Microdomain Calcium Oscillations in *Drosophila* Glia Regulate Seizure Susceptibility and Require NCKX

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Submitted to the Department of Biology on January 15th, 2013 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

**ABSTRACT**

Glial cells exhibit spontaneous and activity-dependent fluctuations in intracellular Ca$^{2+}$, yet it is unclear whether glial Ca$^{2+}$ oscillations are required during neuronal signaling. Somatic glial Ca$^{2+}$ waves are primarily mediated by the release of intracellular Ca$^{2+}$ stores, and their relative importance in normal brain physiology has been disputed. Recently, near-membrane microdomain Ca$^{2+}$ transients were identified in fine astrocytic processes and found to arise via an intracellular store-independent process. Here, we describe the identification of rapid, near-membrane Ca$^{2+}$ oscillations in *Drosophila* cortex glia of the central nervous system. In a screen for temperature-sensitive conditional seizure mutants, we identified a glial-specific Na$^+$/Ca$^{2+}$, K$^+$ exchanger (*zydeco*) that is required for microdomain Ca$^{2+}$ oscillatory activity. We found that *zydeco* mutant animals exhibit increased susceptibility to seizures in response to several environmental stressors, and that *zydeco* is required acutely in cortex glia to regulate seizure susceptibility. We also found that glial expression of calmodulin is required for stress-induced seizures in *zydeco* mutants, suggesting a Ca$^{2+}$/calmodulin-dependent glial signaling pathway is involved in acute glial-neuronal communication. These studies demonstrate that microdomain glial Ca$^{2+}$ oscillations require NCKX-mediated plasma membrane Ca$^{2+}$ flux, and are essential for normal neuronal excitability.
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CHAPTER 1

The Role of Glial Calcium Signaling in the Brain

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Introduction

Glial cells have long been considered to be mere spectators in the brain, playing structural and supportive roles relative to their more electrically excitable neuronal counterparts. However, in the last twenty years our perception of glia has been redefined, in part due to the recognition that glia exhibit calcium excitability. Glial cells oscillate intracellular calcium (Ca\(^{2+}\)) both spontaneously and in response to neuronal activity, and growing evidence indicates that glial Ca\(^{2+}\) signaling influences neuronal physiology on a rapid time scale. In the cortex, glial cells and neurons exist in equal abundance (Azevedo et al., 2009) and are intimately associated. A single astrocytic glial cell contacts multiple neuronal cell bodies, hundreds of neuronal processes, and tens of thousands of synapses (Halassa et al., 2007; Ventura and Harris, 1999). Yet despite the pervasiveness of glia and their Ca\(^{2+}\) excitable nature, we still understand little of their complex physiology. Recent data suggest that glial cells play contributive or even causative roles in several neurological disorders, including epilepsy. A better understanding of how glial cells detect, respond, and actively shape neuronal communication is critical to our understanding of the brain and for developing treatments to neurological disease.

Glial cells exhibit Ca\(^{2+}\) excitability

In the early 1990s, two key observations generated a surge of interest in the role of glial cells in active information processing in the brain. The first was that astrocytes in culture oscillate intracellular Ca\(^{2+}\) in response to a variety of neurotransmitters, including glutamate (Cornell-Bell et al., 1990), and that
glutamate released during normal synaptic transmission was sufficient to induce astrocytic Ca\(^{2+}\) oscillations (Dani et al., 1992). The second key observation was that Ca\(^{2+}\) oscillations in astrocytes trigger Ca\(^{2+}\) elevation in co-cultured neurons (Nedergaard, 1994; Parpura et al., 1994), suggesting that in addition to eavesdropping on synaptic transmission, astrocytes can also communicate with their neuronal partners. These seminal observations introduced an additional layer of complexity to our understanding of the brain by suggesting that glial-neuronal signaling occurs in parallel to neuron-neuron communication, and sparked interest in understanding the complexity of glia and their active roles in neural physiology.

**Neuronal activity stimulates glial Ca\(^{2+}\) oscillations**

The initial observations of glial Ca\(^{2+}\) signaling were performed in astrocytes, the major class of glia in the brain. In acutely isolated hippocampal slices, astrocytic Ca\(^{2+}\) oscillations were found to depend on neuronal activity, as strong electrical stimulation of neuronal afferents caused intra- and intercellular astrocytic Ca\(^{2+}\) elevation (Dani et al., 1992). Hippocampal astrocytes in situ were subsequently shown to respond to glutamate released by neurons in response to electrical stimulation (Porter and McCarthy, 1996). Notably, astrocytic Ca\(^{2+}\) activity was blocked by incubation of the slice with tetrodotoxin (TTX), a voltage gated sodium channel blocker that inhibits action potential propagation (Porter and McCarthy, 1996), indicating neuronal signaling was necessary for astrocytic Ca\(^{2+}\) fluctuations.

Strong electrical stimulation of neurons was clearly sufficient to drive Ca\(^{2+}\) oscillations in astrocytes, yet whether astrocytes responded to physiologically
relevant neuronal activity in vivo remained to be determined. The first demonstration of a glial Ca\textsuperscript{2+} response to a physiologically relevant stimulus was performed in the isolated rat retina, in which light flashes were shown to elicit Ca\textsuperscript{2+} increases in retinal glial cells (Newman, 2005). Subsequently, astrocytes in the exposed cortex of a live, anesthetized mouse were found to oscillate intracellular Ca\textsuperscript{2+} in response to whisker stimulation, indicating that glia respond to sensory input (Wang et al., 2006). Cortical astrocytes imaged by this method were also found to propagate Ca\textsuperscript{2+} over long distances during endogenous neuronal activity, involving hundreds of cells in Ca\textsuperscript{2+} waves termed "glissandi" (Kuga et al., 2011). These large intercellular glial Ca\textsuperscript{2+} waves may represent a means by which astrocytes control blood flow in response to metabolic demand. On a more local scale, hippocampal astrocytes were found to respond to spontaneous synaptic transmission (miniature excitatory postsynaptic potentials, or "minis") with transient increases of intracellular Ca\textsuperscript{2+}, indicating that astrocytes are capable of responding to a variety of neuronal signaling events (Di Castro et al., 2011).

**Glial Ca\textsuperscript{2+} oscillations also occur spontaneously**

Interestingly, glial cells also appear capable of initiating intracellular Ca\textsuperscript{2+} oscillations on their own. Spontaneous, or neuronal activity-independent, glial Ca\textsuperscript{2+} activity was first described in situ in cultured slices from the thalamus and hippocampus (Nett et al., 2002; Parri et al., 2001). Approximately half of the observed astrocytes exhibited Ca\textsuperscript{2+} oscillations when neuronal activity was suppressed by TTX, leading to speculation that glia were even more active
participants in neural physiology than previously appreciated (Nett et al., 2002). An independent group performing similar experiments concurrently found that tissue damage strongly reduces spontaneous astrocytic Ca\textsuperscript{2+} oscillations (Aguado et al., 2002), suggesting spontaneous glial Ca\textsuperscript{2+} activity may be missed in some \textit{in situ} experiments due to damage incurred during slice preparation.

Given that astrocytic activity appeared sensitive to mechanical damage, the spontaneous nature of astrocytic Ca\textsuperscript{2+} signaling needed to be confirmed in intact preparations \textit{in vivo}. Initial attempts to monitor glial Ca\textsuperscript{2+} activity in living animals were performed under anesthesia. Astrocytes imaged on the surface of the cortex exhibited a low rate of cytosolic Ca\textsuperscript{2+} oscillations that increased in frequency when neuronal activity was stimulated pharmacologically (Hirase et al., 2004). These initial, low frequency astrocytic Ca\textsuperscript{2+} oscillations were deemed "spontaneous" because they were observed in the anesthetized animal; however, the possibility that a low level of anesthesia-resistant neuronal activity triggered astrocytic Ca\textsuperscript{2+} responses could not be ruled out. Several years later, the same group applied TTX to the cortex of an anesthetized mouse and found that low frequency astrocytic Ca\textsuperscript{2+} oscillations persisted, indicating that astrocytic Ca\textsuperscript{2+} activity can arise independently of action potential triggered neurotransmitter release (Takata and Hirase, 2008). The general consensus that emerged from these and other studies was that astrocytic Ca\textsuperscript{2+} oscillations occur intrinsically - without neuronal input - but are increased by neuronal activity (reviewed in (Agulhon et al., 2008; Fiacco and McCarthy, 2006)).
**Subcellular glial Ca\(^{2+}\) domains**

In the past two decades, a variety of experimental techniques have been used to study Ca\(^{2+}\) signaling in astrocytes. The majority of data were acquired by fluorescence imaging of Ca\(^{2+}\)-sensitive dyes loaded into the cell body by patch pipette, or applied to tissue by membrane diffusion. Several technical limitations associated with this technique - namely, the inability of dyes to penetrate fine astrocytic processes, and limitations on the sensitivity and resolution of optical imaging - have restricted most studies to measurement of somatic astrocytic Ca\(^{2+}\) oscillations (Rusakov et al., 2011). Although astrocytes *in situ* and *in vivo* exhibit large Ca\(^{2+}\) oscillations that spread throughout the cell body, compartmentalized Ca\(^{2+}\) events also occur in fine glial processes and do not necessarily propagate throughout the cell (Shigetomi et al., 2010). The location, duration, and spread of glial Ca\(^{2+}\) events may reflect differences in the signal initiating the Ca\(^{2+}\) activity and the molecular signaling pathway triggered by astrocytic Ca\(^{2+}\) (Agulhon et al., 2008).

**Somatic vs. microdomain Ca\(^{2+}\) oscillations**

Generally, somatic glial Ca\(^{2+}\) oscillations are slow and long lasting relative to Ca\(^{2+}\) events that are restricted to a small region of the cell. The first *in vivo* studies of Ca\(^{2+}\) oscillations in cortical astrocytes utilized cell permeable fluorescent dyes and reported somatic astrocytic Ca\(^{2+}\) surges that last for several minutes (Hirase et al., 2004; Nimmerjahn et al., 2004). Likewise, intercellular somatic astrocytic Ca\(^{2+}\) waves imaged *in vivo* persisted for tens of seconds to minutes (Kuga et al., 2011). The relatively slow timescale of somatic astrocytic Ca\(^{2+}\) activity was initially thought
to indicate that astrocytes do not participate in rapid signaling in the brain. For example, neuronal synaptic communication takes place on the order of milliseconds. However, analysis of astrocytic morphology revealed that while the average volume of an astrocytic cell body is 1,000 µm$^3$, the total volume occupied by an astrocyte and its fine processes is 66,000 µm$^3$ (Appaix et al., 2012; Bushong et al., 2002), suggesting measurement of cell body Ca$^{2+}$ does not likely reflect the total activity of an astrocyte (Figure 1).

Pioneering work from several groups showed that astrocytic Ca$^{2+}$ signaling takes place in small subcellular domains along astrocytic processes as well as the cell soma. Ultrastructural analysis of Bergmann glia, the astrocyte-like cell type of the cerebellum, revealed the presence of bulbous domains in the glial processes that closely abutted synapses and appeared designed for local glial-neuronal signaling (Grosche et al., 1999). Indeed, Ca$^{2+}$ fluctuations *in situ* were detected in small regions along Bergmann glial processes (encompassing roughly 100 µm$^2$ surface area) that did not propagate to the cell body and lasted several seconds (Grosche et al., 1999). In hippocampal slices, occasional Ca$^{2+}$ oscillations were observed that were restricted to astrocytic processes (Nett et al., 2002). When microdomain Ca$^{2+}$ activity was first observed *in vivo*, it was found that Ca$^{2+}$ oscillations in astrocytic processes occurred much more frequently than Ca$^{2+}$ spikes in cell bodies, suggesting that astrocytes were more active cells than previously appreciated (Wang et al., 2006).

Delineation of astrocytes into smaller functional domains continued with further ultrastructural analysis and refinement of Ca$^{2+}$ imaging techniques. Cultured
rat astrocytes were reported to contain submicrometer structural domains consisting of endoplasmic reticulum tubules and synaptic-like microvesicles closely abutting the plasma membrane (Marchaland et al., 2008). Localized regions of rapid Ca\(^{2+}\) flux were identified in these astrocytes that spread only a couple micrometers from the point of initiation (Marchaland et al., 2008). In 2010, development of a membrane tethered variant of Ca\(^{2+}\)-sensitive GFP (GCaMP) enabled detection of small regions of Ca\(^{2+}\) flux in cultured astrocytes (Shigetomi et al., 2010). These spotty, "microdomain" regions of Ca\(^{2+}\) oscillation spread for \(~5\) \(\mu\)m and exhibited a half-life of a few seconds. Interestingly, membrane-tethered GCaMP also detected global Ca\(^{2+}\) oscillations that persisted for tens of seconds, indicating that somatic and microdomain Ca\(^{2+}\) oscillations arise in the same cell and exhibit different temporal dynamics.

The obvious conclusion from these studies is that astrocytic Ca\(^{2+}\) excitability is heterogeneous, and that unique Ca\(^{2+}\) events may reflect the different types of signals initiating the astrocytic Ca\(^{2+}\) response. Differential encoding of neuronal activity into astrocytic Ca\(^{2+}\) activity has been demonstrated using high resolution two-photon microscopy in the adult mouse hippocampus (Di Castro et al., 2011). Two types of astrocytic Ca\(^{2+}\) activities were recorded: "focal" Ca\(^{2+}\) events spreading \(~4\) \(\mu\)m with an average duration of 700 milliseconds, and "expanded" events that encompassed more than 10-20 \(\mu\)m of cellular process and lasted several seconds (Di Castro et al., 2011). Focal Ca\(^{2+}\) oscillations appeared to reflect the astrocytic response to spontaneous synaptic activity, while expanded events were attributed to action potential-evoked synchronous synaptic release. In support of this
conclusion, small swellings (~1.5 µm in length) of hippocampal astrocytic processes were found by a second group to exhibit compartmentalized Ca\(^{2+}\) oscillations in response to single synapse stimulation (Panatier et al., 2011). These results indicated for the first time that astrocytes respond uniquely to different magnitudes of neuronal activity, and can participate in millisecond time-scale signaling.

**Molecular mechanisms of glial Ca\(^{2+}\) regulation**

In neurons, presynaptic Ca\(^{2+}\) transients are mediated by voltage-gated Ca\(^{2+}\) channels, while postsynaptic Ca\(^{2+}\) influx occurs primarily through neurotransmitter-gated ion channels. However, in astrocytes and other glia, Ca\(^{2+}\) oscillations appear to arise from both intracellular and extracellular sources, and several distinct molecular mechanisms have been identified that likely contribute to the heterogeneity of astrocytic Ca\(^{2+}\) oscillations (Figure 2).

**Somatic glial Ca\(^{2+}\) oscillations**

The vast majority of studies concerning Ca\(^{2+}\) regulation in astrocytes utilized cell body, or somatic, fluorescence imaging to monitor the activity of the astrocyte. These studies implicate organellar Ca\(^{2+}\) stores as essential contributors to intracellular Ca\(^{2+}\) oscillations. The endoplasmic reticulum (ER) is a network of tubules and cisternae that stores Ca\(^{2+}\) at millimolar concentrations (Verkhratsky and Petersen, 2002) and plays a essential role in somatic astrocytic Ca\(^{2+}\) regulation. Pharmacological inhibition of ER Ca\(^{2+}\) release by application of thapsigargin or cyclopiazonic acid, which block the ER Ca\(^{2+}\) ATPase SERCA, fully eliminates somatic
Ca\textsuperscript{2+} oscillations in cultured astrocytes and hippocampal astrocytes in situ (Jeremic et al., 2001; Nett et al., 2002). Ca\textsuperscript{2+} release from astrocytic ER occurs via inositol 1,4,5-triphosphate (IP\textsubscript{3}) gated receptors, of which IP\textsubscript{3}R2 is the primary isoform expressed in astrocytes. In 2008, it was reported that knockout of IP\textsubscript{3}R2 in mouse fully eliminated both spontaneous and activity-dependent somatic Ca\textsuperscript{2+} oscillations in astrocytes, strongly implicating ER Ca\textsuperscript{2+} release as the primary pathway by which astrocytes elevate intracellular Ca\textsuperscript{2+} (Petravicz et al., 2008).

Numerous studies indicate that IP\textsubscript{3} generation is mediated by metabotropic G\textsubscript{q} coupled receptors (GPCR) and phospholipase C activation (Fiacco and McCarthy, 2006). Astrocytes express metabotropic G\textsubscript{q} coupled receptors for both glutamate and ATP, which have been shown by various groups to produce large Ca\textsuperscript{2+} oscillations when applied to astrocytes (Fiacco and McCarthy, 2006). Astrocytes in vitro and in situ also express ligand-gated ionotropic receptors that are Ca\textsuperscript{2+} permeable, including the NMDA and AMPA glutamate receptors, although strong evidence for ionotropic receptor-mediated Ca\textsuperscript{2+} oscillations in glia is lacking (reviewed in (Parpura et al., 2011)).

Although ER Ca\textsuperscript{2+} release through IP\textsubscript{3} gated receptors appears to be the primary mechanism by which astrocytes elevate somatic Ca\textsuperscript{2+}, other sources of Ca\textsuperscript{2+} regulation may modulate the duration and amplitude of the Ca\textsuperscript{2+} transient. One such pathway is store operated Ca\textsuperscript{2+} entry (SOCE). Depletion of ER Ca\textsuperscript{2+} stores triggers influx of extracellular Ca\textsuperscript{2+} through store-operated channels in all cells, including astrocytes (Golovina, 2005). SOCE allows refilling of ER Ca\textsuperscript{2+} that is necessary to sustain cytosolic Ca\textsuperscript{2+} oscillations (Singaravelu et al., 2006), and also appears to
modulate the peak of the astrocytic Ca\(^{2+}\) elevation evoked by G\(_{q}\) GPCR activation (Malarkey et al., 2008). Mitochondria are a second class of organelles that buffer and store large concentrations of Ca\(^{2+}\), and inhibition of mitochondrial Ca\(^{2+}\) buffering was found to increase the peak and duration of cytosolic Ca\(^{2+}\) oscillations in cultured glia (Reyes and Parpura, 2008). Sparse evidence also exists for the involvement of both L-type and T-type voltage-gated Ca\(^{2+}\) channels (VGCCs) in astrocytic Ca\(^{2+}\) influx (MacVicar, 1984; Sontheimer, 1994), although the functional expression of VGCCs in astrocytes in vivo has not been demonstrated.

**Microdomain glial Ca\(^{2+}\) oscillations**

Small regions of localized Ca\(^{2+}\) flux in astrocytes appear to be regulated by mechanisms other than the canonical GPCR-IP\(_3\)-ER Ca\(^{2+}\) release pathway that is critical for large, somatic Ca\(^{2+}\) oscillations. Teasing apart the contribution of various molecular mechanisms to microdomain Ca\(^{2+}\) oscillations is complicated by the fact that no consensus has yet emerged as to how to define a "Ca\(^{2+}\) microdomain." Given that astrocytes elevate Ca\(^{2+}\) in membrane regions from 2-10 \(\mu\)m in length (Di Castro et al., 2011; Panatier et al., 2011) to processes spanning hundreds of \(\mu\)m (Grosche et al., 1999; Wang et al., 2006), diverse molecular mechanisms may regulate different magnitudes of Ca\(^{2+}\) release.

