Modifications of Seizure Susceptibility in Drosophila

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INTRODUCTION

Human seizure disorders are a substantial health problem because of the large number of affected individuals and the variety of different syndromes. For example, an estimated 1% of the United States population is affected by more than 40 different syndromes that make up the epilepsies (Commission 1989; Hauser and Hesdorffer 1990; McNamara 1994). All individuals are potentially vulnerable to seizures; they can occur in anyone after a sufficiently intense insult to the brain (Noebels 1996). Although seizures can occur in most anyone, individuals vary in what constitutes a seizure-inducing stimulus (Sackheim et al. 1987; Walton 1989). Some individuals have high seizure susceptibility and have spontaneous seizures, whereas others have low susceptibility, and even head trauma or certain brain tumors do not lead to seizures (Walton 1989). Understanding what causes this variation in seizure susceptibility remains a fundamental problem in the study of human seizure disorders.

There are many interesting questions regarding what causes changes in seizure susceptibility: What types of genetic defects lead to large changes in susceptibility? How does susceptibility vary within an individual over time? How does it vary across genotypes? These problems can be difficult to resolve in humans; because of ethical considerations and the heterogeneous mechanisms by which seizures are triggered, it is impractical to quantify levels of seizure susceptibility across a diverse human population. An animal model in which seizures could be triggered in a reliable fashion across genotypes and over time would be extremely helpful in understanding what causes seizure susceptibility to vary in a population. We suggest that an especially valuable model for approaching this problem can be provided by the fruit fly, Drosophila.

There are several advantages of studying seizure susceptibility in Drosophila. First, there is the availability of relevant mutants such as the Drosophila bang-sensitive (BS) paralytic mutants, which exhibit high susceptibility to seizures after mechanical and electrical shock (Benzer 1971; Ganetzky and Wu 1982; Pavlidis and Tanouye 1995). These mutants can be used in conjunction with the vast array of excitability and behavioral mutants in Drosophila to examine the types of molecular defects that can suppress or enhance seizure susceptibility. There are also excellent molecular genetic methodologies available for Drosophila, including P-element–mediated cloning methods and the rapidly expanding Drosophila genomic database. Finally, there are a variety of electrophysiological stimulation and recording methods available for the fly, including recordings from the adult giant fiber (GF) system that were used in this study (Tanouye and Wyman 1980; Trimarchi and Murphey 1997).

Although there are many advantages to using Drosophila as a model for human seizure disorders, any system that is useful for studying seizure susceptibility must meet the following criteria: First, there must be a reliable way to test for seizure susceptibility across individuals and genotypes, so that accurate seizure thresholds can be measured. Second, there must be an identifiable baseline level of seizure susceptibility in wild-type strains, so that relative comparisons can be made with mutants and experimentally altered individuals. Finally, seizures in the model system should exhibit characteristics similar to those of seizures in humans. In this study, we demonstrate that Drosophila meets these criteria and is in fact an attractive model for human seizure susceptibility defects. In addition, the data presented here also offer insights into the mechanisms that affect seizure susceptibility.

METHODS

Fly stocks

Drosophila melanogaster strains were reared and studied at room temperature (22–24°C). They were maintained on standard cornmeal...
agar medium. The BS mutants used in this study were easily shocked (eas), slamdance (sda) and bang senseless (bss). The eas allele eas\(^1\) has been described previously (Ganetzky and Wu 1982; Pavlidis et al. 1994) and encodes an ethanolamine kinase involved in one pathway of phosphatidyl ethanolamine synthesis. The allele of bss used in this study, bss\(^2\), has also been previously described (Ganetzky and Wu 1982). The sda\(^{107}S\) allele is the most recent BS mutant identified and has been mapped to 97D (H. Zhang, personal communication). Wild-type flies were either Canton-Special (CS) or Oregon-R (OR) strains. The maleless-no-action potential (ml\(^{n/o/p}\)) mutant strain is a temperature-sensitive paralytic mutation affecting an RNA-helicase–like protein (Kernan et al. 1991; Lee and Hurwitz 1993) and is known to suppress BS paralysis (Ganetzky and Wu 1982; Pavlidis and Tanouye 1995). The BS heterozygotes were generated by crossing the appropriate mutant with CS flies. We used predominately female flies in this study, because their larger size made it easier to perform electrophysiology.

**Behavioral testing**

Behavioral testing was performed on flies that were 2–3 days posteclosion. Flies were allowed to rest 2 hours after exposure to anesthesia before testing. To test flies for the BS phenotype, five flies were placed into a clean vial (Applied Scientific) and allowed to rest for 30 min. They were then vortexed on a VWR vortexer at maximum strength for 10 s. The flies were vortexed again 3–17 min later to determine whether the flies were still refractory. Because of high variability in the behavioral refractory period, the refractory period values listed in the text are ranges of time spanning 2–5 min, depending on the genotype. For each BS genotype tested, >85% of the flies had refractory periods that fell into the range listed.

**GF electrophysiology**

All flies used for electrophysiology were 2–3 days posteclosion. The method used to stimulate and record GF–driven muscle potentials as well as to elicit seizures was as described (Pavlidis and Tanouye 1995; Tanouye and Wyman 1980) with the following modifications: the fly was removed from the vial, another needle attached to a vacuum hose and using this to suction onto the head of the fly. Once anesthetizing the fly. In past studies (Pavlidis and Tanouye 1995), the flies were knocked out with ether before mounting, a technique that appears to reduce the excitability of the flies, which in turn could artificially raise the voltage of the high-frequency (HF) stimulus necessary to elicit a seizure. In addition, we used tungsten stimulating electrodes, uninsulated tungsten wire (WPI 0.075 mm) electrolytically sharpened to a tip of a few micrometers, that were smaller and sharper than those used in previous studies. These electrodes cause less damage to the fly, thereby increasing the stability and reliability of the preparation.

