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Seizures and Failures in the Giant Fiber Pathway of *Drosophila* Bang-Sensitive Paralytic Mutants

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***Drosophila* bang-sensitive paralytic mutants suffer from hyperactivity and paralysis following a mechanical shock; after recovery from paralysis, they cannot be paralyzed for a refractory period lasting up to 1 hr. Previously, we have shown that in *easily shocked* (*eas*), a typical bang-sensitive mutant, electrical shocks delivered to the brain cause seizure-like activity in the dorsal longitudinal flight motor neurons (DLMmns), and failure of giant fiber (GF) stimulation to evoke DLM potentials via the escape response pathway (Pavlidis et al., 1994). Here, we show that seizure and failure in the GF pathway with a refractory period is common to all six members of the bang-sensitive class. This syndrome was not found in any of eight other excitability mutants, including those affecting voltage-gated sodium or potassium-channel function. We show that failure occurs at the synapse between a peripherally synapsing interneuron (PSI) and the DLMmns, while the DLMmn-DLM neuromuscular junctions remain functional. Additionally, failure occurs in all other GF pathway-activated muscles. Failures occurred without seizures in the tergotrochanteral jump muscle (TTM), as was also found in ~10% of DLM tests, suggesting that seizures and failures may be independent events. This hypothesis is supported by the finding that, in double mutant combination with *mle*^{napts}, which suppresses behavioral bang sensitivity, DLM failures, but not seizures, were reduced.**

[Key words:* Drosophila melanogaster, *paralysis, seizure, synaptic transmission, hyperexcitability, giant fiber pathway]

A genetic analysis of behavioral defects in *Drosophila* has proven valuable in identifying genes involved in regulating nervous system excitability, such as ion channel genes (Wu and Ganetzky, 1992). It is thought that the knowledge of nervous system function gained by the study of *Drosophila* mutants can be applied to the study of mammalian nervous system function and pathology (Benzer, 1971). An underlying assumption of this approach is that although there are major differences between insect and mammalian nervous systems at the multicellular organizational level, many of the fundamental cellular and molecular mechanisms regulating excitability are conserved.

The present work focuses on a poorly understood class of *Drosophila* behavioral mutants, the bang-sensitive paralytics, which suffer from intense hyperactivity and temporary paralysis following a mechanical shock, such as a tap of the culture vial on the benchtop or brief vortex mixing (a "bang") (Benzer, 1971; Ganetzky and Wu, 1982a). The hyperactivity, which is a unique feature of this class of mutants, is characterized by intense, uncoordinated motor activity including wing flapping, leg shaking, and abdominal muscle contractions (Benzer, 1971; Pavlidis, 1994; Pavlidis et al., 1994). A comparison of this behavioral defect to a mammalian epileptic seizure has been made (Benzer, 1971), although the hypothesis that the underlying excitability defect in these mutants might have a functional relationship with excitability defects underlying mammalian seizure syndromes has not been explored. More recently, it has been found that the first genetically transmitted mammalian epilepsy for which the responsible gene has been cloned, human myoclonic epilepsy/ragged red fiber (MERRF) disease, is due to a mutation in a mitochondrial lysine tRNA gene (Shoffner et al., 1990), suggesting a similarity with the first bang-sensitive gene to be cloned, *technical knockout* (*tko*), which encodes a mitochondrial ribosomal protein (Royden et al., 1987).

In an examination of the giant fiber (GF) escape response pathway (Fig. 1) of the bang-sensitive *easily shocked* (*eas*), Pavlidis et al. (1994) described a physiological defect that appears to account for *eas* behavior, including the hyperactivity. A short, intense train of electrical stimuli (a 100 msec "buzz") delivered to the brain of the fly could mimic the effects of a mechanical bang on *eas* (Pavlidis et al., 1994). The buzz was followed by intense trains of abnormal, high-frequency (> 100 Hz) dorsal longitudinal muscle (DLM) activity, lasting 1–2 sec, and by failure, lasting ~100 sec, of the DLM excitatory synaptic response to GF pathway stimulation. These events closely follow the characteristics and time course of the *eas* behavior (Pavlidis et al., 1994).

The purpose of the present study is threefold. First, we surveyed the bang sensitives and other excitability mutants for the GF pathway defects found in *eas*. Second, we extended our investigation of this defect to other muscles activated by the GF pathway, and attempted to identify the sites of failure in the

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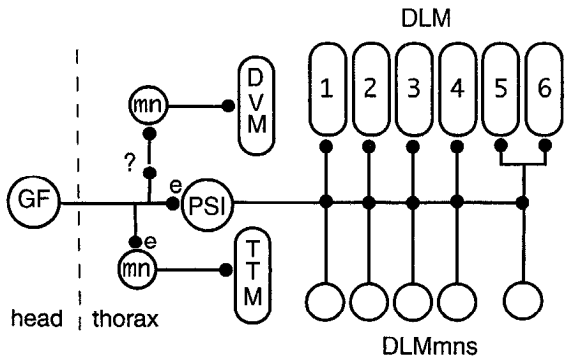

Figure 1. The giant fiber pathway. One of two bilaterally symmetric GF pathways is diagrammed schematically. Neurons are depicted as *circles* and muscles as *ovals*. All synapses (*small black spots*) are chemical except those marked *e* (electrical junctions). The GF is an interneuron that projects from the brain to the thoracic ganglion via the cervical connective. The GF has several outputs in the thorax. First, the GF synapses with the TTMmn, which drives the large tubular TTM. Second, the GF synapses with another interneuron, the peripherally synapsing interneuron (*PSI*), which drives the DLMmns via axoaxonic chemical synapses. The PSI axon does not branch in making these synapses; rather, the synapses are highly localized at the termination of the PSI axon in the posterior dorsal mesothoracic nerve (PDMN), which contains all five DLM motor neuron axons (King and Wyman, 1980). When the GF is stimulated, all six DLM fibers are activated synchronously via the PSI and DLMmns (Tanouye and Wyman, 1980). Note that DLM fibers 5 and 6 are innervated by a single motor neuron (Ikeda et al., 1980). Third, the GF has an output to the DVMs (only one of six fibers is depicted). This output is not well characterized but is probably includes at least one interneuron (Tanouye and Wyman, 1980). The DLM and DVM motor neurons also receive inputs from other pathways responsible for flight activity (not shown).

pathway. Finally, we examined the relationship between bang-sensitive seizures and failures. The results strongly suggest that bang sensitivity is due to a similar physiological defect in all the mutants, giving rise to seizures and synaptic failures. Since there are many genetic loci that can give rise to bang sensitivity, these mutants provide a unique opportunity to dissect complex aspects of neuronal excitability.

