative of decreased frequency of events. Furthermore, the cumulative distribution of IEI showed considerable separation between spontaneous events in astrocytes imaged in the two groups ($p = 5.9665\ e^{-54}$,

Komlogrov Smirnoff (KS) test). We also observed separation in the distributions of basal spontaneous Ca^{2+} event duration of in reactive astrocytes in brain slices prepared from KA treated rats compared to control ($p = 1.720\ e^{-80}$ KS test) (Fig. 4.4b). The duration of calcium events was longer in reactive astrocytes (19.96 ± 20.52 (SD) seconds; 4843 events, from 13 cells, 12 slices, 5 rats) than astrocytes from control tissue (14.41 ± 13.95 (SD) seconds; 4476 events, from 15 cells, 12 slices, 6 rats; $p < .0001$; Mann Whitney). Furthermore, the cumulative distribution of event duration of reactive astrocytes was increased compared to astrocytes from saline-treated rats ($p = 1.720\ e^{-80}$). There were differences in the amplitude between control (59.46 ± 56.96 (SD) $\Delta F/F_0$; 4476 events, from 13 cells, 11 slices, 5 rats) and reactive astrocytes (56.14 ± 52.96 (SD) $\Delta F/F_0$; 4843 events, from 15 cells, 12 slices, 6 rats; $p = .0180$; Mann Whitney)(Figure 4.4c). However, while the differences in the cumulative distributions were statistically significant ($p = 7.9471e^{-4}$) when compared using the KS test, we saw no separation in the traces, suggesting that the differences between the groups may not be biologically significant.

Astrocytes from KA-treated rats respond to application of ATPA

Our published data indicate that all five KAR subunits are expressed on astrocytes during the latent period, with the GluK1 subunit most highly expressed 7 days and 8 weeks post SE (Vargas et al., 2013). To determine if the protein expression of KARs on reactive astrocytes translates to functional KARs, we imaged GCaMP6f activity in astrocytes during direct application of ATPA (500 μM), a selective pharmacological agonist of the Gluk1 KAR subunit (Palygin, Lalo, Verkhratsky, & Pankratov, 2010).

Transfected astrocytes in the stratum radiatum of the hippocampus were identified based on the expression of tdTomato (Figure 4.2) and local application of drug solution was accomplished by focal pressure application with a picospritzer. The ideal location of the pipette tip in relation to the astrocytes being imaged, as well as the duration and pressure, of the picospritzer application, were determined based on astrocyte response using 500 μM ATP, because protoplasmic astrocytes in the hippocampus are known to express metabatropic ATP receptors and respond to changes in the exogenous application of ATP with large calcium transients that are readily observable with GCaMP (Gee et al., 2014; Nakahata, Nabekura, & Murakoshi, 2016).

GCaMP6f and tdTomato were simultaneously excited with a laser emission wavelength of 950 nm. Focal application of ACSF and ATP (500 μM) was also performed with simultaneous calcium imaging as negative and positive controls, respectively. ACSF was applied to determine any fluctuations of calcium in astrocytes was due to response to mechanostimulation from the direct application. Application of ATP was used to confirm health of the slices, as it is a well-characterized agonist that stimulates G-protein-coupled P2Y receptors expressed on the membrane, which results in the mobilization of calcium from internal stores (Pearce, Murphy, Jeremy, Morrow, & Dandona, 1989; Salter & Hicks, 1995). In general, healthy astrocytes returned to baseline calcium levels within 20-30 seconds.

Astrocytes from control brain slices (n = 6 cells; 4 slices, 4 animals) did not respond to application of 500 μM ATPA (Figure 4.5a, Saline-group, Change in area of fluorescence A.U. -0.062 ± .101), consistent with the observation that control astrocytes do not express KAR subunits (Vargas et al., 2013). Application of 500 μM ATP to

astrocytes in brain slices from control rats (n= 6 cells; 4 slices; 4 animals) resulted in a several-fold increase in $\Delta F/F_0$ above baseline, consistent with previous reports (Gee et al., 2015). Application of ATP also resulted in an increase in the total area of fluorescence (Figure 4.5b, Saline-group, Change in area of fluorescence A.U. 1.38 ± 0.548). Also consistent with the literature, application of ACSF had very minimal effects on the area of GCaMP fluorescence in astrocytes from control tissue (Figure 4.5c, Saline-group, Change in area of fluorescence A.U. 0.10 ± 0.040).

Applying 500 μM ATPA to astrocytes in brain slices from KA-treated animals revealed two distinct populations of astrocytes. One population responded to application of ATPA with a large increase in GCaMP fluorescence (1.56 A.U. ± 0.284; n = 6/12 cells; 4/7 slices; 5 animals), while the other population showed relatively no changes in fluorescence area after (0.021 A.U. ± 0.010; n = 6/12 cells; 3/7 slices; 5 animals). We compared the prevalence of astrocytes responding to ATPA (6 in 12), with the prevalence of astrocytes that did not respond (6/12). Fisher's exact test yields a p value that approaches, but does not reach significance (p = .053). The average fluorescence area in responding 0.796 A.U ± 0.27 AU, in kainite-treated rats (Kainate-group, n = 12 cells; 7 slices, 6 animals) (Figure 4.4D, p = 0.0695). The calcium response to application of ATP in astrocytes imaged in brain slices obtained from kainate rats (1.06 ± .491 AU; n = 7 cells, 4 slices, 4 animals) were not statistically different from those imaged in slices obtained from control rats (1.38 ± 0.54 (control)) (Figure 4.5d, p =0.4337). As expected, no response to application of ACSF (n = 10 cells; 5 slices; 4 animals) was observed in astrocytes imaged in slices obtained from kainate tissue (Figure 4.5d, p = 0.6188). Together, these data suggest that KARs are functional in a subset of astrocytes. Studies to

expand and clarify these results are currently ongoing.

Discussion

The main findings in this study are that reactive astrocytes in the hippocampus of KA-treated rats display reduced frequency and increased duration of spontaneous Ca^{2+} transients, independent of neuronal action-potential activity. Our previous finding that reactive astrocytes in the hippocampus increase expression of all KAR subunits 1 week after SE (Vargas et al., 2013) led us to hypothesize that newly expressed KARs on astrocytes would be functional. Focal application of the Gluk1-specific KAR agonist ATPA to reactive astrocytes revealed what appeared to be two distinct populations of reactive astrocytes, those that responded to ATPA, and those that did not. These preliminary findings indicate that increased protein expression of KARs results in the expression of functional receptors on a subset of reactive astrocytes.

Although it is known that astrocytes typically undergo reactive gliosis following KA-induced SE, whether specific astrocyte targets/molecular pathways contribute to the progression of the epilepsy remains largely unknown. In order to better characterize astrocyte-specific changes during the latent period, we employed a GECI adapted for studying astrocytic calcium activity, Lck-GCaMP6f. We used *IUE* to stably transfect cells in the developing rat hippocampus. *IUE* has many advantages over viral injections, in that it requires less invasive means of delivery and confers longer time periods of expression. However, relatively inefficient transfection rates and sparse labeling, combined with several experimental steps with high rates of animal attrition, made this study time- and labor-intensive (Table 4.2). In order to circumvent these challenges in the

future, transgenic mice could be used. Recently, in collaboration with John White and Mario Capecchi's laboratories, our group developed a new mouse line for expressing the GECI, GCaMP5 in specific cell populations in the brain (Gee et al., 2014). By crossing the GCaMP5G-IRES-tdTomato reporter mouse line, with the tamoxifen-inducible GFAP-CreER line, GCaMP5 expression can be achieved specifically in astrocytes (Chow, Zhang, & Baker, 2008). Anthony Umpierre in the Wilcox laboratory recently developed a model of KA-induced TLE in C57BL/6J mice using repeated low-dose injections of KA, which can be applied to genetically-tractable mouse lines such as the Cre-inducible GCaMP5 reporter mouse (Gee et al., 2014; Umpierre et al., 2016). The mouse TLE model presents some limitations, including high rates of animal loss resulting from SE and infrequent spontaneous seizures, but offers the opportunity to significantly expedite the experimental process described here, by eliminating the need for *IUE*. To this end, there are currently ongoing experiments in our laboratory using the low-dose KA mouse model in GCaMP5 reporter mice to investigate if application of ATPA onto KAR's are functional in reactive astrocytes following SE during the latent period.