Two types of stimulation were used, single-pulse stimulation and HF stimulus waversets. Single-pulse stimuli (0.2 ms duration) delivered to the brain were used to drive the GF. To elicit seizures, HF stimuli (0.5-ms pulses delivered at 200 Hz for 300 ms) were delivered to the brain and the intensity (voltage) of the HF stimulus was varied as noted. Frequency of the stimulus also affected seizure susceptibility and was varied to 100 Hz and 50 Hz where noted. With the use of the described techniques, the effective voltages for initiating seizures were much lower than recorded previously (Pavlidis and Tanouye 1995). In all protocols, the GF threshold for 0.2-ms single-pulse stimulation was relatively constant (1.5–3.0 V). For individual experiments in which the GF threshold was well above this range, the preparation appeared to be damaged and the data were discarded. (Under the stimulation conditions used, the long latency presynaptic pathway seen by other investigators was observed <50% of the time (Elkins and Ganetzky 1990; Engel and Wu 1996). The recordings were all taken from muscle fibers, but we believe them to reflect accurately the activity of the motor neurons on a one-to-one basis for reasons described previously (Koenig and Ikeda 1983; Pavlidis and Tanouye 1995).

**Determination of seizure thresholds and suppression thresholds**

To determine seizure thresholds, HF stimuli were initially given to BS mutants at low intensities, 1–6 V depending on the genotype. If the stimulus was unsuccessful at eliciting a seizure, the intensity was subsequently increased in 1-V increments until a seizure was induced. The fly was allowed to rest 5 min between each HF stimulus. Once an initial seizure was induced, the fly was discarded. The seizure thresholds and seizure threshold curves presented represent a population of flies for that specific genotype. Thresholds were determined for individual flies as the lowest intensity at which seizures occurred. Individual thresholds were then compiled for each genotype to determine the mean threshold for that genotype. These threshold values are listed in Table 1. Seizure suppression thresholds were determined in much the same way and represent the mean voltage, above the seizure threshold, at which a particular genotype no longer undergoes seizures. Because higher voltages were needed to determine suppression thresholds as well as seizure thresholds for wild type, eas\(^{+/+}\), sda\(^{+/+}\) and ml\(^{n/o/p}\) strains, in these cases, flies were never given more than two HF stimuli from which individual thresholds were determined. Thresholds for individual flies were then compiled as described for the BS mutants to determine mean thresholds. The graphs found in Figs. 2, 3, and 5 were generated by calculating the percentage of flies that had a seizure at each HF stimulus intensity for a particular genotype. These data points were then plotted and best fit to a sigmoidal (Boltzman) curve using Origin 3.73 software.

**Refractory period determination**

Another set of experiments was conducted to investigate the electrical refractory period length. In these, an initial HF stimulus was delivered just above threshold to elicit a seizure. The HF stimulus intensities used were 7 V for sda, 4 V for eas, and 3.5 V for bss. A subsequent HF stimulus of the same voltage was then given at varying time points after the initial seizure to determine whether the fly was still refractory. The end of the refractory period was determined as the time point at which at least 50% of the flies showed no behavioral evidence of a seizure.

**Table 1. Threshold values for the various strains tested**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Giant Fiber Threshold</th>
<th>Seizure Threshold</th>
<th>Suppression Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>bss</td>
<td>2.3 ± 0.42</td>
<td>3.1 ± 0.7</td>
<td>12.5 ± 3.4</td>
</tr>
<tr>
<td>eas</td>
<td>2.3 ± 0.40</td>
<td>3.6 ± 0.7</td>
<td>10.3 ± 2.9</td>
</tr>
<tr>
<td>sda</td>
<td>2.2 ± 0.38</td>
<td>6.8 ± 1.0</td>
<td>63 ± 8.0</td>
</tr>
<tr>
<td>bss(^+/+)</td>
<td>2.4 ± 0.31</td>
<td>6.7 ± 1.1</td>
<td>59 ± 7.1</td>
</tr>
<tr>
<td>eas(^+/+)</td>
<td>2.3 ± 0.29</td>
<td>30.6 ± 4.5</td>
<td>—</td>
</tr>
<tr>
<td>sda(^+/+)</td>
<td>2.5 ± 0.29</td>
<td>43.2 ± 3.8</td>
<td>—</td>
</tr>
<tr>
<td>CS</td>
<td>2.4 ± 0.44</td>
<td>44.5 ± 4.4</td>
<td>—</td>
</tr>
<tr>
<td>OR</td>
<td>2.4 ± 0.41</td>
<td>48.4 ± 3.6</td>
<td>—</td>
</tr>
<tr>
<td>ml(^{n/o/p})</td>
<td>3.1 ± 0.24</td>
<td>&gt;100</td>
<td>—</td>
</tr>
</tbody>
</table>

Values for the giant fiber threshold, seizure threshold, and suppression threshold (if any) are listed for each genotype that was tested. The four genotypes listed in bold type are sensitive to mechanical shock whereas the others are resistant. CS, OR, sda\(^+/+\), eas\(^+/+\) and ml\(^{n/o/p}\) strains showed no seizure suppression, even at the highest voltage tested, 100 V. (n > 13 for each genotype tested.) See METHODS for description of Drosophila strains. J. Lee and C. F. Wu (1997) have independently observed similar sequences of physiological events in the giant fiber pathway of bss and bss mutant flies that can be correlated with the behavioral phenotype of the BS mutants.
time, rounded to the nearest 30-s interval, at which 50% of the flies had a seizure in response to the second HF stimulus. A similar protocol was used to determine seizure threshold changes for sda during the refractory period. In this case, the intensity of the second HF stimulus was varied from 6 V, which is just below the seizure threshold, up to 100 V. The second HF stimulus was delivered at various time points after the initial seizure. In this way, seizure thresholds were determined, as described previously, for each 60-s time point during the sda refractory period.

