Mutation of a NCKX Eliminates Glial Microdomain Calcium Oscillations and Enhances Seizure Susceptibility

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Glia 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 CNS. In a screen for temperature-sensitive conditional seizure mutants, we identified a glial-specific $\text{Na}^+/\text{Ca}^{2+}$, 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 a variety of environmental stimuli, 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 underlies glial–neuronal communication. These studies demonstrate that microdomain glial Ca^{2+} oscillations require NCKX-mediated plasma membrane Ca^{2+} flux, and that acute dysregulation of glial Ca^{2+} signaling triggers seizures.

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^{2+} activity has been associated with abnormal neuronal excitability in several mammalian models of epilepsy (Tian et al., 2005; Fellin et al., 2006), and cortical astrocytes were found to exhibit Ca^{2+} oscillations immediately preceding a focal seizure event (Gómez-Gonzalo et al., 2010). However, whether increased glial Ca^{2+} activity is a result of seizures, or is a direct cause of seizures, is unclear.

Glial Ca^{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^{2+} activity have been restricted to measurement of somatic Ca^{2+} due to the small volume and inaccessibility of fine glial processes to Ca^{2+} -sensitive dyes (Agulhon et al., 2008). However, recent studies using membrane-targeted Ca^{2+} -sensitive GFP (GCaMP) have shown that small, near-membrane Ca^{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^{2+} oscillations and small, near-membrane Ca^{2+} oscillations in glial processes is critical, and may explain differing results concerning the physiological importance of glial Ca^{2+} signaling in the regulation of neuronal excitability (Fiacco et al., 2007; Petrávicz et al., 2008; Agulhon et al., 2010).

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* glial-specific $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchanger eliminates microdomain cortex glial Ca^{2+} oscillations and predisposes animals to seizures in response to several environmental stressors. In addition, we show that acute, *in vivo* dysregulation of glial Ca^{2+} triggers rapid neuronal seizures. Our findings identify one of the first glial-specific genes responsible for an epileptic phenotype and indicate that a Ca^{2+} /

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calmodulin-dependent glial-derived signal is sufficient to initiate seizure activity in the brain.

Materials and Methods

Drosophila genetics and molecular biology. Flies were cultured on standard medium at 22°C. *zydeco* (*zyd*) mutants were generated by ethane methyl sulfonate (EMS) mutagenesis and identified in a screen for temperature-sensitive (TS) behavioral phenotypes (Guan et al., 2005). *zyd*¹ 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 genomic sequence. We used the UAS/Gal4 system to drive transgenes in glia. UAS-*zyd* was constructed by subcloning the *zydeco* cDNA into pUAST and injected into *w*¹¹¹⁸ by Genetic Services. UAS-*myrGCaMP5* was constructed by subcloning the first 90 aa of Src64b, containing a myristoylation target sequence, into pBI-UASc (creating pBI-UASc-*myr*). GCaMP5G cDNA (Addgene plasmid 31788) was cloned into pBI-UASc-*myr* and injected using ϕ -C-31 transformation.

Behavioral assays. Bang sensitivity of adult female flies was assayed 2–3 d posteclosion. Flies were transferred into empty vials and allowed to rest for 1–2 h. Vials were vortexed at maximum speed for 10 s, and the number of flies that were upright and mobile was counted at 10 s intervals. For assaying temperature-sensitive phenotypes, 1- to 3-d-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 *zyd*¹ 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 the animal fell to the bottom of the vial and exhibited no movement. For assaying seizures in larval animals, third 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.

Immunostaining and Western blots. ZYD polyclonal antibodies were generated in rabbit with a synthetic *zydeco* peptide corresponding to residues 313–329 (DEGRKEEGYSLSPK) and affinity-purified by Yenzym Antibodies. For ZYD immunostaining, dissected third instar larvae of either sex were fixed with 100% ice-cold methanol for 5 min. 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; and 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) with oil-immersion 40× 1.3 numerical aperture (NA) and 63× 0.95 NA objectives. 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).

In vivo Ca^{2+} imaging. UAS-*myrGCaMP5* was expressed in glia with *repo*-Gal4. Second instar male larvae were washed with PBS and placed on a glass slide with a small amount of Halocarbon oil #700 (LabScientific). 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 EM CCD camera at 8–12 Hz with a 40× 1.3 NA oil-immersion objective, using a Volocity Software program. We imaged at a single optical plane within the ventral cortex of the ventral nerve cord (VNC), 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

mm² 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). When indicated, larvae were maintained at 38°C for 1 min before imaging.