Materials and Methods

Fly stocks. Drosophila melanogaster strains were reared and studied at room temperature (22–24°C). A complete list of the strains used in this study is provided in Table 1. Wild-type flies were the Canton-Special (CS) strain. Behavioral mutants were obtained from several sources, primarily the collections of B. Ganetzky (University of Wisconsin, Madison, WI) and C.-F. Wu (University of Iowa, Iowa City, IA). The P-element insertion 2206 was a gift of J. Palka (University of Washington, Seattle, WA). A newly identified bang-sensitive line, iso7.8, was a gift of T. Tully (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The locus defined by iso7.8 has been named *slamdance* (*sda*) and maps to the third chromosome (E. R. Reynolds, personal communication). $sda^{iso7.8}$ has a typical bang-sensitive phenotype (Reynolds, personal communication). *eas* is the only bang sensitive other than *tko*, for which the responsible gene has been cloned; *eas* encodes ethanolamine kinase, which is required for one pathway of ethanolamine phospholipid synthesis (Pavlidis et al., 1994). The other bang-sensitive mutants used in this study were *bss*, *bang sensitive* (*bas*), and *knockdown* (*kdn*).

The other, non-bang-sensitive mutants were chosen to represent a cross-section of the types of behavioral and physiological defects found in *Drosophila*, and which, in particular, had the greatest likelihood of having a relationship to the bang sensitives. Three mutants, *Shaker* (*Sh*; a potassium channel gene defect), *Hyperkinetic* (*Hk*; which probably affects potassium channel function, Stern and Ganetzky, 1989), and *Frequenin* (*Frq*; affects a calcium binding protein), were selected because the larval neuromuscular junctions of these mutants rapidly develop large, prolonged postsynaptic potentials following high-frequency nerve stimulation in a manner similar to *bss* (Jan et al., 1977; Stern and Ganetzky, 1989; Mallart et al., 1991). *seizure* (sei^{ts}) was chosen both because of its presumed effect on sodium channels and the observation of severe DLM hyperactivity at restrictive temperatures (Jackson et al., 1985; Kasbekar et al., 1987). *shibire* (shi^{ts}) affects a dynamin-like protein required for synaptic vesicle membrane recycling (Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991), while *paralyzed* ($para^{ts}$), encodes a major voltage-gated sodium channel (Loughney, et al., 1989). 2206 is a P-element mutation in the α-Na^+-K^+ ATPase gene and displays a weak mechanical shock–sensitive phenotype (Schubiger et al., 1994). *maleless-no-action potential* (mle^{napts}) is a temperature-sensitive paralytic mutation affecting a RNA–helicase-like protein and which suppresses the hyperexcitability defects in mutants such as *Sh* and *Hk*, as well as bang sensitivity (Ganetzky and Wu, 1982a,b; Jackson et al., 1985).

bss; mle^{napts} double mutants were constructed by crosses between *g* bss^{MW1} *f* or bss^2 *f* and mle^{napts1} *cn* flies with subsequent selection for F2 *f*; mle^{napts} *cn* or *g f*; mle^{napts} *cn*. The presence of *bss* was confirmed by back-crosses to *bss* flies. *bss*; mle^{napts} flies had a markedly lower viability and fertility than either *bss* or mle^{napts} alone (Reynolds, personal com-

Table 1. Genotypes of the mutants used, along with an indication of the nature of the defect and the molecular identity of the relevant gene product, if known

Locus	Genotypes used	Affected gene product	Phenotype	Reference
easily shocked	eas^{PC80}, *w* eas^{PC80} *f*	Ethanolamine kinase	bang-sensitive	Pavlidis et al., 1994
bang senseless	bss^{MW1} *f, g* bss^2*f*	?	bang-sensitive	Ganetzky and Wu, 1982a
bang sensitive	*bas, bas;* ry^{506}	?	bang-sensitive	Grigliatti et al. 1973
knockdown	*y cho cv kdn f*	?	bang-sensitive	Ganetzky and Wu, 1982a
technical knockout	tko^{25t}	Mito. ribosomal protein	bang-sensitive	Royden et al., 1987
slamdance	$sda^{iso7.8}$	?	band-sensitive	Present results
Na^+-K^+ ATPase	P[ry^+] *2206* ry^{506}	Na^+-K^+ ATPase	bang-sensitive	Schubiger et al., 1994
Shaker	Sh^{KS133}, Sh^{rK0120}	K^+ channel	leg-shaker	Kamb et al., 1988
Hyperkinetic	Hk^1	?	leg-shaker	Stern and Ganetzky, 1989
Frequenin	T(1; Y) V7,*y w f.*B^s y^+	Ca^{2+}-binding protein	leg-shaker	Pongs et al., 1993
seizure	sei^{ts1}	?	ts paralytic	Jackson et al., 1985
paralyzed	$para^{ts1}$	Na^+ channel	ts paralytic	Loughney et al., 1989
shibire	shi^{ts1}	Dynamin	ts paralytic	van der Bliek and Meyerowitz, 1991
male lethal-no action potential	mle^{napts1} *cn*	RNA helicase	ts paralytic	Kernan et al., 1991

mito, mitochondrial; ts, temperature sensitive.

Table 2. Numbers of flies tested and trials performed, along with data on the effects of buzzes on DLM responses

Strain	*N*			Short buzz			Long buzz failure duration (sec)	Refract-oriness
	Flies	Trials	Seizures	Failures/ short buzzes	Failure duration (sec)	Failures/ long buzzes		
eas	44	176	++++	150/237 (70%)	86 ± 46	ND		++
bss	14	65	++++	58/68 (85)	112 ± 70	ND		+++
bas	11	39	+++	41/62 (66)	46 ± 20	ND		++++
tko	10	40	+++	36/61 (59)	54 ± 14	ND		++++
kdn	10	40	+++	33/57 (58)	58 ± 20	ND		++++
sda	5	19	++++	19/29 (66)	88 ± 37	ND		++
eas; mle^{napts}	5	27	++++	18/49 (36)	27 ± 16	ND		ND
bss; mle^{napts}	6	26	++++	22/32 (68)	37 ± 15	ND		ND
mle^{napts}	6	25	−	19/46 (41)	42 ± 33	6/6 (100%)	25 ± 9	−
2206	19	67	+	44/83 (53)	32 ± 15	7/16 (43)	22 ± 16	−
Frq	7	20	−	7/44 (16)	15 ± 13	21/30 (70)	41 ± 12	−
Hk	11	36	−/+++[a]	9/104 (9)	15 ± 11	29/35 (82)	30 ± 7	−
Sh	5	10	−	6/24 (25)	17 ± 9	7/8 (88)	22 ± 12	−
para^{ts}	4	21	−	23/44 (53)	12 ± 9	13/16 (81)	18 ± 10	ND
sei^{ts}	4	19	−	8/69 (12)	4 ± 3	15/23 (65)	21 ± 9	ND
shi^{ts}	4	8	−	8/29 (28)	13 ± 11	4/4 (100)	16 ± 17	ND
wild type	16	48	−	9/36 (25)	6 ± 7	59/78 (74)	28 ± 13	−

Each trial constituted up to six attempts to get a failure. The "seizures" column provides an indication of the characteristics of seizures, and if seizures occurred, ranging from − for no or infrequent (10% or less), low-frequency activity following a buzz that was not correlated with failure (as in wild type flies, described in the text) to ++++ for high-frequency, common (50–85% of trials) seizures accompanied by failure (such as in *eas,* described in the text). The failure data have been divided into short and long buzzes (20–400 and 500–1000 msec, respectively). The number of failures (numerator) and the total number of buzzes delivered (denominator; including those apparently too short to yield a failure) are listed. The percentage of buzzes that resulted in failures gives a rough indication of how susceptible the flies were to failure. Failure durations are averages with standard deviations. The data in the "refractoriness" column (from separate experiments from the first part of the table) provides an indication of how resistant flies were to failure following recovery (tested in at least five flies of each genotype, at least three trials each). Scale: any measurable refractoriness, +; refractoriness over 2 min; ++; 10–20 min, +++; over 20 min, ++++. ND, not determined.