Seizure length and latency changes

To analyze the difference between seizures elicited by HF stimuli with intensities near the seizure threshold and seizures elicited by HF stimuli with intensities near the suppression threshold, a single bss fly was given two seizures, one with an HF stimulus of 3–4 V and one with an HF stimulus of 12–15 V. The fly was allowed to rest 15 min between each HF stimulus. Half the flies were given the 3–4-V HF stimulus first, whereas the other half were given the 12–15-V HF stimulus first. There was no obvious difference between the two protocols. The latency was measured as the time until the fifth spike after the HF stimulus. This was intended to control for random spikes that occasionally appeared after the HF stimulus but before the seizure. The length of the seizure was measured as the time from the fifth spike to the cessation of spiking activity in the muscle.

Thoracic ganglia stimulation

To stimulate the thoracic ganglia, flies were mounted on a pin in the manner described earlier. The stimulating electrodes were bent at 45° angles and inserted just through the anterior pre-episternum (near the base of the first coxa) with the help of a mirror placed at an angle under the fly. After single-pulse (0.2 ms) stimulation, usually two dorsal longitudinal muscle (DLM) responses could be seen as described previously (Salkoff and Kelly 1976), a short-latency response, stimulation of the DLM motor neuron directly, and a long-latency response. Seizures where induced with a standard 300-ms HF stimulation of 0.5-ms pulses at 100 Hz. Seizure thresholds for thoracic stimulation were calculated as described previously. Thresholds were more variable in the thorax, most likely because of the higher variability of electrode placement in this preparation compared with brain stimulation.

Recordings in hemolymph solution

When recording from the tergoretrochanter muscle (TTM), some experiments were done with the thorax under saline to be sure that the TTM seizure activity was not pick-up from other neighboring muscles. We found that the TTM activity was not pick-up from other muscles, because no differences were seen between TTM seizure activity under saline and that seen under normal conditions. To record unconfused saline, flies were suctioned as previously described, and the wings and legs were removed with dissecting scissors. The fly was then embedded in wax in a Petri dish above a polyethylene tube with a hole cut in it to supply air to the fly. Wax was pressed up against the thoracic cuticle, and the abdomen was covered. Wax was then placed over the thoracic connective so that the head and thorax were separated by a wax barrier. Hemolymph-like solution, HL3, (Stewart et al. 1994) was allowed to cover the thorax, whereas the head was exposed to the air. Insulated tungsten recording electrodes (WPI) were placed into the DLM, dorsoventral muscle (DVM), and TTM muscles and a ground electrode was placed in the bath. The stimulating electrodes were the same as described previously.

Recordings from leg and direct flight muscles

To record from both leg and direct flight muscles, flies were mounted to pins as described above. The tibia levator muscle (TLM) recordings from the mesothoracic leg were obtained as previously described (Trimarchi and Schneiderman 1993), except that glue was used in place of wax to affix the leg. Intercoxlateral levator muscle (ILLM) recordings from the prothoracic leg were prepared in a similar fashion, and then recording electrodes were inserted dorsally along the long axis of the coxa. In recordings of direct flight muscles, the thorax was tilted approximately 90° relative to the head to allow insertion of the recording electrodes into the direct flight muscles based on external cuticle markers (Heide and Gotz 1996; Tanouye and King 1983). The direct flight muscles recorded from this position, the anterior pleural muscle 1 (PA1) and the anterior pleural muscle 3 (PA3) (King and Tanouye 1983), were identified by both external markers and stimulation threshold. When recording from direct flight muscles, simultaneous recordings were made in underlying DVM muscles to determine whether the activity seen in the direct flight muscles was pick-up from the larger DVMs. The activity was never synchronous.

RESULTS

The features of BS mutant seizures to be described are similar to those reported previously (Pavlidis et al. 1994; Pavlidis and Tanouye 1995). A short, intense train of electrical stimuli delivered to the brain causes seizure-like activity, which is observed as abnormal HF (~100 Hz) firing of the DLM motoneurons lasting 1–2 s. The “seizure” is followed by failure of the GF pathway for a period that varies depending on the genotype. The present study shows significant differences in our ability to define seizure susceptibility and the ways in which it may be modulated. This comes about, in large part, from eliminating anesthesia and using smaller diameter tungsten electrodes, both of which appear to keep brain neurons substantially healthier. For example, activation thresholds for the GF neuron in this study are in the 2–3-V range (down from ~10 V) (Pavlidis and Tanouye 1995; Pavlidis et al. 1994; Tanouye and Wyman 1980). Also, seizures are evoked at much lower voltages. For example, in bss mutants, the seizure threshold is 3.1 V (down from ~50 V in Pavlidis and Tanouye 1995). The resolution, reproducibility, and reliability of results allow us, for the first time, to quantify features of seizure initiation in Drosophila and to ascertain how these features vary across genotypes and with past experience.

Seizure susceptibility varies with genotype

In previous studies (Pavlidis and Tanouye 1995), seizures were evoked in BS mutants and were almost never seen in wild-type flies, even at the highest voltage tested (100 V). In the present experiments, seizures are evoked consistently in CS wild-type flies at a threshold of 44.5 ± 4.4 V (Fig. 1, Table 1). Seizures in CS flies were qualitatively similar to BS seizures in the spiking activity observed in the DLM (Fig. 1) and were followed by the usual period of synaptic failure, although the frequency of the spontaneous seizures during recovery was reduced significantly. In addition, the synaptic failure period was shorter in CS flies, 40 ± 12 s (n = 14), than the values previously obtained for different BS mutants that range from 46 ± 20 s to 112 ± 70 s (Pavlidis and Tanouye 1995). A second wild-type strain, OR, was used to verify the ability of wild-type flies to undergo seizure and was found to be similar in phenotype to CS, indicating that the overall response to HF stimuli is similar for both BS mutants and wild-type flies. For OR, the threshold for seizure is 48.4 ± 3.6 V. Not surprisingly, there appears to be some variation in seizure threshold for
different wild-type strains; however, this variation is quite small when compared with the effects of the different genetic mutants discussed here.

