Investigation of Seizure-Susceptibility in a *Drosophila melanogaster* Model of Human Epilepsy with Optogenetic Stimulation

Arunesh Saras,* Veronica V. Wu,* Harlan J. Brawer,* and Mark A. Tanouye*,†,1
*Department of Environmental Science, Policy and Management, Division of Organismal Biology and †Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

ABSTRACT We examined seizure-susceptibility in a *Drosophila* model of human epilepsy using optogenetic stimulation of *ReaChR* (red-activatable channelrhodopsin). Photostimulation of the seizure-sensitive mutant *para*^{bss1} causes behavioral paralysis that resembles paralysis caused by mechanical stimulation, in many aspects. Electrophysiology shows that photostimulation evokes abnormal seizure-like neuronal firing in *para*^{bss1} followed by a quiescent period resembling synaptic failure and apparently responsible for paralysis. The pattern of neuronal activity concludes with seizure-like activity just prior to recovery. We tentatively identify the mushroom body as one apparent locus of optogenetic seizure initiation. The α/β lobes may be primarily responsible for mushroom body seizure induction.

KEYWORDS sodium channel; epilepsy; seizure-suppression; red light activable channelrhodopsin

HUMAN seizure disorders are a substantial health problem because of the large number of people affected, the debilitating nature of seizure episodes, and limitations in treatment options. Recently, there has been considerable interest in using optogenetic approaches to study epilepsy, including investigations of basic seizure mechanisms and new treatment options (Tye and Deisseroth 2012; Paz and Huguenard 2015; Zhao *et al.* 2015). Optogenetics combines optical and genetic methodologies to control and manipulate neuronal excitability with high spatial and temporal resolution. For example, focal seizure-like afterdischarges are driven by photostimulation when excitatory channelrhodopsin2 (ChR2) is expressed in rat hippocampus dentate granule cells (Osawa *et al.* 2013). In the rat hippocampus, inhibitory halorhodopsin expression can reduce the severity of pilocarpine-induced seizures (Sukhotinsky *et al.* 2013).

Here, we use the opsin *ReaChR* (red-activatable channelrhodopsin) to examine seizure-sensitivity in a *Drosophila* model of human epilepsy. *Drosophila* bang-sensitive (BS) mutants are particularly sensitive to seizure; they are 5–10 more susceptible to seizure following electrical shock than wild-type flies (Kuebler and Tanouye 2000; Lee and Wu 2002). Three of the BS mutants examined here are *para*^{bss1}, *eas*, and *sda*, whose genes encode a voltage-gated Na^+ channel, ethanolamine kinase, and aminopeptidase, respectively. All of the mutations are severely seizure-sensitive, especially *para*^{bss1}, suggested as a model for human intractable epilepsy, which it resembles in several aspects (Liao *et al.* 2010; Parker *et al.* 2011; Schwarz *et al.* 2016). *ReaChR* is a red-shifted variant of channelrhodopsin that has been shown to be especially effective for optogenetic activation of neurons in intact freely behaving *Drosophila* adult flies (Lin *et al.* 2013; Inagaki *et al.* 2014). This is thought to be due, in large part, to the higher penetrance of red light through the adult cuticle (5–10% penetrance) compared to blue light used for ChR2 activation (1% penetrance) (Inagaki *et al.* 2014). We show that activation of *ReaChR* in BS flies causes behavioral phenotypes of seizure and paralysis. *ReaChR* optogenetics allows us to examine large numbers of flies with a minimum of mechanical manipulation and under freely behaving conditions. Electrophysiological recording shows that the behavioral phenotypes are due to seizure-like firing of central nervous system neurons followed by synaptic failure. *ReaChR* under GAL4/UAS (upstream activating sequence) control may be used to localize sites of seizure activation. As an initial

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[1]Corresponding author: Department of Environmental Science, Policy and Management, 135 Life Sciences Addition, University of California, Berkeley, CA 94720. E-mail: tanouye@berkeley.edu

example, we show here that activation of mushroom body (MB) neurons alone is sufficient to drive seizure-like neuronal firing. Overall, our study shows that optogenetics can be a powerful method for the study of seizures in the *Drosophila* model, and implicates the αβ lobe of the (MB) as a locus of seizure initiation.