Electrophysiology. Current-clamp recordings of wandering third instar larvae were performed in HL3.1 saline (in mM: 70 NaCl, 5 KCl, 4 MgCl₂, 0.2 CaCl₂, 10 NaHCO₃, 5 Trehalose, 115 sucrose, 5 HEPES-NaOH, pH 7.2) containing 1.5 mM Ca^{2+} using an Axoclamp 2B amplifier (Molecular Devices) at muscle fiber 6/7 of segments A1–A4. Larvae were of either sex, except for recordings made in *zyd*¹ mutant and control 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 min before recording.

Results

zydeco encodes a glial-specific NCKX that regulates seizure susceptibility

We identified mutations in *zyd* in an unbiased screen for recessive TS seizure mutants on the X chromosome (Guan et al., 2005). Using deficiency mapping and positional cloning, we discovered that *zyd* mutations disrupt the CG2893 locus, which encodes a NCKX (Fig. 1A). NCKX proteins are plasma membrane transporters that export intracellular Ca^{2+} by using Na^+ and K^+ electrochemical gradients, and have a high transport capacity important for rapid reduction of elevated cytosolic Ca^{2+} (Altimimi and Schnetkamp, 2007). Two alleles of *zyd* are point mutations in a highly conserved region of NCKX containing residues essential for cation transport (Winkfein et al., 2003) (Fig. 1B), while a third (*zyd*¹) creates an early stop codon that removes 2 of the 11 transmembrane domains.

All three *zyd* mutants are homozygous adult viable and exhibit rapid seizures upon exposure to 38°C (Movie 1). TS *zyd* seizures also occur at the larval stage (Movie 2), consistent with the onset of *zyd* expression during mid-embryogenesis and continuing through adulthood (Winkfein et al., 2004). We recorded central pattern generator output from the neuromuscular junction (NMJ) of third 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 (Fig. 1C,D). 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.

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 (Fig. 1E). 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.

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. Given that *zyd* mRNA is restricted to the CNS and appears specific to glia rather than neurons (Winkfein et al., 2004; Beckervordersandforth et al., 2008), we tested both pan-neuronal and pan-glial Gal4 drivers for rescue of *zyd*. We found that expression of a UAS-*zyd* transgene

Figure 1. Mutation of the NCKX *zgd* causes temperature- and bang-sensitive seizures. **A**, EMS-induced mutations isolated in CG2893/NCKX in three independently generated alleles of *zgd*. Alleles *zgd*² and *zgd*³ are missense mutations in a pore-lining domain of the exchanger (blue), known as the α 1-repeat region. **B**, Alignment of mammalian and invertebrate NCKX α 1-repeat regions, indicating *zgd*² and *zgd*³ mutations (blue = identical, green = similar residues). **C**, **D**, Representative voltage traces of spontaneous central pattern generator activity at larval third instar muscle 6 at 22°C (**C**) and 38°C (**D**) in wild-type and *zgd*¹ mutants ($n > 8$ preparations/genotype). **E**, Recovery time from seizures induced by mechanical stimulation (10 s of vortexing) in WT and *zgd* mutants at 22°C. $n = 100$ flies/genotype.

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

with the pan-glial *repo*-Gal4 driver fully rescues *zyd*¹ mutant TS seizures (Fig. 2A). Pan-neuronal expression with *elav*-Gal4 does not affect the onset or severity of TS *zyd* seizures (Fig. 2A), indicating that ZYD is required in glia, and not neurons, to regulate seizure susceptibility.

To determine whether disruption of ZYD expression in glia is sufficient to elicit seizures, we knocked down *zyd* by RNAi in neurons and glia (Fig. 2B). Pan-glial expression of two unique RNAi hairpins targeting *zyd* produces TS seizures in third instar larvae and is semilethal 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*¹ mutant phenotype (Fig. 2C), indicating that constitutive *zyd* knockdown in mature glia is re-

Movie 2. *Zyd* mutant larvae exhibit temperature-sensitive seizures. Wild-type and *zyd*¹ mutant third instar larvae were transferred to agarose plates heated to 38°C. Video starts 30 s after larvae were transferred to the plate. Video speed is real time.

quired to induce seizure susceptibility. Neuronal knockdown of *zyd* did not cause any temperature-sensitive behavioral or electrophysiological phenotypes in larvae (Fig. 2B) or adults. These results indicate that ZYD function in glia is required to regulate neuronal excitability.