The major difference between wild-type and all BS mutant flies is that the latter have seizures at far lower HF stimulus intensities. For example, in bss, the most susceptible of the BS mutants, seizures occur at only 3.1 ± 0.7 V, or about 13 times lower than that seen for CS (Table 1). Each of the BS mutants studied has a characteristic HF stimulus intensity for eliciting seizures, the seizure threshold, that is much lower than wild type (3.6 ± 0.7 V for eas and 6.8 ± 1.0 V for sda). These mutants demonstrate that seizure susceptibility can be enhanced by genetic mutation. We have also found that the seizure susceptibility can be suppressed in certain mutants. The hypoxicity mutant mle<sup>napt</sup> has a much higher characteristic threshold than wild type. In fact, even at the highest voltages tested (100 V) seizures occurred in only 11% of these flies (n = 18). These data demonstrate that seizure thresholds can be modulated by genetic mutations over a large range in Drosophila.

**Individual neuron excitability is similar in normal and mutant flies**

A possible explanation for genotypic differences in seizure susceptibility is that each individual neuron in BS mutants could be hyperexcitable. In this case, a low-intensity HF stimulus in the BS mutants could be directly activating the same number of neurons as a high-intensity HF stimulus in wild type. 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<sup>+</sup> channel, which would presumably cause membrane hyperexcitability (Smart et al. 1998). Experiments examining GF excitability suggest that this is probably not an explanation for the Drosophila BS mutant phenotype, because the stimulus voltages required for activation of the GF did not differ among genotypes (Table 1). For example, the GF activation threshold for CS (2.4 ± 0.44 V) is virtually identical with that of bss (2.3 ± 0.42 V). GF activation thresholds for other genotypes are also in the same range (Table 1) with the exception of mle<sup>napt</sup> (3.1 ± 0.24), which has a slightly elevated GF threshold. We also tested another group of neurons, the DLM motoneurons, to further examine the excitability of individual neurons. The thresholds for DLM motoneuron activation are 1.52 ± 0.37 V for mle<sup>napt</sup>, 1.31 ± 0.24 V for bss, and 1.35 ± 0.33 V for CS, a further indication that alterations in individual neuron excitability probably does not account for the large differences in seizure susceptibility seen between BS mutant and wild type. We cannot rule out the possibility that there may be other neurons critical to the generation of seizure that have altered excitability.

For bss, the GF activation voltage and the voltage for evoking seizures are very close to each other, and their variability nearly overlaps. However, when examined on a case-by-case basis, the GF activation voltage was lower. Because at its threshold, the GF appears to be the only neuron activated, we interpret this to mean that, despite its extensive motor outputs, stimulation of the GF alone is not sufficient to evoke a seizure. Even in the case of bss, the most susceptible of our mutants, the HF stimulus is recruiting other higher threshold (i.e., smaller diameter) neurons, some of which have been described previously (Tanouye and King 1983; Tanouye and Wyman 1980), and recruitment of these other neurons is necessary to trigger seizures. That low voltages are required in bss flies suggests that relatively few of these other neurons have to be recruited by the HF stimulus to initiate seizures. Higher voltages are required for wild-type flies, suggesting that many more neurons must be recruited for seizure initiation in these flies. Thus, the increased seizure susceptibility seen in BS mutants may correspond to a reduction in the minimum number of neurons that must be recruited by an HF stimulus to initiate a seizure.

**Altering the frequency of pulses within an HF stimulus affects seizure susceptibility**

On the basis of these data, we suggest that single-cell excitability does not appear to play a substantial role in defining the range of seizure susceptibility in normal and mutant Drosoph-
il. Rather, for each genotype, we believe there is a characteristic minimum number of brain neurons that must be driven synchronously by the pulses within an HF stimulus. For some mutants, such as bss, we suggest that this minimum is a very small number of neurons; for wild type a much larger number of neurons must be driven. We do not yet know how many neurons are involved, because there is no good, independent way of measuring this. Nevertheless, by altering the HF stimulus in characteristic ways, we are able to modify seizure susceptibility in a fashion consistent with the hypothesis that we are redefining the minimum number of neurons that must be driven.

One very interesting way in which seizure thresholds may be modulated is by varying the frequency within the HF stimulus. An example of the effect of frequency on seizure initiation can be seen in bss flies. In this case, the seizure threshold for frequencies of 200 Hz, 100 Hz, and 50 Hz increases from $3.1 \pm 0.7$ V, to $5.1 \pm 1.2$ V, to $6.8 \pm 1.1$ V, respectively. Varying the frequency had comparable effects for $sda$ and $eas$ mutants (data not shown). It appears that the individual pulses present in the HF stimulus are somehow integrated by the neurons that are responsible for generating the seizure; some form of temporal summation is taking place. If the number of neurons recruited by a 4-V HF stimulus is always the same, the frequency is expected mainly to affect the firing rate of these neurons. At higher frequencies, the increased rate of firing is temporally summated by the underlying excitatory circuits, and a seizure results. At lower frequencies, temporal summation is not as effective; therefore, a larger number of neurons must be driven to initiate seizure.