Materials and Methods

Fly cultures

Drosophila strains were maintained on standard cornmeal–molasses agar medium at room temperature (24°). For optogenetic experiments, newly eclosed flies were transferred into a vial containing food with 400 μM all-*trans* Retinal (ATR, Sigma [Sigma Chemical], St. Louis, MO). ATR food vials were prepared by heating standard vials to liquefy the food and mixing in the ATR to disperse it evenly. ATR food vials were placed in the dark throughout the preparation and during all genetic crosses. The *paralytic* (*para*) gene is located at map position 14E−15A9 and encodes a voltage-gated Na^+ channel (Loughney *et al.* 1989; Ramaswami and Tanouye 1989). The allele used here is a BS paralytic mutation, $para^{bss1}$, previously named bss^1. It is the most seizure-sensitive of BS mutants, the most difficult to suppress by mutation and by drug, and is a model for human intractable epilepsy (Ganetzky and Wu 1982; Parker *et al.* 2011). The $para^{bss1}$ allele is a gain-of-function mutation caused by a substitution (L1699F) of a highly conserved residue in the third membrane-spanning segment (S3b) of homology domain IV (Parker *et al.* 2011). The *easily shocked* (*eas*) gene is located at 14B on the cytological map and encodes an ethanolamine kinase (Pavlidis *et al.* 1994). The BS allele used in this study is eas^{PC80}, which is caused by a 2-bp deletion that introduces a frameshift; the resulting truncated protein lacks a kinase domain and abolishes all enzymatic activity (Pavlidis *et al.* 1994). The *slamdance* (*sda*) gene is located at 97D and encodes an aminopeptidase N. The allele used in this study is $sda^{iso7.8}$ caused by a 2-bp insertion in the 5′-untranslated region (Zhang *et al.* 2002). The fly lines for a307-GAL4, Gad1-GAL4, 104Y-GAL4, and c232-GAL4 were obtained from Rod Murphey (Florida Atlantic University), Gero Miesenbock (Yale University), and Roland Strauss (University of Wurzburg), respectively, and maintained as stocks in our laboratory. The fly lines for *UAS-ReaChR* (BL #53741), 117Y-GAL4 (BL #30814), 121Y-GAL4 (BL #30815), 7B-GAL4 (BL #7365), c305a-GAL4 (BL #30829), 1471-GAL4 (BL #9465), and Cha-GAL4 (BL #6798) were obtained from the Bloomington *Drosophila* Stock Center.

Behavioral testing for BS phenotypes

Flies were collected using CO_2 anesthesia and 10 flies were placed in fresh food vials with foam plugs for testing the following day (Kuebler and Tanouye 2000). For testing, the food vial was stimulated mechanically with a vortex mixer (VWR International) at maximum speed for 10 sec. The number of flies displaying the phenotype of BS paralysis was tallied immediately after the vortex. Behavioral data for each genotype were pooled ($n \approx 50$). All flies tested were 3 days posteclosion.

Electrophysiology of the giant fiber (GF) system

In vivo electrophysiological assays and recording of seizure-like activity in the dorsal longitudinal muscle (DLM) were performed as described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). Unanesthetized flies were immobilized with dental wax on a glass microscope slide. Uninsulated tungsten electrodes were used for recordings and stimulation. Procedures for determining evoked seizure threshold were described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). Seizure-like activity was evoked by electrical high-frequency stimuli (HFS) delivered to the brain (0.5-msec pulses at 200 Hz for 300 msec) and monitored by recording from the DLM. Evoked seizure-like activity is observed as uncontrolled, high-frequency ($>$ 100 Hz) firing of DLM motorneurons. To assess GF neural circuit function, the GF was stimulated continuously with single-pulse electrical stimuli delivered to the brain (0.2-msec duration, 0.5 Hz). Recordings were obtained with Axoscope software and were analyzed with Axoscope and Stimfit software (Guzman *et al.* 2014). All flies tested were 2–3 days posteclosion.

Optogenetics

The experimental system for delivering high-intensity red light light-emitting diode (LED) stimulation with temporal control was adapted from previous descriptions (Pulver *et al.* 2011; De Vries and Clandinin 2013; Inagaki *et al.* 2014). The LED (627 nm Rebel, Luxeon Star LEDs) was used with optics (29.8° 10 mm Frosted optic, Carclo) and driven with a Buckpuck DC power converter (700 mA, Luxeon Star LEDs). Light intensity and duration were controlled with an electronic stimulator (S-900, Dagan). Heat sinks were utilized to dissipate excess heat (Luxeon Star LEDs: N50-25B). For behavioral experiments, flies were collected using CO_2 anesthesia and 10 flies were placed in each well of a four-well cell culture plate used as a behavioral chamber. Flies were 3 days posteclosion at the time of testing. The behavioral chamber was covered by plastic food wrap to allow penetration of LED illumination and the plastic wrap was perforated to allow fly respiration. After preparation, behavioral chambers were placed in the dark for fly recovery (2 hr). For testing, the behavioral chamber was placed under high-power LEDs mounted on heat sinks. The distance between the behavioral chamber and LED was 1 cm and, during testing, red light illumination was applied continuously for 5 sec. For experiments utilizing photostimulation and electrical recording, flies were mounted for electrophysiology with recording and ground electrodes inserted in the DLMs and abdomen, respectively. The LED was placed 1.5 cm above the fly and triggered by the pulse generator for 500 msec. For stimulation frequency variation, the pulse generator triggered the LED at 1, 10, and 100 Hz frequencies at 50% duty cycle for 1 sec.

Data availability

Drosophila strains are available upon request. All data generated for this study are included in the main text and figures.