ZYD is expressed in cortical glia that encapsulate neuronal cell bodies

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 TS seizures (Fig. 2*B,C*). We also tested for rescue of *zyd*¹ mutant seizures, and found expression of UAS-*zyd* with several cortex glial Gal4 promoters was sufficient to fully suppress seizures in adult *zyd*¹ mutants. Knockdown of *zyd* in other glial populations, including

Movie 3. *Zyd* mutants exhibit seizures upon recovery from cold shock. Wild-type and *zyd*¹ mutant adult flies were placed in prechilled vials on ice for 5 min, then were allowed to recover at room temperature (start of video recording). Video speed is 2× real time.

Figure 2. *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 with control at 38°C, but knockdown of *zyd* by pan-glial- or cortex glial-driven (*NP2222*-Gal4) RNAi causes rapid, seizure-like discharges in the larval muscle. *n* > 5 preparations/genotype. **C**, Use of a collection of glial subpopulation specific Gal4 drivers to rescue *zyd*¹ TS seizures (with UAS-*zyd*), or to knockdown *zyd* (with UAS-*zyd* RNAi). Only Gal4 drivers with expression in cortex glia rescued adult *zyd*¹ seizures at 38°C and phenocopied *zyd* TS seizures by RNAi. NT, Not tested. *n* > 50 animals/genotype.

astrocyte-like glia, ensheathing glia, and surface glia, did not cause TS seizures or other obvious behavioral phenotypes.

Cortex glia are highly lamellated cells that form a honey-combed network in the brain, encapsulating individual neuronal cell bodies (Hoyle et al., 1986; Perea et al., 2005). Cortex glia are involved in guidance of secondary axon tracts and maintenance of cortical structural integrity (Dumstre 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 third instar larval CNS, forming chambers that surround clusters of neuronal cell bodies (Fig. 3A). As development proceeds through metamorphosis, cortex glia in the outer layers of the CNS subdivide these chambers to individually encapsulate the enclosed neurons (Perea et al., 2005). In the third instar ventral nerve cord, ZYD staining closely surrounds each neuronal cell body, as revealed by costaining with antibodies against ELAV, a neuronal nuclei-specific protein (Fig. 3B). ZYD staining also closely colocalizes with pan-glial GFP expression in the CNS (Fig. 3C). ZYD immunoreactivity is strongly reduced in both the central brain hemispheres and ventral nerve cord of *zyd*¹ 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 (Fig. 3D). 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 in cortex glia in the brain.

ZYD is required acutely in the mature CNS to regulate neuronal excitability

To discriminate between an acute versus developmental role for ZYD in the CNS, we conditionally expressed a UAS-*zyd* transgene in *zyd*¹ mutants using the *hsp70*-Gal4 driver (Fig. 4). 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 (Lindquist, 1986; Brand et al., 1994). *zyd*¹ 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*¹ TS seizures in adults 6 h after the heat pulse, consistent with the time course of induced *hsp70*-Gal4 activity measured after a similar heat treatment (Lindquist, 1986). Susceptibility to TS seizures returned in the conditionally rescued *zyd*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 time points tested (Fig. 4, 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*¹ mutants are unlikely to be the result of neuronal miswiring or other developmental defects.

Dynamic microdomain Ca^{2+} oscillations occur in cortex glia *in vivo* and are absent in *zyd* mutants

To observe near-membrane Ca^{2+} dynamics in cortex glia, we generated a myristoylated variant of Ca^{2+} -sensitive GFP (myrGCaMP5), which targets to the inner leaflet of the plasma membrane (Akerboom et al., 2012). Glial expression of myrGCaMP5 revealed small, rapid Ca^{2+} oscillations present in cortex glia in the ventral nerve cord in live, undissected larvae (Fig. 5A; Movie 4). We analyzed cortex glial Ca^{2+} oscillations with *repo*-Gal4 because this driver produced the highest expression of UAS-myrGCaMP5 and

Figure 3. ZYD is expressed in cortex glia. **A**, One hemisphere of the larval CNS in WT and *zyd*¹ 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 occurred. Scale bar, 40 μm . **B**, Optical section within the ventral cortex of the VNC in WT and *zyd*¹ third instar larvae. ZYD staining encapsulates individual neuronal soma. Scale bar, 20 μm . **C**, CNS stained with ZYD antisera in larvae expressing pan-glial GFP. Scale bar, 40 μm . **D**, Optical section of the CNS in larvae expressing pan-neuronal (*elav*-Gal4), pan-glial (*repo*-Gal4), or cortex glial (*NP2222*-Gal4) *zyd* RNAi. Scale bar, 10 μm .