Seizure thresholds in heterozygote flies

The three BS mutants described here are similar in several respects. They have similar behavioral phenotypes, as well as seizure and synaptic failure phenotypes. They are not, however, identical, and one of the most salient differences emerges in heterozygous flies. The situation for each of the mutations, $bss$, $eas$, and $sda$, is completely different as heterozygotes (Table 1). The $eas$ mutation acts as a recessive when examined electrophysiologically. Seizures are evoked in $eas/+ \times$ flies at an HF stimulus intensity of $43.2 \pm 3.8$ V, very close to the wild-type value. This is qualitatively similar to the behavioral phenotype of $eas$, which is completely recessive (Ganetzky and Wu 1982). In contrast, $bss$ acts almost as a completely dominant mutation. Seizures are evoked in $bss/+ \times$ flies at an HF stimulus intensity of $6.7 \pm 1.1$ V, close to the value of $bss$ homozygotes. This also is consistent with what is seen behaviorally (Ganetzky and Wu 1982). The $sda$ mutation, however, has quite an interesting phenotype. It acts as a semidominant mutation in the seizure assay (Fig. 1), whereas behaviorally it acts as a recessive. Seizures are evoked in $sda/+ \times$ flies at an HF stimulus intensity of $30.6 \pm 4.5$ V, between the values for mutant homozygotes and wild type, although somewhat closer to wild type.

Taken together, we have examined nine different genotypes. For each genotype, seizures are elicited by an HF stimulus with a characteristic voltage allowing us to use this as a measure for defining seizure susceptibility. In this way, susceptibility is found to vary over a considerable range, from 3 to 100 V. Genotypes that display a high susceptibility to seizures (i.e., low seizure threshold value: $bss$, $eas$, $sda$, $bss/+ \times$) also have a strong BS behavioral phenotype, and genotypes that display a lower susceptibility to seizure (i.e., higher seizure threshold value: $sda/+ \times$, $eas/+ \times$, CS, OR, mle) are not bang sensitive. Having a characteristic seizure threshold for a particular genotype now allows us to examine changes in seizure susceptibility over time.

Seizure thresholds shift during the refractory period

Behavioral bang sensitivity varies according to genotype; it can also vary according to previous experience. On recovery from paralysis, BS mutants cannot be reparalyzed by a second bang stimulus for a period that varies according to genotype (Ganetzky and Wu 1982; Grigliatti et al. 1973; Judd et al. 1972). This is termed the refractory period. For $bss$, $eas$, and $sda$, the refractory period is 10–15 min, 8–10 min, and 5–7 min, respectively. If the behavioral bang sensitivity is correlated with seizure susceptibility, we reasoned that after a seizure, there might be an increase in the HF stimulus intensity required to elicit a second seizure. That is, the refractory period might be the result of a transient change in seizure susceptibility resulting from a previous bout of seizure. To determine this, $sda$ flies were given an initial seizure at threshold, 7 V. At 60-s intervals after the initial seizure, HF stimuli of various intensities were applied to determine susceptibility to a second seizure. One minute after the initial seizure, the threshold for a second seizure is very high, $94 \pm 6.4$ V. This indicates that there is a large decrease in seizure susceptibility immediately after an initial seizure. In this case, the 94-V threshold indicates that $sda$ is much less susceptible to seizure than wild-type flies. The change in seizure susceptibility is transient; as the time after the first seizure increases, the threshold continuously declines. At 2 min, $sda$ has about the same susceptibility as wild-type flies, $55 \pm 8.6$ V, whereas at 5 to 6 min, the seizure threshold has decreased to near the initial seizure threshold for $sda$ (Fig. 2). Thus, even within a single genotype seizure susceptibility is quite plastic. Immediately after an initial seizure, susceptibility is modulated by some process that causes a large threshold increase. Seizures may still be elicited at this time; however, an HF stimulus of higher intensity must be used. This is presumably because a much greater number of neurons must be stimulated. Whatever this process is, it appears not to involve an increase in individual nerve cell excitability, because there is no change in GF threshold during the refractory period (data not shown).

For $sda$, the changes in seizure susceptibility after an initial seizure resemble what is expected for an explanation of the behavioral refractory period. In the case of $sda$, the duration of the susceptibility change is similar to the duration of the behavioral refractory period, and during most of this time, $sda$ is not very susceptible to seizures, the threshold is in the range of wild type. There are also seizure susceptibility changes in $bss$ and $eas$ after an initial seizure. These changes are generally similar to those seen for $sda$; however, they provide a less satisfying explanation for $bss$ and $eas$ behavioral refractory periods. The duration of the susceptibility change in both of these mutants is not as long as the behavioral refractory period. For $bss$, the seizure susceptibility change lasts 6 min, whereas the behavioral refractory period is 10–15 min. Similarly, the duration of the seizure susceptibility change in $eas$ is 4 min.
also shorter than the behavioral refractory period, 8–10 min. In addition, the change in the seizure threshold is not very great. At 60 s after the initial seizure, a second seizure may be elicited by 8-V HF stimuli for bss and 10-V HF stimuli for eas. These HF stimulus intensities are substantially less than those seen for wild type, and it seems as if these mutants should still be bang sensitive. Taken together, these results suggest that changes in seizure susceptibility may contribute to the behavioral refractory period but do not seem to provide a complete explanation.

This change in susceptibility after a seizure is not limited to the BS mutants but occurs in wild-type strains as well. One minute after an initial seizure, CS flies are highly resistant to seizure; a 100-V HF stimulus triggered a seizure in only one of nine flies tested. Thus, these flies were much less susceptible to seizure than they had been initially.

**Seizures are not seen at high stimulus intensities**

The characteristic HF stimuli that elicit seizures for a given genotype behave in an all-or-nothing manner. That is, below threshold, seizures are never elicited, whereas just above threshold, seizures are elicited and spread throughout all muscle groups examined (description to follow). However, if HF stimulus intensities are increased well above threshold, there are changes in the form of the seizure. These changes consist of the latency to seizure becoming shorter and less variable and the seizure becoming shorter in duration. For example, in bss a 4-V HF stimulus elicits a seizure with a 410 ± 69 ms latency and 730 ± 120 ms duration, whereas a 12-V HF stimulus elicits a seizure with a 190 ± 65 ms latency and 460 ± 155 ms duration (n = 11) (Fig. 3).