Results

ReaChR drives light-sensitive (LS) paralytic behavior in BS mutants

The pan-neuronal driver *elav^{c155}-GAL4* was used to drive *UAS-ReaChR* expression in all *para^{bss1}* neurons (genotype: *elav^{c155} para^{bss1}*/Y; *UAS-ReaChR/+*). In the absence of mechanical or optical stimulation, flies double mutant for *para^{bss1}* and *ReaChR* showed no salient behavior abnormalities: feeding, grooming, mating, and locomotion appeared normal. The presence of *ReaChR* has no apparent effect on the *para^{bss1}* BS paralytic phenotype elicited by mechanical stimulation. A brief mechanical shock given to *elav^{c155} para^{bss1}*/Y; *UAS-ReaChR/+* flies continues to cause characteristic BS paralysis, with 100% of flies paralyzed. When stimulated with red light (5 sec), flies exhibit paralytic behavior (LS paralysis). This is due to *ReaChR* activation of *para^{bss1}* neurons because it is not observed in control flies lacking *UAS-ReaChR* (genotype: *elav^{C155} para^{bss1}/Y*; Figure 1E). Also, LS paralysis did not occur in flies lacking *para^{bss1}* (genotype: *elav^{c155}*/Y; *UAS-ReaChR/+*), flies that were not fed retinal, and flies using *ChiEF* as opsin illuminated with blue light (genotype: *elav^{c155} para^{bss1}/Y*; *UAS-ChiEF/+*) (data not shown). Thus, the higher penetrance of red light for *ReaChR* activation and the *para^{bss1}* defect in neurons are both essential in the generation of LS paralytic behavior.

LS paralysis in *elav^{c155} para^{bss1}*/Y; *UAS-ReaChR/+* flies resembles BS paralysis in most aspects. Optical stimulation causes 100% of flies to be paralyzed ($n = 82$, Figure 1, B and E). Paralysis occurs immediately following illumination, and flies remain immobile for 103 $\pm$ 4.04 sec (mean $\pm$ SEM, $n = 40$). LS paralysis is followed by behavioral seizure-like activity (recovery seizure), and flies recover shortly thereafter. These are prominent features of BS paralytic behavior in *para^{bss1}* mutants recapitulated by *ReaChR* optogenetics. However, there are some small differences in the details between LS paralysis for *para^{bss1}* observed here and BS paralysis reported previously (Parker *et al.* 2011). Mainly, initial BS paralysis in *para^{bss1}* homozygotes is followed by an extended period of tonic-clonic activity. During this period, the fly is usually quiescent (tonic phase), but this is broken up by multiple bouts of clonus-like activity. Tonic-clonic activity in *para^{bss1}* prolongs the time to recovery to $\approx$ 240 sec (Parker *et al.* 2011). For LS paralysis in *para^{bss1}*, tonic-clonic activity is not observed and, thus, resembles *para^{bss1}/+* heterozygotes ($\approx$ 50 sec recovery) and other BS mutants ($\approx$ 80 and $\approx$ 40 sec recovery for *eas* and *sda*, respectively), which all lack clonus-like activity during BS paralysis (Parker *et al.* 2011).

ReaChR also drives LS paralysis in BS mutants other than *para^{bss1}*, for example in an *eas* background (genotype: *elav^{c155} eas/Y*; *UAS-ReaChR/+)*. Optical stimulation (5 sec) also caused complete LS paralysis in these flies (100% LS paralysis, $n = 45$, Figure 1E). Thus, optogenetics using *ReaChR* is an excellent method for studying behavioral abnormalities in freely moving BS flies. LS avoids the massive physical disturbance in these flies ordinarily generated by mechanical BS stimulation via a vortex mixer operating at maximum velocity.

ReaChR elicits seizure-like electrical activity in BS mutants

A prominent feature of *Drosophila* seizure-sensitivity is seizure-like neuronal firing evoked at low threshold by electrical HFS delivered to the brain of BS mutants (Kuebler and Tanouye 2000; Lee and Wu 2002; Parker *et al.* 2011). We show here that similar seizure-like activity is elicited by photostimulation of *ReaChR* in *para^{bss1}* neurons. Seizure-like LS activity was recorded from the DLM of *elav^{c155} para^{bss1}*/Y; *UAS-ReaChR/+* flies during optical stimulation (Figure 1F). Seizure-like activity following LS (5- sec light pulses) consisted of aberrant high-frequency DLM motorneuron firing ($>$ 100 Hz) lasting 8–10 sec. This initial seizure-like activity resembles firing evoked by HFS previously called "initial seizure" or "initial discharge" (Figure 1, C and F and Figure 2; Kuebler and Tanouye 2000; Lee and Wu 2002; Parker *et al.* 2011).

After initial seizure, the next aspect of the LS phenotype is a quiescent period that lasts $\approx$ 25 sec (Figure 1, C and F and Figure 2). This quiescent period resembles a similar HFS phenotype due to transmission failure at many central synapses (Pavlidis and Tanouye 1995). The period of synaptic failure varies among flies of different genotype and age: for example, $\approx$ 38 sec for *sda* mutants and for young and old *para^{bss1}* mutants $\approx$ 45 and 75 sec, respectively (Parker *et al.* 2011). Synaptic failure is thought to be responsible for BS behavioral paralysis, and we infer a similar failure is responsible for LS paralysis (Pavlidis and Tanouye 1995). The final aspect of the patterned electrophysiology phenotype is a prominent recovery seizure observed after both HFS and LS stimulation (Figure 1, C and F and Figure 2; Parker *et al.* 2011).