In this report, we described changes in spontaneous Ca^{2+} dynamics in reactive astrocytes. One group previously reported changes in astrocytic calcium activity following induction of SE with the chemoconvulsant agent, pilocarpine (Ding et al., 2007). However there were several technical drawbacks to this study. They did not monitor vEEG during the induction of SE or the latent period, potentially missing important relevant data about TLE disease progression, including spontaneous seizures. Furthermore, they used a bulk loaded synthetic dye, Fluo4, which does not diffuse throughout the entire cytosolic compartment of astrocytes. In contrast, we induced SE in

rats while monitoring with video EEG to ensure induction of SE and to confirm that no spontaneous seizures had occurred, and thus, that we were studying astrocytes during latent period of TLE disease progression. We subsequently recorded astrocyte calcium dynamics in acute brain slices using Lck-GCaMP6f, a GECI that has been adapted for imaging of astrocyte calcium dynamics. Therefore, we were able to observe calcium dynamics throughout the whole cytosolic compartment, including the fine processes.

We observed clear differences in the distribution of spontaneous event duration and interevent intervals in astrocytes between KA- and saline-treated groups. The Komolgrov-Smirnoff test detected significant differences ($p < .05$) in all distributions measured. However, this statistical test is highly powered, owing to the large number of ROI's and events included in the distribution analysis, and was thus very sensitive to small changes in distribution. Therefore, in order to identify biologically meaningful differences between the two groups, we chose to focus on the distributions that showed clear separation in their traces. We observed that the interevent interval time was decreased, while event durations were longer, in reactive astrocytes from KA-treated rats, as compared to astrocytes from saline-treated rats.

Astrocytes demonstrate spontaneous calcium oscillations in response to neuronal activity, as well as intrinsic Ca^{2+} excitability, or spontaneous increases in Ca^{2+} that are not triggered by neuronal activity (Nett et al., 2002; Parri, Gould, & Crunelli, 2001). We recorded astrocyte activity in acute brain slices in extracellular solution that included 1 μM TTX to block action potential–dependent release of neurotransmitter. This protocol is in contrast to Ding et al. (2007), in which astrocyte calcium activity was measured in vivo without TTX. Inclusion of TTX in our experiment allowed us to focus on changes in

intrinsic spontaneous astrocyte calcium activity.

Spontaneous astrocyte calcium transients are likely due to a range of factors, including changes in extracellular signaling molecules, expression of channels or transporters, regulation of receptor subunit expression, and widespread changes in signaling pathways influenced by inflammatory cytokines- all factors that are known altered during epilepsy. Following SE, and in addition to reactive astrogliosis, widespread changes occur in hippocampal circuitry occur, including massive cell death throughout the hippocampus (Thom, 2014). Even in the presence of TTX, changes in extracellular glutamate can occur through action potential independent neuronal neurotransmitter release. Thus, the increase in interevent interval of spontaneous Ca^{2+} events observed could reflect reduced number of neurons in the hippocampal circuit. Furthermore, a recent study in our laboratory, performed in collaboration with researchers at the University of Pittsburgh, used electron microscopy (EM) to investigate changes in astrocyte enwrapping at CA1 synapses in the hippocampus during the latent period of the low-dose KA model. Our results show dramatic increases in the enwrapping of synapses by astrocytes at the presynaptic terminals and spines and an increase in the number of docked vesicles at presynaptic terminals during the latent period (Rubio et al. in preparation). This work may suggest that the differences in spontaneous activity observed here could reflect changes in the astrocyte ensheathment of synapses following SE.

Some intrinsic astrocytic oscillations (measured in the presence of TTX) appear to be IP3 dependent (Zur Nieden & Deitmer, 2006). Previous studies have suggested that mGluR5 is not expressed by adult astrocytes (Sun et al., 2013). Interestingly, it has been shown that the protein expression levels of mGluRs were increased in reactive astrocytes

in animal models of epilepsy as well as resected TLE tissue from patients, suggesting that increased duration of spontaneous calcium events in reactive astrocyte could be explained by increased signaling through the mGluR pathway (Aronica et al., 2000; Ferraguti et al., 2001; Ulas et al., 2000; Umpierre et al., 2016). Future experiments examining intrinsic calcium signaling in reactive astrocytes could focus on the specific contributions of mGluR receptors in alterations in spontaneous astrocytic activity by examining changes in spontaneous event duration, or frequency (IEI) in the presence of mGluR antagonist.

Although it is well known that astrocytes sense the neurotransmitters in extracellular space, and have intrinsic changes in spontaneous activity in the absence of synaptic transmission, we know very little about the downstream consequences of astrocytic calcium signaling. Experiments done on astrocytes in culture have shown that astrocytic Ca^{2+} signaling is associated with release of gliotransmitters. Several of the best-studied gliotransmitters include ATP, D-serine, TNF-alpha, and arachidonic acid metabolites. Thus, by sensing changes in intracellular Ca^{2+}, astrocytes may actively participate in the information processing in the normal brain. Changes in duration and frequency of spontaneous event activity observed in reactive and healthy astrocytes in this study indicates that intrinsic spontaneous astrocyte calcium signals are changed during the epileptogenic process and that these changes may have important consequences on the development of hyperexcitability of neural networks. Further experimentation is needed to identify the consequences of the changes in frequency and duration of spontaneous events in reactive astrocytes found here.

Previously, KARs were found to be expressed on reactive astrocytes following SE using both immunohistochemistry and Western Blotting on enriched glial hippocampal

membrane fractions 7 days following SE, leading us to hypothesize that KARs would be functional. Our data showed that a population of astrocytes responded to ATPA application with stimulus linked calcium transients, suggesting the presence of functional KARs in brain slices obtained from animals experiencing SE. This observation opens the door to a number of interesting lines of investigation that may have important consequences for our understanding of epileptogenesis.

Studies are currently ongoing in our laboratory to investigate the response of reactive astrocytes to ATPA application in the low-dose KA-induced model of SE in mice. We are confident that switching to this preparation will significantly increase the power of our study and our ability to carefully discern the function of KARs in reactive astrocytes. If KARs on astrocytes in the KA mouse model are functional, we also plan to investigate if activation of KARs in astrocytes can be triggered by endogenously released glutamate, evoked via stimulation of the Schaeffer collateral pathway. Additional pharmacological experiments are also needed to confirm that the response of astrocytes to ATPA is indeed specific. This can be accomplished by repeating puffing experiments while perfusing of the pharmacological blocker of KARs, CNQX, in the extracellular bath. Furthermore, to investigate if KARs are functioning as metabotropic or ionotropic receptors, application of pertussis toxin to block GPCR activity or an extracellular solution with low Ca^{2+} concentration will be helpful in determining the mode of KAR signaling. Finally, an intriguing possibility raised by the data in these experiments suggest that changes in intrinsic calcium signaling during the latent period may be influenced by expression of KARs on reactive astrocytes. An interesting experiment would be to compare spontaneous astrocyte activity in control and reactive astrocytes in

the presence of CNQX, a KAR blocker, and TTX.

