Seizure Suppression by Gain-of-Function *escargot* Mutations

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ABSTRACT

Suppressor mutations provide potentially powerful tools for examining mechanisms underlying neurological disorders and identifying novel targets for pharmacological intervention. Here we describe mutations that suppress seizures in a *Drosophila* model of human epilepsy. A screen utilizing the *Drosophila* *easily shocked* (*eas*) “epilepsy” mutant identified dominant suppressors of seizure sensitivity. Among several mutations identified, neuronal *escargot* (*esg*) reduced *eas* seizures almost 90%. The *esg* gene encodes a member of the snail family of transcription factors. Whereas *esg* is normally expressed in a limited number of neurons during a defined period of nervous system development, here normal *esg* was expressed in all neurons and throughout development. This greatly ameliorated both the electrophysiological and the behavioral epilepsy phenotypes of *eas*. Neuronal *esg* appears to act as a general seizure suppressor in the *Drosophila* epilepsy model as it reduces the susceptibility of several seizure-prone mutants. We observed that *esg* must be ectopically expressed during nervous system development to reduce seizure susceptibility in adults. Furthermore, induction of *esg* in a small subset of neurons (interneurons) will reduce seizure susceptibility. A combination of microarray and computational analyses revealed 100 genes that represent possible targets of neuronal *esg*. We anticipate that some of these genes may ultimately serve as targets for novel antiepileptic drugs.

HUMAN seizure disorders are a significant health concern due to the large number of affected individuals, the potentially devastating ramifications of untreated seizure episodes, and the limitations of antiepileptic drug (AED) options. Seizures are caused by insults to the brain such as head injury, electroconvulsive shock, illness, or fever and are manifest as abnormal, high-frequency, rhythmic firing of neuronal populations in the brain (WALTON 1989). Seizure susceptibility varies considerably among individuals: some individuals have high seizure thresholds and exhibit seizures only after considerable brain injury, whereas other individuals are especially sensitive and exhibit the spontaneous, recurrent seizures that constitute epilepsy (SACKeim *et al.* 1987; WALTON 1989). An estimated 1–2% of the world’s population is affected by epilepsy (McNAMARA 1999). Epilepsy is most commonly treated by AEDs that provide symptomatic relief for a majority of epileptic patients. However, one-third of patients with chronic epilepsy do not respond to any currently available AED, and many responders continue to experience breakthrough seizures (SCHMIDT 2002). Most AEDs work by reducing nervous system excitability and can produce toxic side

effects (BRODIE and DICHTER 1996; McNAMARA 1999). This is a particular problem in children where adverse cognitive and behavioral consequences have been attributed to AEDs (LEVENE 2002).

Seizure-suppressor genes provide a potentially powerful tool for examining seizure disorders and identifying potential AED targets. Surprisingly, the basic approach of utilizing second-site suppressor mutations has not been extensively exploited for neurological syndromes. The general approach is straightforward: starting with an animal genetic model of neurological syndrome, second-site suppressor mutations are evaluated for their ability to revert the phenotype to wild type. Suppressor mutations are used to identify genes that may provide novel insights into the causes and cures of the syndrome. They can also indicate new targets for pharmacological intervention. Suppressor mutations may be identified by reverse genetics, testing likely candidates by double-mutant analysis as described previously (KUEBLER *et al.* 2001). Additionally, forward genetics mutant screens may be used to discover totally unexpected and novel suppressors as described in this article. Suppressor mutations may be identified in mouse or *Caenorhabditis elegans* models of human neurological syndromes, but *Drosophila* models are especially attractive because of the combination of electrophysiology, behavior, genetics, and mass mutagenesis methods available (BENZER 1971; ASHBURNER 1989; PAVLIDIS and TANOUYE 1995).

Seizure-suppressor genes are identified by mutations

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that can revert the seizure phenotype in a *Drosophila* model of epilepsy (KUEBLER *et al.* 2001). The model is based on a collection of mutants that have been found to be especially sensitive to seizures (5–10 times more seizure sensitive than wild-type flies; KUEBLER and TANOUYE 2000; KUEBLER *et al.* 2001). Seizures are induced by electroconvulsive shock using high-frequency (HF) electrical stimulation (200 Hz stimuli, 300 msec wave train) delivered to the brain of *Drosophila*. Wild-type flies have a characteristic seizure threshold for HF stimulation (30.1 ± 3.8 V). Thresholds for seizure-sensitive mutants are considerably lower, typically 3–8 V for the most sensitive strains (KUEBLER *et al.* 2001). Seizure-sensitive mutants include: *easily shocked* (*eas*), which encodes an ethanolamine kinase involved in synthesis of phosphatidyl ethanolamine found in neuronal membranes; *slam-dance* (*sda*), which encodes an aminopeptidase involved in the processing of some neuropeptides; *technical knock-out* (*tko*), which encodes a mitochondrial ribosomal protein; *jitterbug* (*jbug*), which encodes a *Drosophila* homolog of filamin, implicated in periventricular heterotopia that presents with epilepsy in humans; *bangsenseless* (*bss*), and *knockdown* (*kdn*) (ROYDEN *et al.* 1987; PAVLIDIS *et al.* 1994; ZHANG *et al.* 2002; X. REN and M. TANOUYE, unpublished results).

KUEBLER *et al.* (2001) showed that seizure sensitivity can be suppressed by certain *Drosophila* mutations in double-mutant combinations. Loss-of-function mutations that acted as seizure suppressors were identified for several genes whose products had been previously known to influence nervous system excitability. Thus, mutations that affect voltage-gated Na^+ channels, such as *paralytic* (*para*) and *maleless-no-action-potential* (*mle^{naps}*; LOUGHNEY *et al.* 1989; RAMASWAMI and TANOUYE 1989; REENAN *et al.* 2000) are good seizure-suppressor mutations. Other seizure suppressors are *Shaker* (*Sh*), which encodes a voltage-gated K^+ channel (BAUMANN *et al.* 1987; KAMB *et al.* 1987; TEMPEL *et al.* 1987), and *shaking-B* (*shak-B*), which encodes a gap junction channel (KRISNAN *et al.* 1993; CROMPTON *et al.* 1995). These seizure-suppressor mutations, in double mutant combinations with seizure-sensitive mutations, raise seizure threshold often to wild-type levels (KUEBLER *et al.* 2001).

Here, we describe a mutant screen that allowed us to identify novel gain-of-function seizure suppressors. The screen was based on reversion of the seizure-sensitivity phenotype of *eas* mutants by ectopic expression of single genes in all neurons. Of several seizure-suppressor genes identified, we focus on *escargot* (*esg*), the strongest of the suppressors and a gene not previously implicated in seizure susceptibility. The *esg* gene is a member of the snail family of transcription factors that are required for mesoderm and nervous system development in arthropods and chordates (MANZANARES *et al.* 2001). *Drosophila* has three snail family genes, *esg*, *snail* (*sna*), and *wormiu* (*wor*), that are tightly linked at cytological region 35D and appear to bind to the promoters of an overlap-

ping set of target genes whose transcription they repress (ASHRAF *et al.* 1999). The *esg* protein contains five Zn^{2+} -finger DNA-binding domains and recognizes the DNA motif A/GCAGGTG (FUSE *et al.* 1996; ASHRAF *et al.* 1999; CAI *et al.* 2001). The *esg* protein also possesses a P-DLS-K domain, which may bind the dCtBP corepressor (ASHRAF *et al.* 1999; ASHRAF and Ip 2001). The *esg* gene is normally expressed in embryonic neuroblasts and contributes to central nervous system (CNS) development (WHITELEY *et al.* 1992; ASHRAF *et al.* 1999). The gene shows functional redundancy with *sna* and *wor*: a loss-of-function mutation in *esg* alone has no effect on the CNS. However, a deletion removing all three genes causes defects in dividing neuroblasts that ultimately leads to a severely underdeveloped ventral nerve cord and the loss of multiple neuronal markers (ASHRAF *et al.* 1999).

We report that ectopic neuronal expression of *esg* acts as a general seizure suppressor. Expression of *esg* in all neurons suppresses a variety of seizure-sensitive mutants phenotypically and raises seizure threshold at the electrophysiological level. Developmental time-course experiments reveal that *esg* must be ectopically expressed during nervous system development to produce adults that are less seizure prone. Microarray analysis revealed 100 genes whose transcription is induced or repressed by neuronal *esg*. We discuss the possible utility of a dominant, gain-of-function seizure suppressor such as neuronal *esg* for developing novel treatments for epilepsy.

MATERIALS AND METHODS

Fly stocks: A list of *Drosophila* stocks used in this study is given in Table 1. Stocks were maintained on standard cornmeal-molasses medium at room temperature ($\sim 22^\circ$). Crosses were performed at 25° unless otherwise specified. Three bang-sensitive (BS) mutations, *eas*, *bss*, and *sda*, are included. The *eas* gene is located at cytological region 14B and encodes an ethanolamine kinase (PAVLIDIS *et al.* 1994). The recessive *eas^{PC80}* allele carries a frameshift mutation and probably constitutes a null allele. The *bss* gene is located at 1-54.6 (corresponding to approximately cytological region 12F); its gene product has not been described (GANETZKY and WU 1982). The *bss¹* allele is a semidominant mutation. The *sda* gene is located at 97D and encodes an aminopeptidase (ZHANG *et al.* 2002). The recessive *sda^{iso7.8}* mutation occurs in the 5'-noncoding region and greatly diminishes the level of *sda* transcript. The genotypes *w eas^{PC80} f*, *w bss¹ f*, and *w sda^{iso7.8}* are abbreviated as *eas*, *bss*, and *sda*, respectively, in the text. The *elav-GAL4^{3A}* strain was generated by Aaron Di Antonio (UC-Berkeley) by remobilizing the C155 *elav-GAL4* enhancer trap insert to a new position on the third chromosome; D241 was provided by Julie Simpson. GeneSwitch is a conditional, RU486-dependent GAL4-progesterone fusion protein. *elav-GeneSwitch* is the insertion of an *elav-GeneSwitch* expression construct, pP[ELAV-GeneSwitch], into the third chromosome (OSTERWALDER *et al.* 2001) and was provided by Tom Osterwalder and Haig Keshishian (Yale University). The Enhancer P stock collection has been described previously (RØRTH 1996). We obtained the 1878 EP(2) and EP(3) lines screened in this study from the Szeged *Drosophila* Stock Center in Hungary. When the *CyO*