Figure 4. Adult-specific rescue of *zyd* 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*¹ mutants were reared at 22°C until adulthood (2 d posteclosion), then exposed to a brief 37°C heat pulse. TS seizure susceptibility was strongly reduced in *zyd*¹ mutants 6 h 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/time point.

cortex glia are easily identified by morphology in the ventral nerve cord. However, expression of UAS-*myrGCaMP5* with the cortex glial specific drivers *NP2222*-Gal4 and *NP577*-Gal4 revealed similar Ca^{2+} oscillatory activity. Standard fillet dissection of *myrGCaMP5*-expressing larvae in external salines commonly used for electrophysiological recording (HL3.1) (Jan and Jan, 1976; Feng et al., 2004) caused rapid reduction in the frequency of glial Ca^{2+} transients. Cortex glial Ca^{2+} transients were consistently eliminated within 10 to 20 min of dissection despite extreme care taken to prevent damage to the CNS. Therefore, to ensure that the glial Ca^{2+} signals we re-

corded were reflective of nonpathological *in vivo* activity, we performed all imaging experiments in live, undissected larvae gently pressed under a coverslip to reduce movement.

Cortex glial Ca^{2+} transients occur in small microdomains $\sim 4.35 \pm 0.26 \mu\text{m}^2$ ($n = 40$) and appeared to recur frequently in the same region (Fig. 5B), suggesting the presence of specialized structural or functional glial subdomains. The duration of each glial Ca^{2+} transient was 1.35 ± 0.08 s and exhibited a mean $\Delta F/F_{\text{avg}}$ of $35.4 \pm 2.7\%$ ($n = 40$). Ca^{2+} transients generally arose within a small part of the cortex glial sheath surrounding a single neuronal soma (Fig. 5D,E), although occasional Ca^{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.

Microdomain cortex glial Ca^{2+} transients are completely absent in *zyd*¹ mutants at room temperature (Fig. 5C; Movie 5). However, baseline *myrGCaMP5* fluorescence within the cortex glial network is significantly greater in *zyd*¹ mutants compared with wild-type controls (Fig. 5F,G), indicating glial intracellular Ca^{2+} is constitutively elevated in *zyd*¹ mutants. At the restrictive temperature of 38°C, intracellular Ca^{2+} remains significantly elevated in *zyd*¹ cortex glia relative to wild type, although average *myrGCaMP5* fluorescence increases in both wild-type and *zyd*¹ mutant glia with increasing temperature (Fig. 5G). The effect of hyperthermia on resting intracellular Ca^{2+} has been observed previously in *Drosophila* nerve terminals (Klose et al., 2009), and is thought to be due to a reduction in ATP-dependent Ca^{2+} clearance. In addition to constitutively elevated glial intracellular

Figure 5. Microdomain Ca^{2+} oscillations in cortex glia require ZYD. **A**, Ca^{2+} transients occur in small regions of cortex glial membrane in the live larval ventral nerve cord. Scale bar, 10 μm . MyrGCaMP5 fluorescence ($\Delta F/F_{\text{avg}}$) is shown for indicated regions in **B**. **C**, Frequency of microdomain Ca^{2+} transients in VNC cortex glia for wild-type and *zyd¹* mutants at 25°C. $n = 7$ larvae/genotype. **D**, Time-lapse image series of a single cortex glial Ca^{2+} oscillation at 25°C and 38°C in wild-type larvae. Scale bar, 4 μm . **E**, MyrGCaMP5 fluorescence ($\Delta F/F_{\text{avg}}$) for the regions indicated in the first panels of **D**. **F**, Representative images of myrGCaMP5 fluorescence intensity in wild-type and *zyd¹* at 25°C. Scale bar, 20 μm . **G**, Average myrGCaMP5 fluorescence in cortex glia of wild-type and *zyd¹* at 25°C and 38°C. $n = 10$ larvae/condition. Error bars represent the SEM. t test: *** $p < 0.001$, ** $p < 0.01$.