In conclusion, this work provides the foundation for future studies needed to understand the changes in calcium signaling of reactive astrocytes in TLE. Utilizing transgenic mice expressing GCaMP5 in astrocytes will help expedite the experimental process. Experiments to test if focal application of 500 μM ATPA activates KARs on reactive astrocytes in KA-treated mice are currently ongoing. Future studies in this area will hopefully elucidate mechanisms involved in calcium signaling in astrocytes during epileptogenesis.

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Table 4.1. Imaging parameters

Parameter	Value
Image size	512 x 512
Excitation wavelength	950 nm
Resolution	1.25 pixels/um
Dwell time	.4 us
Frame rate	.7-.9 Hz
Objective	20x
Duration	90-110 seconds

Table 4.2. Experimental parameters for spontaneous analysis

Group	Animal #	Slice #	Cells	ROI#	Event#	ROIs/cell
Saline	6	12	15	717	4476	48 ± 7
Kainate	5	11	13	705	4843	53 ± 7

Table 4.3. Animal Numbers

Animal #	Cohort #	Group	Number of days monitored PostSE	Seizures PostSE	Transfections Y/N	Data Collected
5.1	1	KA	11		Y	Spontaneous
5.2	1	KA	7		Y	Spontaneous, ATPA, ACSF, ATP
5.3	1	KA	6		N-thalamus	
5.4	1	KA	13	1 (stage 4)	Undetermined	
5.5	1	KA	10		Y	ATPA, ACSF, ATP
5.6	1	Saline	8		Y	Spontaneous
5.7	1	Saline	9		Undetermined	Removed due to headcap failure
6.1	2	Saline	11		N	
6.2	2	KA	12	3 stage (4/5)	Undetermined	
6.3	2	KA	6		Y	Spontaneous, ATPA, ACSF, ATP
6.4	2	KA	7		N	
6.5	2	KA	8		N	
6.7	2	Saline	9		Y	Spontaneous
8.1	3	Saline	6		Y	ATPA, ACSF, ATP
8.2	3	Saline	7		N	
8.3	3	Saline	9		Y	ACSF, ATP, ATPA
8.4	3	KA	9		N- cortex	
8.5	3	KA	10		Y	Removed due to z drift
8.6	3	KA	12		Y	Spontaneous, ATPA, ACSF, ATP
9.1	4	Saline	12		Y	Spontaneous, ATPA, ACSF, ATP
9.2	4	KA	7		Y	Spontaneous, ATPA
9.3	4	KA	8		N	
9.4	4	KA	8		Y	ATPA
9.5	4	Saline	9		Y	Spontaneous
9.6	4	Saline	9		Y	Spontaneous
9.7	4	Saline	12		Y	Spontaneous

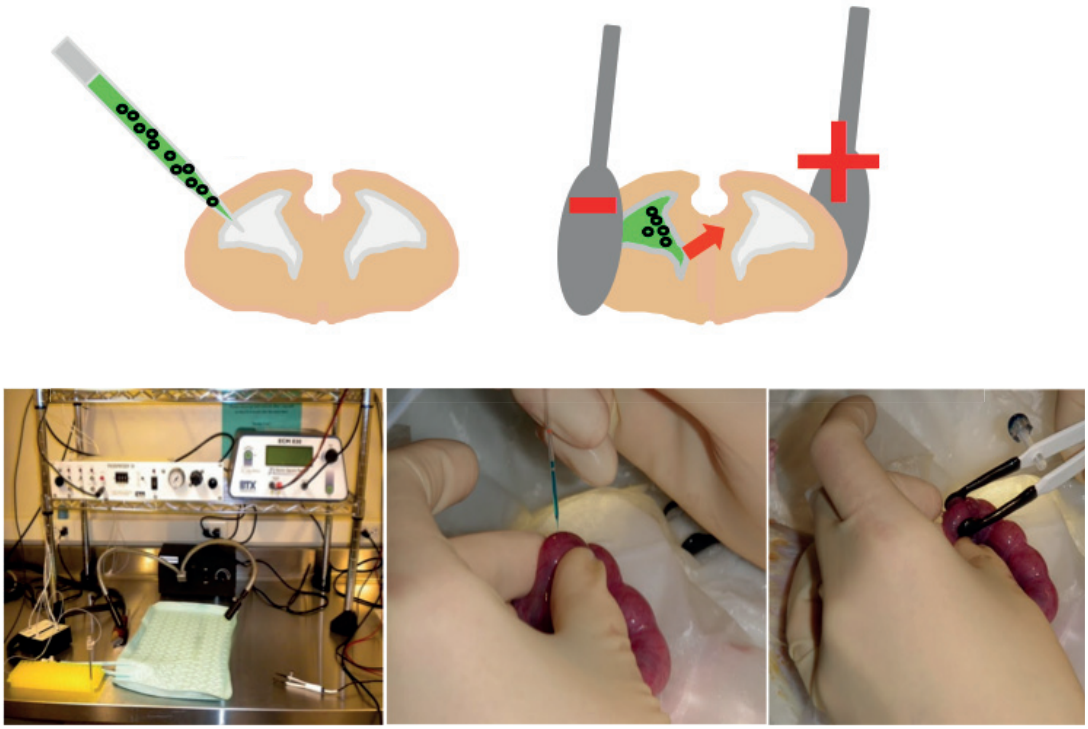

Figure 4.1. In utero electroporation procedure.
To facilitate transfection of DNA into hippocampal progenitor cells, DNA is injected through a glass pipette into the lateral ventricles of embryonic day (E) 14.5 brains and an electrical field is applied across the uterine walls. Embryos are returned to the mother and the dam is allow to give birth. Animals are raised until weaning.