[a]The −/+++ for *Hk* indicates that seizures were only observed in *Hk* flies following long buzzes.

munication). In a similar fashion, *eas*; *mle^{napts}* double mutants were constructed by crosses between *eas f* and *mle^{napts} cn* flies with subsequent selection for F2 *f*; *mle^{napts} cn* flies. Flies of the appropriate phenotype were recovered at a very low rate, were sterile, behaviorally sluggish and uncoordinated, and died within a week of eclosion. Because of the sterility of *eas*; *mle^{napts}* double mutants, we were unable to perform back-crosses to confirm the presence of *eas* in these flies at the behavioral level, though its presence at the physiological level was apparent (see Results). It is not yet clear if the effects of these double mutant combinations on viability are due to *mle^{napts1}* or another locus on the *mle^{napts1} cn* chromosome.

Basic electrophysiology techniques. The GF pathway is illustrated in Figure 1. The basic method used to record GF-driven muscle potentials and to induce paralysis with electrical stimulation (a "buzz") was as described (Tanouye and Wyman, 1980; Pavlidis et al., 1994), with modifications as noted below and in the following sections. A fly is affixed to the recording stage in a position that does not interfere with wing or limb mobility. All stimulating and recording electrodes were uninsulated sharpened tungsten wires (in a small set of experiments testing the effects of buzzes on *eas*, similar results were obtained with 3M KCl-filled glass recording electrodes and stimulation with insulated tungsten wires under saline). Bipolar stimulating electrodes were inserted into the brain for the purposes of delivering GF stimuli and buzzes; a ground electrode was inserted in the abdomen. Two recording electrodes were used to measure potentials from the desired combination of ipsilateral DLMs (usually fibers 3 or 4 and 5 or 6), DVMs, and the TTM. Responses were recorded on a digital storage oscilloscope (Hitachi) and photographed.

Once stable muscle potential responses to suprathreshold test pulses (typically 10–30 V, 0.05 msec, delivered at 1 Hz) delivered to the GF, an electrical "buzz" was delivered with a separate stimulator (Dagan S-900) to the brain via the same simulating electrodes, in the form of a short train of relatively high-voltage stimuli. Typically, we used 100 msec trains of 50 V, 200 Hz stimuli, with a 0.5 msec pulse duration. The duration of buzz, which could give rise to seizure and failure, was determined empirically for each bang-sensitive fly, and could be as brief as a single stimulus pulse in some *eas* flies (not shown). Buzz voltage had to be well above GF threshold to get failures in bang sensitives, suggesting that other neurons besides the GF must be stimulated to cause seizure and failure. If a buzz did not cause response failure, the buzz was increased in duration (often by manually triggering two or three 100 msec buzzes in a row, counted as a single buzz) and repeated, until five or six attempts had been made or until failure occurred. Each attempt, including unsuccessful ones, was included in the calculation of percent failures in Table 2.

Following a buzz, GF stimulation at 1 Hz was resumed to test for the failure and then recovery of evoked muscle potentials. Recovery from failure is defined here by two successive GF test stimuli giving rise to normal (wild-type) latency responses (1.25 msec for the DLMs; 0.8 msec for the TTM; ~2–3 msec for the DVMs, Tanouye and Wyman, 1980) in both fibers being recorded from. Flies were rested at least 15 min between testing in this manner to allow recovery from refractoriness (see Results). Each set of buzzes separated by rest periods constituted a trial. Longer rests were used for bang sensitives with longer refractory periods (*tko*, *kdn*, and *bas*; *bas* required rests of 30–60 min because of refractoriness).

Although all the recordings reported in this article were from muscle fibers, we take the DLM potentials we recorded to reflect motor neuron action potentials on a one-to-one basis, as has been previously demonstrated (Koenig and Ikeda, 1983). Thus, seizure-like activity in the DLM reflects seizures in the DLMmns. This assumption is also supported by the fact that *Drosophila* muscles are not electrogenic except under extreme experimental conditions of prolonged depolarization (Salkoff and Wyman, 1980), and even then the spikes have a characteristic shape and time course (Salkoff and Wyman, 1980) not observed in the bang-sensitive seizures, during which potentials have the shape and time course of a synaptically evoked potential (Pavlidis, 1994). Thus, the seizures are unlikely to arise from intrinsic muscle membrane hyperexcitability. Finally, muscle fibers 5 and 6, which are innervated by the same motor neuron, maintain entirely synchronous activity even

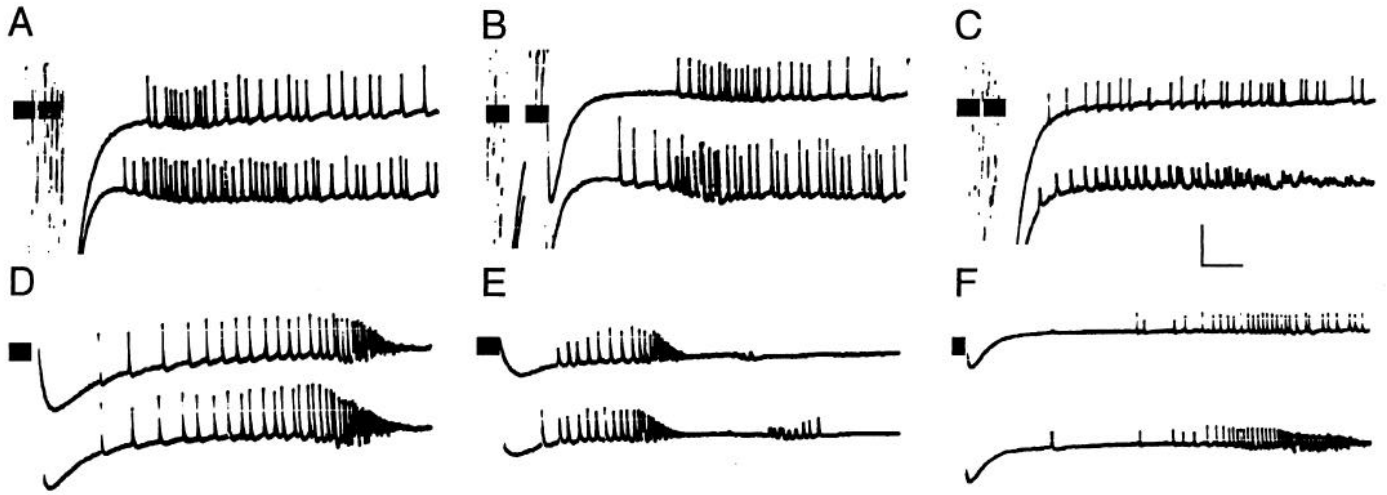

Figure 2. Examples of seizures from *kdn* and *eas*. The pair of traces in each panel are recordings from two ipsilateral DLM fibers. The *short black bars* indicate the timing and extent of buzzes, which generate a large stimulus artifact visible in the beginning of some of the traces (in some cases, multiple 100 msec buzzes were used). *A–C, kdn*. *D* and *E* are typical of *kdn* seizures, which did not generally (> 75% of trials) decrease in potential amplitude or increase in frequency. An exception is shown in *C* (*bottom trace*). *D–F, eas*. *A* and *B* are typical of *eas* seizures. Note the increase in frequency and decrease in potential amplitude as the seizures progress, seen in at least 90% of *eas* seizures. The example in *F* is unusual for *eas* in that the seizure in one DLM fiber (*top trace*) appears to die out without increasing in frequency greatly or decreasing in potential amplitude. *F* also demonstrates a relatively long pause between the buzz and the seizure. Calibration: 20–50 mV, 200 msec.