TABLE 1
Drosophila stocks

Stock no.	Genotype
D502	<i>w eas^{PC80} f; elav-GAL4^{3A}</i>
D241	<i>w; elav-GAL4^{3A}</i>
MR047	<i>w eas^{PC80} f</i>
MR068	<i>w bss¹ f</i>
U036	<i>w; SM5 Cy; TM3/ap^{Xa}</i>
D547	<i>w; SM5 Cy; sda^{iso7.8}/ap^{Xa}</i>
D512	<i>w; elav-GAL4^{3A} sda^{iso7.8}</i>
D510	<i>w bss¹ f; elav-GAL4^{3A}</i>
EP633	<i>w; esg^{EP633}/CyO</i>
EP2009	<i>w; esg^{EP2009}/CyO</i>
EP684	<i>w; esg^{EP684}/CyO</i>
EP2408	<i>w; esg^{EP2408}/CyO</i>
EP2159	<i>w; esg^{EP2159}/CyO</i>
EP683	<i>w; esg^{EP683}/CyO</i>
D544	<i>w; UAS-esg(II)/CyO</i>
D546	<i>w; UAS-sna#61/TM6B</i>
3900	<i>b¹ esg^{35C-1} l(2)CA61^{VS} pr¹ cn¹ bw¹/SM5 Cy</i>
3078	<i>Df(2L)osp29, Adh^{UF} osp²⁹ pr¹ cn¹/CyO</i>
D533	<i>w eas^{PC80} f; esg^{EP2009}/SM5 Cy; elav-GeneSwitch</i>
D552	<i>w eas^{PC80} f; esg^{EP684}/CyO</i>
D255	<i>w; Cha-GAL4</i>
D257	<i>w; OK6-GAL4</i>
D258	<i>w; G14-GAL4/CyO</i>

second chromosome balancer had been lost from a stock of interest, a balanced EP(2) line was regenerated by crossing the *w; EP(2)* males to females from a *w; noc^{Scn}/CyO* stock (obtained from Todd Laverty, UC-Berkeley) and, subsequently, crossing *w; EP(2)/CyO* male and female progeny of this cross to establish a balanced stock. The *UAS-esg(II)* and *UAS-sna#61* insertions (FUSE *et al.* 1999) were obtained from Shigeo Hayashi and are referred to in the text as *UAS-esg* and *UAS-sna*. D544 was produced by crossing the *w y; UAS-esg* males to *w; noc^{Scn}/CyO* females and, subsequently, crossing *w; UAS-esg/CyO* male and *y w/w; UAS-esg/CyO* female progeny of this cross and, finally, crossing a few *w; UAS-esg/CyO* progeny of this second cross to establish a balanced stock. D546 was produced by crossing the *w; UAS-sna* males to *w; TM3/TM6B* females and, subsequently, crossing *w; UAS-sna/TM6B* male and female progeny of this cross to establish a balanced stock. The *esg^{35C-1}* allele is a loss-of-function mutation in the *esg* gene, whereas *Df(2L)osp29* is a deletion that removes all three snail family genes (*esg*, *wor*, and *sna*) in cytological region 35D. Stocks 3900 and 3078 were obtained from the Bloomington *Drosophila* Stock Center. The *Cha-GAL4*, *OK-6-GAL4*, and *G14-GAL4* drivers were obtained from Brian McCabe (UC-Berkeley). In larvae, the *Cha-GAL4* enhancer trap drives expression in interneurons (SALVATERRA and KITAMOTO 2001); *OK6-GAL4* drives it in all motoneurons, salivary glands, wing discs, and a subset of tracheal branches (ABERLE *et al.* 2002), and *G14-GAL4* drives expression in all somatic muscles and salivary glands (ABERLE *et al.* 2002). All three insertions are located on the second chromosome; the *G14-GAL4* insertion is homozygous lethal. D255, D257, and D258 were produced by independently crossing the *w; Driver-GAL4* males to *w; noc^{Scn}/CyO* females and, subsequently, crossing the corresponding *w; Driver-GAL4/CyO* male and female progeny of this cross to establish a balanced stock.

Behavioral testing: Testing for BS paralysis was performed on flies 1–2 days posteclosion unless otherwise specified. Flies

were anesthetized with CO₂ prior to collection and tested the following day. Approximately 15 flies were transferred to a clean vial (Applied Scientific) without agitation and immediately vortexed at maximum speed for 10 sec. Bang-sensitive flies displayed a period of paralysis followed by a period of hyperactivity. The chi-square test was used to determine the *P*-values for differences in percentage bang sensitivity between test flies and their sibling controls.

Genetic analysis: In the initial screen for gain-of-function *eas* suppressors, each of the 1878 EP(2) and EP(3) lines (RØRTH 1996) were independently crossed to D502 females. The resulting unbalanced male progeny would have the genotype *w eas f; EP(2)/+; elav-GAL4^{3A}* or *w eas f; EP(3)/elav-GAL4^{3A}* depending on whether the EP insertion was on the second or third chromosome. These *eas* males would be expected to be bang sensitive unless the neuronally expressed EP gene acted as a dominant *eas* suppressor. Our initial screen revealed 42 lines with potential *elav-GAL4*-activated *eas* suppressors. The screen also identified two other classes of genetic interaction between *eas* and the EP lines: synthetic lethals (5 lines) and *eas* phenotypic enhancers (6 lines); descriptions of these will be presented elsewhere. We then repeated the test crosses of the 42 putative EP suppressor strains to D502 and this time tested both the unbalanced and the balanced male progeny. In 25 cases, we consistently observed that some of the unbalanced male progeny were not bang sensitive. However, for 8 lines similar numbers of unbalanced male progeny (carrying the EP insertion) and balanced male progeny (lacking the EP insertion) failed to show the bang-sensitive behavioral phenotype, indicating that the suppression was not linked to the EP insertion. We then crossed the remaining 17 lines to MR047 and tested unbalanced male progeny (*w eas f; EP(2)/+ or w eas f; EP(3)/+*) for the bang-sensitive behavioral phenotype. In one line, some of the unbalanced male progeny were not bang sensitive, indicating that suppression was independent of *elav-GAL4*. Genomic sequences flanking the EP insertions and associated genes were obtained for the 16 remaining gain-of-function *eas* suppressors from FlyBase (<http://www.flybase.org/transposons/fbinsquery.hform>). To test for *esg^{EP}* suppression of *bss*, EP(2) males were crossed to D510 virgin females and balanced and unbalanced male and female progeny were tested for bang sensitivity. To test for *esg^{EP}* suppression of *sda*, three crosses were required. First, *w; esg^{EP}/CyO* males were independently crossed to U046 virgin females. Next, the resulting *w; esg^{EP}/SM5 Cy; +/TM3* male progeny were crossed to D547 virgin females. Finally, the resulting *w; esg^{EP}/SM5 Cy; sda/TM3* male progeny were crossed to D512 virgin females and the resulting non-TM3 progeny were tested for bang sensitivity. Analogous crosses were done to demonstrate *UAS-esg* suppression of *eas*, *bss*, and *sna* and *UAS-sna* suppression of *eas* and *bss*. To determine whether induction of *esg^{EP684}* expression in various neuronal subpopulations or in nonneuronal tissue suppressed *eas*, D552 virgin females were independently crossed to D255, D257, or D258 males and the balanced and unbalanced male progeny were tested for bang-sensitive paralysis.

Electrophysiology: Electrophysiology was performed on male progeny of crosses of EP(2) males and D502 or D241 females. Flies were tested 1–2 days posteclosion allowing at least 4 hr of recovery from CO₂ anesthesia. The procedures used to stimulate and record giant fiber (GF)-driven muscle potentials and seizures have been described previously (KUEBLER and TANOUYE 2000). Briefly, the fly was immobilized at both its head and its abdomen using separate 23-gauge needles attached to a vacuum line. A cyanoacrylate adhesive was then used to attach the fly to a mounting needle. Both single-pulse stimuli and HF wave trains were delivered to the brain using bipolar tungsten stimulating electrodes. Single-pulse stimuli

(0.5-msec duration, 0.8 Hz) were used to drive the GF, and the GF-driven muscle potentials were recorded from the dorsal longitudinal muscles (DLMs) using tungsten recording electrodes. The GF threshold was considered to be the lowest voltage at which the GF pathway responded to a single pulse stimulus. During the course of each experiment, the GF was stimulated continuously to assess GF system circuit function and flies were discarded if that function appeared compromised. Seizures consist of HF activity in at least seven different muscle groups and >30 muscle fibers in the thorax, reflecting the HF firing of the innervating motoneurons (KUEBLER and TANOUYE 2000). We attempted to elicit seizures by delivering short wave trains of HF electrical stimuli (0.5-msec pulses delivered at 200 Hz for 300 msec) to the brain via bipolar tungsten stimulating electrodes. The minimum intensity (voltage) of the HF stimulus required to elicit a seizure was designated the "seizure threshold." The two-tailed *t*-test was used to determine the *P*-values for differences in seizure threshold between test flies and their sibling controls.

RU486 induction: RU486-containing medium was prepared by adding 10 mg/ml RU486 to rich fly medium (BACKHAUS *et al.* 1984) to a final concentration of 5 µg/ml, as recommended by Thomas Osterwalder (Yale University). We activated the GeneSwitch GAL4 protein in embryos, larvae, and adults in a manner similar to the one described previously (OSTERWALDER *et al.* 2001). To activate GeneSwitch in embryos, mothers were grown on RU486-containing medium for 3 days and then allowed to lay eggs on standard medium for 1 day. GeneSwitch was activated in larvae by transferring females grown on standard medium to the RU486 medium for 1 day and allowing development to continue on the medium. GeneSwitch was activated in adults by transferring newly eclosed flies (<1 day old) to RU486-containing medium and allowing them to remain on the medium for 1–2 days prior to testing.

Microarray analysis: Test larvae expressing *esg* in all postmitotic neurons (genotype *w; esg^{EP2009}/+; elav-GAL4/+*) were produced by crossing D241 virgin females to unbalanced EP2009 males. Control larvae with no *esg* induction (genotype *w; esg^{EP683}/+; elav-GAL4/+*) were produced in parallel crosses by mating D241 virgin females to unbalanced EP683 males. Total RNA was prepared from test and control wandering third instar larvae using TRIzol reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Duplicate 15-µg aliquots of either test or control RNA were used to independently produce the corresponding double-stranded cDNA's via the SuperScript choice system (Invitrogen, Carlsbad, CA). These cDNA's were then used to produce the corresponding biotinylated cRNA's via the ENZO BioArray high-yield RNA transcript labeling kit (Affymetrix). The resulting biotinylated cRNA's were purified and fragmented to 35- to 200-base probe fragments using the GeneChip sample cleanup module (Affymetrix) according to the manufacturer's instructions. The GeneChip Drosophila genome array (Affymetrix) was hybridized with the test or control probes in parallel experiments. Hybridization, staining, washing, and scanning of the GeneChips were performed in the UC-Berkeley Microarray Facility according to the manufacturer's directions.