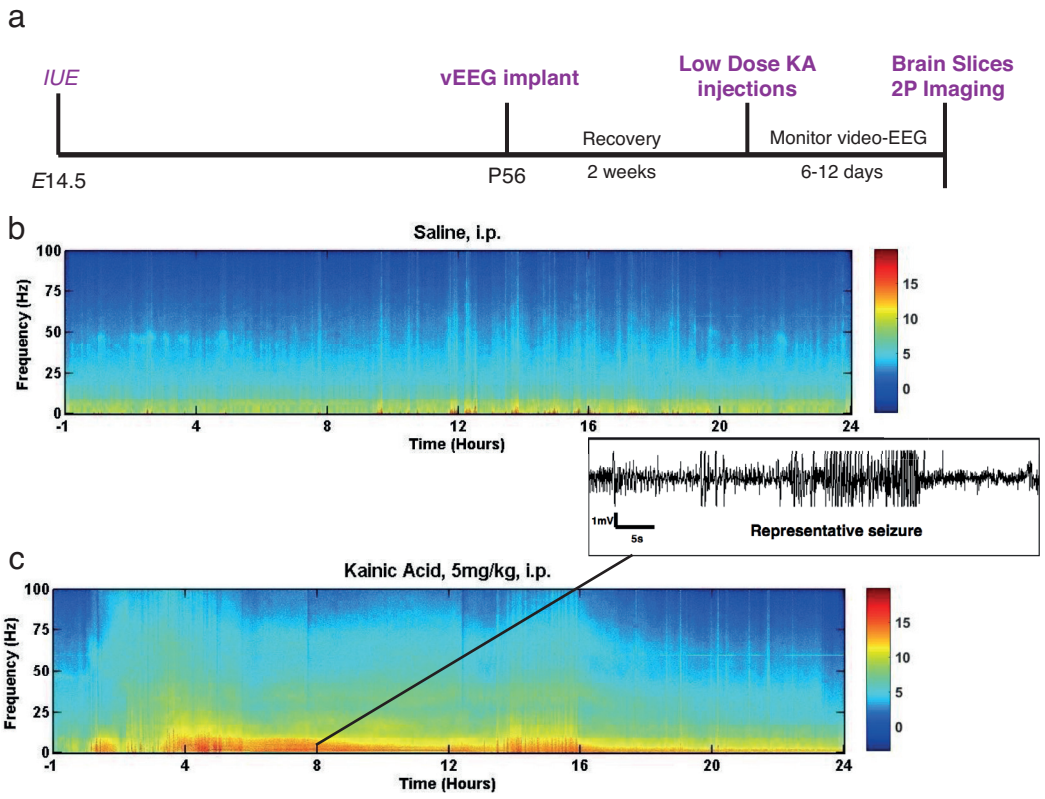

Figure 4.2. Timeline of study and verification of the induction of SE.
(**a)** A timeline of the study showing procedures and sacrifice. (**b**) Saline injections did not result in notable changes in power over the course of recording. (**c**) Multiple KA injections were followed by an increase in power in the 0-25 Hz range, consistent with the occurrence of multiple stage 4/5 seizures in this animal, with a representative trace of a stage 5 seizure (inset). The lines at 60 Hz reflects noise at this bandwidth in both recordings.

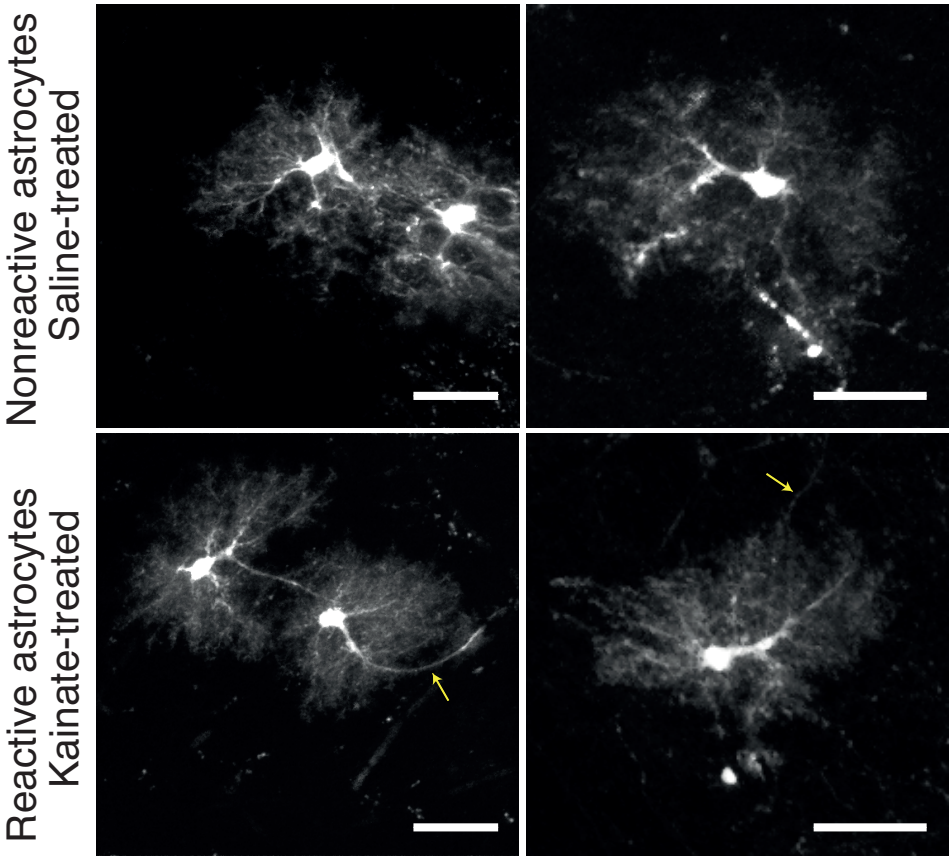

Figure 4.3. TdTomato expression in astrocytes from saline-treated and kainate-treated tissue.
Maximum projections of tdTomato expression in reactive and nonreactive astrocytes in the stratum radiatum of CA1 in the hippocampus approximately 1 week after SE. TdTomato expression throughout the cytoplasm reveals a large, thick processes extending from the soma of reactive astrocytes and a higher density of fine spongiform processes in reactive astrocytes comparable to those of nonreactive astrocytes. Yellow arrows indicate thick processes extending from reactive astrocytes, but not in nonreactive astrocytes. Scale bars = 30 μM.

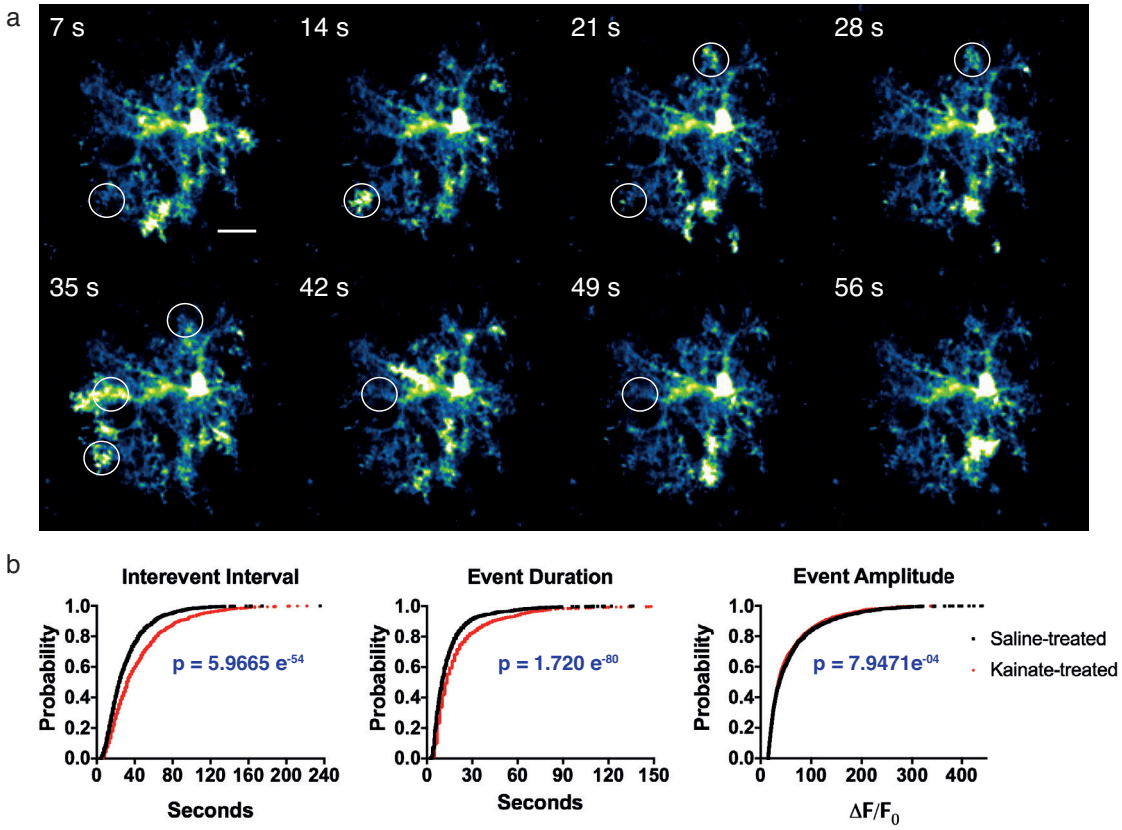

Figure 4.4. Comparison of spontaneous calcium transients in control and reactive astrocytes.