The first report of small regions of astrocytic Ca\(^{2+}\) oscillation spanning less than 10 \(\mu\)m of membrane found that disruption of ER Ca\(^{2+}\) stores by cyclopiazonic acid had no effect on the frequency or magnitude of Ca\(^{2+}\) microdomains (Shigetomi et al., 2010). In addition, Ca\(^{2+}\) microdomains were not evoked by ATP and were not
inhibited by $G_q$ GPCR antagonists, which both stimulated and inhibited somatic $Ca^{2+}$ oscillations, respectively (Shigetomi et al., 2010). Interestingly, extracellular $Ca^{2+}$ was required for microdomain oscillations, indicating small $Ca^{2+}$ oscillations require plasma membrane, rather than organellar, $Ca^{2+}$ flux. A second study examining $Ca^{2+}$ microdomains in astrocytes in situ reported that small ($< 5 \mu m$) $Ca^{2+}$ transients persisted in the IP$_3R2$ knockout mouse, though they were reduced in frequency (Di Castro et al., 2011). Similarly, inhibition of GPCR signaling reduced, but did not eliminate, small $Ca^{2+}$ events, while removal of extracellular $Ca^{2+}$ strongly suppressed $Ca^{2+}$ microdomains (Di Castro et al., 2011). These data therefore support the notion that a plasmalemmal rather than intracellular $Ca^{2+}$ flux pathway is involved in $Ca^{2+}$ microdomains, and also indicates that microdomains may arise via signals distinct from the $G_q$ GPCR agonists that initiate somatic $Ca^{2+}$ elevation.

Given the physical constraints on surface-to-volume ratio in fine astrocytic processes, it seems reasonable that plasma membrane $Ca^{2+}$ flux would play a larger role in $Ca^{2+}$ events restricted to small processes than in $Ca^{2+}$ oscillations that encompass the entire cell body. Electron micrograph analysis of Bergmann glia of the cerebellum revealed that the tips of glial processes closely abutting synapses average less than 300 nm in diameter (Grosche et al., 1999). Likewise, fine astrocytic processes in the brain neuropil can be as thin as 30 to 50 nm (Witcher et al., 2007), which is comparable to the diameter of a single synaptic vesicle. The thinness of fine glial projections would likely exclude the presence of endoplasmic reticulum. Thin glial processes may also provide favorable conditions to compartmentalize signaling cascades triggered by $Ca^{2+}$, since a strong concentration
of signal could be generated within a thin volume with a limited number of participating molecules (Rusakov et al., 2011).

**Functional consequences of glial Ca\(^{2+}\) signaling**

Clearly, glial cells are active signaling components of the brain, and can interact with neurons on a local scale, but how does glial Ca\(^{2+}\) activity affect neuronal function? Determining the effects of astrocytic Ca\(^{2+}\) activity is complicated by the fact that most experimental studies have manipulated global, cell body Ca\(^{2+}\) to investigate astrocytic function. Another potential difficulty in interpreting experimental data is that astrocytic Ca\(^{2+}\) oscillations that appear spatially and temporally similar may trigger distinct downstream signaling mechanisms. For example, Ca\(^{2+}\) oscillations in hippocampal astrocytes were found to cause slow inward currents in neurons when Ca\(^{2+}\) was induced by PAR-1 receptor activation, but did not affect neuronal currents when Ca\(^{2+}\) elevation was induced in astrocytes by P2Y\(_1\) receptor activation (Shigetomi et al., 2008). In both cases, the astrocytic Ca\(^{2+}\) oscillations appeared equivalent, but only PAR-1 dependent Ca\(^{2+}\) oscillations induced neuronal currents (Shigetomi et al., 2008). The variability of astrocytic Ca\(^{2+}\) dynamics and the use of diverse pharmacological and genetic agonists to induce Ca\(^{2+}\) oscillations have therefore generated much debate in the field as to the functional consequences of glial Ca\(^{2+}\) signaling.

**Glial Ca\(^{2+}\) modulation of neuronal activity**

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In early studies, paired recording of astrocytic Ca$^{2+}$ and neuronal activity revealed that elevation of Ca$^{2+}$ in an astrocyte correlated with the appearance of large inward currents in nearby neurons (Parri et al., 2001; Pasti et al., 1997). This observation suggested that astrocytes were not just responsive elements of the brain, but could actively regulate neuronal activity. Cultured astrocytes were also found to modulate spontaneous transmitter release frequency in neurons, indicating astrocytes could affect synaptic transmission (Araque et al., 1998). The discovery that large Ca$^{2+}$ elevations in cultured astrocytes caused release of glial-derived glutamate (Parpura et al., 1994) suggested that Ca$^{2+}$-dependent release of neuroactive transmitters from glia (so-called "gliotransmission") could be a mechanism underlying rapid glial-neuronal communication in the brain.

In the last fifteen years, accumulating in vitro and in situ data indicates that glia are capable of releasing a variety of neuromodulators, including glutamate, ATP, D-serine, GABA, and various neuropeptides (Parpura and Zorec, 2010). However, whether Ca$^{2+}$-dependent gliotransmission occurs in vivo, and under what conditions, is still unclear. The most widely studied gliotransmitter is glutamate, the major excitatory transmitter in the CNS. Multiple studies suggest that Ca$^{2+}$ elevation in astrocytes leads to the exocytic release of glutamate (Parpura and Haydon, 2000; Zhang et al., 2004), which binds to extrasynaptic NMDA receptors on neighboring neurons (Jourdain et al., 2007) and induces slow, inward neuronal currents (Angulo et al., 2004). In addition to glutamate, astrocytes also appear to secrete ATP in a Ca$^{2+}$-dependent manner. Mice expressing a dominant negative SNARE protein to selectively block gliotransmission in astrocytes were found to exhibit substantially
reduced extracellular ATP, which altered properties of synaptic transmission and plasticity (Pascual et al., 2005). Interestingly, astrocytes express purinergic receptors and exhibit large, somatic Ca\textsuperscript{2+} oscillations in response to ATP, suggesting glial ATP release may trigger feed-forward intercellular astrocytic Ca\textsuperscript{2+} waves (Stout et al., 2002). Evidence for astrocytic release of GABA, an inhibitory transmitter (Lee et al., 2010), and D-serine, an NMDA receptor co-agonist (Henneberger et al., 2010), indicate that astrocytes are capable of secreting a variety of neuroactive substances. Determining when and where gliotransmission normally occurs in the brain is the subject of ongoing research.

Glia Ca\textsuperscript{2+} signaling can also lead to changes in neuronal excitability without involving gliotransmission. Somatic astrocytic Ca\textsuperscript{2+} oscillations in the hippocampus were reported to cause a transient (20 to 80 second) decrease in extracellular K\textsuperscript{+} by inducing astrocytic uptake of K\textsuperscript{+} via the Na\textsuperscript{+}/K\textsuperscript{+} ATPase (Wang et al., 2012). Decreased astrocyte Ca\textsuperscript{2+}-dependent K\textsuperscript{+} uptake hyperpolarized neurons by 2-4 mV and reduced the frequency of spontaneous transmitter release (Wang et al., 2012). In addition to regulating K\textsuperscript{+}, glia also recycle synaptically released neurotransmitters, and impairment of this process could alter properties of synaptic transmission. In Bergmann glia, Ca\textsuperscript{2+} signaling was found to be important for glutamate clearance from synapses, as reduced glial IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release caused reduced glutamate reuptake (Mashimo et al., 2010). However, this effect required downregulation of the glutamate transporter GLAST, and is therefore not likely an acutely regulated (second time-scale) signaling event (Mashimo et al., 2010).
Debate regarding the physiological impact of glial Ca\textsuperscript{2+} signaling

In order to directly test the relevance of astrocytic Ca\textsuperscript{2+} signaling in vivo, (Fiacco et al., 2007) reported the generation of a transgenic mouse expressing the novel G\textsubscript{q}-coupled receptor MrgA1 in astrocytes. MrgA1 is not normally expressed in the CNS and is activated by a ligand that does not cross react with endogenous brain receptors, allowing the authors to selectively stimulate astrocytic Ca\textsuperscript{2+}. Focal application of the MrgA1 ligand triggered large Ca\textsuperscript{2+} waves that spread throughout the astrocyte and remained elevated for the duration of drug treatment. Surprisingly, however, elevation of astrocytic Ca\textsuperscript{2+} did not trigger Ca\textsuperscript{2+} responses in nearby neurons, nor did it produce inward neuronal currents or alter neuronal mini release frequency. Contrary to previous work, this result suggested that astrocytic Ca\textsuperscript{2+} elevation does not affect nearby neuronal activity, and instead indicated that prior studies supporting Ca\textsuperscript{2+}-dependent gliotransmission and consequent neuronal modulation were due to experimental error or nonspecific astrocyte stimulation techniques.

Following this result, (Petravicz et al., 2008) reported the generation of an IP\textsubscript{3}R2 knockout mouse that lacked the IP\textsubscript{3}R isoform specifically expressed in astrocytes. Disruption of IP\textsubscript{3}R2 completely suppressed somatic Ca\textsuperscript{2+} oscillations in astrocytes that were normally elicited by a variety of G\textsubscript{q}-coupled receptor agonists. Interestingly, IP\textsubscript{3}R2 knockout mice were viable, did not exhibit changes in mini frequency or NMDA-mediated neuronal currents, and appeared behaviorally wild-type. This result strongly indicated that IP\textsubscript{3}-dependent astrocytic Ca\textsuperscript{2+} elevation was
not necessary for normal neuronal excitatory synaptic activity. These findings were extended by (Agulhon et al., 2010), who utilized both the MrgA1 transgenic and IP₃R2 knockout mice to ask whether astrocytic Ca²⁺ activity affects evoked synaptic transmission or synaptic plasticity. Again, neither selective stimulation nor ablation of astrocytic Ca²⁺ oscillations affected properties of synaptic transmission at hippocampal synapses, indicating that somatic astrocytic Ca²⁺ activity was largely dispensable for neuronal function.

These genetic manipulations of IP₃R2-dependent Ca²⁺ signaling in astrocytes were in contrast to conclusions drawn from studies using pharmacological or patch clamp methods to enhance or suppress endogenous astrocytic Ca²⁺ activity. For example, uncaging IP₃ in a single astrocyte in situ induced large astrocytic Ca²⁺ oscillations and rapidly increased mini release frequency at nearby hippocampal synapses (Fiacco and McCarthy, 2004). One possible explanation for this discrepancy is that similar macroscopic Ca²⁺ waves in astrocytes arise from diverse microscopic signaling cascades (Rusakov et al., 2011). Activation of ectopic MrgA1 in astrocytes may not trigger Ca²⁺ signaling cascades in the right time or place to induce gliotransmission or other signaling mechanisms that modulate neuronal function. Different functional outcomes arising from similar astrocytic Ca²⁺ oscillations was reported by (Shigetomi et al., 2008), who found that glial Ca²⁺ oscillations in response to PAR-1 receptor stimulation triggered inward neuronal currents, but astrocytic Ca²⁺ oscillations induced by P2Y₁ receptor stimulation had no detectable effect on neurophysiology. An understanding of the subcellular
distribution of signaling mechanisms is therefore critical to manipulating astrocytic Ca\textsuperscript{2+} activity.

A second caveat to these experiments is that genetic ablation of IP\textsubscript{3}R2-dependent signaling does not completely inhibit astrocytic Ca\textsuperscript{2+} activity. Optical analysis of Ca\textsuperscript{2+} oscillations in \textit{IP\textsubscript{3}R2\textendash} astrocytes was initially restricted to the glial cell body, which is routinely identified in tissue preparations with astrocyte-specific dyes that concentrate in the cell soma (Appaix et al., 2012; Petravicz et al., 2008). However, fluorescent imaging of astrocytic processes in the \textit{IP\textsubscript{3}R2\textendash} mouse revealed small microdomains of Ca\textsuperscript{2+} flux that persisted in the mutant, indicating some astrocytic Ca\textsuperscript{2+} activity is IP\textsubscript{3}R2-independent (Di Castro et al., 2011). In addition, microdomain Ca\textsuperscript{2+} oscillations observed \textit{in vitro} and \textit{in situ} were found to arise via IP\textsubscript{3}-independent mechanisms (Shigetomi et al., 2010; Shigetomi et al., 2012). Given that somatic Ca\textsuperscript{2+} oscillations are abolished in the IP\textsubscript{3}R2 knockout mouse, it appears likely that microdomain astrocytic Ca\textsuperscript{2+} activity is critical for glial modulation of neuronal function.

\textbf{The role of glia in epilepsy}

Dysregulation of astrocytic function appears to play a role in many neurological diseases, including epilepsy. Epilepsy is a group of recurrent seizure disorders that are caused by genetic and acquired factors and affect over 65 million people worldwide (Ngugi et al., 2010). Although dysregulation of neuronal ion channel function has been implicated in seizure pathogenesis in a subset of idiopathic epilepsy cases (Klassen et al., 2011), the initial cellular events that trigger
a seizure are not well understood. The contribution of glia to epileptogenesis was first considered over fifty years ago, when it was recognized that prolonged seizures induce reactive gliosis in the brain (Binder and Steinhauser, 2006). Reactive glia exhibit striking morphological and functional changes (Ortinski et al., 2010), indicating that astrocytic dysfunction may contribute to seizure pathophysiology.

Several cellular functions performed by astrocytes cause neuronal hyperactivity when dysregulated. The most well studied of these functions is K+ buffering. Astrocytes uptake extracellular K+ released by neurons during action potential firing through inwardly rectifying K+ (Kir) channels (Newman, 1993). Disruption of astrocytic Kir channels enhances neuronal hyperexcitability and seizure susceptibility in both animal models and human patients (Wallraff et al., 2006), although whether defective removal of K+ by astrocytes is a major factor in heritable seizure disorders is unclear (Carmignoto and Haydon, 2012). Astrocytes also recycle synaptically released GABA and glutamate, and disruption of either of these functions promotes seizures in animal models (Cope et al., 2009; Inyushin et al., 2010). In addition to becoming reactive, astrocytes in epileptic brain tissue also exhibit overlapping domains, indicating that structural reorganization of astrocytes may contribute to recurrent excitation in the brain (Oberheim et al., 2008). Despite the identification of various glial functions that influence neuronal excitability, whether structural or metabolic changes in astrocytes are a primary cause of seizures is unclear.

In several mammalian models of epilepsy, increased glial Ca\textsuperscript{2+} oscillations have been associated with the onset of seizure activity (Rouach et al., 2008; Tashiro
et al., 2002). In one study, chemically induced seizures in hippocampal slices were found to cause concomitant oscillations in astrocytic Ca$^{2+}$ that contributed to epileptiform discharges (Tian et al., 2005). Interestingly, common drugs used to treat epilepsy in human patients reduced astrocytic Ca$^{2+}$ activity, suggesting glia may be a direct target of anticonvulsants (Tian et al., 2005). The main proposed mechanism by which enhanced astrocytic Ca$^{2+}$ oscillations promote seizures is through Ca$^{2+}$-dependent release of glutamate. In the hippocampus, (Fellin et al., 2004) and (Angulo et al., 2004) found that Ca$^{2+}$-active astrocytes release glutamate, which binds to extrasynaptic neuronal NMDA receptors and triggers inward currents synchronously in neurons up to 100 µm distant. The ability of astrocytes to induce synchronous excitation in clusters of neurons suggested astrocytic Ca$^{2+}$ signaling may be capable of initiating epileptiform activity. However, synchronous inward neuronal currents produced by astrocytic Ca$^{2+}$ signaling were later shown to be dispensable for the generation of epileptiform activity in situ (Fellin et al., 2006), suggesting astrocytes may contribute to seizures, but are not necessary for their instigation.

Development of an in vitro model for inducing focal seizures in cultured brain slices lead to the observation that neurons and astrocytes participate in feedback loops to promote seizure initiation (Gomez-Gonzalo et al., 2010). By applying small puffs of NMDA, the authors focally increased neuronal excitability to a sub-seizure threshold. Simultaneous elevation of astrocytic Ca$^{2+}$ then triggered seizure-like neuronal discharges in the tissue, indicating that astrocytic Ca$^{2+}$ signaling and elevated basal neuronal activity both contribute to drive neuronal networks to
seizure threshold. A second group studying astrocytic-neuronal feedback signaling in thalamus found that brief electrical stimulation of neuronal afferents triggered astrocytic Ca$^{2+}$ oscillations and slow, inward neuronal currents that persisted for over an hour after the stimulus (Pirttimaki et al., 2011). Dialysis of the Ca$^{2+}$ chelator BAPTA into the astrocytic network blocked in the appearance of neuronal currents, indicating the glutamate source triggering neuronal activity was glial. The prolonged duration of astrocytic glutamate release after stimulus termination suggested that astrocytes integrate neuronal activity in order to induce long-lasting changes in local neuronal excitability. In addition to glutamatergic gliotransmission, Ca$^{2+}$-dependent endocannabanoid signaling in astrocytes has also been linked to the maintenance of epileptiform activity in the hippocampus (Coiret et al., 2012). These in situ studies suggest that astrocytic Ca$^{2+}$ signaling contributes to the probability of a seizure occurring in the brain and increases the severity and frequency of epileptiform discharges. However, many questions remain about the role of astrocytes in epilepsy. For example, it is unknown whether astrocytic activity is sufficient to initiate a seizure in vivo. It is also unclear whether Ca$^{2+}$-dependent gliotransmission is the main mechanism by which astrocytes recruit neurons into synchronous firing, or whether alternative glial-neuronal signaling mechanisms are required. A better understanding of the relationship between astrocytes and neurons during the development of a seizure will likely be informative for developing anti-epileptic therapies.

**NCKX exchangers regulate intracellular Ca$^{2+}$**
Na\textsuperscript{+}, Ca\textsuperscript{2+}/K\textsuperscript{+} exchangers (NCKX) are plasma membrane transporters that utilize the Na\textsuperscript{+} and K\textsuperscript{+} electrochemical gradients of the cell to export intracellular Ca\textsuperscript{2+} (Altimimi and Schnetkamp, 2007). K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was first identified in bovine photoreceptors (Reilander et al., 1992; Schnetkamp et al., 1989), in which rapid reduction of intracellular Ca\textsuperscript{2+} is required to allow the cell to respond to light. In mammalian rod photoreceptors, NCKX exchange is the main mechanism by which Ca\textsuperscript{2+} extrusion occurs under dark conditions (Schnetkamp, 2004). Each turnover of NCKX transports 4 Na\textsuperscript{+} ions in exchange for 1 K\textsuperscript{+} and 1 Ca\textsuperscript{2+} ion, and can occur in either direction depending on the electrochemical gradient (Schnetkamp et al., 1991). NCKX exchangers have a low affinity ($K_d = 1$-$2$ $\mu$M) (Lagnado et al., 1992) for Ca\textsuperscript{2+} relative to ATP-dependent Ca\textsuperscript{2+} pumps such as PMCA and SERCA, which have a Ca\textsuperscript{2+} $K_d$ around $\sim 0.1$ $\mu$M (Magyar et al., 2002). However, while PMCA and SERCA pump Ca\textsuperscript{2+} from the cytosol at a rate of $\sim 150$ ions/sec, NCKX exchangers transport approximately $\sim 5,000$ Ca\textsuperscript{2+} ions/sec (Juhaszova et al., 2000). The high transport capacity of NCKX makes this family of proteins uniquely suited for rapidly reducing $\mu$M levels of intracellular Ca\textsuperscript{2+}.

NCKX exchangers form a five member family in mammals. While NCKX1 expression is restricted to rod photoreceptors, NCKX 2-4 are broadly expressed in the brain and other tissues (Lytton et al., 2002). Ca\textsuperscript{2+} extrusion requiring Na\textsuperscript{+} and K\textsuperscript{+} was measured in neuronal axonal terminals in rats (Lee et al., 2002) and vascular smooth muscle (Dong et al., 2006), indicating NCKX exchange occurs in a variety of tissues. NCKX2, the major neuronally expressed isoform, was knocked out in mouse and found to substantially contribute to cortical neuron Ca\textsuperscript{2+} flux (Li et al., 2006).
Interestingly, NCKX2 knockout mice exhibited reduced hippocampal LTP and enhanced LTD, along with corresponding deficits in behavioral learning and memory, indicating NCKX2-mediated Ca\(^{2+}\) efflux is required for nervous system plasticity (Li et al., 2006). NCKX4 knockout mice were also recently generated and were reported to exhibit prolonged Ca\(^{2+}\) transients in olfactory sensory neurons (Stephan et al., 2012). Both \(NCKX2^{-/-}\) and \(NCKX4^{-/-}\) mice are viable, and deficits in NCKX-mediated Ca\(^{2+}\) transport have been examined in these models only in neurons.