As HF stimulus intensity is increased further, a surprising thing occurs that we have termed “high-voltage seizure suppression.” We have found, in certain genotypes, that high stimulus intensities are not effective at eliciting seizures. For example, in bss, we occasionally observe seizure suppression after HF stimuli of 10 V. As HF stimulus intensity is increased from 10 to 20 V, fewer HF stimuli are effective in eliciting seizures. With HF stimuli of >20 V, seizures are never observed in bss. High-voltage seizure suppression in bss has a threshold of 12.5 ± 3.4 V. This observation describes a very curious situation for bss: There is only a small window of HF stimulus intensities, between ∼3 and 12 V, that are effective in eliciting seizures.
High-voltage seizure suppression is also observed in eas, sda, and bss+/ flies (Fig. 4). For each genotype that shows suppression, there is a characteristic voltage, the suppression threshold, at which it occurs: bss (12.5 ± 3.4 V) ~ eas (10 ± 2.9 V) ~ sda (63 ± 8.0 V) ~ bss+/ (59 ± 7.1 V). There appears to be a relationship between the seizure threshold value and the suppression threshold value. The order of genotypes from the lowest to the highest is roughly the same, with suppression thresholds ~4–9 times higher than seizure thresholds for these genotypes. This suggests that technical limitations are responsible for the genotypes that do not show suppression in these studies: sda+/+, eas+/+, CS, OR, and mlle napts. Extrapolating from their seizure thresholds, these genotypes would all have predicted suppression thresholds of >100 V, too large to be sampled in these experiments.

To investigate the physiological cause of suppression in bss flies, we examined how the GF circuit functions immediately after the HF stimulus. After low-intensity HF stimuli (4–12 V), the GF circuit is functional from the end of the HF stimulus to the onset of seizure. That is, single-pulses are effective in activating the GF because muscle potentials are evoked in the DLMs via the circuit. After high-intensity HF stimuli (~20 V), that lead to suppression of seizure, we find that the GF pathway is not functional. From the earliest times we can measure after a 20-V HF stimulus in these flies (~10 ms), the GF circuit does not support DLM evoked potentials. We cannot be certain that at the early time points after the HF stimulus whether it is synaptic failure, the activation of inhibitory inputs or some other process that limits GF circuit operation; however, it is known that at later time points, it is synaptic failure (Pavlidis and Tanouye 1995). It is clear, however, that increasing the voltage of the HF stimulus decreases the latency to failure of the GF pathway in much the same way that increasing the voltage of HF stimulus causes the latency to seizures to decrease in bss flies (see earlier description).

Seizure suppression interferes with the spread of seizures

When the HF stimulus voltage delivered to the fly is at the seizure or suppression threshold, the results are generally similar. Some HF stimuli are effective, and widespread seizures are elicited; some HF stimuli are not effective and do not elicit seizure. In fact, this is always the case for HF stimuli at the seizure threshold. However, at suppression threshold, a third type of response is occasionally (<10% of stimuli) observed: seizures are observed in some muscles, but not others. Recordings from two ipsilateral DLM fibers show that in every case, the motoneurons innervating these fibers either both undergo seizure or both show suppression (Fig. 5). In contrast, if the same HF stimulus is applied and two contralateral DLM fibers are examined, occasionally seizure occurs in one DLM motoneuron, but not in the other (Fig. 5). We infer that these are cases of partial or incomplete seizure suppression and that the seizure can spread to an ipsilateral, but not to a contralateral, DLM. In the case of partial suppression, we know that by using a slightly higher HF stimulus voltage, we can further suppress the spread of seizure so that it is not seen in any of the DLM motoneurons.
the seizures, probably due to seizures reaching the DLMs by different pathways than for brain stimulation. For bss mutants, ganglion HF stimulus intensities that are effective for eliciting seizures are lower than those for wild type indicating again that the BS mutants are more susceptible to seizures. Using 100-Hz HF stimuli, these three genotypes have seizure thresholds of $3.3 \pm 0.56$ V for bss, $9.5 \pm 2.9$ V for CS, and $23 \pm 4.8$ V for mle$^{nap}$s. Because only 100-Hz HF stimuli were used for thoracic ganglion stimulation, the intensity of the ganglion HF stimulation required to elicit seizure is lower than that seen in brain HF stimulation for all genotypes tested.

The extent of seizures

DLM muscle potentials provide a convenient way to monitor how genotype and experience contribute to seizure susceptibility. In addition, the GF system, particularly the GF-to-DLM pathway provides a convenient way to test for neural circuit properties such as synaptic failure. However, we find that seizures spread throughout a much larger population of thoracic motoneurons. The spread is to all motoneurons that we have recorded from, including both GF-system and non–GF-system outputs. In the GF system, we have observed seizure spread to all known outputs: TTM, DLM, DVM, PA1, and the mesothoracic leg TLM muscles (Fig. 7). We have also observed seizure spread in non–GF-system outputs, including PA3 and prothoracic leg ILLM muscles. Thus, seizures spread extensively throughout the thoracic ganglion motoneuron population; it could be that the entire population is involved.

Because seizures are known to spread through the nervous system along particular pathways (Noebels 1996), we reasoned that we might see latency differences in seizure onset in the various motoneurons. Seizure may spread to some motoneurons by a rather direct route, whereas spread to others may occur via a less direct route. In fact, Fig. 7 shows that the latency to seizure onset in a DLM and a TTM muscle in the same bss fly were not identical. The latency to seizure onset in the DLM is 450 ms, whereas the latency in the TTM is 700 ms. This result is repeatable, because when bss flies are given HF stimuli near the seizure threshold, seizure activity is not seen in