(a) Frames from a movie demonstrating spontaneous calcium transients throughout a reactive astrocyte from a kainate treated rat, including activity in distinct microdomains, as indicated by including activity in distinct microdomains, as indicated by circles Scale bar = 10 μM. (**b**) Cumulative histograms displaying spontaneous event Interevent Interval, Event Duration, and Amplitude are shown, astrocytes from brain slices prepared from kainate-treated rats are shown in red and saline-treated rats are in black. The calculated p-value from a Kolmogorov-Smirnov test is shown in the plot.

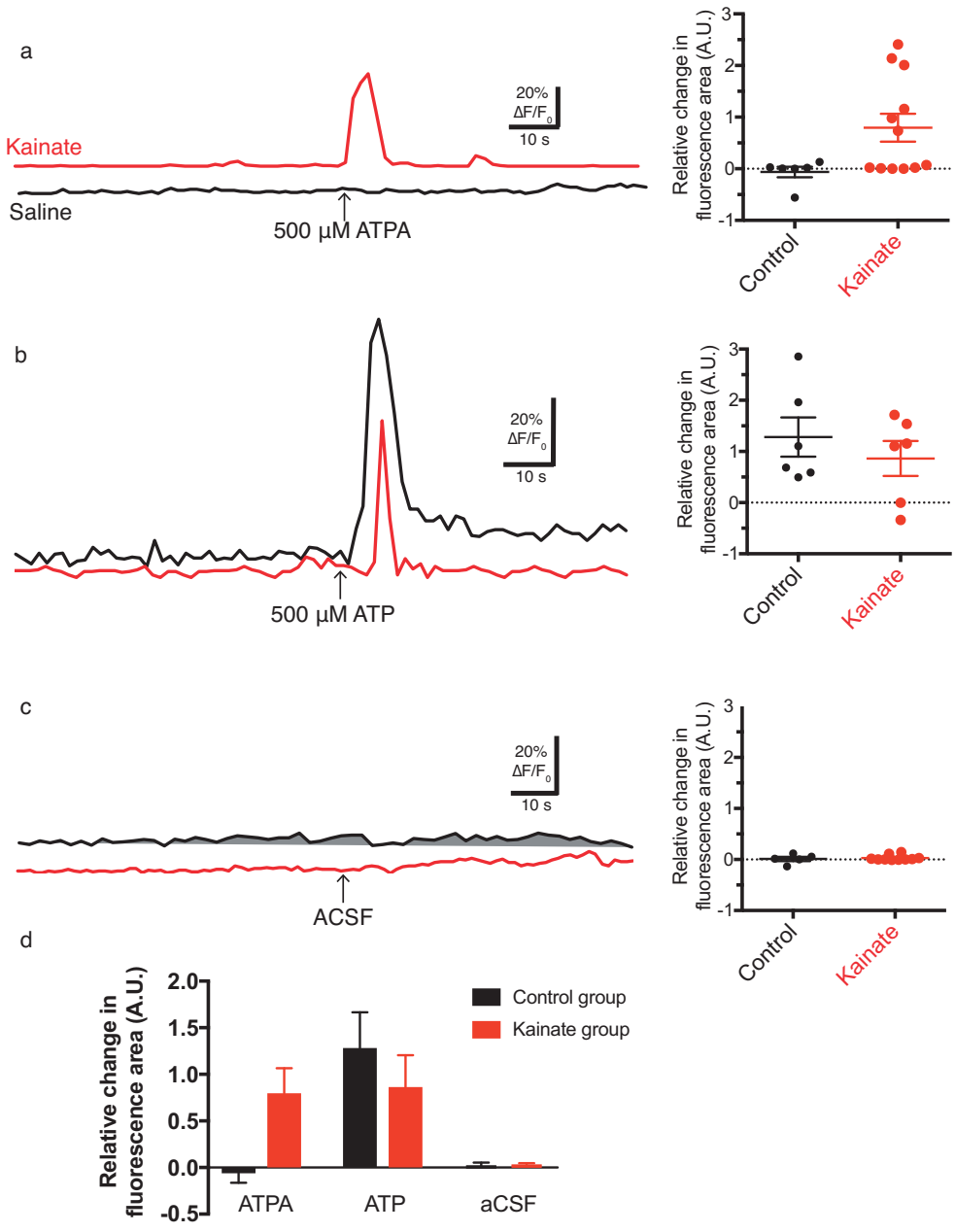

Figure 4.5 Reactive astrocytes respond to ATPA.
(a) An example astrocyte in the CA1 stratum radiatum from a control brain slice (black trace) does not respond to 500 μM ATPA. Half of astrocytes from brain slices from KA-treated rats respond to ATPA (red trace), while the other half exhibited no change in fluorescence. The responses of individual cells with mean and SEM are shown on the far right. **(b)** As a positive control, 500 μM ATP was applied to astrocytes from control (black trace) and kainite-treated rats (red trace), resulting in a change in fluorescence area. (**c**) Application of extracellular solution (ACSF) demonstrated no response in either condition. (**d**) Quantification of the average change in fluorescence area in response to application of ATPA, ATP, or ACSF to astrocytes in the stratum radiatum.

CHAPTER 5

CONCLUSION

Summary of Conclusions

Temporal lobe epilepsy (TLE) is a devastating seizure disorder that is commonly refractory to antiseizure drugs (ASDs), and is also associated with a number of serious comorbidities. Tremendous efforts from the epilepsy research community have generated a wealth of knowledge about many of the changes that occur in neurons during the development of TLE. There are currently over 25 ASD's that physicians can prescribe to treat the seizures that occur in epilepsy and in the last three decades, many new drugs have come to the market and have marked improvements in side-effect profile and tolerability. Despite this progress, the number of patients with TLE that have inadequate control of their seizures has remained around 30% (Serrano & Kanner, 2015). Furthermore, there is currently no way to prevent the development of epilepsy in patients at risk. Perhaps a new approach in our understanding of epilepsy is needed.

Historically, epilepsy research has focused on neuronal subtypes and the dysfunction of neurons in epilepsy. Given that seizures arise from hyperexcitable neuronal circuits, this focus has made sense. However, there is an increasing emphasis on the role of astrocytes in brain circuit function, based on demonstrations that astrocytes play critical roles in determining the balance between neuroprotection and neurotoxicity.

In particular, the growing appreciation of the role of astrocytes in synapse function has led to the term the "tripartite synapse" (Araque, Parpura, Sanzgiri, & Haydon, 1999) which was coined to reflect the role of astrocytes as critical partners at the synapse. Changes at the tripartite synapse have been implicated in epilepsy pathophysiology, but relatively little is understood about functional role of astrocytes in epilepsy.