The patterned DLM electrical activity described here for LS and HFS-induced seizures in *para^{bss1}* mutants differs from seizures seen in some other mutant flies. Most notably, seizure-like DLM activity is observed in *shi^{ts}* and *cac^{TS2}* mutants following a shift to high temperatures (Salkoff and Kelly 1978; Rieckhof *et al.* 2003; Kroll *et al.* 2015; Saras and Tanouye 2016). However, for these mutants, the seizure-like activity does not appear to be patterned into distinct periods of initial seizure, quiescence, and recovery seizure. Temperature-induced seizure-like activity appears to manifest as continuous high-frequency firing of the DLM motorneurons, with occasional interruptions in firing. This appears to decrease in amplitude until complete failure of synaptic transmission at the neuromuscular junction (Siddiqui and Benzer 1976; Koenig and Ikeda 1989; Kawasaki *et al.* 2000).

Long light pulses induce seizure-like activity

Seizure-like activity evoked by electrical HFS shows frequency dependence in stimulus efficacy (Kuebler and Tanouye

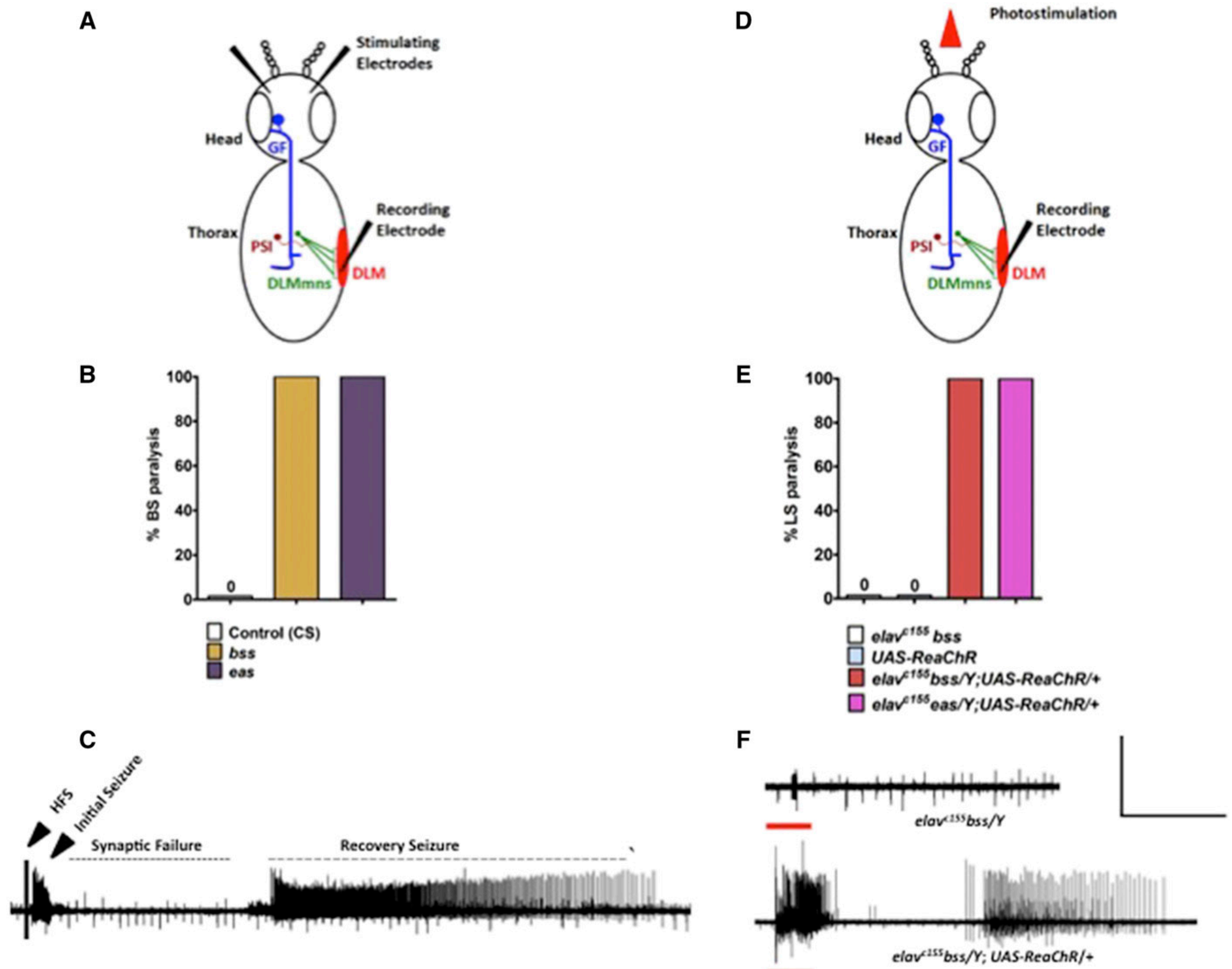

Figure 1 Generation of SLA in BS mutants by optogenetics. (A) Schematic diagram of stimulation and recording model of the GF circuit in *Drosophila*. Stimulation of the brain by electrical HFS activates the GF, which electrically synapses with the PSI. The PSI forms chemical synapses with five mns of the contralateral DLMs, also known as indirect flight muscles. (B) Bang-sensitivity seen in $para^{bss1}$ and *eas* BS mutants after mechanical shock. (C) Recording from a $para^{bss1}$ BS mutant DLM fiber following delivery of a 4 V HFS stimulus that is effective in evoking SLA. (D) Schematic diagram of stimulation and recording model of the GF circuit. Only the recording electrode is inserted in the DLM. (E) LS paralysis behavior observed in $elav^{c155}para^{bss1}$/Y;;UAS-ReaChR/+ and $elav^{c155}$ eas/Y;;UAS-ReaChR/+ double mutants. (F) Upper trace: photostimulation in control $elav^{c155}$bss/Y flies did not evoke any SLA. Lower trace: light-induced seizure showed characteristic initial seizure, synaptic failure, and recovery seizure, similar to the seizure induced by HFS. The red bar represents the time duration of photostimulation. (C and F) Horizontal calibration is 10 sec and vertical calibration is 50 mV. BS, bang-sensitive; CS, control; DLMs, dorsal longitudinal muscles; GF, giant fiber; HFS, high-frequency stimuli; LS, light sensitive; mns, motorneurons; PSI, peripherally synapsing neurons; SLA, seizure-like activity.