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**FIG. 5.** Lateral segregation of seizures in bss. When bss mutant flies are given HF stimuli near the suppression threshold (12 V) some stimuli are ineffective at initiating seizures, and some are effective. Surprisingly, some stimuli elicit seizures in one-half of the fly, whereas seizures are suppressed on the contralateral side. Boxes indicate the length of the HF stimulus. **A**: two pairs of recordings from ipsilateral DLM fibers. These fibers always behave synchronously, either both seizing (top) or both suppressed (bottom). **B**: three recordings from contralateral DLM fibers that show synchronous behavior, both seizing (top) or both suppressed (bottom) and a third class of response, a seizure in the right DLM and suppression of seizure in the left. These unilateral seizures are only observed near the suppression threshold. Calibration: 10 mV; 200 ms.
the TTMs until $303 \pm 64 \text{ ms (n = 12)}$ after seizures begin in the DLMs. These latencies do not appear to be greatly affected by genotype; roughly similar values are observed for *eas*, seizure activity in TTMs was delayed $215 \pm 75 \text{ ms (n = 8)}$ compared with DLMs. This result is opposite to that seen after single-pulse stimulation of the GF pathway where the DLM response has a longer latency than the TTM response (Tanouye and Wyman 1980). This indicates that different pathways to these muscles must exist and can be recruited differentially by different stimulus regimens. Seizures spread to other motoneurons with characteristic latencies. For example, DLMs, DVMs, and ILLMs have a short latency and display seizure activity on the same time scale, whereas TTMs and TLMs have a longer latency.

Although the pathways for seizure spread are not known, there may be ways to dissect them. As described earlier, during high-voltage seizure suppression, seizures are sometimes seen in the ipsilateral, but not the contralateral DLMs. This suggests that seizures spread to these two muscles by different pathways. We infer that the point of divergence must be above the level of the DLM motoneurons because all of the motoneurons innervating the different fibers of the same muscle always behave similarly, and because suppression always occurs in an all-or-nothing manner.

**DISCUSSION**

**Genetic and experience-dependent modifications of seizure thresholds**

Previous studies of seizures in *Drosophila* found that certain mutants, the BS mutants, undergo seizure in response to electrical shock (Pavlidis and Tanouye 1995). Because of experimental limitations in that study (see RESULTS), seizures were rarely seen in other genotypes. Here, we have been able to demonstrate that each genotype, whether wild type or mutant, has a characteristic or signature HF stimulus intensity at which seizures occur. This is the first demonstration that wild-type flies consistently undergo seizure after electrical shock; however, this should not be surprising, because it appears that all higher nervous systems have the capacity for seizure after high-intensity stimuli (Noebels 1996). For example, human subjects have seizures in response to electroconvulsive therapy, whereas repeated high-intensity stimuli can lead to the kindling of seizures in many animals and brain tissue preparations (Alonso-DeFlorida et al. 1958; Fisher 1989; Loscher 1997). The defect then in the BS mutants is not that they have seizures, but rather that the mutation modifies the seizure threshold, in this case making the flies more susceptible to having seizures than wild-type strains. Genetic mutations can also make flies less susceptible to having seizures, as in the case of *mle*mutps. In fact, we have found that seizure thresholds can be modified genetically over a very wide range, from 3 to $>100 \text{ V}$, in this study.

An interesting example of the genetic modification of seizure thresholds is found in looking at heterozygous flies. Unlike the BS mutants whose thresholds are in a narrow range (4 V) the heterozygote thresholds vary over a range of 35 V. These differences cannot always be predicted by their behav-

**FIG. 6.** Seizures after stimulation of either the thoracic ganglion or the brain. Stimulating electrodes were placed into either the thoracic ganglion or the brain, and HF stimuli of various frequencies were delivered. Boxes indicate the length of the HF stimulus. *A*: seizure elicited by a 3.5-V, 100-Hz stimulus delivered to the thoracic ganglion of a *bss* fly. *B*: seizure elicited by a 3.5-V, 200-Hz stimulus delivered to the brain of a *bss* fly. Calibration: 10 mV, 200 ms.

**FIG. 7.** Seizure spread to other giant fiber (GF) muscles. *A*: simultaneous recordings from a DLM fiber and a TTM fiber under saline solution. Boxes indicate the length of the HF stimulus. Calibration: 10 mV, 200 ms. *B*: simultaneous recordings from a DLM fiber and a TLM fiber. The HF stimulus was not recorded on the oscilloscope. Calibration: 5 mV, 200 ms for the TLM; 20 mV, 200 ms for the DLM.
ior, particularly in the case of sdal/+ flies. These flies show how a single copy of the sda mutation can lead to a significant difference in seizure susceptibility compared with wild type (14 V) even though the behavior is normal. This may be in part because different pathways are responsible for the behavioral and electrophysiological phenotypes. The sdal/+ result indicates that mutations that appear behaviorally inconsequential can alter seizure thresholds in perceptible ways. This is also seen in humans in some forms of idiopathic epilepsy (Walton 1989) and in certain knockout mice (Erickson et al. 1996; Signorini et al. 1997).

In addition to genetic mutations, the previous experience of the fly also modifies seizure susceptibility. Immediately after a seizure-inducing HF stimulus there is an abrupt jump in the seizure threshold that does not coincide with any marked change in the threshold of individual neurons. The change in threshold after a seizure is a well-established phenomenon in mammals. In humans the seizure threshold can remain elevated for a few days (Sackeim et al. 1983, 1987), whereas in rats it can remain elevated for a few hours (Green et al. 1982). Because both wild-type and mutant flies exhibit threshold increases after a seizure, it appears to be a normal response to seizure in Drosophila as well.