**NCKX expression in glia**

NCKX members 2-4 are highly expressed in the mammalian CNS and exhibit different regional distributions (Figure 3) (Lytton et al., 2002). Interestingly, the first demonstration of NCKX expression in glia was performed in a rat model of epileptogenesis. NCKX2 expression was found to be transiently upregulated in hippocampal astrocytes of rats treated with kainic acid, which induces generalized seizures by activating ionotropic glutamate receptors (Ketelaars et al., 2004). Transcriptome analysis of acutely purified glia and neurons from the early postnatal mouse brain also identified NCKX expression in glia (Cahoy et al., 2008). In this dataset, NCKX2 exhibited expression in oligodendrocytes, while NCKX3 was enriched in both astrocytes and oligodendrocytes (Cahoy et al., 2008). Recently, NCKX3 expression was also identified in acutely isolated Bergmann glia of the cerebellum (Koirala and Corfas, 2010). Interestingly, NCKX3 was one of the top 20 expressed genes in Bergmann glial cells, suggesting NCKX3 is involved in Bergmann
glial Ca$^{2+}$ regulation (Koirala and Corfas, 2010). However, the role of any NCKX isoform in glial Ca$^{2+}$ regulation has not been determined.

**Using Drosophila to model glial function**

*Drosophila* glia exhibit morphological and molecular similarity to their mammalian counterparts, suggesting investigation of fly glia will aid our understanding of mammalian glial function. Three major morphological classes of *Drosophila* CNS glia have been described (Hartenstein, 2011; Stork et al., 2012) (Figure 4). Surface glia are large, sheet-like cells that form tight intercellular junctions and surround the CNS, mediating the blood brain barrier (DeSalvo et al., 2011). Cortex-associated glia surround individual neuronal cell bodies and are the major glial constituent of the cortex (Spindler et al., 2009). Neuropil-associated glia form two subtypes: ensheathing glia that fasciculate and insulate axons in the CNS, similar to oligodendrocytes, and astrocyte-like glia that interact with synapses (Freeman and Doherty, 2006). The two types of *Drosophila* glia that appear analogous to mammalian astrocytes are cortex and astrocyte-like glia (Awasaki et al., 2008). Both cortex and astrocyte-like *Drosophila* glia are abundant in the CNS and intimately associate with neurons. However, while astrocyte-like glia invade the neuropil and interact with synapses, cortex glia are restricted to the cortical regions of the CNS where they ensheathe neuronal cell bodies with thin membrane processes (Pereanu et al., 2005; Spindler et al., 2009). In mammals, astrocytes occupy spatially segregated domains in the CNS, with each cortical astrocyte encompassing ~4-8 neuronal cell bodies (Bushong et al., 2002; Halassa et al., 2007).
Cortex glia in *Drosophila* exhibit a similar spatial segregation, with each glial cell ensheathing multiple neuronal soma (Pereanu et al., 2005). In comparison to numerous studies describing astrocyte-neuronal signaling at the synapse, the functional implication of the close association between astrocytes and neuronal cell bodies is unknown.

Multiple cellular functions originally identified in mammalian glia are conserved in *Drosophila*. Both fly and mammalian glia are involved in axon pathfinding. In *Drosophila*, peripheral glia direct migrating axons emerging from the ventral nerve cord in developing embryos (Sepp et al., 2001), similar to mammalian Schwann cells that regulate axon emergence in the CNS-PNS transition zone (Fraher, 1997). *Drosophila* axons are ensheathed by glia that fasciculate and insulate them from the extracellular environment (Banerjee et al., 2006; Leiserson et al., 2000), similar to non-myelinating Schwann cell glia. Fly peripheral glia also participate in synaptic remodeling at the neuromuscular junction through phagocytosis of neuronal membrane debris (Fuentes-Medel et al., 2009). In mammals, microglial phagocytosis is required for developmental pruning of synapses (Paolicelli et al., 2011), suggesting glia in the fly also mediate microglial functions despite the absence of a clear "microglial-like" morphological cell type.

*Drosophila* glia have also been linked to regulation of neurotransmission, circuit plasticity, and behavior. Mutation of the glial-specific glutamate transporter *genderblind* reduces ambient extracellular glutamate in the brain, disrupting glutamatergic neuronal signaling and adult courtship behavior (Augustin et al., 2007; Grosjean et al., 2008). A second glial glutamate transporter, EAAT1, regulates
uptake of synaptically released glutamate and larval locomotor activity (Stacey et al., 2010). EAAT1 is homologous to mammalian GLAST and GLT-1, which are the primary glial transporters for glutamate in the mammalian CNS (Anderson and Swanson, 2000). Drosophila glia have been implicated in the formation of long term memory, as flies lacking the glial cysteine proteinase crammer are deficient in an odor-shock learning paradigm requiring 24 hour memory recall (Comas et al., 2004). Circuit plasticity in response to axonal damage requires ensheathing glia in the CNS, which may signal to neurons to modulate their activity in response to deafferentation (Kazama et al., 2011).

Although numerous similarities between Drosophila and mammalian glia have been described, the dynamics of Ca\(^{2+}\) signaling and regulation in Drosophila CNS glia are largely unexplored. Intriguing recent genetic manipulations suggest that Ca\(^{2+}\) signaling in Drosophila glia may be involved in nervous system function. Knockdown of SERCA by RNAi in Drosophila astrocyte-like glia causes behavioral arrhythmicity, suggesting proper glial Ca\(^{2+}\) regulation in astrocyte-like glia is required for neuronal circadian circuitry (Ng et al., 2011). At the adult neuromuscular junction, perisynaptic glia exhibit Ca\(^{2+}\) transients in response to strong electrical stimulation of the synapse (Danjo et al., 2011). However, visualization of Ca\(^{2+}\) activity in Drosophila CNS glia, including cortex glia, has not been previously reported.

Very little is known about the mechanisms of glial Ca\(^{2+}\) regulation in invertebrates. Ca\(^{2+}\) signaling studies in the leech Hirudo medicinalis and the sphinx moth Manduca sexta suggest invertebrate glia mainly employ voltage-gated Ca\(^{2+}\)
channels and Ca\textsuperscript{2+}-permeable, ligand-gated ion channels to respond to neuronal activity (Lohr and Deitmer, 2006; Rose et al., 1995), although whether this is the case in \textit{Drosophila} is unknown. Despite the differences in complexity between mammalian and invertebrate nervous systems, many of the key proteins regulating neuronal signaling are conserved (Littleton and Ganetzky, 2000), suggesting investigation of glial Ca\textsuperscript{2+} signaling in \textit{Drosophila} will shed light on the function of mammalian astrocytes.
Figures
**Figure 1.** A protoplasmic astrocyte (green) enveloping a neuron (red) in the hippocampal CA1 region. Scale bar, 10 μm. Image reproduced from (Allen and Barres, 2009), courtesy of M. Ellisman and E. Bushong.
Figure 2. Mechanisms of Ca\(^{2+}\) regulation in astrocytes. Somatic intracellular elevation of Ca\(^{2+}\) in astrocytes is primarily mediated by Ca\(^{2+}\) release from the ER through inositol 1,4,5-triphosphate (InsP\(_3\)) gated receptors. InsP\(_3\) is generated by activation of PLC by metabatropic G\(_q\)-protein coupled receptors. Store-operated Ca\(^{2+}\) entry (SOCE) replenishes depleted ER Ca\(^{2+}\) by permitting plasmalemmal Ca2+ influx through store-operated channels. ER Ca\(^{2+}\) is refilled by the ER Ca\(^{2+}\) ATPase SERCA. Mitochondria also contribute to cytosolic Ca\(^{2+}\) uptake and release. Intracellular Ca\(^{2+}\) is extruded by the plasmalemmal Ca\(^{2+}\) ATPase PMCA and through Na\(^+\)/Ca\(^{2+}\) exchange (NCX). Image reproduced from (Verkhratsky et al., 2012).
**Figure 3.** NCKX transcript distribution in the mouse CNS. Mammalian NCKX2 is enriched in deep cortical layers and the cerebellum, while NCKX3 exhibits strong expression in the hippocampus and thalamic nuclei. NCKX4 is more uniformly distributed, but is enriched in the olfactory bulb, the hippocampus, and the cerebellum. Image reproduced from (Lytton et al., 2002).
**Figure 4.** Subtypes of glial cells in the *Drosophila* CNS. Glia in the fly are grouped into three classes by morphology and topology. Surface glia (both perineurial and subperineurial) are sheet-like cells that surround the CNS and form the blood brain barrier. Cortex glia are lammeliform cells that extend fine membrane processes and encapsulate each individual neuronal soma in the cortex. Neuropil glia reside at the cortex/neuropil interface and extend their processes into the neuropil. Ensheathing glia stay on the surface of the neuropil and may interact with the tracheal system, while astrocyte-like glia pervade the neuropil and surround axons, dendrites, and synapses. Image reproduced from (Hartenstein, 2011).
References


CHAPTER 2

The NCKX ZYD is Required in Cortex Glia to Regulate Seizure Susceptibility in Drosophila

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Jan Melom performed the majority of the work described in this chapter.
Abstract

Glia exhibit large fluctuations in intracellular Ca\textsuperscript{2+} both spontaneously and in response to neuronal activity, although the influence of glial Ca\textsuperscript{2+} oscillations on neuronal signaling is not well understood. By screening for temperature-sensitive seizure mutations in Drosophila we identified the zydeco mutation, which disrupts a glial-specific K\textsuperscript{-}-dependent, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCKX/ZYD). NCKX proteins are high-capacity, low-affinity exchangers that utilize the electrochemical gradients of the cell to export intracellular Ca\textsuperscript{2+}. We found that zyd mutant animals exhibit increased susceptibility to seizures in response to several environmental stressors. Zyd mutant seizures are rescued by expression of a zyd transgene in cortex glia, the most abundant glial cell type in the CNS that closely associate with neuronal cell bodies. Knockdown of zyd by RNAi in cortex glia also induces temperature-sensitive seizures, indicating that zyd expression in glia is both necessary and sufficient to regulate seizure susceptibility. To determine the temporal requirement for ZYD in glia, we conditionally expressed a zyd transgene using a heat-shock inducible Gal4 driver. We found that adult specific expression of zyd is sufficient to rescue the neuronal seizure phenotype, supporting an acute rather than developmental role for zyd in the nervous system. We verified the importance of glial Ca\textsuperscript{2+} signaling in the Drosophila nervous system by ectopically expressing the heat sensitive dTRPA1 channel in glia, which at elevated temperature allows influx of cations, including Ca\textsuperscript{2+}. Activation of dTRPA1 in cortex glia causes rapid seizures in adult flies, indicating glial Ca\textsuperscript{2+} signaling is acutely required for nervous system function. Our
results indicate that the NCKX ZYD is required in cortex glia to acutely regulate neuronal seizure susceptibility.

Introduction

Epilepsy is defined by incapacitating episodes of hypersynchronous neuronal firing. Although dysregulation of neuronal ion channel function has been implicated in seizure pathogenesis in a subset of idiopathic epilepsy cases (Klassen et al., 2011), the initial cellular events that trigger a seizure are not well understood. Recently, increased glial Ca\textsuperscript{2+} activity has been associated with abnormal neuronal excitability in several mammalian models of epilepsy (Fellin et al., 2006; Tian et al., 2005), and cortical astrocytes were found to exhibit Ca\textsuperscript{2+} oscillations immediately preceding a focal seizure event (Gomez-Gonzalo et al., 2010). However, whether increased glial Ca\textsuperscript{2+} activity is a result of seizures, or is a direct cause of seizures, is unclear.

Glial Ca\textsuperscript{2+} oscillations arise spontaneously (Takata and Hirase, 2008) and in response to physiological neuronal activity (Wang et al., 2006). Most experimental studies of glial Ca\textsuperscript{2+} activity have been restricted to measurement of somatic Ca\textsuperscript{2+} due to the small volume and inaccessibility of fine glial processes to Ca\textsuperscript{2+}-sensitive dyes (Agulhon et al., 2008). However, recent studies using membrane-targeted Ca\textsuperscript{2+}-sensitive GFP (GCaMP) have shown that small, near-membrane Ca\textsuperscript{2+} oscillations arise in astrocytic processes and do not necessarily propagate to the cell body (Shigetomi et al., 2010). The distinction between global, somatic Ca\textsuperscript{2+} oscillations and small, near-membrane Ca\textsuperscript{2+} oscillations in glial processes is critical, and may explain differing results concerning the physiological importance of glial Ca\textsuperscript{2+}
signaling in the regulation of neuronal excitability (Agulhon et al., 2010; Fiacco et al., 2007; Petravicz et al., 2008).

*Drosophila* glia exhibit morphological similarity to their mammalian counterparts (Stork et al., 2012). Mammalian astrocytes occupy spatially segregated domains in the CNS, each encompassing several neuronal cell bodies, and thus appear positioned to regulate local neuronal signaling (Bushong et al., 2002; Halassa et al., 2007). Cortex glia in *Drosophila* exhibit a similar spatial segregation, with each glial cell ensheathing multiple neuronal soma (Pereanu et al., 2005). Multiple functions originally identified in mammalian glia are conserved in *Drosophila*, including regulation of axon pathfinding (Spindler et al., 2009), axonal insulation (Banerjee et al., 2006), synaptic pruning (Fuentes-Medel et al., 2009), and modulation of neurotransmission and behavior (Jackson and Haydon, 2008; Ng et al., 2011). Although numerous functional similarities between *Drosophila* and mammalian glia have been described, the dynamics of Ca\(^{2+}\) signaling and regulation in *Drosophila* CNS glia are largely unexplored.

Here we show that mutation of a *Drosophila* Na\(^+/Ca^{2+}\), K\(^+\) exchanger (NCKX/ZYD) predisposes animals to seizures in response to several environmental stressors. We find that ZYD is specifically required in cortex glia to regulate seizure susceptibility. In addition, we show that acute dysregulation of glial Ca\(^{2+}\) by ectopic dTRPA1 expression triggers rapid (second time-scale) neuronal seizures. Our findings indicate that impairment of glial Ca\(^{2+}\) regulation is sufficient to initiate a seizure in vivo.
Results

zyd encodes a glial-specific NCKX

We identified mutations in *zydeco* (*zyd*) in an unbiased screen for recessive temperature-sensitive (TS) seizure mutants on the X chromosome (Guan et al., 2005). Using deficiency mapping and positional cloning, we discovered that the *zyd*\(^1\) and *zyd*\(^2\) mutations disrupt the CG2893 locus, which encodes a NCKX (Figure 1A). We performed a non-complementation EMS mutagenesis screen with *zyd*\(^1\) to identify additional *zyd* alleles and identified the *zyd*\(^3\) allele, which also disrupts NCKX.

NCKX proteins are plasma membrane transporters that export intracellular Ca\(^{2+}\) by utilizing Na\(^+\) and K\(^+\) electrochemical gradients, and have a high transport capacity important for rapid reduction of elevated cytosolic Ca\(^{2+}\) (Altimimi and Schnetkamp, 2007). The *zyd*\(^2\) and *zyd*\(^3\) alleles are point mutations in a highly conserved region of NCKX containing residues essential for cation transport (Altimimi et al., 2010; Winkfein et al., 2003) (Figure 1B), while a third allele (*zyd*\(^1\)) creates an early stop codon that removes two of the eleven transmembrane domains. All three *zyd* alleles are homozygous adult viable and fertile.

Interestingly, the *zyd*\(^2\) mutant carries an amino acid change (Alanine-80-Valine) at the same residue found at a polymorphic site in the NCKX5 family member that regulates skin color (Lamason et al., 2005). NCKX5 is unlike other mammalian NCKX isoforms in that it is not expressed in the brain, but is present in skin cells, where it regulates melanosome maturation. The Alanine-111-Threonine variant of NCKX5 is found in European-American populations with lighter skin color.
and causes substantial reduction in exchanger activity (Ginger et al., 2008). Mutagenesis of Alanine 111 reduces human NCKX5-mediated Ca\(^{2+}\) flux by \(\sim 50\%\) in heterologous cells (Ginger et al., 2008; Winkfein et al., 2003). Mutagenesis of the Glycine 110, the residue disrupted in \(zyd^{3}\), reduced human NCKX2 function by \(\geq 90\%\) (Winkfein et al., 2003), indicating \(zyd^{2}\) and \(zyd^{3}\) are likely hypomorphic alleles of NCKX.

**zyd mutants are susceptible to stress induced seizures**

All three \(zyd\) mutants are susceptible to seizures triggered by several environmental stressors. We initially identified \(zyd\) in an EMS mutagenesis screen for recessive mutants that exhibit seizures at 38°C. We found that \(zyd\) mutant adult flies transferred to preheated vials and lowered into a 38°C water bath begin to seize after 20 to 30 seconds, whereas wild-type flies do not (Movie 1). Seizures in \(zyd\) mutants manifest as tonic, unpatterned wing and leg contractions that persist for the duration of exposure to the restrictive temperature. Upon return to room temperature, \(zyd\) mutants cease seizing and recover mobility in a matter of minutes. \(Zyd\) mutants are also susceptible to heat shock-induced seizures at the larval stage, consistent with the onset of \(zyd\) expression during mid-embryogenesis and continuing through adulthood (Winkfein et al., 2004). \(Zyd\) mutant 3\(^{rd}\) instar larvae placed on agarose plates preheated to 38°C exhibit spastic muscle contractions within 30 seconds that inhibit normal larval crawling behavior (Movie 2).

At room temperature, \(zyd\) mutants exhibit seizures and temporary paralysis in response to brief vortexing (known as bang-sensitivity), whereas wild-type
controls do not (Figure 2). Bang sensitivity has been identified in several other
_Drosophila_ mutants that exhibit neuronal hyperexcitability and seizures in response
to electrical stimulation (Pavlidis and Tanouye, 1995). We also observed that _zyd^1_
mutants exhibit robust seizures after a brief period of anesthesia on ice (Movie 3). Collectively, these results indicate that _zyd_ mutants are hyperexcitable and prone to
seizure activity triggered by a variety of environmental stimuli.

We recorded central pattern generator output from the neuromuscular
junction (NMJ) of 3rd instar larvae and found that _zyd_ mutants exhibit rapid, unpatterned firing at 38°C, whereas wild-type larvae retain motor neuron bursting
necessary for normal crawling behavior (Figure 3A, B). Cutting the innervating
motor neuron immediately blocks seizure activity recorded at _zyd_ NMJs, indicating
the point of seizure initiation is upstream of the neuromuscular synapse.

**zyd expression in cortex glia regulates seizure susceptibility**

The _Drosophila_ genome encodes two NCKX exchangers: the NCKX encoded by
CG2893 (hereafter referred to as ZYD) and NCKX30C, which is expressed in the CNS
and appears enriched in neurons and photoreceptors (Haug-Collet et al., 1999;
Webel et al., 2002). ZYD was shown to encode a functional NCKX by heterologous
expression of _zyd_ cDNA in cultured cells (Winkfein et al., 2004). _In situ_
hybridizations performed in whole mount embryos showed that _zyd_ mRNA is
exclusively expressed in the nervous system, and appeared present in a subset of
cells tentatively identified as glia (Winkfein et al., 2004). Expression profiling of glial
genes expressed during Drosophila embryogenesis also found that zyd is expressed in glia (Altenhein et al., 2006; Beckervordersandforth et al., 2008).