during intense seizures (not shown), consistent with seizures being neuronally derived. Thus, we use the term "seizure" to denote uncontrolled, high-frequency neuronal activity, though we assay it indirectly with muscle potential recordings.

Motor neuron stimulation. In experiments requiring direct stimulation of motor neurons, a second set of stimulating electrodes driven by a third stimulator (Grass S44) was employed. These electrodes had right-angle bent tips and were positioned in the anterior preepisternum (near the base of the first coxa) with the aid of a mirror placed under the fly. As the stimulus voltage is increased, a GF-initiated TTM or DLM potential was first elicited at ~1 V, 0.05 msec duration, with a shift to a shorter latency (~0.6 msec for the TTM, ~0.8 msec for the DLM) at higher voltages (typically ~3 V); this short latency response is due to direct stimulation of the motor neurons (Salkoff and Kelly, 1976).

Identification of recording sites. When identified recording sites in DLM fibers 5 and 6 were needed, electrode placement was guided by cuticular markers (Levine and Hughes, 1973) and confirmed by the following method, modified from Engel and Wu (1992). After removing the electrodes, a drop of a 2% solution of methylene blue in phosphate buffered saline (PBS) containing 0.1% Tween 20 (to improve wetting) was applied to the dorsal thorax and the fly placed in a humidified chamber. After 10–30 min, the fly was transferred to a solution of 4% formaldehyde, 4% DMSO in PBS for 4–6 hr. This procedure leaves a small blue spot on the cuticle at the electrode insertion sites and allows identification of the underlying fiber by dissection.

Results

Seizure and failure in all bang-sensitive mutants

Electrical buzzes (50–400 msec) applied to the brain of bang-sensitive mutants reveals a complex physiological defect. In most of the experiments to be described this was monitored by recordings of DLM potentials. The first aspect of the physiological phenotype is abnormal spontaneous activity (a "seizure") following the delivery of the buzz. Seizures were observed in every bang-sensitive fly, after 60–85% of buzzes. The seizure is likely to correspond to the preparalysis hyperactivity phase observed in these mutants (Royden, et al., 1987; Pavlidis et al., 1994).

DLM seizures lasted ~0.5–3 sec. Examples of traces demonstrating the variability of seizure appearances are given in Figure 2. From this figure, it can be seen that seizures fell into two loose classes. The first class is exemplified by the seizures seen in at least 75% of trials with *kdn* flies (Fig. 2*A–C*). These seizures typically began with trains of DLM potentials at ~10–30 Hz, which lacked any clear pattern. As the seizures progressed, the frequency of activity changed little until dying out. The second class is exemplified by at least 90% of trials with *eas* flies (Fig. 2*D–F*). These seizures began in a manner similar to those in the first class, but soon show a marked increase in frequency with a concomitant decrease in potential amplitude. The frequency increase and amplitude decrease continued until the potentials were undetectable. Examination of seizures at faster sweep speeds than shown here revealed that the activity near the end of this type of seizure could reach 150 Hz. Examples of typical seizures from the other bang-sensitive mutants (*bss*, *bas*, *tko*, and *sda*) are shown in Figure 3.

The next aspect of the phenotype is the sudden failure of GF stimulation to evoke DLM potentials following the buzz. Such failure in the nervous system is a likely underlying cause of behavioral paralysis in these mutants. Failure occurred in every bang-sensitive fly tested, after 60–85% of buzzes (Table 2). There was considerable variation in the duration of failures in the different bang sensitives, ranging from an average of about 45 sec in *bas* to 112 sec in *bss* (Table 2). In all the bang sensitives, there was a close correlation between the occurrence of seizures and failure: if there was a seizure, there was a failure > 95% of the time. Failures occurred without seizures in about 10% of cases.

Additional common aspects of the bang-sensitive physiological phenotype

In agreement with their behavioral similarity, the bang sensitives had many similarities in their physiological defects in addition to seizure and failure. First, the DLM failure period consisted of two distinct phases in all the bang sensitives: a silent period, with no evoked or spontaneous activity, followed by a period of spontaneous activity. The silent period had a very consistent duration of ~35–40 sec (34 ± 6 sec in *bss*, 35 ± 6 sec in *eas*, 37 ± 6 sec in *bas*, 38 ± 7 sec in *sda*, 41 ± 8 sec in *kdn*, and

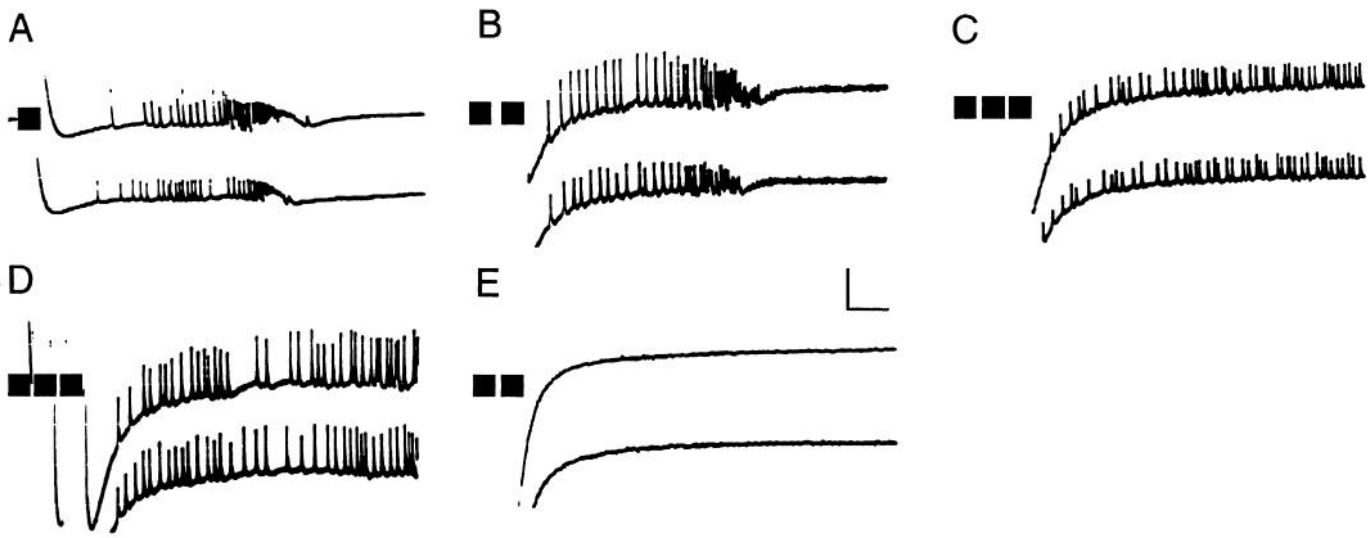

Figure 3. Typical seizures from *bss*, *sda*, *bas*, and *tko*. *A*, *bss*; *B*, *sda*; *C*, *bas*; *D*, *tko*, *E*, control (wild-type). For each bang-sensitive mutant, the example shown is representative of the seizures observed in at least 80% of trials. *Black bars* indicate buzzes as in Figure 2. Calibration: 20 mV, 200 msec.