During TLE, astrocytes become reactive and undergo widespread changes in morphology and protein expression throughout the hippocampus, the main brain region involved in seizure generation. The rat kainic acid model of status epilepticus-induced epilepsy is a well-described model of TLE that recapitulates a number of features seen in patients, including reactive astrocytes and a clinical manifestation that involves an initiating injury, followed by a latent period, then the appearance of spontaneous seizures. The goal of this dissertation was to investigate how changes in astrocytes may affect tripartite synapse function during the development of TLE. While Chapter 1 serves as an introduction and review of the role of astrocytes in epilepsy, Chapter 2 describes the development, construction, and validation of the novel tool, *NASTIE*, to genetically differentiate and label astrocytes and neurons during calcium imaging experiments. We characterized expression of the tool in a variety of preparations, including cell culture, acute brain slice, and examined expression patterns of the reporter fluorescent proteins throughout development in the rat hippocampus. These findings highlight the usefulness of this tool in labeling astrocytes and neurons and demonstrate its versatility for investigating genetically distinct cell types. In Chapter 3, we used this tool alongside 2-photon imaging in acute brain slices to investigate changes in calcium signaling in reactive astrocytes during the latent period in the rat kainate model of TLE. We found

that reactive astrocytes have reduced frequency of spontaneous events and increased duration. Additional experiments in Chapter 3 tested the hypothesis that increased protein expression of kainate receptors (KARs) observed in reactive astrocytes during the latent period resulted in functional KARs. The results from the imaging experiments suggest that a subset of reactive astrocytes do express functional KARs, and that this pathway may mediate a form of pathological neuron-astrocyte signaling during epileptogenesis.

Implications, Limitations, and Future Directions

Features of the in utero electroporation technique

In utero electroporation (*IUE*) was a technical approach used throughout this dissertation. Key features of *IU*E make this technique particularly useful for studying neurons and astrocytes in the context of epilepsy. 1) Compared to viral vectors, *IUE* targeting of the hippocampus avoids invasive injections that can cause reactive astrogliosis and present potentially confounding effects on astrocytes during the study of epileptogenic networks. 2) *IUE* with *piggyBac* plasmids overcome the loss of conventional episomal plasmids in *IUE* and the large carrying capacity makes it possible to deliver several transgenes to the genome (Yusa, Rad, Takeda, & Bradley, 2009). Furthermore, as demonstrated in Chapter 2, the use of hyperactive *piggyBac* transposase significantly increases the efficiency of *piggyback*-mediated integration (Yusa, Zhou, Li, Bradley, & Craig, 2011). *NASTIE*, the novel plasmid developed during this dissertation, contains multiple genes and is larger than 12,000 base pairs, making it too large to deliver with most adenoviral vectors used in gene delivery in the central nervous system (CNS). 3) Transfection of hippocampal precursor cells results in stable transgene expression in

the neural lineage of radial glia, so that both neurons and astrocytes are labeled in the adult animal, thus enabling the study of both cell types (Chen & LoTurco, 2012). 4) Finally, the plasmid system used in *IUE* is inherently flexible. New genetic tools can be employed in vivo as soon as they are available without the need for the relatively long wait time associated with transgenic mouse lines.

Limitations of the *IUE* approach also presented some challenges to the experiments in this dissertation. Many factors, including the site of injection, time of injection, and orientation of paddles, can influence the eventual expression pattern of transfected cells in the adult brain following *IUE*. Thus, it can be difficult to replicate expression patterns between animals and between litters, leading to substantial variation in the ratio of neurons to astrocytes, and the density of cells that are labeled between individual animals and between litters. In some contexts, the sparse labeling of *IUE* is a benefit, as it allows for the unambiguous analysis of the entire extent of a single astrocyte or neuron to be viewed, without overlap from the processes of neighboring cells. In transgenic animals, dense labeling can pose a problem and some groups have taken to titrating the doses of tamoxifen in order to balance expression levels with the ability to view individual cells (Agarwal et al., 2017). However, with *IUE*, sparse labeling made finding labeled neurons and astrocytes that were closely situated a challenge in investigating neuronal-glial interactions. Inconsistencies in the *IUE* approach also added significantly to the workload and difficulty of experiments in Chapter 4. Preparing animals for imaging and pharmacological application experiments required months of groundwork, and if the *IUE* targeting to the hippocampus was not successful, then months of work were lost.

To avoid these pitfalls, we are currently following up on the observation that KARs are functionally expressed in a subset of reactive astrocytes by repeating drug application experiments in the transgenic GCaMP5 mouse line that has been crossed with a GFAP-Cre line (Gee et al., 2014). Expression of GCaMP5 in this mouse line is easily induced by interperitoneal injections of tamoxifen and results in GCaMP5 expression in the vast majority of astrocytes in the brain. This approach has already significantly expedited data collection in reactive astrocytes. We anticipate that future experiments designed to dissect the mode of kainate receptor signaling in reactive astrocytes will benefit from this approach.

Using *NASTIE* to investigate neuron-glial interactions

Chapter 2 described the construction, validation, and characterization of a novel genetic tool to label astrocytes and neurons during Ca^{2+} imaging experiments. This tool was designed to facilitate Ca^{2+} imaging simultaneously in astrocytes and neurons, in order to investigate the interactions between these cell types at the tripartite synapse. We were never able to demonstrate this capability for a few reasons, which I will discuss here. First, astrocytes display a remarkably complex meshwork of processes, which penetrate the neuropil in three dimensions and can sometimes enwrap synapses. Electron microscopy (EM) studies indicate that astrocyte processes can be as thin as 30–50 nanometers (Rusakov, 2015). This is beyond the optical defraction of the conventional 2-photon microscopy approaches used in this dissertation and currently, EM is the only traditional imaging tool capable of resolving cellular architecture on this scale.

It is also clear that astrocytes behave very differently from neurons at the tripartite

synapse. Not only do astrocytes cover much different spatial territory, their activation and output responses (measured by Ca^{2+}transient via GCaMP6f) occur on temporal and spatial scales distinct from those of neurons (Araque et al., 2014). These fundamentally different characteristics have led some to hypothesize that astrocytes may act as integrators of circuit activity, and that they contribute to information processing by linking activities of neurons that occur on different spatial and temporal dimensions to achieve higher order integration across brain circuits. In a recent review article written by a number of prominent glial biologists in the field, it is proposed that astrocytes may be capable of enriching neuronal activity by incorporating information coming from outside the synaptic world (i.e., from vascular, immune and other cells) to fine tune the synaptic circuitry according to the environmental state (Araque et al., 2014). In light of this, *NASTIE* may be beneficial in imaging studies of neuronal-glial interactions but may need to be implemented alongside other techniques, and over longer imaging sessions than were performed in this dissertation, in order to fully appreciate the full range of astrocyte-neuron interactions.

Astrocyte heterogeneity

In Chapter 2, we discuss the unexpected observation that *NASTIE* detected a population of astrocytes that likely expressed Synapsin I during their lineage, resulting in Cre-mediated recombination of tdTomato. By P35, this population accounted for ~25% of all transfected GFAP-expressing cells and led us to ask whether this population of Cerulean-positive astrocytes reflect a functionally unique subpopulation of astrocytes. For now, the answer to this question remains elusive. We demonstrated subtle differences

in spontaneous Ca^{2+} activity between tdTomato-positive and Cerulean-positive astrocytes, and further studies are clearly needed to explore these two astrocyte groups in more detail.