2000). Single-pulse electrical stimulations (0.2-msec duration) are never effective in evoking seizure-like activity. Decreasing the stimulus pulse frequency within an HF wavetrain from 200 to 100 to 50 Hz decreased the likelihood of evoking a seizure event (Kuebler and Tanouye 2000). It was proposed previously that electrical stimulation in the HFS wavetrain drives populations of neurons synchronously and that temporal synaptic summation occurs in the underlying excitatory seizure circuits; this summation is not as effective at lower stimulation frequencies (Kuebler and Tanouye 2000).

We investigated the electrophysiology of seizure-like activity in $elav^{c155}$ $para^{bss1}$/Y; UAS-ReaChR/+ flies with different frequencies of red light stimulation. We tested 1, 10, and 100 Hz frequencies, along with continuous red light exposure. The $elav^{c155}$ $para^{bss1}$/Y; UAS-ReaChR/+ double mutants were exposed to continuous red light exposure to 1, 10, and 100 Hz light stimulation frequency. There were no apparent differences in seizure induction by any of these different stimulation frequency treatments (Figure 2). However, we found that a minimum light exposure was necessary to induce seizure-like activity; light pulses with durations shorter than

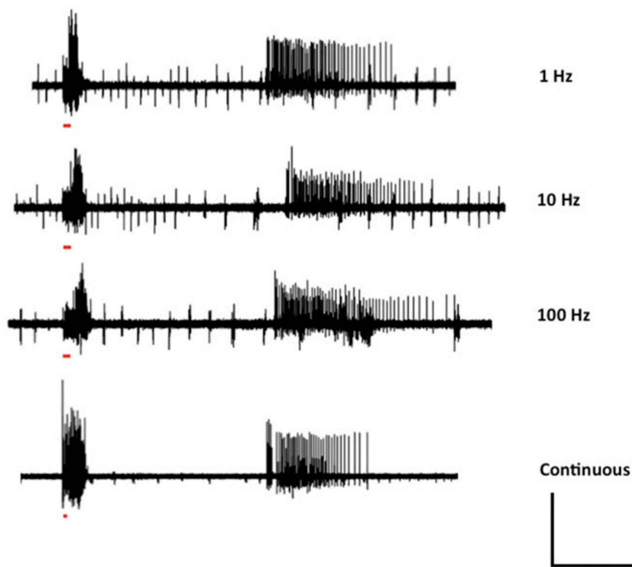

Figure 2 Optimization of photostimulation frequency. Maximum intensity of red light for 500 msec with 50% duty cycle of frequency range from 1 to 100 Hz was delivered to *elav^{c155} para^{bss1}/y; UAS-ReaChR/+* double mutants. There was not any significant difference in seizure-like electrical activity in double mutants at different frequency compared to continuous red light photostimulation. The red bar represents the time duration of photostimulation. In the continuous photostimulation trace, the red bar is smaller than the other frequency traces as in this case we applied continuous photostimulation with 100% duty cycle. Horizontal calibration is 10 sec and vertical calibration is 100 mV.