Analysis of the microarray data was performed using Microarray Suite, Version 5.0 (Affymetrix). Since test and control probes were independently prepared and Drosophila GeneChips were processed on two separate occasions, we obtained four data sets (Test1, Control1, Test2, and Control2). A comparison expression analysis was performed to produce four normalized data sets: Test1 normalized to Control1, Test1 normalized to Control2, Test2 normalized to Control1, and Test2 normalized to Control2. We concluded that a gene was differentially expressed in test and control larvae if we

obtained a consistent change of at least 1.5-fold in all four comparisons that was highly statistically significant (change *P*-value <10⁻³). This analysis revealed 72 genes with increased expression and 84 with decreased expression in the test larvae expressing neuronal *esg*.

The noncoding regions of all 156 genes displaying differential expression in test *vs.* control larvae were examined for the presence of repeated copies of the *esg*-binding motif (RCAGGTG) using the SeqSeek program (<http://flycompute.uoregon.edu/cgi-bin/seqseek.pl>; FREEMAN *et al.* 2003). The noncoding regions examined included 3000 bp upstream, 2000 bp downstream, and 7000 intronic bp closest to the start of each gene. We required a minimum of five *esg*-binding sites in 500 bp and included a degeneracy mask that accepted a single substitution in each motif. This computational analysis revealed that 47 of 72 upregulated genes and 53 of 84 downregulated genes carried repeated *esg* motifs in their noncoding regions. Each of these 100 genes represents a potential transcriptional target of *esg* in larval neurons.

RESULTS

Screen for *eas* suppressors reveals multiple *esg* alleles:

We performed a large-scale screen of Drosophila autosomal Enhancer *P*-element (EP) lines (RØRTH 1996) to identify genes that, when expressed in neurons, render Drosophila less prone to seizures. The screen involved examination of *eas* flies carrying one chromosomal copy of *elav-GAL4* and one copy of a particular UAS-bearing EP insertion (genotype *eas; UAS-EP/+; elav-GAL4/+*). The *eas* mutants are highly prone to seizures and have a low seizure threshold in electrophysiological tests (KUEBLER and TANOUYE 2000). They also display a behavioral bang-sensitive phenotype: in response to mechanical stimulation (a "bang"), 100% of *eas* mutants show behavioral paralysis (PAVLIDIS *et al.* 1994). As an initial screen for seizure sensitivity, we tested for reversal of the *eas* bang-sensitive behavioral phenotype. The *elav-GAL4* construct leads to neuronal expression of the GAL4 transcriptional activator (LIN and GOODMAN 1994). This drives expression of the EP-associated gene in all neurons. It was our expectation that high-level neuronal expression of some EP genes would render *eas* flies less prone to seizures, resulting in reduced bang sensitivity.

A screen of 1878 EP(2) and EP(3) insertions (RØRTH 1996) identified 25 strains that, in the presence of *elav-GAL4*, consistently reduced the bang sensitivity of *eas* flies. In eight of these lines, the suppressor was not linked to the EP insertion and, in one other, it was independent of *elav-GAL4*. The remaining 16 EP insertions produced partial (*i.e.*, incompletely penetrant) suppression of *eas* in conjunction with *elav-GAL4*. Table 2 shows the results for six of these EP lines. Line EP(2) 2589 shows moderate reversal of the *eas* bang-sensitive phenotype (78% bang sensitivity), but its *P*-insertion site at 52B is not immediately associated with an identified gene. Line EP(2)310 shows 84% bang sensitivity. The *P* insertion of this line is in the 3'-untranslated region of the *polyA-binding protein* (*pAbp*) gene. Poly(A)-binding

TABLE 2
Gain-of-function *eas* suppressors

EP line	% bang sensitivity		Map position	Gene	Molecular function
	Test (n)	Control (n)			
EP(2)684	4 (229)	100 (136)	35D	<i>escargot</i> (<i>esg</i>)	Zn ²⁺ -finger transcription factor
EP(2)2009	11 (171)	100 (253)	35D	<i>escargot</i> (<i>esg</i>)	Zn ²⁺ -finger transcription factor
EP(2)633	20 (163)	100 (194)	35D	<i>escargot</i> (<i>esg</i>)	Zn ²⁺ -finger transcription factor
EP(2)2589	78 (147)	100 (149)	52B	Unknown	NA
EP(2)310	84 (139)	100 (120)	55B	<i>polyA-binding protein</i> (<i>pAbp</i>)	RNA-binding protein
EP(2)2235	98 (179)	100 (175)	39A	β^v -integrin (β Int- v)	Heterophilic cell adhesion molecule

The results of a screen for Enhancer P (EP) mutations that suppress the behavioral bang-sensitive phenotype of *eas* are presented. The flies tested (Test) were males of the genotype *eas*; *EP*/+; *elav-GAL4*/+. Control flies of the genotype *eas*; +/CyO; *elav-GAL4*/+ were male siblings of the test flies. Test and control flies resulted from crosses between *eas*; *elav-GAL4* virgin females and *w*; *EP*/CyO males. The number of flies tested is shown for each EP line. Results for the five EP lines that produced a highly significant ($P < 0.001$) fraction of non-bang-sensitive test progeny are shown in the first five rows; results for an additional line that produced a small but significant ($P < 0.05$) fraction of non-bang-sensitive test progeny is shown in the last row. The cytological map position of each EP insertion is given. When known, the corresponding gene and its molecular function are noted. NA, not applicable.

protein is enriched at subsynaptic regions of the *Drosophila* larval neuromuscular junction (SIGRIST *et al.* 2000). Altering the level of *pAbp* expression has been shown to change the levels of postsynaptic proteins involved in neuronal activity and connectivity (SIGRIST *et al.* 2000). EP(2)2235 shows modest, but statistically significant, reversion of the *eas* bang-sensitivity phenotype. The *P*-element insertion of this line occurs in the 5'-untranslated region of the β^v -integrin gene (β Int- v), which encodes a heterophilic cell adhesion molecule. Expression of β Int- v ^{EP2235} under the control of *elav-GAL4* results in larvae with reduced or abnormal synapses and neuronal pathfinding defects (KRAUT *et al.* 2001).

The three strongest gain-of-function *eas* suppressors identified in our screen all carried *P* insertions in the same gene, *esg* (Table 2). Whereas the *eas* control flies (genotype *eas*; +/CyO; *elav-GAL4*/+) were completely bang sensitive (100% bang sensitivity), their *eas* siblings (genotype *eas*; *esg*^{EP}/+; *elav-GAL4*/+) showed marked reversion of the *eas* bang-sensitive phenotype. For EP(2)684, 4% of flies showed the bang-sensitive phenotype; for EP(2)2009, 11% of flies were bang sensitive; and for EP(2)633, 20% of flies were bang sensitive. The EP684, EP2009, and EP633 insertions all occur in the 5' upstream region of the *esg* gene (Figure 1) at -442, -333, and -460, respectively, from the translational start (ABDELILAH-SEYFRIEND *et al.* 2000).

Multiple bang-sensitive mutations are suppressed by neuronal *esg*: Additional genetic experiments revealed that *elav-GAL4*-activated *esg*^{EP} interacts with other BS mutations suggesting that it functions as a general seizure suppressor (Figure 2). Among BS mutations, *eas* is considered intermediate in terms of both the degree to which it reduces seizure threshold and the ease with which it can be suppressed by secondary mutations

(KUEBLER and TANOUYE 2000; KUEBLER *et al.* 2001). By these criteria, *sda* is a weaker BS mutation than *eas* whereas *bss* is a stronger one. As shown in Figure 2B, the bang-sensitive phenotype of *sda* was strongly suppressed by *elav-GAL4*-activated *esg*^{EP} (2, 4, and 11% bang sensitivity for EP684, EP2009, and EP633, respectively). In contrast, the *elav-GAL4* *sda* control siblings of these

FIGURE 1.—Screen for *eas* suppressors reveals multiple *escargot* alleles. The three strongest gain-of-function *eas* suppressors identified in our screen (EP684, EP2009, and EP633) all carry EP insertions in the same gene, *esg*. All three of these *esg*^{EP} insertions (solid triangles) occur in the 5'-flanking region of the *esg* gene. Two additional *esg*^{EP} insertions (shaded triangles) also show some suppression of bang sensitivity. One of these (EP2408) occurs in the 5'-flanking region of the *esg* gene, whereas the other (EP2159) is in the 5'-noncoding region of the *esg* transcript. A sixth *esg*^{EP} insertion (EP683, open triangle) does not show any suppression of bang sensitivity. The EP element in *esg*^{EP683} is located in the 5'-flanking region of *esg* but is inserted in the opposite orientation of the other five *esg*^{EP} insertions; *GAL4* induction of *esg*^{EP683} should not induce the level of *esg* transcript. The *escargot* ORF (large solid box) encodes a transcription factor with five Zn²⁺-finger DNA-binding domains (open stripes). The *escargot* protein also possesses a P-DLS-K domain (shaded stripe), which probably binds the dCtBP corepressor.

FIGURE 2.—Neuronal expression of *esg*^{EP} suppresses a variety of bang-sensitive mutants. Flies of different genotypes were examined for the bang-sensitive behavioral phenotype in response to a 10-sec mechanical bang. The data reveal that *elav-GAL4*-driven *esg*^{EP} acts as a general suppressor of bang-sensitive paralysis: several *esg*^{EP} alleles act as suppressors, and different bang-sensitive mutations are suppressed. (A) Ectopic expression of different *esg*^{EP} alleles suppresses the bang-sensitive behavioral phenotype of the *eas* mutation. In the case of *esg*^{EP684} (EP684, solid bar, genotype *eas*; *esg*^{EP684}/+; *elav-GAL4*/+), ectopic expression driven by *elav-GAL4* produces considerable suppression of the *eas* phenotype: only 4% of flies show paralysis compared with 100% of their sibling controls (open bar, genotype *eas*; +/CyO; *elav-GAL4*/+). Substantial suppression is also seen for the *esg*^{EP} alleles EP2009 and EP633 (11 and 20% bang sensitivity, respectively). For all genotypes, *n* > 135. (B) Ectopic expression of different *esg*^{EP} alleles also suppresses the bang-sensitive behavioral phenotype of the *sda* mutation. In the case of *esg*^{EP684} (EP684, solid bar, genotype *esg*^{EP684}/+; *elav-GAL4* *sda/sda*), ectopic expression driven by *elav-GAL4* produces marked suppression of the *sda* phenotype: only 2% are bang sensitive compared with 100% of the sibling control flies (open bars, genotype +/CyO; *elav-GAL4* *sda/sda*). Substantial suppression is also seen for the *esg*^{EP} alleles EP2009 and EP633 (4 and 11% bang sensitivity, respectively). For all genotypes, *n* > 135. (C) The behavioral phenotype of the *bss*/+ semidominant genotype is suppressed by neuronal expression of different *esg*^{EP} alleles. For *bss*/+ sibling control flies (open bars, genotype *bss*/+; +/CyO; *elav-GAL4*/+) >50% displayed bang sensitivity. Note that this behavioral response differs somewhat from others described in this article in that flies demonstrate paralysis, but not hyperactivity. This has sometimes been referred to as “stress sensitivity” (HOMYK 1977). In contrast, 0% bang-sensitive paralysis is observed in *bss*/+ siblings carrying *esg*^{EP684} (solid bars, genotype *bss*/+; *esg*^{EP684}/+; *elav-GAL4*/+). Substantial suppression is also seen for the *esg*^{EP} alleles EP2009 and EP633 (5 and 10% bang sensitivity, respectively). For all genotypes, *n* > 95.