Modification of seizures: suppression and spatial segregation

After high-voltage HF stimuli, we have found that seizures can be suppressed in certain genotypes. One possibility is that these high-intensity stimuli somehow cause widespread synaptic failure that inhibits the generation or spread of a seizure. This synaptic failure may be similar to that seen after seizure in the GF pathway (Pavlidis and Tanouye 1995). The second possibility is that high-voltage HF stimuli may disrupt positive feedback loops that are thought to be involved in the generation of the oscillations seen during seizures (Huguenard and Prince 1994; Traynelis and Dingledine 1988; Warren et al. 1994). If multiple neurons within a positive feedback loop are recruited simultaneously by the high-voltage HF stimulus, it is possible the loop would be disrupted so that the seizure would be suppressed or possibly shortened. The third possibility is that suppression is a result of synaptic inhibition; it is possible that the HF stimulus, in addition to recruiting excitatory (seizure) circuits, is also recruiting inhibitory circuits, which can, at certain voltages, suppress seizures. For example, after 12-V HF stimuli in bss flies, seizures are very short, possibly because they are short-circuited by inhibitory input. However, after 20-V HF stimuli in bss flies, seizures are suppressed, and the GF immediately fails to support evoked potentials indicating the possible abrupt activation of inhibitory inputs. A fluctuation in the recruitment of these putative inhibitory circuits could account for the suppression of seizures and the shortening of seizures we see near the suppression threshold.

It is also possible to generate seizures in the BS mutants that are spatially segregated, i.e., limited to a specific region of the fly CNS, after HF stimuli that are near the suppression threshold. This suggests a fundamental difference between the seizure threshold and the suppression threshold. The seizure threshold defines the minimum number of neurons required to initiate a seizure. Once seizure is initiated, there is no barrier to its spread; it spreads throughout the nervous system regardless of genotype. In contrast, we propose that high-voltage seizure suppression occurs because seizures do not spread properly from the site of initiation (i.e., probably near the stimulating electrodes in the brain) to the monitoring point (i.e., the DLM recording electrodes in the thorax). During partial seizure suppression, the spread of the seizure is limited in space, possibly depending on the kinetics and the level of inhibitory activity on either side of the fly. This is similar to the spatial limitation or amplification of seizures seen in humans, in which the spread of seizure depends on the ability of synaptic connections to amplify or suppress the seizure as it spreads from the site of initiation (Noebels 1996; Privitera et al. 1991; Serles et al. 1998).

Amplification of neuronal activity leads to seizures

In attempting to understand how seizures are activated and suppressed in Drosophila we have considered a neuronal circuitry model that involves an interplay between excitatory or seizure initiating circuits and inhibitory or seizure suppression circuits. The excitatory circuits may contain circuit elements that are linked through reciprocal excitatory synapses that provide positive feedback, as described in other seizure models (McNamara 1994; Traynelis and Dingledine 1988). Similarly, the inhibitory circuits may contain circuit elements that feed back inhibition through presynaptic or postsynaptic inhibitory synapses. Another interesting possibility for these circuits is that they trigger synaptic failure in a pathway essential for seizure initiation similar to the operation of a household circuit breaker. Seizures then would occur after sufficient activation of the excitatory circuits alone; however, when the two types of circuits are sufficiently coactivated, seizures would be either minimized or entirely suppressed.

We assume that the HF stimuli, on average, are driving a very similar population of neurons in every genotype, because there are no significant changes in individual neuronal excitability between genotypes for the GF neuron and the DLM motoneurons. This raises an interesting question: Why are different responses seen in different genotypes after stimulation of the same population of neurons? For example, after a 20-V HF stimulus, bss/+ flies exhibit seizures, whereas CS flies do not. We believe that the neurons that are activated in bss/+ mutants amplify the signal via subtle alterations in the underlying excitatory circuits—i.e., changes in positive feedback loops or altered membrane kinetics, so that a seizure occurs. This amplification and summation of a 20-V HF stimulus does not occur in CS flies and therefore no seizures are seen. One piece of evidence supporting this hypothesis is the fluctuations in seizure latency seen in bss flies. Low-voltage HF stimuli, which would recruit few neurons, give rise to seizures with long latencies, presumably because it takes some time for the activity in these few neurons to be amplified within the excitatory circuits to generate a seizure. When higher voltage HF stimuli are used, more neurons are recruited in the excitatory circuits, and therefore it takes less time for this activity to be amplified and summated into a seizure.

We believe that the inhibitory circuits are activated in a similar manner, although higher voltages are required. When a 20-V HF stimulus is delivered to a bss fly, no seizures occur because of high-voltage seizure suppression. We interpret this to mean that within the population of neurons excited by a 20-V HF stimulus, there are elements of both the excitatory...
and inhibitory circuits. In *Drosophila* flies, the input into the inhibitory circuits is rapidly amplified, as described, so that it suppresses the excitatory signals and results in seizure suppression. In the case of *bs*s/+ and CS, these inhibitory neurons are also activated, but the activity is not amplified in these genotypes.

This example illustrates the major features of *Drosophila* seizure susceptibility, which is that for a given HF stimulus intensity (20 V) the stimulation of a similar population of neurons can give three completely different outputs, depending on the genotype of the fly. To account for this, the circuits in the *bs*s and *bs*s/+ genotypes must have subtle alterations, which, although they do not significantly affect behavior, allow for the amplification and summation of certain levels of synchronous activity not seen in wild-type CS flies.

**Similarities with human seizures**

There are many reasons *Drosophila* offers an attractive model for understanding seizure susceptibility in humans. For example, one BS mutant, *tko*, has been linked with the gene product involved in human myoclonic epilepsy-ragged red fiber (MERRF) disease, because they both encode proteins involved in mitochondrial protein synthesis (Royden et al. 1987; Shoffner et al. 1990). In addition, both the BS defect and the seizure disorders associated with epilepsy are conditional defects, in that seizures occur interspersed within periods of normal nervous system function, during which often no obvious behavioral or neuronal defects can be detected.

The authors thank S. Faulhaber for assistance in maintenance of *Drosophila* stocks; R. Vance for assistance in starting this project; and H. Zhang, J. Lee, and C. Oh for discussions throughout this project.

Part of this work was supported by National Institute of Neurological Disorders and Stroke Grant NS-31231 to M. A. Tanouye.

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Received 10 June 1999; accepted in final form 18 October 1999.

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