Ca^{2+} , *zyd¹* 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.

Acute cortex glial Ca^{2+} influx via ectopic TRPA1 activation triggers seizures

Given that *zyd* mutants lack microdomain Ca^{2+} transients and also exhibit increased global intracellular Ca^{2+} , we hypothesized that seizures may arise in *zyd* mutants due to either a constitutive lack of regulated glial Ca^{2+} signaling, or acute elevation of glial Ca^{2+} triggered by environmental stimuli. To discriminate between these two possibilities, we investigated the requirement for acute glial Ca^{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^{2+} influx with moderate increases in temperature (Rosenzweig et al., 2005; Hamada et al., 2008) (Fig. 6A). Previous studies have shown that ectopic expression of dTRPA1 in motor neurons elicits action potentials above 26°C and does not affect mem-

brane properties at 22°C (Pulver et al., 2009), indicating dTRPA1 is a useful tool for acutely manipulating intracellular Ca^{2+} . We assessed the behavioral effect of activating dTRPA1 in cortex glia, and found that adult flies exhibited strong, immobilizing seizures within seconds of a temperature shift to 30°C (Movie 6). 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 (Fig. 6B). 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.

Movie 4. Cortex glial Ca^{2+} oscillations detected *in vivo* with myrGCaMP5. Ca^{2+} oscillations within cortex glia in the ventral nerve cord of an undissected wild-type second instar larvae. Video speed is $2\times$ real time.

Movie 5. Cortex glial Ca^{2+} oscillations are absent in *zyd* mutants. Microdomain Ca^{2+} transients are present in wild-type, but not *zyd*¹ mutant, second instar larvae. Images were acquired through the cuticle of an undissected second instar larvae. Video speed is $3\times$ real time.

Glial expression of calmodulin is required for seizure initiation in *zyd* mutants

To probe the mechanism by which altered glial Ca^{2+} regulation in *zyd*¹ mutants induces seizures, we performed an RNAi screen for suppression of *zyd*¹ mutant TS seizures. We knocked down candidate genes in *zyd*¹ mutants that are implicated in vesicular trafficking and regulation, given recent evidence that Ca^{2+} -dependent exocytosis in mammalian glia alters neuronal excitability (Parpura and Zorec, 2010). We found that pan-glial knockdown of calmodulin (*cam*) in *zyd*¹ mutants fully suppresses TS seizures in both larvae and adults (Fig. 7A,C), indicating a Ca^{2+} /calmodulin-dependent glial signaling pathway is required for seizure initiation. Calmodulin is an essential Ca^{2+} -binding protein that regulates multiple Ca^{2+} -dependent cellular processes and is expressed in *Drosophila* glia (Altenhein et al., 2006), although its role in glia is unknown. We verified that expression of *cam* RNAi reduces calmodulin protein by Western blot using the eye-specific driver *gmr*-Gal4 (Fig. 7B). Knockdown of *cam* with *repo*-Gal4 did not significantly reduce total calmodulin brain protein, likely due to high expression of calmodulin in the *Drosophila* compound eye and neuronal tissue (Chintapalli et al., 2007).

Given that calmodulin is required for seizures in *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^{2+} influx via dTRPA1 causes immediate paralysis in adult flies (Movie 6) that persists until animals are returned to room temperature. Concomitant expres-

sion 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 (Fig. 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 min 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 calmodulin knockdown indicates that calmodulin plays a central role in mediating a glial Ca^{2+} -dependent process that acutely affects nervous system function.

Discussion

The findings reported here indicate that mutation of the NCKX *zyd* eliminates microdomain Ca^{2+} oscillations in glia and predisposes animals to seizures. *In vivo* imaging of live, unanesthetized animals revealed that *Drosophila* cortex glia exhibit rapid and frequent microdomain Ca^{2+} fluctuations in discrete domains encompassing neuronal cell bodies. Disruption of cortex glial Ca^{2+} transients by mutation of *zyd* enhances seizure susceptibility to several environmental triggers, which is restored upon acute expression of ZYD in the adult CNS. Our results also indicate that a Ca^{2+} /calmodulin-dependent signaling pathway underlies acute glial modulation of neuronal activity. Previous studies have generated conflicting data regarding the importance of astrocytic Ca^{2+} signaling to neuronal physiology (Fiacco et al., 2007; Petrávicz et al., 2008; Agulhon et al., 2010; Gómez-Gonzalo et al., 2010; Henneberger et al., 2010). Our data demonstrate *in vivo* that endogenous microdomain Ca^{2+} oscillations in glia acutely influence neuronal function, and are involved in the pathophysiology of seizures in *Drosophila*.