TABLE 3

Neuronal induction of *esg* suppresses *sda*

<i>esg</i> construct	% bang sensitivity		<i>P</i> -value
	Test (<i>n</i>)	Control (<i>n</i>)	
EP684	2 (178)	100 (485)	7.7×10^{-141}
EP2009	4 (300)	100 (255)	3.9×10^{-112}
EP633	11 (135)	100 (137)	1.3×10^{-48}
EP2408	19 (101)	100 (198)	2.1×10^{-49}
EP2159	91 (472)	100 (190)	2.1×10^{-5}
EP683	100 (267)	100 (127)	0.51
UAS- <i>esg</i>	86 (255)	100 (117)	2.9×10^{-5}

The flies tested (Test) had the genotype *esg*^{EP}/+; *elav-GAL4* *sda/sda* or UAS-*esg*/+; *elav-GAL4* *sda/sda*. Control flies of the genotype +/CyO; *elav-GAL4* *sda/sda* were siblings of the test flies. Neuronal induction of five of the six *esg*^{EP} insertions, as well as UAS-*esg*, produced a highly significant (*P* < 0.001) reduction in the bang sensitivity of *sda* homozygotes.

flies were all bang sensitive (100% bang sensitivity). The bang-sensitive phenotype of *bss* was not suppressed by any of the three *esg*^{EP} alleles identified here. Thus, *bss*; *esg*^{EP}/+; *elav-GAL4*/+ flies showed the same 100% bang sensitivity as their *bss*; +/CyO; *elav-GAL4*/+ control siblings. However, additional observations suggest that *esg*^{EP} does interact weakly with *bss* (Figure 2C). The *bss* mutation is semidominant, and *bss*/+; +/CyO; *elav-GAL4*/+ control flies show >50% bang sensitivity. In contrast, their *bss*/+; *esg*^{EP}/+; *elav-GAL4*/+ siblings displayed little or no bang sensitivity (0, 5, and 10% bang sensitivity for EP684, EP2009, and EP633, respectively).

The reduced seizure susceptibility of the *elav-GAL4* + *esg*^{EP} lines likely reflects the neuronal induction of *esg* in these flies. The EP(2) collection includes six *esg*^{EP} lines: three of these (EP684, EP2009, and EP633) were identified in our screen for gain-of-function *eas* suppressors whereas the other three (EP2408, EP2159, and EP683) were not detected by our screen (Figure 1). Since *elav-GAL4*-activated *esg*^{EP} suppresses *sda* more strongly than *eas* (Figure 2), we subsequently examined all of the *esg*^{EP} insertions, in addition to a UAS-*esg* construct inserted elsewhere on chromosome II, for *elav-GAL4*-mediated suppression of *sda*. These experiments revealed that *elav-GAL4* activation of five of the six *esg*^{EP} insertions, as well as the UAS-*esg* construct, produced significant *sda* suppression (Table 3). The one *esg*^{EP} insertion that failed to suppress *sda* (EP683) is inserted in the opposite orientation, so that *elav-GAL4* induction of *esg*^{EP683} would not be expected to induce *esg* transcription. Neuronal induction of *esg* expression using the UAS-*esg* construct produced some suppression of the bang sensitivity of *eas* and *bss*/+ flies (Table 4). The *sna* transcription factor shares 76% identity with *esg*, and the corresponding genes are functionally redundant (WHITELEY *et al.* 1992; ASHRAF *et al.* 1999). We observed that *elav-GAL4* activation of the snail transcription factor

TABLE 4
Both *UAS-esg* and *UAS-sna* can suppress bang sensitivity

Genotype	% bang sensitivity		<i>P</i> -value
	Test (<i>n</i>)	Control (<i>n</i>)	
<i>ees</i> ; <i>UAS-esg</i> /+; <i>elav-GAL4</i> ^{3A} /+	78 (174)	100 (138)	4.4×10^{-9}
<i>ees</i> ; <i>UAS-sna</i> /+; <i>elav-GAL4</i> ^{3A} /+	88 (40)	100 (54)	4.9×10^{-3}
<i>bss</i> /+; <i>UAS-esg</i> /+; <i>elav-GAL4</i> ^{3A} /+	48 (683)	70 (148)	2.4×10^{-6}
<i>bss</i> /+; <i>UAS-sna</i> /+; <i>elav-GAL4</i> ^{3A} /+	39 (123)	82 (146)	2.3×10^{-13}

Genotypes for flies tested (Test) are indicated; control flies were gender-matched siblings that carried a balancer chromosome rather than the UAS construct. Results are highly significant ($P < 0.001$).

using a *UAS-sna* construct also produced a statistically significant reduction of bang sensitivity in either *ees* or *bss*/+ flies (Table 4). By contrast, a presumptive reduction of functional *esg* produced neither seizure suppression nor enhancement (Table 5). We found that heterozygous *ees*/+ or *bss*/+ flies that were also heterozygous for either a null mutation in *esg* or a deletion that removed all three snail-related genes (*esg*, *wor*, and *sna*) showed no statistically significant difference in bang sensitivity relative to their sibling controls (Table 5).

Suppression by *esg* must occur during nervous system development: Expression of *esg* could act directly on adult nervous system function to revert the *ees* bang-sensitive phenotype. Alternatively, it could act during nervous system development to produce a neuronal organization that is less susceptible to seizures. We used conditional expression of *esg*^{EP2009} to distinguish between these two possibilities. Experimental control of *esg*^{EP2009} expression was obtained by using the *elav-GeneSwitch* construct (OSTERWALDER *et al.* 2001). GeneSwitch is a conditional, RU486-dependent, GAL4-progesterone fusion protein (BURCIN *et al.* 1998). The *elav* promoter directs synthesis of inactive GeneSwitch in the neurons of embryos, larvae, pupae, and adults (ROBINOW and

WHITE 1988, 1991); GeneSwitch is activated at the desired point in development via administration of RU486.

The seizure-suppressor function of *esg*^{EP2009} in adults is dependent on its expression during nervous system development occurring in the larval stage. That is, an examination of *esg*^{EP2009} induction during different developmental stages showed that larval expression is critical for seizure suppression in adults (Table 6). Furthermore, larval induction of *esg*^{EP2009} produced a greater degree of suppression in adults tested at 2–3 days of age than in those tested at only 1–2 days (Table 6). RU486 induction of GeneSwitch maintained throughout embryonic, larval, and adult stages suppressed bang sensitivity (47% bang sensitivity for flies tested at 2–3 days) in flies of the genotypes *ees*; *esg*^{EP2009}; *elav-GeneSwitch* and *ees*; *esg*^{EP2009}/CyO; *elav-GeneSwitch*. This indicates a substantial amount of seizure suppression although somewhat less than that for *ees*; *esg*^{EP2009}/+; *elav-GAL4*/+ (11% bang sensitivity; Table 2). A reduced level of bang sensitivity was also observed if GeneSwitch induction occurred only during larval and adult stages (53% bang sensitivity for flies tested at 2–3 days) or only during the embryonic and larval stages (47% bang sensitivity for flies tested at 2–3 days). Little reduction

TABLE 5
Reduced *esg* does not affect bang sensitivity

Genotype	% bang sensitivity	
	Test (<i>n</i>)	Control (<i>n</i>)
<i>ees</i> /+; <i>esg</i> ^{35^{ac1}} /+	0 (136)	0 (138)
<i>ees</i> /+; <i>Df</i> (2L) <i>osp29</i> /+	0 (107)	0 (124)
<i>bss</i> /+; <i>esg</i> ^{35^{ac1}} /+	54 (316)	58 (291)
<i>bss</i> /+; <i>Df</i> (2L) <i>osp29</i> /+	34 (324)	33 (323)

The *esg*^{35^{ac1}} allele is a loss-of-function mutation in the *esg* gene, whereas *Df*(2L)*osp29* is a deletion that removes all three snail family genes (*esg*, *wor*, and *sna*). Genotypes for female flies tested (Test) are indicated; control flies were female siblings that carried a balancer chromosome rather than the corresponding *esg* mutation. No statistically significant differences in bang sensitivity were observed between the test and control progeny. In all four crosses, 100% of the corresponding *ees* or *bss* male siblings were bang sensitive.

TABLE 6
Critical period for *esg*^{EP} suppression of *ees*

<i>esg</i> ^{EP} induced in neurons			% bang sensitivity		
Embryo	Larvae	Adult	1–2 days	2–3 days	<i>n</i>
–	–	–	100	100	85
+	–	–	84	95	173
+	+	–	70	51	88
–	+	+	75	50	130
–	–	+	98	100	51
+	+	+	73	47	90

Expression of *esg* was induced in neurons at various developmental times using *esg*^{EP2009} and *elav-GeneSwitch*. Adult flies were tested for bang sensitivity at 1–2 days and again at 2–3 days. The number of flies tested (*n*) includes both *esg*^{EP2009} homozygotes and *esg*^{EP2009}/SM5 heterozygotes, except in the second row (embryos induced), which includes only *esg*^{EP2009} homozygotes.