To determine whether the seizures in zyd are caused solely by mutation of CG2893, we used the UAS/Gal4 system to perform tissue-specific rescue of zyd mutants. We tested both pan-neuronal and pan-glial Gal4 drivers, and found that expression of a UAS-zyd transgene with the pan-glial repo-Gal4 driver fully rescues zyd\textsuperscript{1} mutant TS seizures (Figure 4A). Pan-neuronal expression with elav-Gal4 does not affect the onset or severity of TS zyd seizures (Figure 4A), indicating that ZYD is required in glia, and not neurons, to regulate neuronal excitability.

To determine whether ZYD expression in glia is sufficient to regulate neuronal excitability, we knocked down zyd by RNAi in neurons and glia (Figure 4B). Pan-glial expression of two unique RNAi hairpins targeting zyd produces TS seizures in 3\textsuperscript{rd} instar larvae and is semi-lethal in adults. Adult escapers expressing glial zyd RNAi are ataxic and die within several days, indicating that glial zyd expression is critical for nervous system function. Interestingly, expression of zyd RNAi with gcm-Gal4, a pan-glial driver expressed only during early stages of development (Hosoya et al., 1995), did not recapitulate the zyd\textsuperscript{1} mutant phenotype (Table 1), indicating that constitutive zyd knockdown in mature glia is required to induce seizure susceptibility. Neuronal knockdown of zyd did not cause any temperature-sensitive behavioral or electrophysiological phenotypes in larvae (Figure 4B) or adults. These results indicate that ZYD function in glia is necessary and sufficient to regulate seizure susceptibility.
**ZYD immunoreactivity labels cortex glial membranes in the CNS**

*Drosophila* glia exhibit distinct morphological subtypes that can be transgenically targeted with specific Gal4 drivers (Awasaki et al., 2008; Doherty et al., 2009), allowing determination of whether ZYD is required in a particular subpopulation of glia. We found that knockdown of *zyd* by RNAi using four unique Gal4 drivers that express in cortex glia caused strong temperature-sensitive seizures (Figure 4B, Table 1). We also tested for rescue of *zyd* mutant seizures, and found expression of UAS-*zyd* with several cortex glial Gal4 promoters was sufficient to fully suppress seizures in adult *zyd* mutants. Knockdown of *zyd* in other glial populations, including astrocyte-like glia, ensheathing glia, and surface glia, did not cause temperature-sensitive seizures or other obvious behavioral phenotypes (Table 1).

Cortex glia are highly lamellated cells that form a honeycombed network in the brain, encapsulating individual neuronal cell bodies (Hoyle et al., 1986; Pereanu et al., 2005). Cortex glia are involved in guidance of secondary axon tracts and maintenance of cortical structural integrity (Dumstrei et al., 2003; Spindler et al., 2009), but little is known about their function in the mature nervous system. To verify ZYD expression in cortex glia, we generated antisera to the protein. ZYD immunoreactivity uniformly labels cortex glial membranes in 3rd instar larvae, forming chambers that surround clusters of neuronal cell bodies (Figure 5A). As development proceeds through metamorphosis, cortex glia in the outer layers of the CNS subdivide these chambers to individually encapsulate the enclosed neurons (Pereanu et al., 2005). In the 3rd instar ventral nerve cord, ZYD staining closely
surrounds each neuronal cell body, as revealed by co-staining with antibodies against ELAV, a neuronal nuclei specific protein (Figure 5A). ZYD staining also closely colocalizes with pan-glial GFP expression in the CNS (Figure 6A). ZYD immunoreactivity is strongly reduced in both the central brain hemispheres and ventral nerve cord of zyd<sup>1</sup> mutants.

To verify the specificity of the RNAi hairpins targeting zyd, we examined ZYD expression in the CNS of larvae expressing either pan-neuronal, pan-glial, or cortex glial zyd RNAi (Figure 6B). Neuronal expression of zyd RNAi with elav-Gal4 does not reduce ZYD immunoreactivity, while both pan-glial and cortex glial zyd RNAi abolishes ZYD expression, indicating that ZYD is expressed exclusively on cortex glial membranes in the CNS.

**Acute rescue of zyd mutants reduces seizure susceptibility**

To discriminate between an acute versus developmental role for ZYD in the CNS, we conditionally expressed a UAS-zyd transgene in zyd<sup>1</sup> mutants using the hsp70-Gal4 driver (Figure 7). The conditional hsp70-Gal4 driver is not transcribed at room temperature, but is induced above 30°C, and is maximally expressed at 36-37°C (Brand et al., 1994; Lindquist, 1986). zyd<sup>1</sup> mutants were raised at 22°C from embryogenesis to adulthood, and then subjected to a brief 37°C heat pulse to induce expression of UAS-zyd. Conditional UAS-zyd transgene expression rescued zyd<sup>1</sup> temperature-sensitive seizures in adults six hours after the heat pulse, consistent with the time course of induced hsp70-Gal4 activity measured after a similar heat treatment (Lindquist, 1986). Susceptibility to temperature-sensitive seizures
returned in the conditionally rescued zyd\textsuperscript{1} flies several days after the heat pulse, likely reflecting the rate of ZYD protein turnover. Genetically identical control animals not subjected to a brief heat pulse exhibited TS seizures at all timepoints tested (Figure 7, blue bars). These data indicate that restoration of ZYD function in the mature nervous system reduces neuronal excitability, and suggests that seizures arising in zyd\textsuperscript{1} mutants are unlikely to be the result of neuronal miswiring or other developmental defects.

**Acute cortex glial Ca\textsuperscript{2+} influx via ectopic TRPA1 activation triggers seizures**

We hypothesized that seizures may arise in zyd mutants due to either a constitutive lack of regulated glial Ca\textsuperscript{2+} signaling, or acute elevation of glial Ca\textsuperscript{2+} triggered by environmental stress. To discriminate between these two possibilities we investigated the requirement for acute glial Ca\textsuperscript{2+} regulation *in vivo* by ectopic expression of the heat-activated dTRPA1 cation channel. *Drosophila* TRPA1 is normally restricted to a small number of thermotactic neurons and is permeable to Ca\textsuperscript{2+} influx with moderate increases in temperature (Hamada et al., 2008; Rosenzweig et al., 2005) (Figure 8A). Previous studies have shown that ectopic expression of dTRPA1 in motor neurons elicits action potentials above 26°C and does not affect membrane properties at 22°C (Pulver et al., 2009), indicating dTRPA1 is a useful tool for acutely manipulating intracellular Ca\textsuperscript{2+}. We assessed the behavioral effect of activating dTRPA1 in cortex glia, and found adult flies exhibited strong, immobilizing seizures within seconds of a temperature shift to 30°C (Movie 4). These seizures were similar in onset and appearance to seizures observed in
animals expressing pan-neuronal dTRPA1 with *elav-Gal4*. Interestingly, pan-glial dTRPA1 activation with *repo-Gal4* causes immediate paralysis in adult flies, possibly due to impairment of blood brain barrier glia, which are required for axonal insulation and action potential conduction (Baumgartner et al., 1996).

We recorded central pattern generator activity in the muscle of larvae expressing ectopic dTRPA1 while applying a temperature ramp, and found that cortex glial dTRPA1 larvae exhibited increased motor neuron bursting at 27°C, followed by continuous seizure activity at 30°C (Figure 8B). Similar seizure progression was observed in pan-neuronal dTRPA1 expressing larvae. These results indicate that acute, *in vivo* disruption of Ca\(^{2+}\) regulation in cortex glia is sufficient to initiate neuronal seizure activity.

**Discussion**

Our results show that mutation of the cortex glial NCKX zyd predisposes animals to seizures in response to several environmental stressors. We found that acute expression of ZYD in the adult CNS rescues heat shock-induced seizures within hours, indicating ZYD dynamically modulates neuronal network excitability. We also found that acute, *in vivo* disruption of cortex glial Ca\(^{2+}\) regulation by ectopic dTRPA1 activation triggers seizure on a rapid (second) time scale. These results indicate that glial Ca\(^{2+}\) regulation by a novel NCKX mechanism is important for regulating neuronal excitability.

**The NCKX ZYD is required in cortex glia to regulate seizure susceptibility**
We isolated mutations in the NCKX zyd in a screen for temperature-sensitive behavioral phenotypes in *Drosophila*. This type of forward genetic screen has previously enabled identification of conditional alleles in genes required for nervous system function, including the voltage-gated Na⁺, K⁺, and Ca²⁺ channels (*para*, *shaw*, *cac*), proteins involved in synaptic vesicle exocytosis (*NSF*, *syntaxin*), and endocytosis (*dynamin*, *dap160*), among others. Surprisingly, we found that *zyd* is not expressed in neurons, but is localized to cortex glia, a large population of cells in the CNS that associate closely with neuronal cell bodies. Cortex glia ensheathe neuronal soma with extremely thin membrane projections and form a honey-combed network in the brain (Pereanu et al., 2005), yet the function of these cells in modulation or support of acute neuronal signaling is not understood.

*Zyd* has been shown to encode a functional K⁺-dependent Na⁺/Ca²⁺ exchanger (Winkfein et al., 2004), and is homologous to a family of vertebrate NCKX exchangers that have been well described for their role in regulating Ca²⁺ levels in rod photoreceptors (Schnetkamp, 2004) and cortical neurons (Li et al., 2006). In contrast to the slower, high affinity Ca²⁺ ATPases that are thought to fine tune resting Ca²⁺ concentration in the nM range, NCKX transporters rapidly extrude Ca²⁺ that reaches mM concentrations (Altimimi and Schnetkamp, 2007), and are activated during sharp peaks in intracellular Ca²⁺.

The mutations we isolated in *zyd* are likely hypomorphic rather than null alleles. Glial knockdown of *zyd* by RNAi is semi-lethal, and adult escapers are ataxic and die within several days, whereas the *zyd* mutants identified in our screen are adult viable and exhibit no obvious behavioral defects at room temperature. In
addition, mutation of the residues disrupted in zyd2 and zyd3 in human NCKX2 reduces Ca^{2+} exchange by 50-90%, suggesting zyd2 and zyd3 are NCKX hypomorphs (Winkfein et al., 2003).

The closest mammalian homologs to Drosophila ZYD are NCKX3 and NCKX4, which are expressed broadly in the CNS, but are relatively uncharacterized (Lytton et al., 2002). Recently, mRNA from NCKX3 was found to be highly expressed in acutely isolated Bergmann glial cells from the adult mouse cerebellum (Koirala and Corfas, 2010), and NCKX2 expression was identified in oligodendrocytes (Cahoy et al., 2008), indicating that mammalian NCKX exchangers may regulate Ca^{2+} in these glia as well.

**Disruption of cortex glial Ca^{2+} regulation acutely triggers seizures**

Although seizures and enhanced astrocytic Ca^{2+} activity have been concurrently observed in situ (Fellin et al., 2006; Fellin et al., 2004; Gomez-Gonzalo et al., 2010; Tashiro et al., 2002; Tian et al., 2005), a causative role for glia in seizure initiation has not been previously demonstrated. We found that acute cortex glial Ca^{2+} influx mediated by ectopic expression of dTRPA1 causes immediate (second time-scale) seizures in vivo. This result indicates that astrocytic dysfunction is sufficient to induce neuronal seizures without additional elevation of basal neuronal excitability, as has been previously suggested (Gomez-Gonzalo et al., 2010).

The requirement for regulated glial Ca^{2+} signaling in the mature CNS suggests that seizures in zyd mutants arise due to acute glial dysfunction. Consistent with this hypothesis, we found that acute expression of a UAS-zyd transgene in adult zyd1
mutants reduced seizure susceptibility within hours, indicating that restoration of ZYD function in the adult CNS is sufficient to restore normal neuronal excitability. The absolute time required between functional ZYD expression and reduced seizure susceptibility is unclear, as the heat shock-driven induction of ZYD first required translation of the Gal4 transcription factor. However, given that seizure rescue occurred in adult animals in less than six hours, seizures in zyd mutants are unlikely to be due to neuronal miswiring or other developmental defects. Since ZYD is not required in surface glial that constitute the blood-brain barrier, we can rule out a defect in ion balance in these glial subtypes. ZYD is also not required in ensheathing glia, that insulate axons, or neuropil glia that associate with synapses. We find that ZYD expression is restricted to cortical glia that interact specifically with neuronal cell bodies, forming glial subdivisions in the CNS which are reminiscent of the spatial segregation of mammalian astrocytes (Awasaki et al., 2008). As such, Ca\(^{2+}\) activity in cortex glia may normally modulate local excitability of small, interconnected neuronal networks.

**Methods**

**Homology searches and protein alignments**

NCBI BLAST (blastp) was used to identify NCKX homologs. Alignment and visualization was performed using CLC DNA Workbench 4.0 based on a progressive neighbor joining algorithm (Feng and Doolittle, 1987). The following GenBank accession numbers were used in this analysis: *Caenorhabditis elegans* ncx-4, CAB03047.2; *Drosophila melanogaster* ZYD, EAA46191.4; *D. mel* NCKX30C,
AAF52801; *Mus musculus* NCKX4, AA171939; *Homo sapiens* NCKX1, AAI43376; *H. sap* NCKX2, AAI43890; *H. sap* NCKX3, CAC36052.2; *H. sap* NCKX4, AAH69653; *H. sap* NCKX5, AAI13631.

**Drosophila genetics and molecular biology**

Flies were cultured on standard medium at 22°C. *zyd* mutants were generated by EMS mutagenesis and identified in a screen for temperature-sensitive behavioral phenotypes (Guan et al., 2005). *zyd* was mapped to the X heterochromatic region containing CG2893 by recombination and complementation analysis with deficiency chromosomes. Mutation of CG2893 in three independently generated *zyd* alleles was identified by sequencing and comparison to control (CS) genomic sequence. We used the UAS/Gal4 system to drive transgenes in glia (Brand et al., 1994). A list of *Drosophila* stocks used in this analysis can be found in Table 2. UAS-*zyd* was constructed by amplifying full-length *zydeco* cDNA with primers containing 5' EcoRI and 3' XhoI restriction enzyme sites. *zyd* cDNA was cloned into pUAST by double digestion and ligation with EcoRI and XhoI, and injected into *w*¹¹¹³ by Genetic Services, Inc. (Cambridge, MA).

PCR primers for mapping the *zyd*² and *zyd*³ point mutations (954 bp genomic product):

5' - TGGAACCATTGGCAGTATGA - 3'  
5' - TCCGACTAATGAGAAGTCGATTG - 3'

PCR primers for mapping the *zyd*¹ point mutation (586 bp genomic product):
5' - CGACGATTTCCAGAATTAACG - 3'
5' - CGCAGACCAATTTTCATGATC - 3'

PCR primers for cloning the zyd cDNA into pUAST (1608 bp product):
5' - CCGGAATTCATGGAAGACTATTGGGGTCT - 3'
5' - TCATGATCGACCGCAGGTGG - 3'

Behavioral assays

Bang sensitivity of adult female flies was assayed 2-3 days post-eclosion. Flies were transferred into empty vials and allowed to rest for 1-2 hours. Vials were vortexed at maximum speed for 10 seconds, and the number of flies that were upright and mobile was counted at 10 second intervals. For assaying temperature-sensitive phenotypes, 1-3 day old flies were transferred into preheated vials in a water bath held at the indicated temperature with a precision of 0.1°C. Flies of either sex were analyzed, except in the case of zyd¹ mutant UAS/Gal4 rescue animals, in which only males were used. Seizures were defined as the condition in which the animal lies incapacitated on its back or side with legs and wings contracting vigorously. Paralysis was defined as the condition in which animal fell to the bottom of the vial and exhibited no movement. For assaying seizures in larval animals, 3rd instar larvae of either sex were gently washed with PBS and transferred to 1% agarose plates heated to 38°C using a temperature-controlled stage. Larval seizures were defined as continuous, unpatterned contraction of the body wall muscles that prevented normal crawling behavior.
**Electrophysiology**

Current clamp recordings of wandering 3\textsuperscript{rd} instar larvae were performed in HL3.1 saline (in mM: 70 NaCl, 5 KCl, 4 MgCl\textsubscript{2}, 0.2 CaCl\textsubscript{2}, 10 NaHCO\textsubscript{3}, 5 Trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2) containing 1.5 mM Ca\textsuperscript{2+} using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) at muscle fiber 6/7 of segments A1-A4. Larvae were of either sex, except for recordings made in *zyd\textsuperscript{1}* mutant UAS/Gal4 rescue animals, which were always male. For recording the output of the central pattern generator, the CNS and motor neurons were left intact with the ventral nerve cord. Temperature was controlled with a Peltier heating device and continually monitored with a microprobe thermometer. Preparations were maintained at the indicated temperature for 2 minutes before recording.

**Immunostaining**

ZYD polyclonal antibodies were generated in rabbit with a synthetic zydeco peptide corresponding to residues 313-329 (DEGRKEEGYSSLSSYPKD) and affinity-purified by Yenzym Antibodies, LLC (San Francisco, CA). For ZYD immunostaining, dissected 3\textsuperscript{rd} instar larvae of either sex were fixed with 100% ice-cold methanol for 5 minutes. Antibodies were used at the following dilutions: rabbit anti-ZYD, 1:1000; rat anti-ELAV 7E8A10 (Developmental Studies Hybridoma Bank), 1:50; rat anti-GFP (Nacalai Tesque), 1:1000; Cy3- and Cy2- conjugated anti-rabbit and anti-rat (Jackson ImmunoResearch), 1:3000. Larvae were mounted in 70% glycerol in PBS.
and imaged on a Zeiss Pascal confocal microscope with Pascal software (Carl Zeiss MicroImaging, Inc.) with oil immersion 40X 1.3 NA and 63X 0.95 NA objectives.
Figures

A  ZYD / NCKX

Extracellular

Intracellular

zyd<sup>0</sup>
G79S
zyd<sup>2</sup>
A80T

B

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zyd<sup>0</sup>  zyd<sup>2</sup>
G79S   A80T

C

[Tree diagram with species names and distances]
Figure 1. zyd mutants are disrupted in NCKX. A) Three independently generated alleles of zyd encode point mutations in NCKX, a multi-transmembrane domain protein. zyd² and zyd³ are missense mutations in the first pore-lining domain of the exchanger (blue) while zyd¹ encodes an early stop codon. B) Alignment of the pore-lining domain of NCKX in flies, mice, and humans. zyd² and zyd³ mutations occur in highly conserved residues (blue = identical, green = similar residues). C) Phylogeny of NCKX homologs. Drosophila ZYD is most homologous to mammalian NCKX3 and 4. Bootstrap values (confidence indicator) are shown for each branch.
Figure 2. *zyd* mutants exhibit bang-sensitive seizures. Adult wild-type and *zyd* mutant flies were placed in empty vials and vortexed for 10 seconds at high speed at 22°C. Wild-type flies immediately begin crawling up the vial sides upon termination of the stimulus, whereas *zyd* mutants exhibit seizures and paralysis at the bottom of the vial. Flies were considered recovered if they were upright and moving off the vial bottom. *n* = 100 flies/genotype. Def = Genomic deletion spanning the *zyd* locus.
**Figure 3.** Representative voltage traces of spontaneous central pattern generator activity at larval 3rd instar muscle 6 at 22°C (A) and 38°C (B) in wild-type and *zyd*¹ mutants (*n* > 8 preparations/genotype).
Figure 4. *zyd* is required in cortex glia to regulate seizure susceptibility. **A)** Pan-glial *repo-Gal4* driven expression of a wild-type UAS-zyd transgene rescues temperature-sensitive seizures in *zyd*\(^1\) mutants, but pan-neuronal *elav-Gal4* expression does not. **B)** Knockdown of *zyd* by pan-neuronal RNAi does not affect central pattern generator activity compared to control at 38°C, but knockdown of *zyd* by pan-glial or cortex glial (*NP2222-Gal4*) driven RNAi causes rapid, seizure-like discharges in the larval muscle. \(n > 5\) preparations/genotype.
Figure 3