42 ± 3 sec *tko*; n = 12–27 trials on four to six flies for each mutation). This silent period probably corresponds to the paralysis phase of the behavior, which lasts 30–40 sec (Pavlidis, 1994; E. R. Reynolds and P. Pavlidis, unpublished observations). The subsequent spontaneous DLM activity varied greatly in intensity from fly to fly and trial to trial, but often resembled the seizure (not shown). During this period, evoked responses were still absent. This phase apparently reflects the behavioral hyperactivity observed during recovery from behavioral paralysis (Royden et al., 1987; Pavlidis et al., 1994).

Second, abnormal evoked responses were observed during recovery. In wild-type flies, and in bang sensitives prior to a buzz, GF stimulation always gives rise to synchronized responses in all the DLM fibers (Fig. 4*A*,*B*; Tanouye and Wyman, 1980). However, in all bang sensitives tested, evoked potentials from individual DLM fibers appeared independently and irregularly during recovery, in > 95% of trials. Examples of independent recovery are shown for each bang sensitive in Figure 4. Additionally, in at least 50% of all trials in all the mutants, the DLM responses early in recovery had abnormal, long, variable latencies. The latencies were commonly different for the two DLMs being studied, as in Figure 4*E*; abnormal latency is also apparent in Figure 4*F*. In ~10% of trials, abnormal-looking potential amplitudes and shapes were also observed (not shown). As recovery proceeded, these abnormalities became progressively less severe (e.g., latencies became gradually shorter and responses) until reaching a normal state (as in Fig. 4*A*,*B*).

A third common aspect of the defect was a refractory period following recovery, during which a buzz was less effective at producing seizures and failure (Table 2). This is likely to reflect the behavioral refractoriness that has been previously noted in all bang sensitives (Ganetzky and Wu, 1982a). For example, we found it necessary to rest *bas* flies at least 30 min between trials before buzzes could induce seizure and failure again (n = 5 flies, at least three trials each), in agreement with observations that *bas* flies are refractory to vortexing for up to an hour (Grigliatti et al., 1973). In *eas* flies buzzing immediately following recovery usually did not cause seizure or failure, but short rests (2–10 min) restored the ability of a buzz to cause seizure and failure (n = 5 flies, at least three trials each); again, this agrees well with behavioral observations (Pavlidis et al., 1994).

The conclusions from these experiments are that all six bang-sensitive mutants have qualitatively similar phenotypes at the level of the DLM responses to a buzz, and furthermore, that this syndrome seems to reflect all the different stages of the bang-sensitive behavioral phenotype, including preparalysis hyperactivity, paralysis, postparalysis hyperactivity, recovery, and the refractory period (Pavlidis et al., 1994). On this basis, we suggest that the response of the DLM to a buzz is probably representative of what occurs in many other parts of the bang-sensitive nervous system following a bang, and can thus be used as a starting point in analyzing the physiological basis of bang sensitivity.

Failure of synaptic transmission in the GF-DLM pathway

We next asked whether the failure in the GF pathway is due to failure of the GF itself or to other elements in the pathway. The independent recovery of GF-evoked DLM potentials following a buzz (Fig. 4) suggests that failure has occurred downstream of the functional branch point in the pathway. This indicates that the limiting site of recovery in the GF pathway is either the peripherally synapsing interneuron (PSI)-DLM motor neuron (DLMmn) synapse, the DLMmn action potential, or the DLM nmj, and not at the level of the GF or PSI action potentials, which would result in all-or-none recovery in all six DLM fibers (Fig. 1).

The spontaneous activity during recovery suggests that DLMmns can still fire action potentials while GF-evoked responses are still absent. To confirm this, we directly stimulated the motor neurons of *eas* flies by placing a second set of stimulating electrodes in the thorax (n = 15 flies, 46 trials). As expected, GF stimulation failed to yield DLM responses after a buzz (Fig. 5*A*) until recovery occurred with the time course expected for *eas* (60–100 sec). During this period, stimulation of the motor neurons still evoked DLM responses (Fig. 5*A*), although in 30% of trials, the responses did fail briefly, or the DLM potentials changed size. Such mn-evoked response changes lasted an average of 5 sec, and persisted for no more

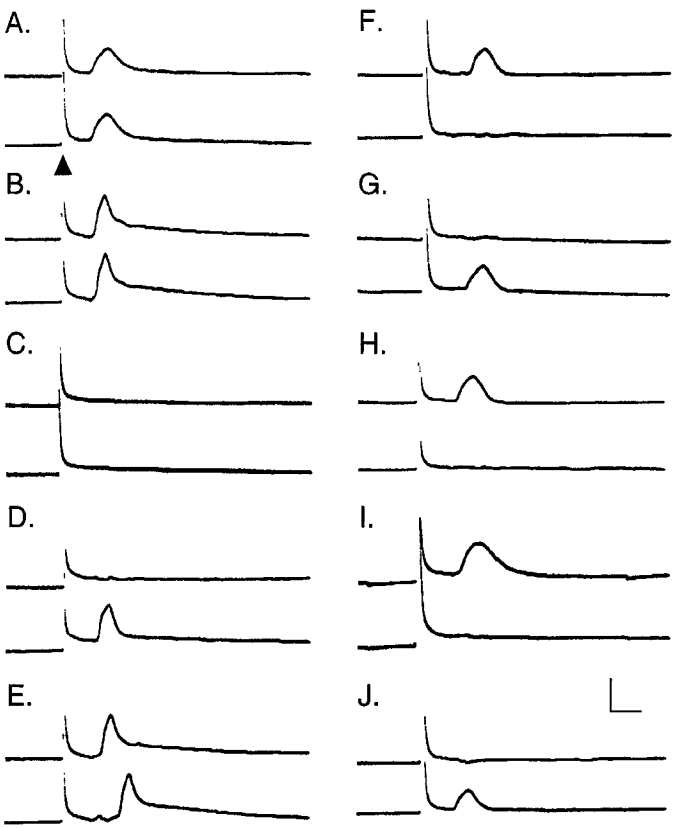

Figure 4. Abnormal DLM responses following buzzes in bang sensitives. *A,* Wild-type DLM recordings showing the normal, synchronous response to GF stimulation (the stimulus artifact is indicated by an *arrowhead*), with a invariable latency (1.2 msec; Tanouye and Wyman, 1980). *B,* Normal responses in *eas* prior to a buzz. These responses are indistinguishable from those in wild-type flies, as shown in *A* (other bang-sensitive mutants also have wild-type–like responses before a buzz, not shown). *C,* Failure of GF stimulus-evoked DLM responses in *eas* following a buzz, typical of what was observed in all bang-sensitive flies. *D,* Independent recovery of individual DLM fibers in *eas*, indicating failure at a branch point in the GF pathway. *E,* Abnormally long, independently varying DLM response latencies during recovery in an *eas* fly. *F–J,* Examples of independent recovery of individual DLM fibers in *tko*, *kdn*, *sda*, *bss*, and *bas*, respectively. Calibration: 50 mV, 2 msec.