TABLE 7

Induction of *esg* in interneurons or muscle suppresses *eas*

GAL4 driver	% bang sensitivity		P-value
	Test (n)	Control (n)	
<i>elav-GAL4</i> (all neurons)	4 (229)	100 (136)	1.5×10^{-73}
<i>Cha-GAL4</i> (interneurons)	88 (429)	100 (188)	4.1×10^{-6}
<i>OK6-GAL4</i> (motor neurons)	100 (165)	100 (121)	
<i>G14-GAL4</i> (muscle)	78 (101)	100 (107)	7.0×10^{-7}

Flies tested (Test) were male flies of the genotype *eas; esg^{EP684}/Driver-GAL4*. Control flies were *eas; Driver-GAL4/CyO* male siblings of the Test flies. Results for the *elav-GAL4*, *Cha-GAL4*, and *G14-GAL4* drivers are highly significant ($P < 0.001$).

of bang sensitivity was observed when RU486 feeding was limited to the adult stage (98% bang sensitivity at 1–2 days, 100% bang sensitivity at 2–3 days). Consequently, we presume that the approximately twofold reduction in bang sensitivity produced by RU486 feeding during the larval and adult stages was overwhelmingly due to the larval induction of *esg^{EP2009}*. A slight decrease in bang sensitivity was obtained by GeneSwitch induction in embryos (85% bang sensitivity at 1–2 days, 95% bang sensitivity at 2–3 days). We suspect that this modest reduction in bang sensitivity could actually reflect larval expression of *esg^{EP2009}*, because GeneSwitch activation in embryos persists in first and (to a lesser degree) second instar larvae (OSTERWALDER *et al.* 2001). Control flies grown on normal food without RU486 were all bang sensitive (100% bang sensitivity) indicating that the inactive GeneSwitch does not induce transcription of *esg^{EP2009}*.

Induction of *esg* in larval interneurons reduces bang sensitivity: To better understand why induction of *esg* in larval neurons mediates seizure suppression in adults, we induced *esg* in specific subpopulations of larval neurons and monitored the bang sensitivity of the resulting adults. These experiments revealed that induction of *esg* in larval interneurons using *Cha-GAL4* produced a 12% reduction in adult bang sensitivity (Table 7). Hence, expression of *esg* in larval interneurons alone can reduce seizure susceptibility in adults. A significantly greater reduction in bang sensitivity (96%) occurred when *esg* was induced in all neurons; we do not know whether this is because *esg* must be induced in an additional neuronal subpopulation for high-level suppression or whether the *elav-GAL4* driver produces a higher level of *esg* induction in larval interneurons than does *Cha-GAL4*. However, induction of *esg* in another larval interneuron subpopulation, motoneurons, using *OK6-GAL4* had no discernible effect on adult bang sensitivity: 100% were bang sensitive (Table 7). We were unable to ascertain whether induction of *esg* in larval sensory neurons influences adult bang sensitivity as *sca-GAL4*-driven *esg^{EP684}* and *esg^{EP2009}* were lethal in our crosses; however, the single *eas; esg^{EP684}/sca-GAL4* escaper was bang sensi-

tive (data not shown). Induction of *esg* in all larval somatic muscles using the *G14-GAL4* driver produced a 22% reduction in adult bang sensitivity (Table 7). This observation is consistent with the notion that *esg* induced in neurons is acting in a non-cell-autonomous manner to reduce susceptibility to seizures.

Neuronal *escargot* raises seizure threshold: The bang-sensitive behavioral phenotype has been a useful indirect measure of seizure sensitivity in *Drosophila*: flies that show a strong behavioral phenotype are most sensitive to seizure, whereas those that lack the phenotype are less seizure sensitive (PAVLIDIS and TANOUYE 1995; KUEBLER and TANOUYE 2000; KUEBLER *et al.* 2001). To examine seizure susceptibility directly, we used electrophysiology methodologies in suppressed and unsuppressed *eas* flies. Seizures are evoked by a wave train of HF electrical stimuli (0.5-msec pulses delivered at 200 Hz for 300 msec) delivered to the *Drosophila* brain (PAVLIDIS *et al.* 1994). Seizures in *Drosophila* consist of uncontrolled firing of many, if not all, neurons in the CNS at firing frequencies of >100 Hz. Recordings of seizures are most conveniently made from muscle fibers in the thorax and reflect seizure activity of the motoneurons that innervate them. At least 30 motoneurons innervating seven muscle groups have shown seizure activity (KUEBLER and TANOUYE 2000). Figure 3A shows a seizure recorded from the dorsal longitudinal muscle of an *eas; +/CyO; elav-GAL4/+* control fly in response to an 8-V HF stimulus. Abnormal HF muscle potentials (>100 Hz) are observed reflecting seizure activity of the single motoneuron innervating this muscle fiber (KUEBLER and TANOUYE 2000). This seizure activity is similar in appearance and time course to those previously observed in *eas* mutants (PAVLIDIS and TANOUYE 1995). Figure 3B shows a dorsal longitudinal muscle recording from an *eas; esg^{EP2009}/+; elav-GAL4/+* fly in response to an 8-V HF stimulus. In this instance, no seizure activity can be seen, indicating that the HF stimulus was not of sufficient strength to elicit a seizure. Figure 3C shows a seizure resulting from a higher-intensity 20-V HF stimulus delivered to the *eas; esg^{EP2009}/+; elav-GAL4/+* fly. Taken together, these results indicate that

the stimulus is of sufficient intensity to exceed the higher seizure threshold of this fly. (D) HF stimuli of varying voltages were delivered to *eas* control flies or their siblings that were *eas* and carried the *esg*^{EP2009} suppressor to determine their seizure thresholds. The threshold curves show the percentage of flies that have seizures following an HF stimulus of a particular voltage. Fifteen *eas* control flies and 20 of their *esg*^{EP2009}-carrying siblings were tested. Note that the *esg*^{EP2009} curve is broader than the control one, indicating a greater range of threshold values. (E) The *esg*^{EP} suppressors raise the average seizure threshold for *eas* by approximately twofold. The mean seizure threshold of *eas*; +/CyO; *elav-GAL4*/+ control flies is 7.6 ± 1.9 V. The mean seizure threshold of *eas* flies carrying any one of the three *esg*^{EP} alleles (genotype *eas*; *esg*/+; *elav-GAL4*/+) is higher (EP684, 15.5 ± 5.7 V; EP2009, 15.5 ± 6.8 V; and EP633, 13.3 ± 3.2 V).

the seizure sensitivity of *eas* is suppressed by ectopic expression of *esg*^{EP2009}: seizures are evoked by low-intensity HF stimuli when *esg*^{EP2009} is not present in the *eas*; +/CyO; *elav-GAL4*/+ fly, indicating seizure sensitivity, whereas when *esg*^{EP2009} is present, a higher-intensity HF stimulus is necessary to evoke a seizure, indicating a suppression of seizure sensitivity.

We quantified the effectiveness of *esg*^{EP}-mediated seizure suppression by comparing mean threshold voltages needed to elicit a seizure in suppressed and unsuppressed flies. It was shown previously that flies of different genotypes have characteristically different mean threshold voltages, with seizure-prone, bang-sensitive flies displaying seizure thresholds 5–10 times lower than those of wild-type control flies (KUEBLER *et al.* 2001). The mean seizure threshold for unsuppressed flies of the genotype *eas*; +/CyO; *elav-GAL4*/+ is 7.5 ± 1.9 V (Table 8). This value is comparable to, although slightly higher than, the seizure threshold reported previously for the *eas* mutant strain (3.4 ± 0.05 V; KUEBLER *et al.* 2001). In contrast, ectopic neuronal expression of *esg*^{EP2009} raises seizure threshold approximately twofold. Thus, suppressed flies of the genotype *eas*; *esg*^{EP2009}/+; *elav-GAL4*/+ have a mean seizure threshold of 15.5 ± 5.7 V. This is a substantial improvement in *eas* seizure susceptibility, although not quite to wild-type levels. For example, seizure threshold for Canton-Special is 30.1 ± 3.8 V (KUEBLER *et al.* 2001). The *eas* *shak-B2* double

mutant has a seizure threshold of 15.3 ± 3.2 V (KUEBLER *et al.* 2001), indicating that the *esg*^{EP2009} gain-of-function mutation suppresses *eas* seizures to about the same extent as the *shak-B2* loss-of-function mutation.

Figure 3D shows a plot of the percentage of flies seizing at different HF stimulus intensities. The *eas*; *esg*^{EP2009}/+; *elav-GAL4*/+ curve is shifted to the right relative to that of the *eas*; +/CyO; *elav-GAL4*/+ sibling control curve. This right shift reflects the seizure suppression by *esg*^{EP2009}: most *eas* flies carrying *elav-GAL4*-activated *esg*^{EP2009} require a significantly higher HF stimulus intensity to elicit a seizure than do the sibling controls. Note also that the *esg*^{EP2009} curve is broader than the control curve, reflecting a larger range of seizure threshold values. Some *eas*; *esg*^{EP2009}/+; *elav-GAL4* flies have seizure threshold values comparable to those of their control siblings. This may account for the observation that suppression of the *eas* behavioral phenotype by *esg*^{EP2009} is incompletely penetrant: flies with seizure thresholds below ~ 10 V would likely be bang sensitive.

The increased seizure threshold of *eas* flies expressing *elav-GAL4*-activated *esg*^{EP2009} apparently reflects a general reduction in seizure-sensitivity mediated by neuronal *esg*. Electrophysiological measurements of *eas*; *esg*^{EP}/+; *elav-GAL4*/+ flies carrying either of the two other *esg* alleles (EP684 and EP633) reveal that these flies also have significantly elevated seizure thresholds (15.5 ± 5.7 V and 13.3 ± 3.2 V, respectively; Figure 3E). Electro-

FIGURE 3.—Neuronal expression *esg*^{EP} raises the seizure threshold of *eas* flies. (A) A seizure is elicited in an *eas* sibling control fly (genotype *eas*; +/CyO; *elav-GAL4*/+) by a high-frequency stimulus of low strength (8 V). The HF stimulus (box) is a short wave train (0.5-ms pulses at 200 Hz for 300 msec) of electrical stimuli delivered to the brain. The recording is from a DLM and reflects the abnormal HF firing of the innervating DLM motoneuron. The vertical calibration bar is 20 mV and the horizontal bar is 200 msec. (B) A low-voltage HF stimulus (8 V) fails to elicit a seizure in an *eas* fly carrying the *esg*^{EP2009} suppressor (genotype *eas*; *esg*^{EP2009}/+; *elav-GAL4*/+) because the stimulus is below the intensity required for a higher seizure threshold. (C) A higher-voltage HF stimulus (20 V) elicits a seizure in an *eas* fly carrying the *esg*^{EP2009} suppressor (genotype *eas*; *esg*^{EP2009}/+; *elav-GAL4*/+) because