500 msec were not reliable at seizure induction. These optogenetic results presented here suggest that synchronous driving of neuronal populations may not be as important as previously believed (Kuebler and Tanouye 2000), although some form of summation may be occurring via *UAS-ReaChR* membrane depolarization.

A role for the Drosophila mushroom body in seizure induction

The use of *UAS-ReaChR* with GAL4 drivers of different spatial expression patterns provides a powerful way to identify brain regions contributing to initiation of *Drosophila* seizures. Here, we present an initial example of investigation: evidence for an MB role in seizure genesis. *UAS-ReachR* was driven using several brain-specific GAL4 drivers. The *a307-GAL4* driver, specific for the adult GF system (Allen *et al.* 2006), did not elicit LS paralytic behavior via *UAS-ReaChR* (genotype: *para^{bss1}/Y; UAS-ReaChR/a307-GAL4*). Thus, within the limitations of using a single GAL4 driver, it appears that activation of the GF system alone via *UAS-ReaChR* may not be capable of driving seizure-like activity. Two other GAL4 drivers also failed to elicit LS paralytic behavior via *UAS-ReaChR* in a *para^{bss1}* background: *104Y-GAL4* and *c232-GAL4* that drive UAS expression in the fan-shaped body and the ellipsoid body of the central complex, respectively (Young and Armstrong 2009). *UAS-ReaChR* expression in excitatory interneurons by *Cha-GAL4* (genotype: *para^{bss1}/Y; UAS-ReaChR/Cha-GAL4*) was positive for eliciting LS behavioral paralysis by photostimulation. In contrast, *UAS-ReaChR* expression in inhibitory interneurons by *Gad1-GAL4* failed to elicit LS behavioral paralysis.

Interestingly, when *UAS-ReaChR* was driven using the MB driver *c739-GAL4* in a *para^{bss1}* background (genotype: *para^{bss1}/Y; UAS-ReaChR/c739-GAL4*), photostimulation elicited LS paralytic behavior in 78% of animals tested (n = 36, Figure 3A). Electrophysiology recordings showed that photostimulation elicited seizure-like activity in all flies tested (n = 5, Figure 3B). A second MB driver, *117Y-GAL4*, gave similar results (genotype: *para^{bss1}/Y; UAS-ReaChR/117Y-GAL4*). LS paralytic behavior was observed in 90% of flies tested (n = 64, Figure 3A); electrophysiology showed seizure-like activity induced by photostimulation (n = 5). Taken together, these positive results utilizing two different MB GAL4 drivers indicate that the MB appears sufficient to initiate seizures in *para^{bss1}* mutants by *ReaChR*.

Identification of αβ lobe as the locus for MB seizure induction

The MB is composed of different lobes: αβ, α'β', and γ. Since different GAL4 drivers are capable of distinguishing these lobes, this allows the possibility of refining the MB localization for seizure initiation. Here, we find evidence for a MB αβ role in seizure genesis. *UAS-ReaChR* was driven with the MB αβ driver *7B-GAL4* in a *para^{bss1}* background (genotype: *para^{bss1}/Y; UAS-ReaChR/7B-GAL4*). Photostimulation elicited LS paralytic behavior in 85% of flies tested (n = 41, Figure 3C). Electrophysiology recordings showed that photostimulation elicited seizure-like activity in all flies tested (n = 5, Figure 3D). A second MB αβ driver, *121Y-GAL4*, gave similar results (genotype: *para^{bss1}/Y; UAS-ReaChR/121Y-GAL4*). LS paralytic behavior was observed in 82% of flies tested (n = 46, Figure 3C). Electrophysiology recordings showed seizure-like activity induced by photostimulation (n = 5, Figure 3D). Taken together, these positive results utilizing two different MB αβ drivers indicate that the αβ lobe appears sufficient to initiate seizures in *para^{bss1}* mutants by *ReaChR*.

We tested two additional MB GAL4 drivers that did not induce LS paralysis behavior via *UAS-ReaChR*. LS paralytic behavior was not observed using *c305a-GAL4* (genotype: *para^{bss1}/Y; UAS-ReaChR/c305a-GAL4*, 0% LS paralysis, n = 44, Figure 3C) and *1471-GAL4* (genotype: *para^{bss1}/Y; UAS-ReaChR/1471-GAL4*, 0% LS paralysis, n = 59, Figure 3C). Thus, we can conclude that not all MB GAL4 drivers are capable of driving LS paralytic behavior in *para^{bss1}* via *ReaChR*. However, from negative results, we are not able to make further conclusions. It may be that *c305a-GAL4* and *1471-GAL4* drive lower *ReaChR* expression levels than the other drivers utilized in this study. It could be that the failure to drive seizures comes from differences in neuroanatomy or connectivity; *c305a-GAL4* and *1471-GAL4* are specific for the MB α'β' lobe and the γ lobe, respectively. Resolution of these