The NCKX ZYD is required for endogenous glial Ca^{2+} oscillations

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 Ca^{2+} channels (*para*, *shaw*, *cac*), proteins involved in synaptic vesicle exocytosis (*NSF*, *syntaxin*), and endocytosis (*dynammin*, *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 ensheath neuronal soma with extremely thin membrane projections and form a honeycombed 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.

We found that ZYD is required for microdomain Ca^{2+} transients in cortex glia, as *zyd* mutant glia fail to exhibit small Ca^{2+} oscillations observed *in vivo* in wild-type animals. *zyd* has been shown to encode a functional K^+ -dependent $\text{Na}^+/\text{Ca}^{2+}$ 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 Ca^{2+} levels in rod photoreceptors (Schneekamp, 2004) and cortical neurons (Li et al., 2006). In contrast to the slower, high-affinity Ca^{2+} ATPases that are thought to fine tune resting Ca^{2+} concentration in the nanomolar range, NCKX transporters rapidly extrude Ca^{2+} that reaches micromolar con-

Figure 6. 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 $\geq 26^\circ\text{C}$, 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 third instar larval muscle during application of a temperature ramp. Preparations were held at the indicated temperature for 2 min before recording. $n > 6$ larvae per genotype.

Movie 6. Acute dysregulation of cortex glial Ca^{2+} causes seizures. dTRPA1-mediated Ca^{2+} 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 $2\times$ real time.

centrations (Altimimi and Schnetkamp, 2007) and are activated during sharp peaks in intracellular Ca^{2+} .

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. 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-

Figure 7. Knockdown of calmodulin suppresses seizures in *zyd* mutants and paralysis due to ectopic glial TRPA1 activation. **A**, Central pattern generator activity recorded in larval muscle in control and following pan-glial expression of *cam* RNAi in *zyd*¹ 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 suppression in adult *zyd*¹ 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.

American populations with lighter skin color and causes substantial reduction in exchanger activity (Ginger et al., 2008). 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+} activity in these glia as well.

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

Seizures in *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 in addition to lacking microdomain Ca^{2+} transients, *zyd* mutant glia also exhibit constitutively elevated intracellular Ca^{2+} at room temperature and the seizure-inducing temperature of 38°C. One possibility is that the *zyd* mutant nervous system is “primed” for seizures due to deregulation of a Ca^{2+} -dependent glial process that modulates neuronal activity. Increasing temperature has been shown to enhance neuronal activity in *Drosophila* (Peng et al., 2007), possibly inducing runaway excitation in *zyd* mutants. Another possible model is that stressful environmental stimuli trigger seizures by acutely increasing intracellular glial Ca^{2+} . We found that hyperthermia causes a global increase in glial Ca^{2+} that is significantly elevated in *zyd* mutants relative to wild type. This observation is consistent with previous studies on hyperthermia-induced Ca^{2+} increase 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 hypothesis 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.

Given that ZYD is not required in surface glia 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, which insulate axons, or neuropil glia, which 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 that are reminiscent of the spatial segregation of mammalian astrocytes (Awasaki et al., 2008). As such, Ca^{2+} oscillations in cortex glia may normally regulate the activity of small, interconnected neuronal networks. A second clue to how glial Ca^{2+} oscillations regulate excitability is the observation that calmodulin is required in glia for seizure induction in *zyd*¹ mutants. Calmodulin acts as a Ca^{2+} sensor in many signaling pathways and is predicted to have a limited spatial range of action (Solà et al., 2001; Saucerman and Bers, 2012), suggesting calmodulin mediates a local signaling function in cortex glial Ca^{2+} microdomains.

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 (Tashiro et al., 2002; Gómez-Gonzalo et al., 2010) and *in vivo* work demonstrating several anti-epilepsy drugs reduce glial Ca^{2+} oscillations (Tian et al., 2005). The observation that astrocytic, Ca^{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^{2+} signaling were found to be dispensable for the generation of epileptiform activity *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 (*zyd*) responsible for an epileptic phenotype suggests that at least in some cases, Ca^{2+} dysregulation in an astrocyte-like cell population is sufficient to initiate a seizure.

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

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