TABLE 8
Giant fiber electrophysiology of *esg^{EP}* and control flies

Genotype	Giant fiber threshold (V)	Seizure threshold (V)	P-value
<i>esg</i> ; +/CyO; <i>elav-GAL4</i> /+	1.3 ± 0.20	7.6 ± 1.9	
<i>esg</i> ; <i>esg^{EP684}</i> /+; <i>elav-GAL4</i> /+	1.6 ± 0.43	15.5 ± 5.7	8.18 × 10 ⁻⁶
<i>esg</i> ; <i>esg^{EP2009}</i> /+; <i>elav-GAL4</i> /+	1.3 ± 0.26	15.5 ± 6.8	5.11 × 10 ⁻⁵
<i>esg</i> ; <i>esg^{EP633}</i> /+; <i>elav-GAL4</i> /+	1.3 ± 0.45	13.3 ± 3.2	4.39 × 10 ⁻⁴
<i>w</i> ; +/CyO; <i>elav-GAL4</i> /+	1.6 ± 0.29	36.7 ± 6.5	
<i>w</i> ; <i>esg^{EP2009}</i> /+; <i>elav-GAL4</i> /+	1.3 ± 0.45	55.5 ± 13.2	1.52 × 10 ⁻²

Values for the giant fiber threshold and seizure threshold (V) are given for each of the genotypes tested. The CyO control flies (first and fifth rows) were siblings of the *esg^{EP2009}*/+ flies. The *esg^{EP684}*/+ and *esg^{EP633}*/+ flies had no bang-sensitive phenotype when subjected to behavioral tests. Giant fiber threshold was determined using single-pulse stimuli. Seizure threshold was determined using HF stimuli. The P-values for differences in seizure threshold between the *esg^{EP}* and corresponding control strains are indicated.

physiological recordings showed that *elav-GAL4*-activated *esg^{EP}* also changes seizure threshold in non-BS flies (Table 4). Flies carrying *elav-GAL4*-activated *esg^{EP2009}* (genotype *w*; *esg^{EP2009}*/+; *elav-GAL4*/+) have a seizure threshold of 55.5 ± 13.2 V, which is higher than that of control flies that lack *esg^{EP2009}* (genotype *w*; +/CyO; *elav-GAL4*/+; threshold 36.7 ± 6.5 V). Note that neuronal *esg* does not appear to be fully epistatic to *esg*: the seizure threshold of *esg*; *esg^{EP2009}*/+; *elav-GAL4*/+ flies (15.5 V) is higher than that of *esg*; +/CyO; *elav-GAL4*/+ flies (7.5 V) but lower than that of *w*; *esg^{EP2009}*/+; *elav-GAL4*/+ flies (55.5 V).

Individual neuron excitability is not changed by *esg*: A possible explanation for genotypic differences in seizure susceptibility is that each individual neuron in *esg* control flies (*esg*; *elav-GAL4*/+) could be hyperexcitable compared to the corresponding neuron in their suppressor-carrying siblings (*esg*; *esg^{EP2009}*/+; *elav-GAL4*/+). In this case, a lower-intensity HF stimulus (*i.e.*, 7.5) in *esg* control BS could be activating the same number of neurons as a higher-intensity HF stimulus (*i.e.*, 15.5-V HF) in the suppressor-carrying sibling. The implication would be that the same number of neurons is activated to evoke the seizure. There is some precedent for thinking that this may occur because one source of seizure disorder in mice corresponds with the knockout of a voltage-gated K⁺ channel, which would presumably cause membrane hyperexcitability (SMART *et al.* 1998).

Experiments examining giant fiber (GF) threshold suggest that altered single-cell excitability is probably not an explanation for seizure suppression. Stimulus voltages required for activation of the GF did not differ between suppressed and unsuppressed flies (Table 4). The GF activation threshold for *esg*; *esg^{EP}*/+; *elav-GAL4* flies is virtually identical with the 1.3 ± 0.20-V GF threshold of the *esg*; +/CyO; *elav-GAL4*/+ control flies (EP684, 1.6 ± 0.43; EP2009, 1.3 ± 0.26; and EP633, 1.3 ± 0.45). Thus, *elav-GAL4*-driven *esg^{EP}* does not appear to suppress seizures by altering the excitabilities of individual neu-

rons. A more likely possibility is that *elav-GAL4*-activated *esg^{EP}* suppresses seizures by requiring a larger number of neurons to be recruited to trigger a seizure. Note that we cannot rule out the possibility that there may be other neurons not examined here that are critical to the generation of seizures and are altered in excitability.

Microarray analysis of potential *esg* targets: The *esg* transcription factor normally functions as a repressor (FUSE *et al.* 1994; ASHRAF *et al.* 1999). Consequently, the expectation is that *esg^{EP}*-mediated seizure suppression arises from reduced expression of one or more *esg* target genes and the lowered level of their corresponding gene products. However, ectopically expressed neuronal *esg* could conceivably act as a transcriptional activator to induce one or more critical target genes. The gene product(s) of the relevant *esg* target(s) could potentially be used in screens for novel AEDs. As a first approach in determining the relevant target genes for neuronal *esg*, we performed microarray analysis to determine which of >13,500 transcripts were either induced or repressed in larvae expressing *esg* in all neurons. In these experiments, test larvae had the genotype *w*; *esg^{EP2009}*/+; *elav-GAL4*/+, whereas control larvae had the genotype *w*; *esg^{EP683}*/+; *elav-GAL4*/+. Both the EP2009 (test) and the EP683 (control) insertions are located in the 5' upstream region of the *esg* gene and are separated by only 19 bp. However, they are inserted in opposite orientations. Consequently, *elav-GAL4* induces *esg^{EP2009}*, producing suppression of bang sensitivity (Figure 2), whereas *elav-GAL4* fails to induce *esg^{EP683}*, producing no change in bang sensitivity (Table 3).

Our microarray analysis (MATERIALS AND METHODS) revealed 156 genes that demonstrated a reproducible and statistically significant difference in expression level between test and control larval RNAs. Of these 156 genes, 100 also displayed clustered repeats of the *esg* target sequence (RCAGGTG) within their noncoding sequences (Table 9). This list includes 47 upregulated genes and 53 downregulated ones. One or more of these

TABLE 9
Microarray analysis of transcripts altered in larvae with pan-neural escargot expression

Gene	Fold change	P-value	Comments
Neurotransmitters/neuropeptides			
<i>ebony (e)</i> CG3331	↑ 3.6	2×10^{-6}	β-Alanyl-dopamine synthase
<i>black (b)</i> CG7811	↑ 2.5	1.1×10^{-5}	Glutamate decarboxylase
CG3999	↑ 1.7	2.6×10^{-5}	Glycine dehydrogenase
<i>Nplp2</i> CG11051	↓ 2	2×10^{-6}	Neuropeptide hormone
Ca²⁺-binding proteins			
CG7447	↑ 1.7	2.7×10^{-4}	Has a Ca ²⁺ -binding EGF-like domain
<i>Amy-d</i> CG17876	↓ 2	2×10^{-6}	Ca ²⁺ -binding α-amylase
<i>Troponin C @ 47D (TpnC47D)</i> CG9073	↓ 1.8	2×10^{-6}	Ca ²⁺ -binding protein that regulates muscle contraction
CG6514	↓ 1.7	8.4×10^{-5}	Ca ²⁺ -binding protein
Transporters			
CG6600	↑ 3.7	9×10^{-6}	Organic cation transporter
<i>slimfast (slif)</i> CG11128	↑ 1.6	9.3×10^{-5}	Cationic amino acid transporter
Serine protease inhibitors			
CG7906	↑ 3.9	1.7×10^{-5}	Kazal type
CG7924	↑ 3.6	2×10^{-6}	Kazal type
CG1342	↑ 1.7	5×10^{-5}	Serpin type
<i>Spn43Aa</i> CG12172	↑ 1.6	2.5×10^{-4}	Serpin type
CG6687	↓ 1.6	1.3×10^{-4}	Serpin type
<i>E(spl)-m1</i> CG8342	↓ 1.5	2.5×10^{-6}	
Proteases			
<i>SP1029</i> CG11956	↑ 1.8	2×10^{-6}	Aminopeptidase
CG6298	↓ 2.8	2.5×10^{-6}	Serine protease
CG10472	↓ 1.9	6×10^{-6}	Serine-type endopeptidase
CG8773	↓ 1.9	1.6×10^{-5}	Glutamyl aminopeptidase
CG4847	↓ 1.7	2×10^{-6}	Cysteine protease
<i>Ser99Dc</i> CG17951	↓ 1.7	2.5×10^{-6}	Serine-type endopeptidase
CG8299	↓ 1.6	3.5×10^{-6}	Serine-type endopeptidase
CG10081	↓ 1.6	1.8×10^{-5}	Zn ²⁺ -dependent exopeptidase
CG9673	↓ 1.6	1.8×10^{-4}	Serine protease
Other enzymes			
CG5171	↑ 1.9	1.7×10^{-5}	Trehalose-phosphatase
<i>Nmdmc</i> CG18466	↑ 1.7	8.5×10^{-6}	Methenyltetrahydrofolate cyclohydrolase
CG7115	↑ 1.6	6.3×10^{-5}	Serine/threonine phosphatase
<i>Asph</i> CG18658	↑ 1.5	7.5×10^{-6}	Peptide-aspartate β-dioxygenase
<i>Est-P</i> CG17148	↑ 1.5	4.4×10^{-4}	Carboxylesterase
CG15534	↓ 3.0	9.8×10^{-4}	Sphingomyelin phosphodiesterase
<i>Uro</i> CG7171	↓ 2.6	8.5×10^{-6}	Peroxisomal urate oxidase
CG9149	↓ 2.5	2.5×10^{-6}	Acetyl-CoA C-acetyltransferase
CG9466	↓ 2.2	2×10^{-6}	α-Mannosidase
CG9468	↓ 2.1	2×10^{-6}	α-Mannosidase
CG6839	↓ 1.9	4×10^{-6}	DNA/RNA endonuclease
CG3264	↓ 1.7	3×10^{-6}	Alkaline phosphatase
CG1809	↓ 1.6	9.1×10^{-5}	Alkaline phosphatase
<i>Cyp6a18</i> CG13977	↓ 1.5	1.5×10^{-4}	Mitochondrial electron transporter
Defense/immunity			
<i>LysX</i> CG9120	↑ 4.4	3×10^{-6}	Lysozyme
<i>dro5</i> CG10812	↑ 3.9	3×10^{-6}	
CG12789	↑ 1.8	2.5×10^{-5}	Scavenger receptor
<i>AttC</i> CG4740	↓ 5.1	3.5×10^{-6}	

(continued)

TABLE 9
(Continued)