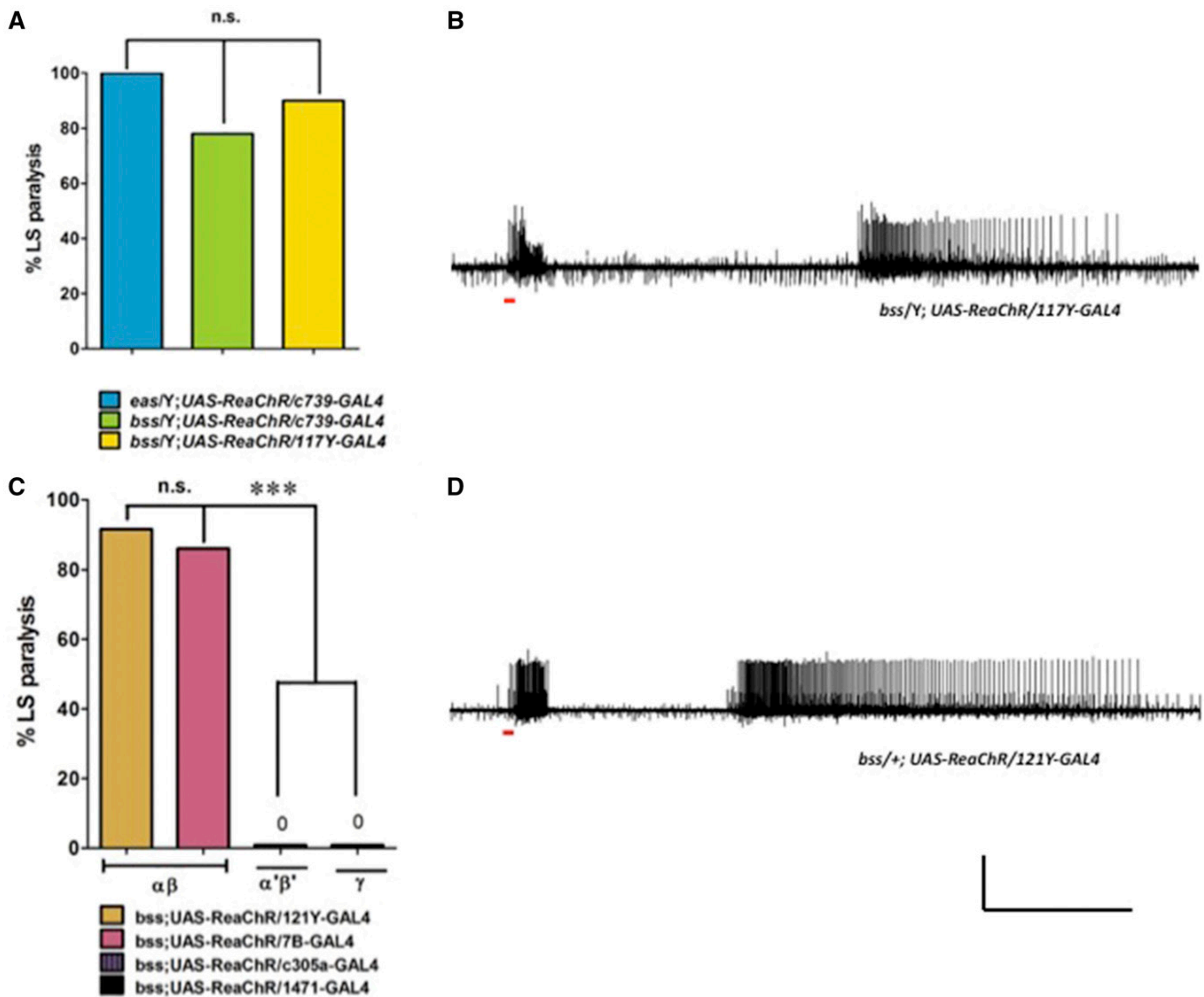

Figure 3 Characterization of MB and its αβ lobe as a seizure initiation site in *Drosophila* brain. (A) Quantification of LS paralysis behavior elicited in MB by expression of *UAS-ReaChR*, using different MB-GAL4 drivers. (B) Expression of *UAS-ReaChR* in MB by *117Y-GAL4* driver and subsequent optical stimulation by red light-elicited seizures in BS mutants. (C) Quantification of LS paralysis behavior by different MB lobe GAL4 drivers. Neither α′β′ nor γ lobe GAL4 drivers produced any LS paralysis behavior. (D) Expression of *UAS-ReaChR* in MB αβ lobe using *121Y-GAL4* is sufficient to trigger seizures by light stimulation. (B and D) Horizontal calibration is 10 sec and vertical calibration is 50 mV. BS, bang-sensitive; LS, light-sensitive, MB, mushroom body.

issues awaits further analyses with additional drivers and/or positive findings.