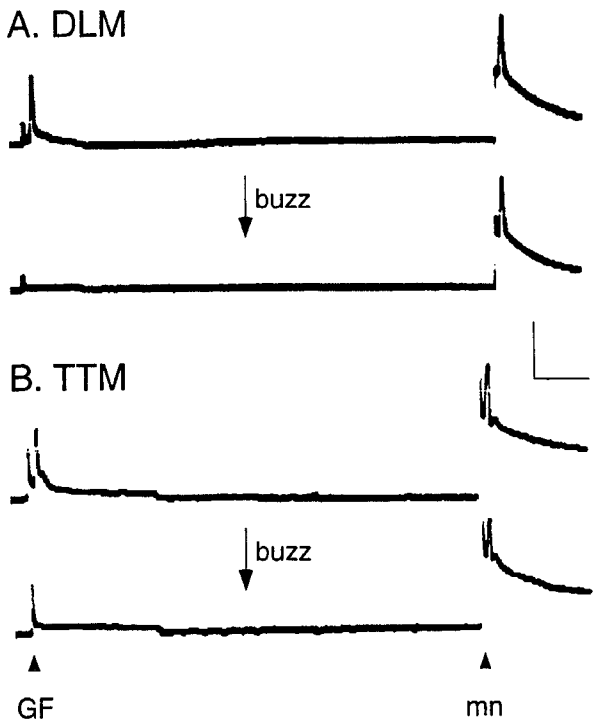

Figure 5. Motor neuron function during GF-evoked response failure. For each trace, a stimulus (indicated by *arrowheads at bottom*) was applied first to the brain to evoke a response initiated at the GF, then, after a pause of ~100 msec, to the thoracic ganglion to evoke a response initiated at the motor neuron (the mn-evoked response rides on a large stimulus artifact). Typical responses before (*top traces of each pair*) and after a buzz (*bottom traces of each pair*) are shown. Identical results were obtained if the stimulus order was reversed (thoracic stimulus first; not shown). Between each such pair of test pulses there was a 1 sec rest. *A,* Recording from a DLM. After a buzz, GF stimulation fails to evoke a DLM response, but motor neuron stimulation does. *B,* Recording from a TTM (a different fly than in *A*). As for the DLM motor neuron, stimulation still evokes a DLM response after a buzz. Calibration: *A,* 50 mV, 20 msec; *B,* 20 mV, 20 msec.

than 10 sec. Similar results were obtained in *bss* flies (n = 2 flies, six trials). This shows that for most of the failure period, the DLMmns, the DLM neuromuscular junctions, and the DLMs are functional. It confirms that the site in the pathway limiting recovery lies upstream of the DLMmn action potential.

In another set of experiments, we recorded simultaneously from *eas* DLM fibers 5 and 6, which are innervated by the same motor neuron (Fig. 1). In this case, recovery was never independent (n = 10 trials in four *eas* flies; compared to 13 independent recoveries in 14 trials in four *eas* flies when recording from other muscle pairs). This is further evidence that the site of failure is not individual DLM nmjs. More importantly, it shows that independent recovery is not an artifact caused by an effect on the DLMmns (i.e., irregular transmission), but is, in fact, due to a defect upstream in the pathway. The only points upstream of the DLMmn action potentials that could result in independent recovery of DLMs are the PSI-DLMmn synapses (Fig. 1). The changes in response latency commonly observed during recovery (Fig. 4) are also consistent with PSI-DLMmn synaptic failure.

Failure in other GF-activated muscles

If the events observed in the DLM reflect a widespread phenomenon in the fly, one would expect other muscles in the fly to undergo seizures and failure following a buzz. As a first examination of this hypothesis, we tested the effects of buzzes on the other muscles which respond to GF stimulation, the dorsal ventral indirect flight muscle (DVM), and the tergotrochanteral jump muscle (TTM) (Fig. 1). These studies were carried out primarily on *eas* flies, with some experiments on *bss*. Delivery of a buzz causes the DVM fibers to undergo a seizure that appears qualitatively similar to that in the DLMs (Fig. 6, top). In contrast, seizures were never observed in the TTM (Fig. 6, bottom). Following the buzz, the DVMs and the TTM fail to respond to GF stimulation. Recovery of the DVMs followed a very similar time course as the DLMs (*eas*: 92 ± 27 sec, n = 5 flies, 10 trials; *bss*: 111 ± 71 sec, n = 3 flies, 8 trials). The TTMs recovered somewhat more quickly, in an average of 73 ± 33 sec in *eas* (n = 15 flies, 46 trials), and in 66 ± 21 sec in *bss* (n = 5 flies, 15 trials). Also, in 58% of the trials with *eas*, TTM responses that were normal in appearance and usually normal in latency briefly reappeared much earlier than DLM or DVM recovery. In *bss*, early recovery occurred in 40% of the trials. Early recovery was always coincident with the beginning of spontaneous activity (30–40 sec after the buzz). These early

Figure 6. Responses of DVM and TTM to buzzes in *eas*. The DVM (*top trace*) undergoes a seizure like that observed in the DLMs, while the TTM (*bottom trace*) does not. This example is atypical since there was some TTM activity, but was selected to show that TTM potentials could be recorded. Similar results were obtained with *bss* flies. Buzzes are indicated as in Figure 2. Calibration: *DVM*, 20 mV, 200 msec; *TTM*, 10 mV, 200 msec.

responses were unstable (in all but five *eas* trials), decreasing in amplitude and disappearing, recovering again with a normal time course (not shown). We also tested TTM motor neuron (TTMmn) function following buzzes in *eas*. As for the DLMmn, TTMmn stimulation could still yield a normal evoked TTM response (Fig. 5*B*). Failures of the TTMmn- derived response occurred more frequently than for the DLMmn (56% of TTMmn trials vs 30% of DLMmn trials). Failures of the TTMmn-derived response were also much longer lasting (TTMmn average failure 18 sec, $n = 6$ flies, 19 trials; DLMmn average failure 5 sec, $n = 15$ flies, 46 trials). These results indicate that the effects of buzzes on the GF-TTM pathway may be more complex than those on the GF-DLM (see Discussion).