Gene	Fold change	P-value	Comments
<i>DptB</i> CG10794	↓ 5.1	4.1×10^{-5}	Peptidoglycan recognition protein
<i>Dpt</i> CG12763	↓ 3.0	1.1×10^{-5}	
<i>PGRP-SB1</i> CG9681	↓ 2.5	2.1×10^{-5}	
<i>Mtk</i> CG8175	↓ 1.9	7.5×10^{-5}	
Structural constituents			
<i>Lcp65Ad</i> CG6955	↑ 6.5	9×10^{-6}	Larval cuticle
CG2330	↑ 1.8	4.7×10^{-4}	Extracellular matrix
<i>Lcp65Ag1</i> CG10530	↓ 2.5	9.4×10^{-5}	Larval cuticle
CG11142	↓ 1.9	2×10^{-6}	Peritrophic membrane
<i>Peritrophin-A</i> CG17058	↓ 1.9	1.3×10^{-5}	Peritrophic membrane
CG9077	↓ 1.8	1.2×10^{-5}	Larval cuticle
CG17145	↓ 1.7	2.5×10^{-6}	Peritrophic membrane
CG2555	↓ 1.5	8.5×10^{-6}	Larval cuticle
CG10725	↓ 1.5	9.5×10^{-6}	Peritrophic membrane
Larval/pupal development			
<i>Eig71Ek</i> CG7325	↑ 61.8	2×10^{-6}	Involved in salivary gland cell death
<i>Eig71Ed</i> CG7350	↑ 46.7	2.5×10^{-6}	
<i>Edg78E</i> CG7673	↑ 7.2	7.6×10^{-5}	
<i>Eip78C</i> CG18023	↑ 2.8	6.3×10^{-5}	Transcription factor
<i>ImpE2</i> CG1934	↑ 1.5	1.8×10^{-4}	
Other			
<i>MtnB</i> CG4312	↑ 3.7	2.4×10^{-4}	Metallothionein
<i>Hsp26</i> CG4183	↑ 2.1	2×10^{-6}	Heat-shock protein
<i>MtnC</i> CG5097	↓ 9.5	3.7×10^{-5}	Metallothionein
<i>pastrel</i> (<i>pst</i>) CG8588	↓ 2	1.3×10^{-5}	Mutants show memory defects
Novel			
CG2150	↑ 12.1	2×10^{-6}	
CG4151	↑ 10.9	5.5×10^{-6}	
CG9083	↑ 9.2	3.5×10^{-6}	
CG10853	↑ 9.2	4×10^{-6}	
CG12164	↑ 8.3	7×10^{-6}	
CG14534	↑ 5.1	2.3×10^{-4}	
CG17325	↑ 4.9	2×10^{-6}	
CG15901	↑ 4.4	1.8×10^{-4}	
CG9822	↑ 2.6	2×10^{-6}	
CG14664	↑ 2.6	2.5×10^{-6}	
CG4783	↑ 2.6	6.5×10^{-6}	
CG7377	↑ 2.6	1.2×10^{-5}	
CG13947	↑ 2.3	2×10^{-5}	
CG10112	↑ 2.1	1.6×10^{-4}	
CG14265	↑ 1.9	9.5×10^{-6}	
CG2016	↑ 1.9	7.7×10^{-5}	
CG2444	↑ 1.6	2×10^{-6}	
CG13314	↑ 1.6	8.9×10^{-5}	
CG1124	↑ 1.6	1.4×10^{-4}	
CG11350	↓ 10.6	1.8×10^{-5}	
CG11584	↓ 2.6	3.5×10^{-6}	
CG5765	↓ 2.5	1.2×10^{-5}	
CG7465	↓ 2.2	2×10^{-6}	
CG5866	↓ 2.1	4×10^{-5}	
CG9757	↓ 1.9	1.5×10^{-5}	
CG7876	↓ 1.9	6.6×10^{-5}	
CG11300	↓ 1.8	1.2×10^{-4}	
CG10650	↓ 1.7	2×10^{-6}	
CG14963	↓ 1.7	1.4×10^{-4}	

(continued)

TABLE 9
(Continued)

Gene	Fold change	P-value	Comments
CG3984	↓ 1.7	2.6×10^{-4}	
CG5084	↓ 1.6	2×10^{-6}	
CG13043	↓ 1.6	8.2×10^{-5}	
CG10953	↓ 1.5	1.5×10^{-5}	
CG13805	↓ 1.5	6.1×10^{-5}	
CG7272	↓ 1.5	2.5×10^{-4}	

Microarray analysis revealed that transcript levels for all 100 genes shown displayed a reproducible and significant (P -value $< 10^{-3}$) change of at least 1.5-fold in larvae with pan-neural escargot expression (genotype *esg^{EP2009}/+*; *elav-GAL4/+*) relative to control larvae (genotype *esg^{EP683}/+*; *elav-GAL4*). All of these 100 genes also carry escargot binding site repeats within their noncoding regions.

genes is likely the *esg* transcriptional target that actually mediates its seizure suppression. The list of genes that displayed significant levels of induction in larvae with pan-neural *esg* includes two (*ebony* and *black*) that encode enzymes involved in the biosynthesis of neurotransmitter (dopamine and glutamate, respectively), two (*CG6600* and *slif*) that encode cationic transporters, and four for serine protease inhibitors (*CG7906*, *CG7924*, *CG1342*, and *Spn43a*). The *esg* gene itself showed modest induction (1.8-fold); however the induced level was still too low to be reliably quantified, and the change P -value was only 0.043. Several genes involved in larval/pupal development displayed striking induction; this likely reflects the greater number of contaminating prepupae included with the test larvae than with the control larvae. The list of genes that showed significant repression in larvae expressing neuronal *esg* includes one (*Nplp2*) that encodes a neuropeptide, several Ca^{2+} -binding proteins, and five serine proteases. A number of the potential *esg* target genes that displayed particularly striking differences in expression levels between test and control larvae encode proteins of unknown function. Further genetic experiments will be needed to determine which of our 100 potential targets of neuronal *esg* actually mediates seizure suppression.

DISCUSSION

The *Drosophila* model—comparisons with humans:

Drosophila has been an important model for examining fundamentally important problems in biology, especially developmental biology and neurobiology (RUBIN and LEWIS 2000). An important lesson from these studies is that findings are generally applicable to other experimental systems such as *C. elegans* and mouse due to conservation of fundamental processes and essential gene products (VERAKSA *et al.* 2000; TICKOO and RUSSELL 2002). An implication from cross-species conservation is that *Drosophila* has the potential to be a powerful system for modeling human pathologies. As for other

biological problems, the *Drosophila* system carries with it advantages arising from advanced genetic and molecular biology methodologies, particularly methods for screening large numbers of mutants to identify candidates for subsequent evaluation and analysis (ASH-BURNER 1989). In this article we are concerned with this general issue and, in particular, the study of *Drosophila* seizure susceptibility and its application to human seizure disorders.

Seizures in flies and humans have several similarities providing support for the utility of this type of investigation. Previous investigations have shown that for *Drosophila*: (1) all individuals have a seizure threshold; (2) genetic mutations can modulate seizure susceptibility; (3) electroconvulsive shock therapy in flies raises the threshold for subsequent seizures; (4) seizures spread through the fly CNS along particular pathways that are dependent on functional synaptic connections and recent electrical activity; (5) seizures in flies can be spatially segregated into particular regions of the CNS; (6) *Drosophila* epilepsy can be ameliorated by the human AEDs sodium valproate, phenytoin, gabapentin, and potassium bromide; and (7) mutations affecting *Drosophila* Na^+ channels are excellent seizure suppressors, consistent with the notion that many AEDs are targeting Na^+ channels (KUEBLER and TANOUYE 2000, 2002; KUEBLER *et al.* 2001; REYNOLDS *et al.* 2003; TAN *et al.* 2004). Also, mutations of two fly genes provide a molecular genetic link with human epilepsy. The *jbug* mutant is a seizure-sensitive mutant, and the gene encodes fly filamin-1 (X. REN and M. TANOUYE, unpublished results). Mutations in human filamin-1 cause periventricular heterotopia that has a neuronal migration defect and presents with epilepsy (FOX *et al.* 1998). The *tho* gene encodes a ribosome-associated protein and may be akin to myoclonic epilepsy ragged-red fiber disease, which is caused by a mutation in a human tRNA gene (ROYDEN *et al.* 1987).

Seizure-suppressor mutations: Suppression of seizures has heretofore been done in the context of ameliorating epilepsy by directly altering electrical excitability

of the nervous system. Pharmacological agents that act as AEDs typically reduce nervous system excitability by inhibiting voltage-gated Na⁺ channels (*e.g.*, phenytoin, carbamazepine, lamotrigine, and fosphenytoin) or increase nervous system inhibition via intervention in the GABA neurotransmitter system (sodium valproate, gabapentin, and topiramate; SCHMIDT 2002). Similarly, in a previous study of genetic seizure-suppressor genes (KUEBLER *et al.* 2001), *Drosophila* mutations affecting electrical excitability were chosen to test by reverse genetic analysis: Na⁺ channel genes (*para* and *mle^{nap1b}*), a K⁺ channel gene (*Sh*), and a gap junction channel gene (*shak-B*). Interestingly, although the *Drosophila* double mutants showed seizure suppression, behavioral defects were also manifest as side effects of the suppressor mutations. These included sluggish behavior, temperature-sensitive paralysis, abnormal twitching and shaking, infertility, and shortened life span (D. KUEBLER and M. TANOUYE, unpublished results). These side effects observed in flies are roughly analogous to those occasionally seen in humans in response to certain AEDs.

The identification of *esg* as a seizure suppressor is surprising because the biological functions with which it has been associated are not obviously electrical excitability functions. In this regard, it differs from the kinds of ion channel and gap junction mutants that we previously identified as suppressors from double-mutant analyses, candidates originally inspired by conventional drug studies (KUEBLER *et al.* 2001). The mechanism underlying *esg* suppression of seizures remains to be elucidated in further experiments. For example, *esg*-mediated seizure suppression could be affecting nervous system electrical excitability indirectly through functions not previously suspected for *esg*. Alternatively, *esg* could be suppressing seizures in ways that do not involve modifications of electrical excitability. This would be consistent with the emerging observation from molecular studies that many syndromes that present with epilepsy, including human syndromes, mouse knockout mutations, and *Drosophila* mutations, are not obviously affecting electrical excitability functions (ROYDEN *et al.* 1987; PAVLIDIS *et al.* 1994; PURNAM and MCNAMARA 1999; MCNAMARA 1999; ZHANG *et al.* 2002). One might similarly expect that many seizure-suppression mechanisms would exist that are not working via alteration of electrical excitability; *esg* could be the first of these to be identified. Thus, it is possible that by examining *esg* gain-of-function seizure suppression, we may gain new insight into mechanisms by which the nervous system can be constructed in ways that reduce seizure sensitivity independent of effects on electrical excitability. Some of the genes whose expression is influenced by neuronal *esg* may ultimately serve as targets for AEDs, particularly in epileptic neonates and children whose nervous systems are undergoing rapid remodeling. These might be expected to have few nervous system excitability-related side effects.