Over 100 years ago, Ramon y Cajal initially described extensive morphological heterogeneity of astrocyte populations in the brain (Garcia-Marin, Garcia-Lopez, & Freire, 2007). Protoplasmic astrocytes found in grey matter, fibrous astrocytes in white matter, Bergmann glia in the cerebellum, and Müller cells of the retina display very different morphologies and expression of astrocyte-enriched proteins such as GFAP (Ben Haim & Rowitch, 2017). More recently, there is a growing appreciation of the different functional characteristics of astrocyte populations throughout different brain regions. For example, cortical astrocytes have been shown to express NMDA receptors, while those in the hippocampus do not appear to express these receptor gated ion channels (Matyash & Kettenmann, 2010). While many researchers have suspected that additional layers of cellular heterogeneity exist among astrocytes within the same circuits and brain regions, only recently have the genetic and imaging tools been available to investigate this. Two studies in the past year have revealed very interesting and unexpected aspects of astrocyte heterogeneity. One recent paper elegantly demonstrated that the release of sonic hedgehog (SHH) by neurons acts as a critical signaling factor to determine the transcriptional profiles of nearby mature astrocytes. Furthermore, they found that astrocytes in different brain regions responded to SHH differently. Cerebellar Bergmann glia responded to SHH by increasing expression of glutamate receptors, while protoplasmic astrocytes from the hippocampus respond to SHH with changes in expression of the potassium channel Kir4.1, which altered their potassium buffering

capabilities(Farmer et al., 2016). Another recent study used a form of fluorescent activated cell sorting (FACS) to identify 5 unique populations of astrocytes in the brain, each with its own unique molecular profile and functions related to development and synaptogenesis (John Lin et al., 2017). Based on these two high profile studies, astrocyte heterogeneity appears to be having its moment in the spotlight, and a tool like *NASTIE*, which not only labels genetically distinct populations, but also can deliver optogenetic tools to study function, may prove useful going forward.

To investigate if Cerulean-positive astrocytes might represent a functionally unique genetic subtype, we investigated one aspect of astrocyte physiology-spontaneous Ca^{2+} activity. However, many other important functional properties of these astrocytes remain unexplored; therefore, further work is needed to investigate the origin and characteristics of Cerulean-positive and tdTomato-positive astrocytes. I am particularly intrigued about the origin of Cerulean-positive astrocytes. Because Cre-recombination acts as a lineage tracer, it is impossible to determine, based on expression of the Cerulean fluorophore alone, whether Cerulean-positive cells constitutively express Synapsin I, expressed Synapsin I transiently at some point in their past, or are derived from a cell that expressed Synapsin I at some point. One possibility for future experiments is to do FACS sorting of tdTomato-positive and Cerulean-positive astrocytes, followed by gene expression profiling to look closely at the cellular differences between these two groups. However, one potential pitfall of this experiment is that if *IUE* yields sparse labeling, there may not be sufficient cell numbers after FACS sorting. As an alternative, single cell RNA sequencing could be done on individual tdTomato-positive/Cerulean-positive astrocytes that are isolated in brain slices. This could be a potentially efficient method to

determine where Cerulean-positive and tdTomato-positive exhibit distinct molecular profiles.

How should changes in astrocyte Ca^{2+} activity be interpreted?

Over the past two decades, much emphasis has been placed on recording Ca^{2+} signals within astrocytes as a basis to understand their roles in neural circuits. Difficulties in detecting and interpreting astrocyte Ca^{2+} signals have led to much debate and controversy within the field (Bazargani & Attwell, 2016). The focus on Ca^{2+} is based on the fact that Ca^{2+} is a ubiquitous second messenger and intracellular Ca^{2+} signals are thought to be the primary signal by which astrocytes interact with neurons and blood vessels. Astrocyte Ca^{2+} transients are widely considered to be a form of cellular excitability; however, these transients are extremely diverse and do not exhibit stereotyped electrical activity like action potentials. Therefore, it is likely that the functional consequences of astrocyte Ca^{2+} are much more diverse and complex than neuronal action potentials (Nimmerjahn & Bergles, 2015). GECIs optimized for astrocytes, such as Lck-GCaMP6, have made detecting calcium transients in astrocyte processes relatively simple. However, because only a small region of the astrocyte, and thus only a fraction of microdomains, are visible in a given cell and can be measured within the focal plane of a 2-photon microscope, it is difficult to fully appreciate their complete spatiotemporal profiles (particularly in the z-direction). Furthermore, fine astrocyte processes can be extremely small and therefore undetectable by conventional 2-photon microscopy. We were able to detect Ca^{2+} transients in very small processes throughout astrocytes. However, it is possible that even with the newest, most sensitive

and brightest, GCaMP6f, we are missing potentially nanoscopic subtle Ca^{2+} events. Indeed, physiological Ca^{2+} activity in astrocytes may extend beyond what can be detected with currently available Ca^{2+} indicators (Zheng et al., 2015). Therefore, there may be very subtle physiological changes that link particular types of Ca^{2+} signals and their downstream consequences.

Interpreting the downstream consequences of Ca^{2+} activity in astrocytes is difficult. It is known that intracellular Ca^{2+} can activate many transcription factors and downstream enzymes including PKC, MEF-2, NFAT, NF-kB, CaMKII and CREB, so Ca2+ activity invariably plays a role in gene expression (Mellstrom, Savignac, Gomez-Villafuertes, & Naranjo, 2008). Within the glia field, there has also been a significant controversy in the field as to the role of these Ca^{2+} signals in the release of signaling molecules, called gliotransmitters from astrocytes (Hamilton & Attwell, 2010). While it now seems clear that astrocytes do indeed release signaling molecules such as ATP and D-serine (Dallerac, Chever, & Rouach, 2013), whether this happens in a Ca^{2+}-dependent manner in processes is still unsettled (Hamilton & Attwell, 2010).

A recent study convincingly showed that one function of Ca^{2+} signaling in astrocyte microdomains is to provide metabolic support, by sensing increasing energy demands and activating oxidative phosphorylation in mitochondria (Agarwal et al., 2017). Using GCaMP3 expressing mice, they demonstrated that neuronally evoked astrocyte Ca^{2+} transients initiated in the astrocyte processes propagate into mitochondria-rich somatic regions, which stimulate oxidative phosphorylation. Then, ATP produced by mitochondria in the soma in turn diffuse back into the perisynaptic processes and supplements ATP produced by local glycolysis during periods of intense glutamate

uptake.

Role of KARs in epileptogenesis

We demonstrated that KARs expressed on reactive astrocytes during the development of epilepsy are functional in a subset of cells, suggesting a new pathway that could represent and important functional alteration contributing to the development of epilepsy. Due to experimental limitations associated with *IUE*, discussed above, the statistical power of these experiments was rather low. We are currently conducting similar experiments in our laboratory, using transgenic mice that express GCaMP5 in astrocytes throughout their brain, and applying ATPA to investigate the responses of KARs on reactive astrocytes (Gee et al., 2014). Because in this transgenic mouse model, every astrocyte in the stratum radiatum expresses GCaMP5, placement of pipettes for agonist application is simplified. However, there are a few important caveats to keep in mind, as we repeat these experiments in the low-dose kainate mouse model of TLE. First, Western Blot and IHC needs to be performed, in order to confirm that reactive astrocytes express KARs during the latent period. We currently do not know if reactive astrocytes during the latent period in the mouse express KAR subunits, but a good indication will be if we see a Ca^{2+} response upon application of ATPA. Furthermore, unlike the rat model of KA-induced TLE, where the vast majority of rats treated with KA go on to develop epilepsy, the rate of spontaneous seizures (i.e., development of chronic epilepsy) in the mouse model is low (Umpierre et al., 2016). Future experiments to pharmacologically dissect the pathway involved in KAR activation in reactive astrocytes during epileptogenesis are planned. Because KARs can signal as ionotropic and metabotropic

receptors, it will be important to use pharmacology to parse the specific mode of signaling. Furthermore, we plan to investigate if KARs on reactive astrocytes can be activated in response to endogenous glutamate release (i.e., high frequency stimulation) of the Schaeffer collateral pathways.