Relationship between seizure and failure

A simple explanation for the cause of failure is that the intense activity of the seizure could result in the inactivation of labile sites in neurons, such as the PSI-DLMmn synapses. However, as described above, DLM failures sometimes occurred without the observation of a seizure (that seizures do not occur in the TTM also demonstrates that seizures are not an obligate feature of the phenotype). This shows that PSI synaptic failure is independent of seizures in the DLMmns, and raises the question of whether PSI seizures occur at all.

We further explored the relationship between seizure and failure by testing the effects of a suppressor of bang sensitivity, mle^{napts}, on bang-sensitive physiology. Since mle^{napts} decreases neuronal excitability (Wu and Ganetzky, 1980), we hypothesized that mle^{napts} might suppress seizures in double mutant combination with bang-sensitive mutations. However, buzzes could still induce DLM seizures in *bss*; mle^{napts}, while failures were greatly reduced in duration (Table 2; similar results were obtained using either bss^{MW1} or bss^{2}). The seizures were qualitatively similar to those seen in *bss* alone (Fig. 7*A*). Even more dramatic than the effects on *bss*, in *eas*; mle^{napts} seizures were not consistently accompanied by failures (39 seizures but only 18 failures in five flies). Again, seizures were not obviously reduced in severity (Fig. 7*B*). Failures in these flies, when they occurred, were also reduced in duration (Table 2). Thus, seizures in the DLMs are at least partially separable from PSI synaptic failure in these double mutants.

Alone, mle^{napts} DLMs did not have seizures following buzzes, although there were sometimes significant failures (Table 2). These failures were distinct from bang-sensitive failures in that there was no refractory period ($n = 14$ tests of refractoriness on two flies), spontaneous activity during recovery, or independent recovery.

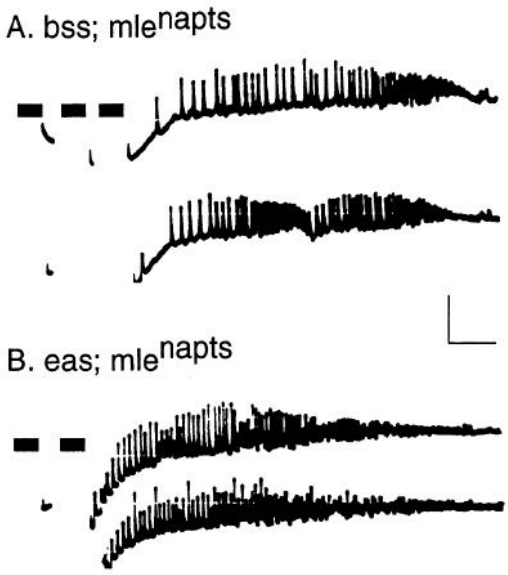

Figure 7. Seizures in *bss*; mle^{napts} and *eas*; mle^{napts} double mutants. *A*, *bss*; mle^{napts}; *B*, *eas*; mle^{napts}. These seizures are indistinguishable from those seen in *bss* and *eas*. Calibration: 20 mV, 200 msec.

Effects of buzzes on other excitability mutants

In wild-type flies (Pavlidis et al., 1994), short buzzes (<500 msec) have no effect on the DLM responses other than an occasional slight, temporary threshold increase. To examine the possibility that seizures and failures might not be specific to bang sensitives, we tested the responses of a number of other excitability mutants to short buzzes. The results are summarized in Table 2. We tested the three mutants (*Sh, Hk, Frq*), which have larval nmj defects similar to *bss*. We also tested several temperature-sensitive paralytic mutants, sei^{ts}, $para^{ts}$, and shi^{ts}. There was no significant effect of short buzzes on any of these mutants (Table 2).

We tested the 2206 strain (the Na^+-K^+ ATPase mutant), which has a mild mechanical shock-sensitive behavioral phenotype (Schubiger et al., 1994). The 2206 behavioral phenotype is significantly different from the bang sensitives; for example, they are paralyzed for only a few seconds and lack a refractory period (Pavlidis, 1994; Schubiger et al., 1994). We found that in many 2206 flies, short buzzes could cause failure lasting ~30 sec (Table 2). Such failures were sometimes accompanied by activity following the buzz (20% of trials) (Fig. 8*A*); however, spontaneous activity could also occur following single test stimuli and without failure (not shown).

We also examined the effects of longer buzzes on these excitability mutants, since in wild-type flies, long buzzes (500–1000 msec) can cause failures of about 30 sec (Pavlidis et al., 1994; Table 2). Failures in wild-type flies were different from those in bang sensitives. First, there was spontaneous activity following only ~10% of long buzzes, ranging from 1–10 potentials to the initiation of flight-like activity (~10 Hz), compared to the routine observation of seizures in the bang sensitives (after at least 60% of all buzzes). Such activity was not well correlated with failure (failure in only ~50% of trials in which there was spontaneous activity; compared to a correlation of > 95% in the bang sensitives). There was also no refractory

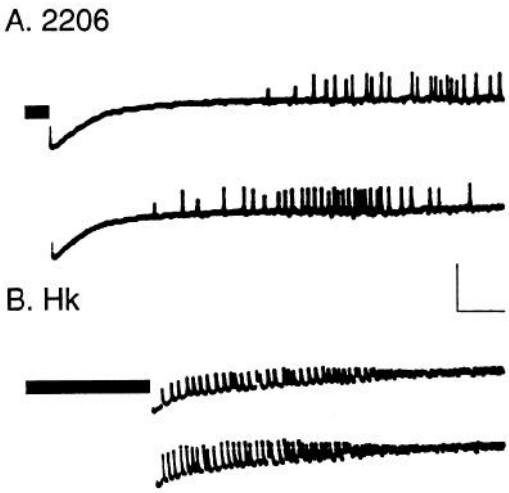

Figure 8. DLM hyperactivity following buzzes in non-bang-sensitive strains. *A,* DLM hyperactivity in a 2206 fly following a 100 msec buzz. In 2206 flies, such activity was only seen after about 20% of buzzes, which resulted in failure. *B,* A *Hk* DLM seizure following a 500 msec buzz. In this example, the potential size declined as the seizure progressed. Calibration: 50 mV, 200 msec.

period for failures in wild-type flies, as a second buzz delivered immediately after recovery always causes a second failure (n = 5 flies, at least three trials each).

With two exceptions, all the additional excitability mutants had responses to long buzzes indistinguishable from wild-type flies (Table 2). *Frq* mutant DLM responses failed for slightly longer periods after long buzzes as compared to wild-type flies (p = 0.05; Student's t test), but did not have seizures. Interestingly, *Hk* flies had a much higher tendency for seizure-like activity following long buzzes than wild-type (~70% of trials). Additionally, in several of the *Hk* flies examined, the DLM potentials decreased in size during the seizure, similar to what was observed in some bang sensitives (Fig. 8*B*). Failure durations following long buzzes in *Hk* were similar to wild-type flies (Table 2).

Because they displayed some aspects of the bang-sensitive phenotype (behaviorally or physiologically), we tested *Hk, Frq,* and 2206 flies for refractoriness following DLM response recovery. In each case, no significant refractoriness was detectable. After recovery from a first buzz, a second 500 msec buzz still induced DLM failures in *Hk, Frq,* and 2206 flies (tested in at least four flies each, three trials per fly). In addition, there was no measurable refractory period for failures following short buzzes in 2206 flies (n = 8 flies, at least three trials each).