Evaluation of *esg* as a seizure suppressor: We define *esg* as a seizure-suppressor gene on the basis of gain-of-function mutations that (1) revert the bang-sensitive behavioral phenotype associated with *eas*, *sda*, and *bss*/+ flies and (2) cause an increase in the seizure threshold of *eas* mutants. This conclusion is bolstered by the identification of five different *esg^{EP}* alleles (all with independently derived *P*-element insertions) and one *UAS-esg* construct (located in a distinct cytological location) that all act as *sda* suppressors. On the basis of the lack of allele specificity of the *esg-sda* interaction, mutations of *esg* appear to be general seizure suppressors. This is expected since neither the *esg^{EP}* alleles nor *UAS-GAL4* would be expected to produce structurally altered gene products; suppression is presumably due to the ectopic expression of a structurally normal protein. It is expected that the five *esg^{EP}* mutations identified here, as well as the *UAS-esg* insertion, show similar ectopic expression patterns under *elav-GAL4* control and thereby produce seizure suppression in a similar gain-of-function manner.

Neuronal induction of *esg^{EP}* appears to reduce the fly's overall seizure susceptibility. This assertion is supported by the observation that wild-type, non-BS flies carrying *elav-GAL4*-activated *esg^{EP}* display an increased seizure threshold (Table 8). A general reduction in seizure sensitivity would also explain why neuronal *esg^{EP}* suppresses a variety of BS mutations (Figure 2). Two of the BS mutations examined (*eas* and *sda*) encode very different products: *eas* is an ethanolamine kinase involved in synthesis of the phosphatidyl ethanolamine in neuronal membranes, and *sda* encodes an aminopeptidase (PAVLIDIS *et al.* 1994; ZHANG *et al.* 2002). The third BS mutation is likely to encode yet another very different product (J. TAN and M. TANOUYE, unpublished results). These three BS mutations may well reduce the fly's seizure threshold by different mechanisms. The *elav-GAL4* activation of *esg^{EP}* suppresses *sda* best of all; suppression of *eas* is intermediate and suppression of *bss* is the weakest (Figure 2). This is consistent with previous observations on general seizure suppressors that *bss* is the strongest of the three mutations (in terms of both its reduction of seizure threshold and the facility with which it can be suppressed by secondary mutations that reduce nervous system excitability) and *sda* is the weakest, with *eas* being intermediate (KUEBLER and TANOUYE 2000; KUEBLER *et al.* 2001).

The gain-of-function *esg^{EP}* mutations that act to suppress seizures cause no other obvious phenotypes whether in a wild-type or an *eas* background. Thus, *esg* mutant flies show no obvious nervous system excitability defects: they are not temperature-sensitive paralytics (hypoexcitability) and they do not shake their legs under ether anesthesia (hyperexcitability). Other behaviors also appear to be normal: flies groom, court, mate, jump, and fly. Flies that are *eas*⁺; *esg^{EP2009}*; *elav-GAL4*

have a seizure threshold that is near the wild-type range (55.5 ± 13.2 V).

Mechanism of *esg* suppression: In the experiments presented here, the combined features of *esg^{EP}*, *elav-GAL4*, and GeneSwitch begin to give us a picture of seizure suppression. We suggest that *esg^{EP}* produces seizure suppression via its effect on immature postmitotic larval neurons that differentiate into interneurons of the adult CNS. We suggest further that this could be due to cytoskeletal organization or reorganization that underlies the elaboration and strengthening of synaptic interconnections.

Several other possible explanations of seizure suppression are not supported by the experiments presented here. For example, seizure suppression cannot occur by *esg^{EP}* ameliorating some acute property of mature neurons in adults. Thus, *esg^{EP}* suppression is probably not by manipulation of neurotransmitter metabolism, ion channel maintenance, or by other steady-state mechanisms used for maintaining or sustaining nervous system function or structure. This is because the bang-sensitive phenotype of *eas* is not suppressed by expression of *esg^{EP}* in the adult nervous system as shown by the GeneSwitch experiment (Table 6). Another alternative explanation is also not supported by the experiments presented here: *esg^{EP}*-mediated seizure suppression must be unrelated to *esg*'s normal role in facilitating neurogenesis (HAYASHI *et al.* 1993) or its role in polyploidization (HAYASHI *et al.* 1993; FUSE *et al.* 1996, 1999). This is because *elav-GAL4* does not induce *esg^{EP}* expression in either embryonic or larval neuroblasts (ROBINOW and WHITE 1988, 1991). The GeneSwitch experiment also shows that embryonic expression of *esg^{EP}* most likely does not account for its seizure suppression in adults. Furthermore, reducing the dosage of *esg*, which is normally expressed only in neuroblasts, has no effect on bang sensitivity (Table 5).

The GeneSwitch experiments show that seizure suppression in adult *eas* flies is apparently due primarily to *esg^{EP}* induction in the larval stage (Table 6). In larvae, there are four main classes of neurons (TRUMAN 1990), all of which should have *esg^{EP}* expression driven by *elav-GAL4*:

- i. Some larval neurons undergo programmed cell death and make no contribution to the adult nervous system. These neurons would have no impact on seizure suppression in adults.
- ii. Some larval neurons subserve similar functions in the adult and pass through metamorphosis with little or no restructuring. These neurons may be analogous to mature neurons of adults, suggesting that *esg^{EP}* induction would have little effect on seizure suppression.
- iii. Some larval neurons undergo considerable restructuring during metamorphosis because their adult function is much different from their larval role.

The neurons in classes ii and iii appear to form the core of motor and neuromodulatory systems in the adult (TRUMAN 1990).

- iv. Some adult-specific neurons have no function in the larva and differentiate after pupariation. Many of these adult-specific neurons appear to be interneurons in the sensory system (TRUMAN 1990).

Seizure suppression by *esg^{EP}* could be due to its effects in several of these classes; however, the class of adult-specific neurons (class iv) is an especially attractive candidate. These interneurons are involved in integrating sensory signals such as those arising from mechanical "bang" stimulation. Interneurons in the sensory system are numerous and are probably the neurons most greatly affected by electrical stimuli delivered to the brain by HFS. The observation that induction of *esg^{EP}* in larval interneurons, but not motoneurons, produces adults with reduced seizure susceptibility (Table 7) is also consistent with the notion that *esg^{EP}* is acting primarily in the class iv neurons. Interneurons of class iv undergo considerable development late in third instar larvae, but can also continue development after eclosion. This development may account for the continued progression of seizure suppression through days 2–3 of the adult stage (Table 6).

One possibility is that larval *esg* induction in developing interneurons affects their synaptic connections and thereby interferes with the spread of seizures. Class iii larval neurons undergo both new outgrowth and pruning of their dendritic and axonal processes during metamorphosis, and arrested adult-specific neurons (class iv) begin to extend processes after pupariation (TRUMAN 1990). Since *esg^{EP}* induced in larvae may well persist in pupae, it could affect the expression of genes whose products influence synapse formation or strengthening shortly after pupariation. Pupae do not take up the RU486, which might explain why the degree of suppression by *esg^{EP}* is lower when activated by *elav-GeneSwitch* than when activated by *elav-GAL4*.

An intriguing possibility is that cytoskeletal elements are playing an important role in seizure sensitivity and resistance in *Drosophila*. Snail family transcription factors such as *esg* normally promote cell-cell separation during development, at least in part by inhibiting the expression of cadherin, a homophilic cell-cell adhesion molecule linked to components of the actin network (TANAKA-MATAKATSU *et al.* 1996; HEMAVATHY *et al.* 2000). Filamin mutants are seizure sensitive in flies (*jbug*; X. REN and M. TANOUYE, unpublished results) and humans with periventricular heterotopia (FOX *et al.* 1998); β -integrin was identified as a suppressor of *eas* (Table 2); collagen type IV is a prominent target of aminopeptidase N (*sda*) in metastasis (SAIKI *et al.* 1993); and E-cadherin is downregulated by *sna* and potentially *esg* (BATTLE *et al.* 2000; CANO *et al.* 2000). Indeed, cytoskeletal reorganization is probably critical in morpho-

logical changes in dendritic spines associated with synaptic plasticity (YUSTE and BONHOEFFER 2001) and epilepsy (HOLMES and BEN-ARI 1998; ISOKAWA 2000; MEREDITH *et al.* 2000; SWANN *et al.* 2000). Cytoskeletal organization and reorganization also plays a prominent role as scaffolding for proteins subserving membrane excitability. Thus, signaling efficiency, reliability, and stability appear to be greatly influenced by the subcellular colocalization of excitability proteins in the photoreceptor and the presynaptic terminal and at the post-synaptic membrane of the neuromuscular junction. Defects in this scaffolding function via abnormal cytoskeletal elements may contribute substantially to the kinds of excitability instability thought to underlie seizure disorders.

The *esg* gene encodes a presumptive transcriptional repressor with restricted temporal and spatial expression in the nervous system (WHITELEY *et al.* 1992; FUSE *et al.* 1994). Presumably, ectopic expression of *esg* in larval interneurons mediates seizure suppression in adults via the activation or repression of one or more target genes. A combination of microarray and computational analyses revealed 100 genes that represent possible targets of neuronal *esg* (Table 9). Although none of these genes encode cytoskeletal elements, a number of them encode serine protease inhibitors (induced by neuronal *esg*) or serine proteases (repressed by neuronal *esg*) that could act on cytoskeletal elements or other proteins that influence synaptic interconnections. Further experiments will be needed to determine which of the 100 genes actually mediates seizure suppression. Such a gene could ultimately serve as target for novel antiepileptic drugs.

Isolation and examination of suppressor mutations is a potentially powerful approach to seizure disorders. It allows the identification of biological processes not previously associated with seizures through genes such as *esg*. In addition, *esg* has several properties that make it attractive as a candidate for new AED development: it reduces seizure susceptibility without apparent side effects. While it appears to be effective only during a window of time during neuronal development, this window may serve as an advantage in treating cases in which epilepsy develops early. Nevertheless, *esg*, or a seizure-suppressor gene with similar properties identified in this or future screens, may allow the development of powerful new treatments for the devastating effects of intractable epilepsy.

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