A

WT

zyd

B

WT

zyd

ZYD / ELAV

ZYD / ELAV
Figure 5. ZYD is expressed in cortex glia. A) One hemisphere of the CNS in WT and zyd<sup>1</sup> mutant larvae stained with antisera to ZYD (purple) and ELAV (green), which labels neuronal nuclei. ZYD immunoreactivity outlines large chambers in the outer cortex where glial ensheathment of secondary neurons has not yet occurred. Scale bar, 40 μm. B) Optical section within the ventral cortex of the ventral nerve cord in WT and zyd<sup>1</sup> mutant 3<sup>rd</sup> instar larvae. ZYD staining encapsulates individual neuronal soma. Scale bar, 20 μm.
Figure 3

(A) 

(B) 

elav-Gal4; UAS-zyd RNAi

repo-Gal4; UAS-zyd RNAi

NP2222-Gal4; UAS-zyd RNAi

ZYD / GFP

ZYD / ELAV
**Figure 6.** ZYD uniformly labels cortex glial membranes. **A)** One hemisphere of the 3rd instar CNS stained with ZYD antisera in larvae expressing pan-glial GFP. Scale bar, 40 μm. **B)** Optical section of the CNS in larvae expressing pan-neuronal (elav-Gal4), pan-glial (repo-Gal4), or cortex glial (NP2222-Gal4) UAS-zyd RNAi. Scale bar, 10 μm.
Flies exhibiting seizures (%)

Time after 37°C pulse (hours)

0 20 40 60 80 100

0 3 6 12 24 48 72

22°C

Hatching → Adulthood

37°C

zyd¹; hsp70-Gal4; UAS-zyd

Flies exhibiting seizures (%)

Time after 37°C pulse (hours)

0 3 6 12 24 48 72 120

No heat pulse

37°C 5 min heat pulse
Figure 7. Adult specific rescue of zyd<sup>1</sup> mutants by conditional transgene expression rapidly reduces seizure susceptibility. Expression of UAS-zyd was controlled with the heat-inducible hsp70-Gal4 driver that is not expressed at room temperature and is activated above 30°C. zyd<sup>1</sup> mutants were reared at 22°C until adulthood (2 days post-eclosion), then exposed to a brief 37°C heat pulse. Seizure susceptibility tested at 38°C was strongly reduced in zyd<sup>1</sup> mutants six hours after transgene expression with hsp70-Gal4 (red bars). Genetically identical controls not receiving a heat pulse exhibited seizures at all time points (blue bars). n = 30 flies/timepoint.
**Figure 8.** Acute Ca$^{2+}$ dysregulation in cortex glia causes seizures. **A)** The heat-activated dTRPA1 cation channel is closed at 22°C and open at temperatures ≥ 26°C, allowing Ca$^{2+}$ influx. **B)** Ectopic expression of dTRPA1 in neurons (with *elav*-Gal4) and in cortex glia (with *NP2222*-Gal4) triggers seizure-like output from the central pattern generator. Voltage traces were recorded from the 3$^{\text{rd}}$ instar larval muscle during application of a temperature ramp. Preparations were held at the indicated temperature for 2 minutes before recording. *n > 6* larvae per genotype.
Table 1. Use of a collection of glial subpopulation specific Gal4 drivers to rescue zyd<sup>1</sup> seizures at 38°C (with UAS-zyd), or to knockdown zyd and test for seizures (with UAS-zyd RNAi). Only Gal4 drivers with expression in cortex glia rescued adult zyd<sup>1</sup> seizures at 38°C and phenocopied zyd seizures by RNAi. NT = not tested. n > 50 animals/genotype.

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<td>\text{UAS-zyd RNAi}</td>
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<td>Vienna Drosophila RNAi Center (#40987, 40988)</td>
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<td>\text{UAS-zyd RNAi}</td>
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\textbf{Table 2.} \textit{Drosophila} stocks used in this analysis.
Movie Legends

Movie 1. *zyd* mutant adults exhibit temperature-sensitive seizures. Wild-type and *zyd* mutant adults were transferred into vials preheated in a 38°C water bath. Video speed is 3X real-time.

Movie 2. *zyd* mutant larvae exhibit temperature-sensitive seizures. Wild-type and *zyd* mutant 3rd instar larvae were transferred to agarose plates heated to 38°C. Video starts 30 seconds after larvae were transferred to the plate. Video speed is real-time.

Movie 3. *zyd* mutants exhibit seizures upon recovery from cold shock. Wild-type and *zyd* mutant adult flies were placed in pre-chilled vials on ice for 5 minutes, then allowed to recover at room temperature (start of video recording). Video speed is 2X real-time.

Movie 4. Acute dysregulation of cortex glial Ca²⁺ causes seizures. dTRPA1-mediated Ca²⁺ influx into cortex glia (with NP2222-Gal4) causes rapid onset seizures in adult flies, similar in time course to pan-neuronal dTRPA1 activation (with *elav*-Gal4). Pan-glial dTRPA1 expression (with *repo*-Gal4) causes paralysis. Video speed is 2X real-time.
References


CHAPTER 3

Microdomain Cortex Glial Calcium Oscillations Require ZYD and Modulate Excitability via Calmodulin-Dependent Signaling

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Jan Melom performed the majority of work described in this chapter. Dina Volfson, Eben Bein, and Nia Dasul-Jin contributed to the RNAi screen. Belgin Yalcin contributed to the cloning of UAS-\textit{myrGCaMP3}. 
Abstract

Astrocytes exhibit microdomain fluctuations of intracellular Ca\textsuperscript{2+}, yet the mechanism regulating Ca\textsuperscript{2+} oscillations and their functional relevance in the brain is not well understood. We have found that a glial-specific NCKX exchanger in *Drosophila* (ZYD) is required for microdomain fluctuations in glial Ca\textsuperscript{2+} that acutely regulate seizure susceptibility. We identified *zyd* in a screen for conditional temperature-sensitive seizure mutants, and found that ZYD is expressed in cortex glia, a population of neuronal cell body-associated glia. We investigated the contribution of ZYD to glial Ca\textsuperscript{2+} regulation by expressing myrGCaMP5, a membrane-targeted Ca\textsuperscript{2+}-sensitive GFP, in cortex glia of the CNS. We found that cortex glia in live, intact larvae exhibit highly dynamic and complex microdomain Ca\textsuperscript{2+} oscillations. Cortex glial Ca\textsuperscript{2+} fluctuations occur in small patches of membrane partially enwrapping a neuronal cell body. In *zyd* mutants, cortex glial Ca\textsuperscript{2+} oscillations are absent, while baseline GCaMP fluorescence throughout the cortex glial network is significantly elevated. To determine the mechanism by which altered glial Ca\textsuperscript{2+} regulation affects neuronal excitability, we performed an RNAi screen for suppression of *zyd* mutant seizures. We found that glial knockdown of calmodulin fully suppresses the temperature-sensitive seizures in *zyd*, indicating a Ca\textsuperscript{2+}/calmodulin dependent signaling pathway is involved in acute glial-neuronal communication. Intriguingly, we also found that glial-specific knockdown of several genes involved in neurotransmitter reception and vesicle trafficking suppress *zyd* mutant seizures, suggesting Ca\textsuperscript{2+}-dependent gliotransmission is required for seizure initiation. We are continuing to characterize additional genetic suppressors of *zyd* in
order to define the mechanism by which cortex glia acutely modulate neuronal seizure susceptibility.

**Introduction**

Although Ca$^{2+}$ oscillations in cultured astrocytes were first observed over two decades ago (Cornell-Bell et al., 1990), we still know relatively little about the complexity of glial Ca$^{2+}$ dynamics *in vivo*. Most studies have utilized fluorescent Ca$^{2+}$-sensitive dyes to monitor astrocytic Ca$^{2+}$ activity in the cell body, which is easier to visualize in tissue than fine cellular processes (Hirase et al., 2004; Nimmerjahn et al., 2004). However, development of more sensitive Ca$^{2+}$ indicators and optics has revealed Ca$^{2+}$ fluctuations in astrocytic processes that are restricted to several micrometers of membrane and rarely encompass the cell body (Di Castro et al., 2011; Marchaland et al., 2008; Panatier et al., 2011; Shigetomi et al., 2010). These fluctuations, termed "Ca$^{2+}$ microdomains," are likely also molecularly and functionally heterogeneous (reviewed in (Rusakov et al., 2011)).

Somatic glial Ca$^{2+}$ oscillations were initially found to occur in response to synaptic stimulation and glutamate release in neuron-astrocyte co-cultures (Charles et al., 1991; Cornell-Bell et al., 1990) and *in situ* (Dani et al., 1992). However, spontaneous (or neuronal activity-independent) glial Ca$^{2+}$ oscillations were also observed (Aguado et al., 2002; Parri et al., 2001), indicating somatic glial Ca$^{2+}$ oscillations may be functionally heterogeneous. Similarly, microdomain glial Ca$^{2+}$ oscillations have been found to be both neuronal activity-dependent (Di Castro et al., 2011; Panatier et al., 2011) and spontaneous (Shigetomi et al., 2010; Wang et al.,
One study found that different spatial subtypes of microdomain astrocytic calcium oscillations reflect different types of neuronal activity (Di Castro et al., 2011), suggesting the signals initiating glial calcium oscillations are diverse.

The molecular mechanisms regulating glial calcium oscillations appear to be similarly heterogeneous. While somatic glial calcium oscillations depend on IP$_3$ generation and ER calcium store release (Petravicz et al., 2008), microdomain calcium oscillations appear to require plasmalemmal rather than intracellular calcium flux (Di Castro et al., 2011; Shigetomi et al., 2010). Microdomain calcium oscillations are also not activated by G$_q$ GPCR agonists commonly used to elevate somatic glial calcium (Shigetomi et al., 2010). The distinction between compartmentalized versus global calcium oscillations in glia is likely critical. Multiple groups have found that glial calcium activity robustly regulates aspects of synaptic transmission and neuronal excitability (reviewed in (Halassa and Haydon, 2010)). However, several studies have also reported that genetic manipulations that either ablate (Petravicz et al., 2008) or enhance (Fiacco et al., 2007) somatic calcium oscillations have no effect on neuronal physiology. This apparent discrepancy may be due to an incomplete understanding of microdomain calcium activity and the diverse molecular mechanisms that regulate intracellular glial calcium (Rusakov et al., 2011). Given that astrocytic calcium signaling appear to be involved in several neurological disorders, including epilepsy (Carmignoto and Haydon, 2012), a better understanding of how glial calcium activity is regulated in vivo is essential.

Here we show that mutation of the Drosophila glial-specific NCKX ZYD eliminates microdomain glial calcium oscillations that occur rapidly and frequently in
wild-type cortex glia in vivo. We identified multiple genes involved in vesicle trafficking and neurotransmitter reception that appear to be required in glia to trigger seizures in zyd mutants. Our findings demonstrate that microdomain Ca$^{2+}$ oscillations acutely regulate seizure susceptibility, and indicate that a Ca$^{2+}$/calmodulin-dependent glial derived signal is sufficient to initiate seizure activity in the brain.

**Results**

**Dynamic microdomain Ca$^{2+}$ oscillations occur in cortex glia in vivo**

Cortex glia pervade the *Drosophila* CNS, forming a network around neuronal cell bodies and secondary axon tracts (Pereanu et al., 2005; Spindler et al., 2009). Fine, lamelliform cortex glial processes encapsulate individual neurons and average less than 100 nm in thickness (Pereanu et al., 2005). We initially attempted to monitor Ca$^{2+}$ in cortex glia with GCaMP3, a genetically encoded Ca$^{2+}$-sensitive variant of GFP (Nakai et al., 2001). GCaMP3 has been used in *Drosophila* to observe Ca$^{2+}$ transients in neurons evoked by sensory stimulation (Tian et al., 2009). However, we did not observe fluctuations in the fluorescence of GCaMP3 expressed in CNS glia (with repo-Gal4) in either intact or filleted 3rd instar larval preparations. In addition, GCaMP3 signal was faint and difficult to detect in cortex glial processes surrounding neuronal soma.

To enhance the effective concentration of GCaMP in cortex glial processes, we generated a membrane-targeted variant of GCaMP3 by fusing the myristoylation signal from *Drosophila* Src64b to the N-terminus of GCaMP3 (myrGCaMP3). We
found that myrGCaMP3 was targeted to the plasma membrane and labeled the surface of glia to a much greater extent than soluble GCaMP3 (Figure 1). During our initial analysis, an improved variant of GCaMP (GCaMP5) became available that exhibited a higher signal-to-noise ratio over its predecessor (Akerboom et al., 2012). We therefore generated UAS-myrGCaMP5 to use for analysis of near-membrane glial Ca\textsuperscript{2+} activity.

Glial expression of myrGCaMP5 revealed small, rapid Ca\textsuperscript{2+} oscillations present in cortex glia in the ventral nerve cord in live, undissected larvae (Figure 2, Movie 5). We analyzed cortex glial Ca\textsuperscript{2+} oscillations with repo-Gal4 because this driver produced the highest expression of UAS-myrGCaMP5. Cortex glia were easily identified by morphology, as they are the only glial cells with processes ensheathing neurons in the ventral nerve cord cortex. Expression of UAS-myrGCaMP5 with the cortex glial specific drivers NP2222-Gal4 and NP577-Gal4 revealed similar Ca\textsuperscript{2+} oscillatory activity.

Cortex glial Ca\textsuperscript{2+} transients arise in small microdomains approximately 4.35 ± 0.26 µm\textsuperscript{2} (n = 40) and appeared to recur frequently in the same region (Figure 2A, B), suggesting the presence of specialized structural or functional glial subdomains. The duration of each glial Ca\textsuperscript{2+} transient was 1.35 ± 0.08 seconds and exhibited a mean ΔF/F\text{avg} of 35.4 ± 2.7% (n = 40). Ca\textsuperscript{2+} transients generally arose within a small part of the cortex glial sheath surrounding a single neuronal soma (Figure 2C, D), although occasional Ca\textsuperscript{2+} waves appeared to pass through glial membrane encompassing multiple neurons. As a single cortex glial cell of the early larval
ventral nerve cord encompasses dozens of neurons (Ito et al., 1995), it is unclear whether the observed Ca\(^{2+}\) waves pass between neighboring glia.

We found that standard fillet dissection of repo-Gal4; UAS-myrGCaMP5 larvae in external salines commonly used for electrophysiological recording (HL3.1, Jan & Jan) rapidly reduced the frequency of glial Ca\(^{2+}\) transients. Cortex glial Ca\(^{2+}\) transients were consistently eliminated within ten to twenty minutes of dissection despite extreme care taken to prevent damage to the CNS. Therefore, to ensure glial Ca\(^{2+}\) signals we recorded were reflective of non-pathological, in vivo activity, all imaging experiments were performed in live, undissected larvae gently pressed under a coverslip to reduce movement.

**Microdomain Ca\(^{2+}\) oscillations are eliminated in zyd mutants**

Microdomain cortex glial Ca\(^{2+}\) transients are completely absent in zyd\(^{1}\) mutants (Figure 3C, Movie 6). We found that zyd\(^{1}\) mutants fail to exhibit microdomain Ca\(^{2+}\) oscillations at either room temperature or 38°C (n = 10 larvae), while wild-type controls exhibit frequent and dynamic microdomain Ca\(^{2+}\) fluctuations under both conditions. No apparent fluctuations in myrGCaMP5 fluorescence were observed in zyd\(^{1}\) mutants during 38°C-induced seizure activity, which manifested as unpatterned muscle contractions in the immobilized larvae.

Interestingly, we observed large Ca\(^{2+}\) oscillations in glia ensheathing larval motor axons in the peripheral nervous system. Ensheathing glia are a separate glial subtype from cortex glia, and are thought to be analogous to vertebrate non-myelinating Schwann cell glia (Freeman and Doherty, 2006). Based on our genetic
rescue and immunostaining data, zyd does not appear to be expressed in ensheathing glia. We found that both wild-type and zyd	extsuperscript{i} mutants exhibit similarly large Ca\textsuperscript{2+} oscillations in ensheathing glia (Movie 7), indicating that Ca\textsuperscript{2+} activity in other glial subtypes is unaffected in zyd mutants.

**Basal Ca\textsuperscript{2+} is persistently elevated in zyd mutant cortex glia**

In addition to lacking microdomain Ca\textsuperscript{2+} oscillations, we found that zyd	extsuperscript{i} mutants exhibit persistently elevated intracellular Ca\textsuperscript{2+} throughout the cortex glial network. Basal myrGCaMP5 fluorescence in cortex glia of zyd	extsuperscript{i} mutants was more than 1.5 fold greater in intensity relative to wild-type controls (Figure 3A). At the restrictive temperature of 38°C, intracellular Ca\textsuperscript{2+} remains significantly elevated in zyd	extsuperscript{i} cortex glia relative to wild-type glia (Figure 3B). Interestingly, we observed that myrGCaMP5 fluorescence increased in both wild-type and zyd	extsuperscript{i} mutant glia with increasing temperature (Figure 3B). Elevation of resting intracellular Ca\textsuperscript{2+} in response to hyperthermia has been observed previously in *Drosophila* nerve terminals, and is thought to be due to reduction in the availability of ATP, which reduces ATP-dependent Ca\textsuperscript{2+} clearance (Klose et al., 2009).

**Disruption of Ca\textsuperscript{2+} oscillations in zyd mutant LBD cells**

We also identified large, cytosolic Ca\textsuperscript{2+} oscillations in several cells of the peripheral nervous system known as lateral bipolar dendritic (LBD) cells. LBD cells are located on the posterior side of muscle 8 in the larval hemisegments T3-A3, and extend long processes that associate with the transverse nerve (Figure
The nature of the LBD cell is unknown, as it expresses both the glial-specific transcription factor *repo* (Halter et al., 1995) as well as the neuron-specific factor *elav*. LBD cells contain numerous vesicles and may have a neurosecretory function (Hoshino et al., 1999). We observed that LBD cells in wild-type larvae exhibit large, periodic oscillations of cytosolic GCaMP3 fluorescence (Figure 4B)(Movie 8). Unlike cortex glia of the CNS, Ca$^{2+}$ oscillations in LBD cells persist for up to 30 minutes after larval dissection and occur simultaneously in the cell body and cellular processes. Ca$^{2+}$ oscillations in LBD cells can also be detected with soluble GCaMP3 as well as the membrane-tethered myrGCaMP variant, indicating Ca$^{2+}$ elevation occurs throughout the cell cytosol. We found that somatic Ca$^{2+}$ oscillations in LBD cells require physiological concentrations of external Ca$^{2+}$ (Figure 4C), indicating that oscillations involve plasma membrane Ca$^{2+}$ flux.