Discussion

Bang sensitives: a distinct class of excitability mutants

Historically, *Drosophila* excitability mutants have been classified as either hyperexcitable (e.g., *Sh*) or hypoexcitable (e.g., *para*) (Hall, 1985). The bang sensitives have been generally considered hyperexcitable. This is based on the observation of abnormally rapidly developing giant potentials at *bss* larval nmjs (Jan and Jan, 1978), the suppression of bang sensitivity by mle^{napts} (which also suppresses hyperexcitability mutants such as *Sh*, Ganetzky and Wu, 1982a,b), and the behavioral hyperexcitability during recovery (Royden et al., 1987). The seizures we have observed are also indicative of hyperexcitability. Despite their apparent hyperexcitability, some aspects of the phenotype are more suggestive of hypoexcitability: paralysis [including temperature-sensitive paralysis (Ganetzky and Wu, 1982a; Royden, 1988), also found in hypoexcitable mutants such as mle^{napts} and $para^{ts}$], reduced mechanoreceptor responses to bristle deflection (Engel and Wu, 1994), and a 20-fold decrease in the rate of spontaneous transmitter release observed in *bss* larvae (Jan and Jan, 1978). Although the work presented here tends to align the bang sensitives with the hyperexcitable mutants, the apparent activity dependence of the defect sets them apart. The cause of this activity dependence and the cellular and molecular events leading to seizures, failure, and refractoriness remain unclear.

Relationship between bang sensitives and hyperexcitable mutants

We found that some, but not all, aspects of the bang-sensitive physiological phenotype can be found in certain hyperexcitability mutants. Following long buzzes, *Hk* flies could have DLM seizures and *Frq* flies failed for slightly longer periods than wild-type flies. 2206 flies were more sensitive than wild-type flies to short buzzes. Although consistent with hyperexcitability in bang sensitives, the differences between these mutants and the bang sensitives makes it unclear whether these similarities to bang sensitives are merely superficial or if they represent similarities at the cellular or molecular level. Thus, it is difficult to determine on this basis if the molecular defects in *Hk,* 2206, or *Frq* are relevant to those in the bang sensitives.

The 2206 strain warrants further discussion because of its mechanical shock sensitivity, which has been compared to a bang-sensitive phenotype (Schubiger et al., 1994). This P-element-mediated mutation results in a two-thirds decrease in Na^+-K^+ ATPase α-subunit protein levels and causes marked behavioral hyperexcitability (Pavlidis, 1994; Schubiger et al., 1994). Given their weak phenotype and lack of a refractory period, it is unclear whether they belong within the bang-sensitive class of mutants. Since we found some physiological similarities between 2206 and the bang sensitives, we consider this an open question. However, because of the weak 2206 phenotype, and that *eas* and *tko* encode other proteins, we consider it unlikely that the other bang sensitives affect the Na^+-K^+ ATPase itself. It is possible, nevertheless, that other bang sensitives affect a process that is also sensitive to Na^+-K^+ ATPase activity.

Synaptic transmission defects in bang sensitives

The independent recovery of the DLMs (Fig. 4) and the ability of motor neuron stimulation to elicit DLM responses following a buzz (Fig. 5) show that DLM failure occurs at the level of individual PSI-DLMmn synapses. Similar logic has been applied in determining the cause of independent DLM responses in two other (non-bang-sensitive) mutants, Cha^{ts} and *gfA* (Gorczyca and Hall, 1984; Thomas and Wyman, 1984). It is unlikely that a failure of action potentials to invade individual PSI synapses could explain our findings, since it has been shown in anatomical studies that the PSI-DLMmn synapses are highly localized in a nonbranching PSI process in the posterior dorsal mesothoracic nerve (PDMN) (legend to Fig. 1; King and Wyman, 1980).

The behavior of the TTM is more difficult to explain in terms of synaptic failure. That TTMmn stimulation could still evoke responses after GF-evoked responses have failed appears to indicate the GF as the primary site of failure, or less likely, the GF-TTMmn electrical synapse. It is conceivable that the GF does fail but that this is only limiting for recovery of the TTM, since DLM recovery was not limited by GF responsiveness.

However, this does not explain the "early recovery" of TTM responses we saw in many experiments. Without more data, we consider it possible that the current model of the GF-TTM pathway is incomplete. There are already suggestions that there may be multiple TTM motor neurons (King and Wyman, 1980) and that there are multiple GF-TTM pathways (Krishnan et al., 1993). Resolution of these issues will be important to our understanding of TTM physiology in bang sensitives.

Despite these problems, currently the best explanation for our data is that in bang-sensitive adults synapses fail following a bang or buzz. At this time we cannot determine if the site of failure is presynaptic or postsynaptic. The defects in *bss* larvae, while not including synaptic failure, are indicative of a presynaptic component to the phenotype (Jan and Jan, 1978; Ganetzky and Wu, 1982a). However, the relationships among adult motor neuron seizures, altered larval motor neuron excitability, and failure of the PSI-DLMmn synapses remains unclear.

Seizures and the causes of failure

Contrary to our initial expectation that seizures cause failure in bang sensitives, seizures were not an obligate feature of the defect, as evidenced by the occasional GF-DLM response failure without seizure, as well as by the lack of seizures in the TTM. This suggests that PSI failure can occur without a PSI seizure, since one might expect some of such activity to be transmitted to the DLM even in the absence of DLMmn seizures. The finding that mle^{napts} suppresses bang-sensitive failures, but not seizures, is another indication that seizures are not a prerequisite for failures. Further support for this idea comes from experiments showing that *eas* flies, which are also temperature sensitive, do not undergo behavioral hyperactivity prior to cold-induced paralysis (Pavlidis, 1994). These findings suggest that seizures and failures are independent manifestations of the defect. In any case, seizures that can be triggered by exogenous stimulation of the nervous system are a novel phenomenon in *Drosophila*. More work will be required to determine if there is any relationship between bang-sensitive seizures and seizures in mammals. That both a human epilepsy and a bang-sensitive mutation have been traced to mitochondrial translational machinery defects is additionally suggestive that there might be some connection between seizures in flies and in mammals (Royden et al., 1987; Shoffner et al., 1990).

Conclusion

The present work shows that all bang-sensitive mutants have similar, though not necessarily identical, adult physiological defects that can account for the behavioral phenotype. Despite their similarities, molecular studies have thus far indicated diversity in the gene products affected in these mutants (Royden et al., 1987; Pavlidis et al., 1994). We propose that the bang sensitives all affect a common complex physiological process, capable of being affected by a range of genetic defects but with a similar end effect. The molecular diversity of the genes affected might explain some of the differences in phenotypes among the bang sensitives (e.g., differences in refractory period and DLM failure durations). For example, some of the differences among the bang sensitives might exist because some mutants have more or less severe defects in the common process. Alternatively, there may be additional processes affected in some mutants but not in others. Answers to these questions must wait until the bang-sensitive physiological and molecular defects are better understood.

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