Discussion

Channelrhodopsin2 (ChR2) has been used previously for optogenetic stimulation in *Drosophila* third instar larvae. (Schroll *et al.* 2006; Zhang *et al.* 2007; Pulver *et al.* 2009; Zimmermann *et al.* 2009). Blue light appears to penetrate the larval cuticle sufficiently for ChR2 activation. In adult flies, ChR2 use has been limited mainly to dissected nervous system preparations and the GF system (Lima and Miesenbock 2005; Zhang *et al.* 2007; Gordon and Scott 2009; Zimmermann *et al.* 2009; Pulver *et al.* 2011). Interestingly, a seizure-like response has been described for adult wild-type flies carrying four copies of *UAS-ChR2*, but not one or two copies (Zimmermann *et al.* 2009). We have previously been unsuccessful in generating seizure-like responses in BS mutants with *UAS-ChR2* and *UAS-Chief*.

ReaChR is activated by red light that penetrates the *Drosophila* adult cuticle more readily than blue light (Suh *et al.* 2007; Lin *et al.* 2013; Inagaki *et al.* 2014). Inagaki *et al.* (2014) demonstrated the advantages of *ReaChR* in an elegant study of male courtship behavior in freely moving flies. In the present study of *ReaChR* in BS mutants, overhead LED illumination of the entire animal causes LS behavioral paralysis in 100% of flies. The red light is apparently penetrating the adult cuticle and causing extensive activation of neurons

throughout the brain and thoracic ganglion. It is difficult to determine the actual extent of nervous system activation because the entire repertoire of LS behavioral phenotypes is observed with activation of only the MB; we expect that actual nervous system activation is more extensive. In future experiments, we will express *ReaChR* exclusively in the thoracic ganglion to determine if overhead illumination penetrates through the thoracic cuticle and indirect flight muscles to initiate seizure-like activity in the ventral nervous system. HFS electrical stimulation of the thoracic ganglion has previously been shown to evoke seizure-like activity (Kuebler and Tanouye 2000), but little is known about these thoracic seizures. An analysis would provide additional insight into mechanisms responsible for seizure initiation and spread from another locus in the *Drosophila* model.

Neurological dysfunction in BS mutants has been elicited in three ways: (1) mechanical BS stimulation, (2) HFS electrical stimulation, and now (3) *ReaChR* optogenetics. It had been presumed that electrophysiological phenotypes resembling those evoked by electrical HFS stimulation (initial seizure, synaptic failure, and recovery seizure) were responsible for the behavioral phenotypes caused by BS stimulation (seizure-like behavior, paralysis, and recovery seizure behavior) (Benzer 1971; Ganetzky and Wu 1982; Kuebler and Tanouye 2000; Lee and Wu 2002). This had been difficult to demonstrate directly because BS paralysis is a behavior seen in freely moving adult flies, whereas HFS electrical stimulation is on tethered flies mounted for electrophysiology. The relationship is made clearer in the present study using optogenetic stimulation. Remarkably, there are no salient differences among the phenotypes induced in the different ways. That is, BS paralytic behavior and LS paralytic behavior appear the same; both HFS and LS seizure-like electrophysiological activity appear nearly the same in their firing patterns. Taken together, these results confirm that seizure-like neuronal firing drives BS and LS paralytic behavioral phenotypes in BS flies.

We performed an initial analysis to map out brain locations where seizures initiate. *UAS-ReaChR* is an outstanding method for this type of mapping because specific spatial expression of opsin can be directed with different GAL4 drivers. We find that $para^{bss1}$ MB $\alpha\beta$ neurons are sufficient to initiate seizure-like activity when driven by *ReaChR*. The *Drosophila* MB is especially well-studied for its essential role in olfactory learning and memory (Heisenberg 2003; Keene and Waddell 2007; Berry *et al.* 2008; Waddell 2016). It is thought that the orderly arrangement of axons and neuropile of MB Kenyon cells facilitate learning and memory (Heisenberg 2003). In flies, this can provide in flies the type of anatomical substrate essential for seizures in mammals (Hauser and Hesdorfer 1990; Traub and Miles 1991). The MB was implicated previously in seizure initiation (Hekmat-Scafe *et al.* 2010). However, in another study, the MB was not markedly more seizure-sensitive than other brain regions (Rusan *et al.* 2014). Taking together from the combined results of these studies, we propose a model whereby the total amount of brain tissue that is BS mutant determines whether or not the nervous system is seizure-sensitive or not. For the seizure-sensitive brain, one site of seizure initiation is the $\alpha\beta$ lobe of the MB.

The optogenetic approaches described here with *UAS-ReaChR* add a powerful new tool for investigating the circuitry responsible for seizures in *Drosophila*. For seizure-like behavior associated with BS mutants, there are several circuit questions that need to be resolved. What is the neurocircuitry responsible for seizure initiation? Following initiation, what circuitry is responsible for spreading seizure activity throughout the nervous system? And most interestingly, what is the circuitry responsible for terminating seizures? Future experiments using GAL4 drivers specific for different brain regions will aid substantially in resolving these issues. Another valuable use for optogenetic stimulation is the potential to be developed into an effective assay for antiepileptic drug screening. It is desirable to utilize minimally intrusive LS stimulation of MB $\alpha\beta$ neurons in conducting screens, especially compared to the traumatic use of vortex mixing to provide mechanical BS stimulation. This will facilitate the testing of large numbers of compounds in high-throughput drug screens.

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