ZYD immunoreactivity is present in LBD cells and reduced in *zyd*$^1$ mutants (Figure 5), suggesting ZYD contributes to LBD intracellular Ca$^{2+}$ regulation. We examined Ca$^{2+}$ oscillations in *zyd*$^1$ mutant and *zyd* RNAi expressing larval LBD cell bodies (Figure 4D) and found that both exhibit a reduction in Ca$^{2+}$ oscillation frequency (Figure 4E). Both *zyd*$^1$ mutant and *zyd* RNAi expressing LBD cells also exhibited an increase in Ca$^{2+}$ peak amplitude ($\Delta F/F_{\text{avg}}$) relative to wild-type LBD cells at 22°C (Figure 4F). We found that in the dissected 3rd instar larvae, GCaMP3 fluorescence diminished considerably in control LBD cells held at 38°C, which prevented analysis of LBD Ca$^{2+}$ oscillations in *zyd*$^1$ mutants at the seizure-inducing temperature.
Given that Ca\textsuperscript{2+} oscillations in LBD cells exhibit a high ΔF/F\textsubscript{avg} and persist in the dissected larvae, we chose to determine the effect of ectopic dTRPA1 expression on LBD cell Ca\textsuperscript{2+} oscillations. We previously observed that dTRPA1-mediated Ca\textsuperscript{2+} influx in cortex glia induced rapid seizures in adult flies. We found that dTRPA1 activation at 30°C robustly altered Ca\textsuperscript{2+} oscillatory behavior in LBD cells (Figure 6A, B), indicating dTRPA1 expression influences endogenous Ca\textsuperscript{2+} dynamics. dTRPA1-mediated Ca\textsuperscript{2+} influx increased the frequency of GCaMP3 fluorescence oscillations (Figure 6C) while reducing the total change in GCaMP3 fluorescence over time (Figure 6D). The peak amplitude of Ca\textsuperscript{2+} oscillations (ΔF/F\textsubscript{avg}) was also reduced in dTRPA1 LBD cells at 30°C (Figure 6E). Ca\textsuperscript{2+} oscillations in control and dTRPA1 expressing LBD cells were identical at 22°C, the condition in which the dTRPA1 channel is closed. These results indicate that activation of dTRPA1 at 30°C is sufficient to alter endogenous Ca\textsuperscript{2+} activity.

A glial-specific RNAi screen to identify suppressors of zyd mutant seizures

The \textit{in vivo} Ca\textsuperscript{2+} imaging data indicate that endogenous Ca\textsuperscript{2+} oscillatory activity and basal Ca\textsuperscript{2+} regulation are disrupted in zyd mutants. To probe the mechanism by which altered glial Ca\textsuperscript{2+} regulation in zyd mutants affects neuronal excitability, we performed an RNAi screen for suppression of zyd\textsuperscript{1} mutant temperature-sensitive (38°C) seizures. We utilized the UAS/Gal4 system to express RNAi hairpins to candidate genes in zyd\textsuperscript{1} mutant glia in order to identify gene products required in the glial Ca\textsuperscript{2+}-dependent signaling pathway that triggers seizures. We knocked down 269 candidate genes in zyd\textsuperscript{1} glia that are implicated in
vesicular trafficking and regulation, given recent evidence that Ca\textsuperscript{2+}-dependent exocytosis in mammalian glia alters neuronal excitability (Parpura and Zorec, 2010). We targeted a further 258 genes that have been found to be enriched in \textit{Drosophila} glia and were not selected for on the basis of molecular function (Altenhein et al., 2006; Egger et al., 2002; Freeman et al., 2003). Finally, we tested 295 genes encoding membrane receptors and secreted signaling ligands in an effort to identify essential components of the glial-neuronal signaling pathway disrupted in \textit{zyd}.

\textbf{Glial calmodulin is required for seizure initiation in \textit{zyd} mutants}

In our initial screen, we found that pan-glial knockdown of \textit{calmodulin} (\textit{cam}) fully suppresses temperature-sensitive seizures in both \textit{zyd\textsuperscript{1}} mutant larvae and adults (Figure 7A, C). Calmodulin is an essential Ca\textsuperscript{2+}-binding protein that regulates multiple Ca\textsuperscript{2+}-dependent cellular processes (Wang et al., 2003) and is expressed in \textit{Drosophila} glia (Altenhein et al., 2006), although its role in glia is unknown. We verified that expression of \textit{cam} RNAi reduces calmodulin protein by Western blot using the eye specific driver \textit{gmr-Gal4} (Figure 7B). Knockdown of \textit{cam} with \textit{repo-Gal4} did not significantly reduce total calmodulin brain protein, likely due to high expression of calmodulin in the \textit{Drosophila} compound eye and neuronal tissue (Chintapalli et al., 2007).

Given that calmodulin is required for seizures in \textit{zyd} mutants, we asked whether knockdown of calmodulin could suppress paralysis observed in animals expressing ectopic glial dTRPA1. We observed that acute, pan-glial Ca\textsuperscript{2+} influx via dTRPA1 causes immediate paralysis in adult flies (Movie 4) that persists until
animals are returned to room temperature. Concomitant expression of UAS-cam RNAi significantly lengthens the time required to induce paralysis in glial dTRPA1 adults at 30°C, and the majority of flies were found to recover from paralysis during incubation at the restrictive temperature (Figure 7D). We also observed that pan-glial dTRPA1 flies expressing cam RNAi exhibited wild-type activity levels immediately upon return to room temperature, whereas control dTRPA1 flies remained immobilized for several minutes after a 3 minute exposure to 30°C. The suppression of glial dTRPA1-induced paralysis was not due to a titration of available Gal4, as expression of other UAS-RNAi hairpins did not produce a similar rescue effect. The strong suppression of glial dTRPA1-induced paralysis by cam knockdown indicates that cam plays a central role in mediating a glial Ca^{2+}-dependent process that acutely affects nervous system function.

Knockdown of genes involved in vesicle trafficking and neurotransmitter reception suppresses zyd seizures

Multiple genes involved in vesicular trafficking and neurotransmitter signaling altered the seizure susceptibility of zyd^i mutants when knocked down in glia. Of the 822 candidate genes we tested, 18 genes (2.19%) fully eliminated seizures in zyd^i mutants, 14 genes (1.70%) reduced the severity or penetrance of seizures, and 4 genes (0.49%) were synthetic lethal (Table 1). Several RNAi-mediated rescue phenotypes were verified with additional unique RNAi hairpins, although for most genes, seizure suppression was observed with a single hairpin.
Multiple genes that suppressed *zyd*\(^1\) seizures when knocked down in glia are directly involved in Ca\(^{2+}\)-dependent exocytosis, such as *syt4* and *caps*. Syt4 is a SNARE-binding Ca\(^{2+}\) sensor involved in vesicular release at synapses (Barber et al., 2009) that has also been implicated in glutamate release from mammalian astrocytes (Zhang et al., 2004). *Drosophila caps* (Ca\(^{2+}\)-activated protein for secretion) plays an essential role in dense core vesicle exocytosis from neuroendocrine cells (Renden et al., 2001). Dense core vesicles have been observed to undergo Ca\(^{2+}\)-dependent membrane fusion in cultured mammalian astrocytes (Prada et al., 2011), causing secretion of neuropeptide Y (NPY), a regulator of synaptic transmission (Ramamoorthy and Whim, 2008). Interestingly, we found that glial knockdown of *dNPFR1*, the *Drosophila* homolog of the NPY receptor, also reduced seizures in *zyd*\(^1\) mutants (Table 2). These findings suggest that Ca\(^{2+}\)-dependent gliotransmission is likely an important component of *zyd* mutant seizures.

We also found that glial-specific knockdown of metabatropic GABA\(_B\) receptors (GABA\(_B\)R) eliminated seizures in *zyd*\(^1\) adults. Three genes encode GABA\(_B\)Rs in *Drosophila*. GABA\(_B\)R1 and GABA\(_B\)R2 are obligate heterodimers that activate G\(_{ai}\) and the inwardly rectifying K\(^+\) channel GIRK, while GABA\(_B\)R3 appears to be an insect-specific GABA\(_B\)R variant with no known ligand or functional interaction (Mezler et al., 2001). We found that knockdown of either GABA\(_B\)R1 or 3 in glia eliminated seizures in *zyd*\(^1\) mutants, while knockdown of GABA\(_B\)R2 strongly reduced seizure severity, indicating that glial detection of GABA is required for *zyd*\(^1\) seizures. Interestingly, gain-of-function mutations and pharmacological activation of
mammalian GABA\(_B\)Rs have been linked to temporal lobe epilepsy and absence seizures (Hosford et al., 1992; Motalli et al., 1999; Salzmann et al., 2005). Mammalian GABA\(_B\)R activation has also been shown to elevate Ca\(^{2+}\) in hippocampal astrocytes (Serrano et al., 2006). These results suggest that GABAergic activation of *Drosophila* cortex glia is required for *zyd*\(^1\) seizures, and that blocking glial GABA detection may prevent glial Ca\(^{2+}\) influx triggering neuronal hyperactivity. However, additional genetic and molecular analysis is required to confirm these interactions, and to fully understand the glial-neuronal signaling pathway dysregulated in *zyd* mutants.

### SERCA and SOCE are essential components of glial Ca\(^{2+}\) regulation

While screening for genetic suppression of *zyd*\(^1\) seizures, we found that *zyd*\(^1\) mutants are synthetic lethal in combination with cortex glial (*NP2222-Gal4*) expression of *ca-p60a* RNAi (Table 2). *ca-p60a* encodes the only *Drosophila* SERCA homolog, a Ca\(^{2+}\)-dependent ATPase that pumps Ca\(^{2+}\) from the cytosol into the endoplasmic reticulum. Knockdown of SERCA has been shown to raise intracellular cytosolic Ca\(^{2+}\) in *Drosophila* S2 cells (Zhang et al., 2006), which in combination with constitutively elevated Ca\(^{2+}\) in *zyd*\(^1\) glia triggers lethality. Knockdown of *ca-p60a* in cortex glia in otherwise wild-type animals does not cause lethality (Table 2).

SERCA knockdown has also been shown to reduce Ca\(^{2+}\) induced Ca\(^{2+}\) release from intracellular stores (CICR) and store-operated Ca\(^{2+}\) entry (SOCE) (Zhang et al., 2006). Both CICR and SOCE have been identified as mechanisms of Ca\(^{2+}\) entry into mammalian astrocytes (Malarkey et al., 2008; Singaravelu et al., 2006; Verkhratsky
et al., 1998). To test whether SOCE is involved in *Drosophila* glial Ca\(^{2+}\) regulation, we expressed RNAi to the top ten genes identified in a *Drosophila* genome-wide screen for involvement in SOCE (Zhang et al., 2006). Surprisingly, pan-glial knockdown of six of the ten genes caused adult lethality, including knockdown of Orai, the plasma membrane channel gating store-dependent Ca\(^{2+}\) entry (Table 2). Knockdown of five of the top ten SOCE genes specifically in cortex glia also resulted in adult lethality. The critical requirement for multiple components of SOCE in *Drosophila* glia indicates that glial Ca\(^{2+}\) regulation also requires intracellular store Ca\(^{2+}\), and is essential for nervous system function.

**Discussion**

These findings demonstrate that dynamic microdomain Ca\(^{2+}\) oscillations occur in cortex glia *in vivo*, and that ZYD is required for glial Ca\(^{2+}\) oscillatory activity. We imaged glia expressing membrane-tethered GCaMP5 in live, unanesthetized animals and found that microdomain Ca\(^{2+}\) fluctuations arose in discrete domains encompassing neuronal cell bodies. Glial Ca\(^{2+}\) oscillations appeared to recur in the same region over time and were extremely rapid (averaging 1.3 seconds in duration). Mutation of *zyd* eliminated microdomain Ca\(^{2+}\) fluctuations and also increased basal Ca\(^{2+}\) within the cortex glial network.

We screened for suppressors of *zyd* seizures with glial-specific knockdown of candidate genes by RNAi, and found that calmodulin is required in glia to trigger neuronal seizures. We also identified genes involved in vesicle trafficking and GABA reception that are required in glia to initiate seizures in *zyd* mutants. Previous
studies have generated conflicting results regarding the importance of astrocytic Ca$^{2+}$ signaling to neuronal physiology (Agulhon et al., 2010; Fiacco et al., 2007; Gomez-Gonzalo et al., 2010; Henneberger et al., 2010; Petravicz et al., 2008). Our data demonstrate \textit{in vivo} that endogenous Ca$^{2+}$ oscillations in glia acutely influence neuronal function, and indicate a glial-neuronal signaling pathway is involved in the pathophysiology of seizures in \textit{Drosophila}.

**Dysregulation of cortical glia Ca$^{2+}$ promotes seizures**

Seizures in \textit{zyd} mutants are triggered by temperature shifts (both high and low) and mechanical stimulation. How does disruption of ZYD predispose animals to seizures? We found that \textit{zyd}$^1$ mutant glia lack dynamic microdomain Ca$^{2+}$ oscillations that occur frequently and rapidly in wild-type glia \textit{in vivo}. We also observed that \textit{zyd}$^1$ mutant glia exhibit constitutively elevated intracellular Ca$^{2+}$ at room temperature and the seizure-inducing temperature of 38°C. One possibility is that \textit{zyd} mutants may be susceptible to seizures due to constitutive loss of a Ca$^{2+}$-dependent glial process that modulates baseline neuronal excitability. If microdomain Ca$^{2+}$ oscillations are critical for triggering a downstream glial signaling pathway in the correct temporal and spatial context, then loss or reduction of this process in \textit{zyd}$^1$ mutant glia may "prime" the nervous system for seizures. Environmental triggers such as hyperthermia have been shown to enhance endogenous neuronal activity in \textit{Drosophila} (Peng et al., 2007), possibly inducing runaway excitation in \textit{zyd} mutants.
Another possibility is that stressful environmental stimuli trigger seizures by acutely increasing intracellular glial Ca\(^{2+}\) and inappropriately activating a glial Ca\(^{2+}\)-dependent signaling pathway that initiates seizures. In support of this hypothesis, we found that hyperthermia causes a global increase in glial Ca\(^{2+}\) that is significantly elevated in zyd mutants relative to wild-type animals. This observation is consistent with previous studies on hyperthermia-induced Ca\(^{2+}\) elevation in *Drosophila* nerve terminals (Klose et al., 2009). Although zyd mutant glia exhibit elevated intracellular Ca\(^{2+}\) at room temperature, additional Ca\(^{2+}\) elevation at 38°C may push the cell past a threshold that triggers a seizure-inducing process. Consistent with this model is our observation that acute cortex glial Ca\(^{2+}\) influx mediated by ectopic expression of dTRPA1 causes immediate seizures in adult animals. The requirement for regulated glial Ca\(^{2+}\) signaling in the mature CNS suggests that seizures in zyd mutants arise due to acute glial dysfunction.

We also analyzed the contribution of ZYD to Ca\(^{2+}\) regulation in several large cells in the peripheral nervous system that exhibit high amplitude, somatic Ca\(^{2+}\) oscillations. Ca\(^{2+}\) oscillations in LBD cells are reduced in frequency and increased in amplitude in the zyd mutant, indicating disruption of ZYD impairs but does not eliminate somatic Ca\(^{2+}\) oscillations. Knockdown of zyd by RNAi in LBD cells impaired the frequency and amplitude of Ca\(^{2+}\) oscillations to a similar extent. Given that Ca\(^{2+}\) oscillations in LBD cells persist in the zyd mutant, other Ca\(^{2+}\) regulatory mechanisms may contribute to oscillatory activity in these cells.

**Evidence for the molecular mechanism underlying seizures in zyd mutants**
We performed an RNAi-based suppressor screen for genes expressed in glia that are required for seizures in zyd mutants. Of the candidate genes we tested, 36 genes (4.4%) appeared to modulate seizure severity in the zyd\textsuperscript{I} mutant. One of the genes that fully eliminated zyd seizures when knocked down in glia is \textit{calmodulin} (CaM). CaM acts as a Ca\textsuperscript{2+} sensor in many signaling pathways and modulates the activity of several Ca\textsuperscript{2+}-dependent enzymes (Sola et al., 2001). CaM is also predicted to have a limited spatial range of action (Saucerman and Bers, 2012), suggesting CaM may mediate a local signaling function in cortex glial Ca\textsuperscript{2+} microdomains. Interestingly, mammalian CaM is required for seizure induction elicited by a variety of convulsant agents (Sola et al., 2001), indicating that Ca\textsuperscript{2+}/CaM signaling is critically required to modulate neuronal excitability. Suppression of zyd\textsuperscript{I} seizures by \textit{cam} knockdown also suggests that disruption of Ca\textsuperscript{2+}, Na\textsuperscript{+}, or K\textsuperscript{+} ionic gradients due to mutation of NCKX is not sufficient to trigger seizures in the brain.

We identified multiple genes that suppress seizures in zyd\textsuperscript{I} mutants that are involved in vesicular exocytosis (\textit{syt4}, \textit{caps}), neurotransmitter signaling (\textit{gaba-b-r1-3}, \textit{nmdar2}), neuropeptide signaling (\textit{dnprfr1}, \textit{nep4}, among others) and intracellular Ca\textsuperscript{2+} regulation (\textit{ca-p60a}, \textit{ca-\textalpha1T}, \textit{pmca}). Collectively, these results suggest that multiple Ca\textsuperscript{2+}-dependent processes may become dysregulated in zyd\textsuperscript{I} mutants and contribute to seizures. In order to verify the involvement of these genes in glial Ca\textsuperscript{2+} signaling, further genetic and molecular analysis is required. Generation of double mutants between zyd\textsuperscript{I} and candidate genes will be important for understanding the molecular mechanism that triggers seizures when glial Ca\textsuperscript{2+} is dysregulated.
**Intracellular store Ca\(^{2+}\) regulation is required in glia for viability**

Knockdown of SERCA, an ATP-dependent Ca\(^{2+}\) pump that sequesters Ca\(^{2+}\) in the endoplasmic reticulum, is synergistically lethal in combination with *zyd*, indicating intracellular stores are involved in *Drosophila* glial Ca\(^{2+}\) regulation. The role of Ca\(^{2+}\) release from the ER is well documented in mammalian astrocytes, and SERCA has been shown to be required for cytosolic astrocytic Ca\(^{2+}\) oscillations and Ca\(^{2+}\)-dependent glial glutamate release (Parpura et al., 2011), although whether SERCA contributes to the shaping of microdomain Ca\(^{2+}\) transients is unclear. We also found that knockdown of genes involved in store-operated Ca\(^{2+}\) entry causes adult lethality in *Drosophila*. Intracellular store Ca\(^{2+}\) is sensed by the ER protein STIM, which translocates to the plasma membrane and triggers refilling of store Ca\(^{2+}\) through the ORAI channel. SOCE is present in mammalian glia, though its functional role is relatively uncharacterized (Parpura et al., 2011). Recently, mammalian STIM1 was found to be enriched in astrocytes and upregulated in a rat model of chronic epilepsy (Steinbeck et al., 2011), suggesting that perturbation of multiple modes of glial Ca\(^{2+}\) regulation affects neuronal excitability.

**A role for glia in seizure pathophysiology**

These data suggest a direct role for glia in seizure generation, indicating that at least some epileptic pathologies might have a glial rather than neuronal origin. Evidence for a non-neuronal induction of seizures has been suggested in several cases, including *in situ* studies correlating elevated astrocytic Ca\(^{2+}\) oscillations with seizure initiation (Gomez-Gonzalo et al., 2010; Tashiro et al., 2002) and *in vivo* work
demonstrating several anti-epileptic drugs reduce glial Ca\textsuperscript{2+} oscillations (Tian et al., 2005). The observation that astrocytic, Ca\textsuperscript{2+}-dependent glutamate release elicits synchronous currents in neighboring neurons suggests a possible mechanism by which glia may control the excitability of small neuronal circuits (Angulo et al., 2004; Fellin et al., 2004; Tian et al., 2005). However, synchronous inward neuronal currents produced by astrocytic Ca\textsuperscript{2+} signaling were found to be dispensable for the generation of epileptiform activity \textit{in vitro} (Fellin et al., 2006), suggesting glia may play more of a modulatory role in seizure activity. The identification of a glial-specific gene (\textit{zyd}) responsible for an epileptic phenotype suggests that at least in some cases, Ca\textsuperscript{2+} dysregulation in an astrocyte-like cell population is sufficient to initiate a seizure. Our genetic, electrophysiological and biochemical results suggest a model by which ZYD is required in cortex glia to regulate intracellular glial Ca\textsuperscript{2+} and glial modulation of neuronal activity (Figure 8).