Closing Remarks and Outlook

The concept of the tripartite synapse reflects a new understanding of brain physiology. Astrocytes have traditionally been considered a passive, homogenous cellular population, but accumulating evidence places them as functionally diverse, critical players in the brain in both health and disease. The need for innovative, new approaches to studying changes in circuits during the development of epilepsy are desperately needed in order to provide improved therapies for patients. This dissertation lays the groundwork for future imaging experiments at the tripartite synapse using the novel genetic tool, *NASTIE*, and points to a kainate-receptor mediated signaling pathway that may reflect an important functional change in reactive astrocytes during the development of epilepsy.

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APPENDIX

COULD ASTROCYTES BE USED TO BEAT EPILEPSY? EXPERIMENTS IN dnSNARE MICE DRUM UP NEW HOPE[1]

Historically, epilepsy research has focused on understanding the dysfunction of neuronal cell types in the progression of TLE. More recently, a subset of glial cells, known as astrocytes, have become implicated in the process of epileptogenesis. Following status epilepticus (SE), astrocytes undergo a process termed reactive gliosis wherein these cells exhibit dramatic changes in morphology and protein expression (Binder & Steinhauser, 2006). These changes are a hallmark of TLE; however, the functional consequences of this process on disease progression remain unclear (Steinhauser & Boison, 2012). Reports have demonstrated that astrocytes begin to increase expression of a variety of receptors including metabotropic glutamate receptors (Aronica et al., 2000) and kainate receptors (Vargas, Takahashi, Thomson, & Wilcox, 2013) during SE-induced epilepsy, suggesting that astrocytes may be responding to changes in extracellular glutamate in the synaptic cleft during seizures. Astrocytes are considered an integral part of the tripartite synapse and have been hypothesized to be capable of modulating synaptic transmission via the vesicular release of neurotransmitters including glutamate (Perea & Araque, 2007), though this remains a controversial topic

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within the glial biology field (Nedergaard & Verkhratsky, 2012). This model suggests that astrocytes could contribute to the excitation of surrounding neurons through a direct mechanism, and thus may offer a new potential therapeutic target in epilepsy.

By far, the greatest challenge faced by those studying the role of astrocytes in epilepsy has been a lack of astrocyte-specific tools to dissect the contribution of these cells to neuronal networks. Fortunately, in the last few years, the increased accessibility of cell-specific inducible transgenic animals and optogenetic sensors, such as the GCaMP family of proteins, offers hope that exciting breakthroughs lay just beyond the horizon (Li, Agulhon, Schmidt, Oheim, & Ropert, 2013). In this vein, a recent study by Clasadonte and colleagues used astrocyte-specific transgenic animals to investigate the role that astrocyte-mediated transmitter release may play in the progression of TLE.

Previously this group developed a line of tamoxifen-inducible transgenic mice called the dominant negative SNARE (dnSNARE) mice wherein the vesicular machinery required for the release of transmitters is selectively disrupted in astrocytes (Pascual et al., 2005). In the current study, 2-month-old dnSNARE and WT male littermate mice were administered low-dose injections of pilocarpine and allowed to undergo status epilepticus (SE) for 90 minutes before terminating the SE with diazepam. After a latent period following SE, these animals went on to develop TLE, characterized by spontaneous recurrent seizures. Appropriately, this progressive development of seizures was tracked using long-term video EEG recording. dnSNARE animals had a longer latency to the development of spontaneous recurrent seizures than wildtype age-matched controls. Furthermore, during the chronic epilepsy period, the dnSNARE mice had less severe seizures, a slower progression of seizure severity over time, and a reduction in the

number of interictal spikes as compared to wildtype controls, suggesting that the loss of vesicular release of signaling molecules in the dnSNARE mice could modify epileptogenesis.

The investigators also looked at behavioral and histological markers associated with TLE 8 months after SE. They assessed locomotor activity using an open field behavior test. Intriguingly, wildtype animals treated with pilocarpine showed less locomotor activity than their dnSNARE counterparts. Clasadonte et al. also quantified the relative number of neurons using an antibody for NeuN. Profound neuronal cell death was associated with SE in the hilar region of the dentate gyrus in WT animals, but not dnSNARE animals, suggesting that the dnSNARE mutation is neuroprotective. The authors also investigated reactive gliosis using immunohistochemistry for glial fibrillary acidic protein (GFAP, a protoplasmic astrocytic marker) and found less GFAP expression in the dnSNARE mice after SE, compared to controls. These studies pointed to a reduced pathology of brain regions that are normally quite sensitive to SE.

They then turned to an in vitro acute brain slice model of epileptiform activity to investigate changes in synaptic physiology. In brain slices prepared from dnSNARE animals, there was a reduced latency to onset of epileptiform activity, and a decrease in the number of ictal events compared to WT animals. Furthermore, patch-clamp recordings from CA1 pyramidal neurons 5 months after pilocarpine treatment indicated that the NMDA currents were significantly reduced in the dnSNARE mice. Finally, Clasadonte and coauthors found that treatment with D-AP5, an NMDA receptor antagonist, during the latent period of epileptogenesis was sufficient to phenocopy the long-term effects of astrocytic dnSNARE expression, suggesting that vesicular released

glutamate exerts pro-epileptogenic effects selectively on NMDA receptors.

This investigation offered a valuable new perspective on understanding the role of astrocyte-derived chemical transmission on the process of epileptogenesis. A particular strength of the study was the use of astrocyte-specific inducible genetic techniques, as selectively distinguishing astrocyte vs. neuronal contributions to networks has been a continuous limitation in other studies attempting to understand astrocyte function in epileptogenesis.

Furthermore, the use of long-term chronic EEG and behavioral monitoring was another considerable strength of the study and provided important relevant data on the various phases of TLE disease progression. One concerning aspect of the experimental design was the lack of electrographic monitoring during pilocarpine-induced SE for all animals in the study. Although behavioral seizures were assessed, this evidence alone is inadequate to conclude that the severity of the SE was the same in both the dnSNARE and their wildtype counterparts. Thus, it cannot be ruled out that the severity of SE was reduced in the dnSNARE animals, possibly explaining the reductions in seizures, as well as pathogenic and behavioral differences between the two groups. In addition, analysis of seizures was performed only on 4-6 individual mice. Given the variability in number of seizures among the different cohorts, forthcoming studies should include larger sample sizes when assessing behavioral and electrographic seizure measures.

Future directions will hopefully address the selective mechanisms that control glutamate release from astrocytes in wildtype animals, as this still remains a contentious topic within the field of astrocyte physiology. In addition, caution should be taken to not conflate phenocopying with the elucidation of a direct mechanism. Furthermore, specific

elaboration of the NMDAR-dependent pathway in which astrocyte-selective inhibition of vesicle release is effective in diminishing progression of TLE is warranted. While the approach taken in this study suggests that glutamate released from astrocytes may be contributing to the progression of TLE, the authors did not rule out contributions from other gliotransmitters such as D-serine, a required co-agonist of the NMDA receptor.

Most excitingly, the study by Clasadonte and colleagues provides evidence suggesting that astrocytes could serve as potential disease modifying targets in TLE, the Holy Grail for epilepsy researchers. Thus, our goal should be to conduct experiments that provide nuanced information about the mechanistic role of astrocytes during epileptogenesis. With so many new and exciting emerging technologies, obtaining robust data that clarifies and strengthens our models is well within reach.

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