In summary, these findings demonstrate a direct role for glial Ca\textsuperscript{2+} in regulating neuronal network excitability. Additional genetic screens for suppressors of the temperature-sensitive \textit{zyd} seizure phenotype should uncover how Ca\textsuperscript{2+} oscillations in glia signal to neighboring neurons, as well as generate new insights into the pathophysiology of epilepsy.

\section*{Methods}

\textbf{\textit{Drosophila} genetics and molecular biology}

Flies were cultured on standard medium at 22°C. We used the UAS/Gal4 system to drive transgenes in glia. UAS-\textit{myrGCaMP3} was constructed by subcloning
the first 90 amino acids of src64b, containing a myristoylation (myr) target sequence, into pBI-UASc (creating pBI-UASc-myr). The myr fragment of src64b was amplified from genomic DNA from transgenic UAS-myrRFP flies using primers containing 5’ EcoRI and 3’ BglIII restriction enzyme sites. GCaMP3 cDNA (AddGene plasmid 22692) was cloned into pBI-UASc-myr with primers containing 5’ NotI and 3’ XbaI restriction enzyme sites. UAS-myrGCaMP3 was injected using phiC31 site-directed transformation into the attP40 (2nd chromosome) landing site by Genetic Services, Inc. (Cambridge, MA). UAS-myrGCaMP5 was constructed by replacing the GCaMP3 sequence with GCaMP5 cDNA (Addgene plasmid 31788, courtesy of Loren Looger, Janelia Farms) using an identical strategy, and injected into the attP40 phiC31 site. GCaMP5 differs from GCaMP3 at three residues (T302L, R303P, and D380Y), which were confirmed by sequencing.

Primers used for construction of pBI-UASc-myr:

\[
\text{SRC64b 5’ EcoRI: } 5’ - \text{GAATTCA}TGGGAACAAATGCTGC - 3’ \\
\text{SRC64b 3’ BglIII: } 5’ - \text{AGATCT}ACCGTTGGTGGTGGTGC - 3’
\]

Primers used for construction of UAS-myrGCaMP3 and UAS-myrGCaMP5:

\[
\text{GCaMP 5’ NotI: } 5’ - \text{GCGGCCG}C\text{aATGATGGTTTCATCAT} - 3’ \\
\text{GCaMP 3’ XbaI: } 5’ - \text{TCTAGATTACTTCGTGCATCAT} - 3’
\]

**Immunostaining**
Dissected 3\textsuperscript{rd} instar larvae of either sex were fixed with 100% ice-cold methanol for 5 minutes. Antibodies were used at the following dilutions: rabbit anti-ZYD, 1:1000; rat anti-GFP (Nacalai Tesque), 1:1000; Cy3- and Cy2- conjugated anti-rabbit and anti-rat (Jackson ImmunoResearch), 1:3000. Larvae were mounted in 70\% glycerol in PBS and imaged on a Zeiss Pascal confocal microscope with Pascal software (Carl Zeiss MicrolImaging, Inc.) with oil immersion 40X 1.3 NA and 63X 0.95 NA objectives.

\textbf{Western blotting}

Western blotting of adult whole head lysates was performed using standard laboratory procedure. Nitrocellulose membranes were probed with rabbit anti-calmodulin clone EP799Y (Abgent), 1:500. Equal loading was assayed using anti-tubulin clone B-5-1-2 (Sigma-Aldrich), 1:60,000. Primaries were detected with Alexa Fluor 680-conjugated anti-rabbit and anti-mouse (Invitrogen). Western blots were visualized in an Odyssey infrared scanner (Li-Cor).

\textit{In vivo Ca\textsuperscript{2+} imaging}

For imaging cortex glia in the CNS, UAS-\textit{myrGCaMP5} was expressed in glia with \textit{repo-Gal4}. 2\textsuperscript{nd} instar male larvae were washed with PBS and placed on a glass slide with a small amount of Halocarbon oil #700 (Lab Scientific). Larvae were turned ventral side up and gently pressed with a coverslip and a small iron ring to inhibit movement. We acquired images with a PerkinElmer Ultraview Vox spinning disk confocal microscope and a high speed EMCCD camera at 8-12 Hz with a 40X 1.3
NA oil immersion objective, using Volocity software. We imaged at a single optical plane within the ventral cortex of the ventral nerve cord, in the dense cortical glial region immediately below the surface glial sheath. Average myrGCaMP5 signal in cortex glia was quantified in the central abdominal neuromeres of the VNC within a manually selected ROI excluding the midline glia. Ca$^{2+}$ oscillation frequency was quantified within the first minute of imaging at room temperature in a 2 mm$^2$ region of VNC cortex per larvae. The average area of a Ca$^{2+}$ oscillation was quantified during the peak of an oscillatory event ($\Delta F/F_0 \geq 10\%$). Temperature was controlled using a Tempcontrol 37-1 analog temperature regulated stage (Carl Zeiss Microscopy) and monitored with an Ultra Fine flexible microprobe attached to a BAT-12 thermometer (Physitemp Instruments, Inc.). Larvae were maintained at 38°C for 1 minute prior to imaging.

For imaging LBD cells of the peripheral nervous system, UAS-GCaMP3 was expressed with repo-Gal4. Wandering 3rd instar larvae were dissected according to standard technique and the CNS was removed. Preparations were bathed in HL3.1 Drosophila saline (in mM: 70 NaCl, 5 KCl, 4 MgCl$_2$, 0.2 CaCl$_2$, 10 NaHCO$_3$, 5 Trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2) containing 1.5 mM Ca$^{2+}$. Fluorescence within the LBD cell body was averaged in each image over a 1-min interval, and background fluorescence was subtracted. Peak amplitude (max $\Delta F/F_{avg}$), peak frequency, and total peak area were calculated with Igor Pro software (WaveMetrics) using a fitted baseline. For perfusion of Ca$^{2+}$ during imaging, HL3.1 saline with 0 mM Ca$^{2+}$ was replaced with 0.2 mM Ca$^{2+}$ containing HL3.1 by gravity perfusion.
Electrophysiology

Current clamp recordings of wandering 3\textsuperscript{rd} instar larvae were performed in HL3.1 saline containing 1.5 mM Ca\textsuperscript{2+} using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) at muscle fiber 6/7 of segments A1-A4. Larvae were of either sex, except for recordings made in zyd\textsuperscript{1} mutant UAS/Gal4 rescue animals, which were always male. For recording the output of the central pattern generator, the CNS and motor neurons were left intact with the ventral nerve cord. Temperature was controlled with a Peltier heating device and continually monitored with a microprobe thermometer. Preparations were maintained at the indicated temperature for 2 minutes before recording.
Figures

A

myristoylation M13 GFP (149-238) GFP (1-144) CaM

B repo-Gal4; UAS-GCaMP3

C repo-Gal4; UAS-myrGCaMP3
**Figure 1.** Expression of membrane-tethered GCaMP in *Drosophila* peripheral LBD cells. **A**) A myristoylation target sequence was fused to the N-terminus of GCaMP to generate myrGCaMP5. GCaMP consists of circularly permutated GFP fused to a domain of myosin light chain kinase (M13) and calmodulin (CaM). Upon binding Ca\(^{2+}\), GCaMP undergoes a conformational change and increases GFP fluorescence. **B**) Expression of soluble GCaMP3 in the lateral bipolar dendritic cell (LBD), a large peripheral glial cell in the 3\(^{rd}\) instar larvae. **C**) Expression of myrGCaMP3 in the LBD cell of a 3\(^{rd}\) instar larvae. Scale bar, 20 \(\mu m\).
**Figure 2.** Microdomain Ca$^{2+}$ transients occur in cortex glia *in vivo.* **A)** MyrGCaMP5 fluorescence in cortex glia in the wild-type larval ventral nerve cord. Scale bar, 10 μm. **B)** Regions of myrGCaMP5 fluorescence (ΔF/F$_{avg}$) labeled in (A) are shown for a 40 second interval. **C)** Time-lapse image series of a single cortex glial Ca$^{2+}$ oscillation at 25°C and 38°C in wild-type 2$^{nd}$ instar larvae. Scale bar, 4 μm. **D)** MyrGCaMP5 fluorescence (ΔF/F$_{avg}$) for the regions indicated in the first panels of (C).
A. 

WT

zyd

B. 

Cortex glial myrGCaMP5 fluorescence (a.u.)

WT zyd

WT zyd

25°C 38°C

C. 

Ca\textsuperscript{2+} transient frequency (Hz per mm\textsuperscript{2})

WT zyd

25°C
Figure 3. Microdomain Ca\textsuperscript{2+} oscillations in cortex glia require ZYD. A) Representative images of myrGCaMP5 fluorescence intensity in wild-type and zyd\textsuperscript{1} at 25°C. Scale bar, 20 μm. B) Average myrGCaMP5 fluorescence in cortex glia of wild-type and zyd\textsuperscript{1} at 25°C and 38°C. n = 10 larvae/condition. C) Frequency of microdomain Ca\textsuperscript{2+} transients in cortex glia for wild-type and zyd\textsuperscript{1} mutants at 25°C. n = 7 larvae/genotype. Error bars represent the SEM. t-test: ***p < 0.001, **p < 0.01.
A. Diagram showing the structure of a tissue with labeled cells.

B. Image showing a labeled cell with the notation "repo-Gal4; UAS-GCaMP3" and "HRP / GFP" in the background.

C. Graph showing the change in calcium concentration over time, with different conditions labeled as "0 mM Ca\(^{2+}\), 0.2 mM Ca\(^{2+}\), and 0 mM Ca\(^{2+}\).

D. Graph showing the oscillation frequency relative to WT, with different conditions labeled as "WT", "zyd\(^{1}\)", "UAS-zyd RNAi; repo-Gal4".

E. Bar graph showing the peak amplitude relative to WT, with different conditions labeled as "WT", "zyd\(^{1}\)", "UAS-zyd RNAi; repo-Gal4".

F. Bar graph showing the oscillation frequency relative to WT, with different conditions labeled as "WT", "zyd\(^{1}\)", "UAS-zyd RNAi; repo-Gal4".
Figure 4. Disruption of LBD cell Ca^{2+} oscillations in zyd\textsuperscript{i} mutants. A) Diagram of a dissected Drosophila larvae with repo-positive lateral bipolar dendritic (LBD) cells indicated. B) A LBD cell expressing GCaMP3 (green) stained with antisera to horse radish peroxidase (purple), which labels neuronal membranes. Scale bar, 5 μm. C) GCaMP3 fluorescence in an LBD cell with perfusion of 0.2 mM extracellular Ca^{2+}. D) Sample traces of GCaMP3 fluorescence in WT, zyd\textsuperscript{i}, and zyd RNAi expressing 3\textsuperscript{rd} instar larval LBD cells at 22°C. E and F) Ca^{2+} oscillations are less frequent (E) and larger in amplitude (F) in zyd\textsuperscript{i} and zyd RNAi animals (n > 20 cells/genotype). Statistical analysis by two-tailed student’s t-test, all data presented as mean ± s.e.m.
zyd\textsuperscript{1} / UAS-GFP

zyd\textsuperscript{1}; repo-Gal4; UAS-GFP
Figure 5. ZYD is expressed in LBD cells. Wild-type and zyd^{1} mutant LBD cells expressing GFP driven by repo-Gal4 (green) were stained with anti-ZYD antisera (purple). Scale bar, 20μm.
Fig. 4

A. dTRPA1

B. 

repo-Gal4; UAS-GCaMP3

30°C

repo-Gal4; UAS-GCaMP3; UAS-dTrpA1

C. Frequency (Hz)

P < 0.0001

22°C  30°C

D. Peak area (a.u.)

P < 0.0001

22°C  30°C

E. Peak ΔF/Fl (%) 

P < 0.0001

22°C  30°C
**Figure 6.** Ectopic expression of dTRPA1 alters somatic Ca²⁺ oscillations in LBD cells. 

**A)** Diagram of the heat-activated dTRPA1 cation channel closed (22°C) and open (≥26°C). 

**B)** Sample traces of GCaMP3 fluorescence in control (blue) and dTRPA1 expressing LBD cells (red) at 30°C. 

**C to E)** Ca²⁺ peaks in dTRPA1 expressing LBD cells are more frequent (C), have smaller total area (D), and smaller amplitude (E) relative to wild-type at 30°C. Ca²⁺ oscillations in WT and dTRPA1 LBD cells at 22°C are not significantly different. (n = 20 cells/genotype).
**Figure 7.** Knockdown of *cam* suppresses seizures in *zyd* mutants and paralysis due to ectopic glial TRPA1 activation. **A** Central pattern generator activity recorded in larval muscle in control *zyd* mutants and following pan-glial expression of *cam* RNAi in *zyd* mutants at 38°C. **B** Western blot of adult heads expressing *cam* RNAi in the eye with *gmr*-Gal4. Tubulin was used as loading control. **C** Quantification of seizure susceptibility tested at 38°C in adult *zyd* animals expressing pan-glial *cam* RNAi. *n = 100 flies/genotype*. **D** Time course of paralysis upon exposure to 30°C in adult flies expressing pan-glial dTRPA1 (blue line) and flies expressing pan-glial dTRPA1 and *cam* RNAi (red line). *n > 50 flies/genotype.*
**Figure 8.** Model of ZYD function in glial-neuronal Ca$^{2+}$-dependent signaling. Cortex glia (green) encapsulate neuronal cell bodies (blue) in the CNS. Our results support a model by which microdomains of Ca$^{2+}$ elevation in cortex glia trigger a Ca$^{2+}$/calmodulin-dependent signaling event in glia that alters neuronal activity. ZYD is required to reduce intracellular glial Ca$^{2+}$ by transporting Ca$^{2+}$ across the plasma membrane. Disruption of ZYD impairs Ca$^{2+}$ regulation in cortex glia and dysregulates glial modulation of neuronal activity.
### Tables

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<td>No seizures Ca(^{2+})-binding effector protein</td>
<td>TRiP #HMS01318</td>
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<td>syt4</td>
<td>No seizures Ca(^{2+}) sensor for exocytosis, SNARe binding</td>
<td>TRiP #JF02272</td>
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<td>ca- (\alpha)1T</td>
<td>No seizures Partial seizures T-type voltage gated Ca(^{2+}) channel</td>
<td>TRiP #JF02150 VDRC #31961 VDRC #31963</td>
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<td>No seizures Metabatropic GABA receptor, obligate heterodimer</td>
<td>BDSC #28353</td>
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<td>BDSC #26729</td>
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<td>hrs</td>
<td>No seizures Downregulation or activation of multiple RTK signaling pathways; partner of STAM</td>
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<td>No seizures Signal transducing adaptor molecule; partner of HRS</td>
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<td>No seizures Early endosome Rab GTPase</td>
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<td>ER Ca(^{2+}) ATPase</td>
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<td>Vacuolar H(^{+}) ATPase, calmodulin binding</td>
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Table 1. Suppressors and enhancers of zyd\(^{t}\) temperature-sensitive (38°C) seizures identified by pan-glial RNAi expression. UAS-RNAi hairpins targeting candidate genes were expressed in zyd\(^{t}\) mutants with co-expression of UAS-Dcr2 to enhance knockdown efficacy. Adult flies (3-4 days post eclosion) were placed in preheated vials in a 38°C waterbath, and their behavior was observed for 5 minutes. RNAi hairpin stock IDs producing suppression or enhancement are listed. "No seizures" = wild-type behavior, "Partial seizures" = Sporadic/weak seizures with intermittent recovery. BDSC = Bloomington Drosophila Stock Center, VDRC = Vienna Drosophila RNAi Center, TRiP = Transgenic RNAi Project (Harvard).
### Table 2

Knockdown of components of store-operated Ca\(^{2+}\) entry (SOCE) by RNAi in glia causes adult lethality. RNAi hairpins to multiple genes associated with SOCE (Zhang et al., 2006) cause lethality when expressed with pan glial (repo-Gal4) or cortex glial (NP2222-Gal4) drivers. Knockdown of SERCA in cortex glia is lethal in the zyd\(^i\) mutant background.

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<tr>
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<th>repo-Gal4; UAS-Dcr2; UAS-RNAi</th>
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\(^*\) At least two unique RNAi hairpins confirm lethality phenotype
Movie Legends

Movie 5. Cortex glial Ca\textsuperscript{2+} oscillations detected \textit{in vivo} with myrGCaMP5. Ca\textsuperscript{2+} oscillations within cortex glia in the ventral nerve cord of an undissected wild-type 2\textsuperscript{nd} instar larvae. Video speed is 2X real-time.

Movie 6. Cortex glial Ca\textsuperscript{2+} oscillations are absent in zyd mutants. Microdomain Ca\textsuperscript{2+} transients are present in wild-type, but not zyd\textsuperscript{1} mutant, 2\textsuperscript{nd} instar larvae. Video speed is 3X real-time.

Movie 7. Ca\textsuperscript{2+} oscillations in peripheral ensheathing glia are unaffected in zyd\textsuperscript{1} mutants. Ensheathing glia associated with larval motor neurons exhibit Ca\textsuperscript{2+} oscillatory activity in both wild-type and zyd\textsuperscript{1} mutant larvae. Video speed is 1X real-time.

Movie 8. Somatic Ca\textsuperscript{2+} oscillations in the LBD cell of a wild-type 3\textsuperscript{rd} instar larvae. UAS-GCaMP3 was expressed in LBD cells with repo-Gal4. Video speed is 1X real-time. Scale bar, 5 μm.


CHAPTER 4

Conclusions and Future Directions

Jan Elizabeth Melom

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Summary

Our results indicate that glial Ca$^{2+}$ signaling is acutely required in the CNS to regulate neuronal excitability, and that dysregulation of glial Ca$^{2+}$ triggers seizures. We identified zydeco, a glial-specific Na$^+$/Ca$^{2+}$, K$^+$ exchanger, in an unbiased screen for temperature-sensitive Drosophila seizure mutants. NCKX proteins are high-capacity, low-affinity exchangers that utilize the electrochemical gradients of the cell to export intracellular Ca$^{2+}$. We identified multiple alleles of zyd that exhibit seizures in response to several environmental stressors, including temperature shifts (high and low) and mechanical shock. We found that zyd expression in cortex glia, a cell type analogous to mammalian astrocytes, is required to regulate seizure susceptibility. Cortex glia ensheath neuronal cell bodies in the CNS and are the major glial constituent of the cortex, yet their function in the brain is unclear.

By imaging glial Ca$^{2+}$ in vivo with the membrane-tethered Ca$^{2+}$ sensor myrGCaMP5, we found that cortex glia dynamically oscillate Ca$^{2+}$ in small subcellular membrane domains. Whereas wild-type glia exhibit frequent and rapid microdomain Ca$^{2+}$ oscillations, zyd mutant cortex glia lack all detectable oscillatory activity. In addition, zyd mutant glia exhibit constitutively elevated basal intracellular Ca$^{2+}$, indicating that loss of the Ca$^{2+}$-export protein ZYD critically disrupts glial Ca$^{2+}$ regulation. We tested whether acute dysregulation of glial Ca$^{2+}$ is sufficient to trigger seizures by ectopically expressing the heat-activated Ca$^{2+}$-influx channel dTRPA1 in glia. We found that acute glial Ca$^{2+}$ influx mediated by dTRPA1 triggered rapid (second time-scale) seizures in adult animals in vivo, indicating that glial Ca$^{2+}$ regulation is acutely required in the CNS to maintain neuronal signaling. In
support of this finding, we found that zyd mutant seizure susceptibility was rescued within hours of expression of a wild-type zyd transgene in adult flies. Adult-specific temporal rescue of zyd mutants indicates that seizures in these animals are unlikely to be due to neuronal miswiring or other developmental defects.

We investigated the molecular mechanism underlying zyd mutant seizures by performing an in vivo RNAi screen for glial-specific seizure suppressors. We found that calmodulin, a ubiquitous Ca\(^{2+}\) binding effector protein, is required in glia for seizures in zyd mutants. This result suggests that a glial Ca\(^{2+}\)/calmodulin-dependent signaling pathway is disrupted in zyd mutants and contributes to seizure activity. We expanded the RNAi screen to test ~850 candidate genes for their involvement in zyd mutant seizures, and found that several genes involved in vesicle trafficking and neurotransmitter reception are required in glia for seizure initiation. We also identified other Ca\(^{2+}\) regulatory mechanisms in glia that modulate zyd seizure susceptibility and viability, including the T-type voltage gated Ca\(^{2+}\) channel, the ER Ca\(^{2+}\) ATPase SERCA, and the store-operated Ca\(^{2+}\) entry pathway. How various Ca\(^{2+}\) regulatory mechanisms interact in cortex glia to regulate acute glial-neuronal signaling is a topic of ongoing research.

Ca\(^{2+}\) oscillations in mammalian astrocytes have been studied for over twenty years, yet we are just beginning to understand the complexity and heterogeneity of astrocytic Ca\(^{2+}\) oscillations in vivo. Astrocytes exhibit large, somatic Ca\(^{2+}\) oscillations both spontaneously and in response to neuronal activity (Fiacco and McCarthy, 2006) that are critically dependent on ER Ca\(^{2+}\) store release (Petravicz et al., 2008). Recently, microdomain Ca\(^{2+}\) oscillations in astrocytes were identified and found to
arise via a novel, intracellular store-independent mechanism (Di Castro et al., 2011; Shigetomi et al., 2010). Our finding that Drosophila zyd critically regulates microdomain Ca\textsuperscript{2+} oscillations in cortex glia suggests that mammalian NCKX proteins may contribute to small Ca\textsuperscript{2+} oscillations in astrocytes. NCKX exchangers in mammals are expressed throughout the brain (Altimimi and Schnetkamp, 2007), and their function in glia is unknown. Our data further suggests that glial cells can be central players in the development of seizures. Previous research has generated debate surrounding the role of glial Ca\textsuperscript{2+} signaling in the brain (Agulhon et al., 2010; Agulhon et al., 2008; Fiacco et al., 2007; Henneberger et al., 2010; Petravicz et al., 2008) that may be due to manipulation of somatic rather than microdomain glial Ca\textsuperscript{2+} activity. We find that genetic dysregulation of endogenous glial Ca\textsuperscript{2+} signaling is sufficient to initiate seizures in Drosophila, suggesting that a better understanding of glial-neuronal signaling will aid the development of anti-epileptic therapies.

**Future Directions**

**Characterization of glial genes required for seizures in zyd**

In a candidate screen of 822 genes knocked down in glia by *in vivo* RNAi, we identified 36 genes that appear to suppress or enhance zyd\textsuperscript{1} mutant seizures (Table 2). The majority of these genes have known molecular functions and are involved in various cellular pathways, including vesicle trafficking, neurotransmitter detection, neuropeptide signaling, endocytic trafficking, and regulation of gene expression, among others. Despite the small fraction of genes from the candidate screen that
fully eliminated temperature-sensitive seizures in zyd\(^1\) (2.19%), a clear molecular mechanism underlying zyd mutant seizures has not yet emerged.

One possible explanation for this result is that multiple Ca\(^{2+}\)-dependent cortexglial processes are involved in triggering zyd mutant seizures. Since zyd\(^1\) mutants exhibit constitutively elevated basal glial Ca\(^{2+}\), cortex glial Ca\(^{2+}\) signaling may become globally dysregulated. We found that glial expression of cam is essential for seizures in zyd\(^1\) mutants, yet cam regulates a variety of signaling pathways and Ca\(^{2+}\)-dependent enzymes (Sola et al., 2001). Another possible explanation is that some of the candidate genes we identified are false-positives or false-negatives. RNAi-mediated gene knockdown occasionally introduces false-positive results due to off-target effects (Mohr et al., 2010). By using additional, unique RNAi hairpins to target the same gene, false-positives can be eliminated; however, potential false-negative results due to insufficient knockdown of the target gene makes elimination of false-positive results difficult.

The majority of genes we found that suppressed or enhanced zyd\(^1\) seizures were knocked down with a single RNAi hairpin. In order to verify that our RNAi-knockdown phenotypes were not due to an off-target effect, we screened additional RNAi hairpins against strong suppressors of zyd\(^1\) mutant seizures. For several genes, additional hairpins partially recapitulated the seizure suppression phenotype, and previously described null mutations were available (Table 1). We generated double mutants between zyd\(^1\) and the candidate suppressor genes syt4, ca-\(\alpha1T\), and nep4 in order to test whether genetic lesions in these genes would also reduce zyd\(^1\) mutant seizure susceptibility. We found that all three double mutant combinations
exhibited 38°C seizures indistinguishable from zyd\(^1\) single mutants (Table 1). This result may indicate that RNAi-mediated seizure suppression for these genes was due to off-target effects. However, this possibility is unlikely given that seizure suppression by glial knockdown of both ca-\(\alpha\)1T and nep4 was recapitulated with more than one unique RNAi hairpin. Another possibility is that glial-specific, rather than whole-organism, removal of ca-\(\alpha\)1T or nep4 is required to suppress seizures in zyd\(^1\) mutants. ca-\(\alpha\)1T, nep4, and syt4 are expressed in neurons and other tissues (Adolfsen et al., 2004; Meyer et al., 2009; Ryglewski et al., 2012), suggesting that the glial-specific function of these genes may be difficult to assess in null mutants.

Whether the candidate genes we identified by glial RNAi are involved in the zyd\(^1\) mutant seizure-inducing pathway will need to be verified with additional molecular and cellular analysis. To determine whether the targeted genes are being knocked down efficiently by RNAi, we will assay total brain protein extracts by Western blot. For example, we previously found that cam RNAi reduces calmodulin protein, indicating that cam RNAi efficiently knocks down its target. Additional double mutant analysis will also be performed with candidate suppressors or enhancers of zyd\(^1\) mutant seizures. Unfortunately, mutations in several strong zyd\(^1\) seizure suppressors, such as cam, cause embryonic or early larval lethality, making analysis of zyd\(^1\) seizure suppression unfeasible. To overcome this difficulty, genetic mosaic analysis with FLP/FRT mediated mitotic recombination (Golic and Lindquist, 1989) or Cre/loxP (Nakazawa et al., 2012) can be used to knockout a target gene specifically in glia. Genetic mosaic analysis of genes also expressed in neurons (such
as syt4, ca-α1T, and nep4) may be useful for determining whether reducing these genes specifically in glia suppresses zyd1 mutant seizures.

**Genetic screen for neuronal regulators of zyd mutant seizures**

A key question in glial-neuronal signaling is how neurons detect and respond to glial-derived signals. To address this question, we will express RNAi hairpins with elav-Gal4 to knockdown genes specifically in neurons in the background of the zyd1 mutant. Similar to our glial-specific RNAi suppressor screen, we will look for genes that suppress temperature-sensitive (38°C) seizures in the adult zyd1 mutant fly. We expect that any neuronal protein required to receive a glial-derived signal or trigger elevated neuronal activity that drives seizures in the zyd mutant will be recovered in the screen, providing that knockdown of the gene in neurons is not lethal. We will initially screen 295 genes selected for encoding cell-surface receptors, including GPCRs, RTKs, classical neurotransmitter receptors, neuropeptide receptors, and aminergic receptors, among others. We will verify RNAi hits in the manner described above. This approach can also be extended to a genome-wide RNAi screen in order to identify neuronal components of the seizure-inducing signaling pathway in an unbiased manner.

**Pharmacological and genetic dissection of glial Ca\(^{2+}\) oscillations**

It is unknown whether cortex glial microdomain Ca\(^{2+}\) oscillations are generated spontaneously or in response to neuronal activity. We observed that anesthesia of *Drosophila* larvae with desflurane, a volatile anesthetic administered
to human patients, immediately eliminated glial Ca$^{2+}$ activity. Blockade of glial Ca$^{2+}$ activity by anesthesia in mammals has been previously reported (Hirase et al., 2004; Takata and Hirase, 2008), and may indicate that glial microdomain Ca$^{2+}$ oscillations arise in response to neuronal activity. We will determine whether cortex glial Ca$^{2+}$ oscillations require neuronal activity by exposing pan-glial myrGCaMP5 expressing larvae to pharmacological agents that enhance (high K$^+$, TEA) or decrease (TTX) neuronal activity. If glial Ca$^{2+}$ oscillations are activated by enhanced neuronal activity, we would expect the frequency of Ca$^{2+}$ oscillations to increase with factors that drive neuronal firing and decrease with blockers of neuronal activity. If, on the other hand, glial Ca$^{2+}$ oscillations are generated spontaneously, we would expect glial activity to remain unperturbed. We will also determine whether plasma membrane Ca$^{2+}$ influx is required for microdomain oscillations by imaging glial activity in Ca$^{2+}$-free saline.

To pharmacologically dissect glial Ca$^{2+}$ activity, we may need to develop an ex vivo larval preparation amenable to long-term Ca$^{2+}$ imaging. We previously found that standard 3$^{rd}$ instar larval fillet dissections and saline perfusion used for long-term (~1 hour) electrophysiological recording caused glial Ca$^{2+}$ activity to reduce in frequency and terminate within 5-10 minutes. The reason for glial Ca$^{2+}$ sensitivity to dissection and saline perfusion is unknown. Drosophila glia may be exquisitely sensitive to pH or ionic concentrations, given that we found that perfusion with several Drosophila salines with varied pH and [K$^+$] (HL3.1 (Feng et al., 2004), Jan & Jan (Jan and Jan, 1976), and "external saline" (Rohrbough and Broadie, 2002)) altered the time course of glial Ca$^{2+}$ rundown. Systematic variation of saline
components may be necessary to maintain glial Ca\textsuperscript{2+} oscillations in a dissected preparation, which will allow application of pharmacological agents. Alternatively, a method to microinject larvae with drugs could be developed, which would allow glial Ca\textsuperscript{2+} imaging to be performed in the intact animal.

A second question we will address is how microdomain glial Ca\textsuperscript{2+} activity is regulated within the cell. Our in vivo RNAi data indicate that components of the store-operated Ca\textsuperscript{2+} entry (SOCE) pathway are required in glia for adult viability, suggesting SOCE contributes to glial Ca\textsuperscript{2+} regulation. We will explore the requirement for SOCE in cortex glia by imaging Ca\textsuperscript{2+} oscillations in larvae expressing glial RNAi to SOCE components. If SOCE is required to maintain or shape glial Ca\textsuperscript{2+} oscillations, we would expect Ca\textsuperscript{2+} activity to be correspondingly altered. We also identified several other Ca\textsuperscript{2+} regulatory genes in our RNAi screen that suppressed zyd\textsuperscript{4} mutant seizures. One of these genes, ca-\textalpha\textsuperscript{1}T, encodes a T-type voltage-gated Ca\textsuperscript{2+} channel (VGCC). T-type VGCCs are unique in that they can open at negative membrane potentials, and are involved in neuronal oscillatory activity (Zamponi et al., 2010). Whether T-type VGCCs are involved in glial Ca\textsuperscript{2+} influx is unknown, but may represent a mechanism by which glial Ca\textsuperscript{2+} influx occurs at resting (negative) membrane potential. We will explore whether ca-\textalpha\textsuperscript{1}T is involved in glial microdomain Ca\textsuperscript{2+} oscillations by imaging glial myrGCaMP5 in ca-\textalpha\textsuperscript{1}T mutant and ca-\textalpha\textsuperscript{1}T RNAi expressing larvae. If ca-\textalpha\textsuperscript{1}T is involved in glial Ca\textsuperscript{2+} influx, we expect that microdomain glial Ca\textsuperscript{2+} oscillations will be reduced in amplitude or eliminated.

**Relationship between neuronal activity and glial Ca\textsuperscript{2+} oscillations**
If we find that glial Ca\textsuperscript{2+} oscillations are responsive to neuronal activity, we will directly determine the relationship between glial and neuronal signaling with concurrent Ca\textsuperscript{2+} imaging and channelrhodopsin 2 (ChR2) mediated neuronal stimulation. ChR2 is a light-activated cation channel that can be genetically targeted to neurons with UAS/Gal4, allowing non-invasive optogenetic manipulation of neuronal firing (Zhang et al., 2007). We will generate a transgenic construct in which myrGCaMP5 is expressed under control of the repo (pan-glial) promoter, which has been previously cloned and validated (Awasaki et al., 2008). Expression of UAS-ChR2 with elav-Gal4 (pan-neuronal) and repo-myrGCaMP5 will allow us to manipulate neuronal firing rates while concurrently imaging cortex glial Ca\textsuperscript{2+} activity. ChR2 can be precisely targeted with blue light, enabling spatial control over single neuron activity. We will depolarize single neurons in the ventral nerve cord and monitor Ca\textsuperscript{2+} activity in the surrounding cortex glial sheath. Concurrent glial Ca\textsuperscript{2+} imaging and manipulation of neuronal activity will be a powerful experimental technique for testing future hypotheses about glial-neuronal signaling mechanisms. For example, if we identify a gene expressed in glia that suppresses zyd\textsuperscript{1} mutant seizures, we can ask whether neuronal activity stimulates glial Ca\textsuperscript{2+} oscillations in that mutant background. We hypothesize that any gene required for glial Ca\textsuperscript{2+} influx or signal transduction downstream of Ca\textsuperscript{2+} would be recovered in the screen for zyd\textsuperscript{1} seizure suppressors. We can also express the light-activated chloride pump halorhodopsin in neurons to optogenetically silence neuronal activity (Inada et al., 2011) and determine whether Ca\textsuperscript{2+} activity in the ensheathing cortex glial cell is
affected. This approach will be complimentary to pharmacological experiments to enhance or suppress neuronal activity in ex vivo preparations described above.

**Investigation of cortex glial development**

Our results indicate that cortex glial Ca\(^{2+}\) activity dynamically regulates neuronal function, but why acute glial-neuronal signaling is required in the brain is unclear. The development and morphology of cortex glia in the CNS may offer insight into how glia regulate the excitability of neuronal circuitry. In early *Drosophila* embryogenesis, approximately 100 neuroblasts give rise to individual lineages of 10-16 primary neurons and glia (Larsen et al., 2009). During larval development, each neuroblast produces an additional \(\sim 100-150\) secondary neurons. The cell bodies of primary and secondary neurons deriving from the same neuroblast remain clustered in the cortex, while the axons fasciculate with each other and project to the same region of the brain (Larsen et al., 2009) (Figure 1A). Approximately 160 cortex glia are in the larval brain (Pereanu et al., 2005), similar to the number of neuronal lineages. Our hypothesis is that a cortex glial cell specifically associates with neurons of the same lineage, therefore encapsulating neurons with identical projections. From single cell MARCM analysis, a cortex glial cell wraps dozens of local neurons, and its spatial domain does not overlap other cortex glia, indicating one cortex glial cell will have unique effects on a specific cluster of neurons (Doherty et al., 2009) (Figure 1B, C). An intriguing possibility is that a cortex glial cell could sense activity within a neuron in its spatial domain and respond by mobilizing internal Ca\(^{2+}\) stores and releasing a signal to boost the
activity of neighboring neurons. If clustered neurons form part of the same circuit, coactivation of neighboring neurons would boost circuit output. Alternatively, cortex glia may silence neighboring neurons in response to activity detected in a single neuron, thus enhancing the signal-to-noise ratio within a circuit.

It is currently unknown whether cortex glia associate with individual neuronal lineages. Interestingly, during the second wave of neurogenesis in larvae, large cortex glial chambers surround mitotically active neuroblasts (Pereanu et al., 2005)(Figure 1D). During metamorphosis, cortex glial processes infiltrate these chambers and encapsulate individual neurons (Figure 1E). If a single cortex glial cell formed the one larval cortical chamber, it would be developmentally positioned to encapsulate neurons deriving from the same neuroblast. We will test this hypothesis by performing MARCM labeling of single neuroblasts with UAS-mRFP in developing embryos to label neuronal lineage progeny. We can then dye fill individual cortex glia with Lucifer Yellow and determine whether a single cortex glial cell occupies a single neuroblast domain. An alternative approach would be to use the newly developed Drosophila Brainbow to stochastically express several fluorescent proteins in cells of common origin (Hampel et al., 2011). By expressing Brainbow in a neuroblast, we may recover cortex glia and neurons labeled in different colors, which would allow us to determine whether cortex glia cover the domain of a single neuroblast clonal unit.

We will also utilize MARCM to express UAS-myrGCaMP5 in single cortex glial cells in the larval ventral nerve cord. This will allow us to visualize Ca$$^{2+}$$ activity within a single glial cell and allow us to determine whether activity within a single
cell is (or is not) synchronized across the cell.

**Conclusion**

We identified *zyd*, a glial-specific NCKX that regulates cortex glial microdomain Ca\(^{2+}\) oscillations and seizure susceptibility in *Drosophila*. To our knowledge, *zyd* is one of the first glial-specific genes responsible for an epileptic phenotype, and indicates that glia play a central role in seizure generation. We plan to investigate the molecular mechanism underlying seizures in *zyd* mutants by *in vivo* RNAi screening of candidate genes for suppression or enhancement of *zyd* mutant seizures, both in neurons and glia. We hypothesize that a glial-neuronal signaling pathway is required in *zyd* mutants to trigger seizures in neurons in response to dysregulated glial Ca\(^{2+}\). We also plan to investigate the biology of microdomain glial Ca\(^{2+}\) oscillations. We will use genetic and pharmacological approaches to determine whether glial activity is responsive to neuronal activity, and how other Ca\(^{2+}\) regulatory mechanisms contribute to glial Ca\(^{2+}\) oscillations. Finally, we will investigate cortex glial development and morphology in the CNS in relation to neuronal lineage units. We hypothesize that cortex glia may interact with functionally related neurons, which would provide a novel mechanism in the brain for glial control of neuronal circuit excitability. We expect that these experiments will shed light on the functional complexity of mammalian astrocytes and provide new understanding of the pathophysiology of epilepsy.
Figures

A

B

C

cell body/cortex

Cortex

D

E

OL

np

nc

an

nb

pn

np

cx

GFP

syntaxin

nerve2

nerve2

nerve2
**Figure 1.** Neuronal lineages and cortex glia in the CNS. **A)** Diagram of a neuroblast clone in the larval cortex. The cell bodies of primary and secondary neurons deriving from the original neuroblast remain clustered in the cortex, while their axons fasciculate and project into the neuropil. **B)** A single cortex glial cell in the adult CNS expressing GFP. **C)** A single cortex glial cell expressing GFP in the larval CNS (arrow) located next to a neuroblast (triangle). **D)** Cortex glia (green) in the 3rd instar larval CNS form large chambers on the surface of the cortex encompassing recently generated secondary neurons (red). **E)** In the adult CNS, cortex glia have infiltrated the large chambers and ensheathed individual neuronal soma. Part (A) reproduced from (Spindler and Hartenstein, 2010). Part (B) reproduced from (Doherty et al., 2009). Parts C-E reproduced from (Pereanu et al., 2005).
### Tables

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**Table 1.** Candidate genes identified by glial RNAi that suppress zyd\(^I\) mutant seizures. Multiple RNAi hairpins for each gene were expressed in glia (with repo-Gal4) in the zyd\(^I\) mutant background. The behavior of adult zyd\(^I\) mutant flies expressing glial RNAi is listed in column 3 ("Phenotype"). Null (*syt4*, *ca-α1T*) or presumptive null (*nep4*) mutations in the candidate genes were crossed into the zyd\(^I\) mutant background, and double mutants were assayed for seizure susceptibility at 38°C (column 5).
References


