DOI: 10.1534/genetics.106.057463

Metabolic Disruption in Drosophila Bang-Sensitive Seizure Mutants

Tim Fergestad, Bret Bostwick and Barry Ganetzky[1]

Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706

Manuscript received February 21, 2006
Accepted for publication April 18, 2006

ABSTRACT

We examined a number of Drosophila mutants with increased susceptibility to seizures following mechanical or electrical stimulation to better understand the underlying factors that predispose neurons to aberrant activity. Several mutations in this class have been molecularly identified and suggest metabolic disruption as a possible source for increased seizure susceptibility. We mapped the bang-sensitive seizure mutation *knockdown* (*kdn*) to cytological position 5F3 and identified citrate synthase as the affected gene. These results further support a role for mitochondrial metabolism in controlling neuronal activity and seizure susceptibility. Biochemical analysis in bang-sensitive mutants revealed reductions in ATP levels consistent with disruption of mitochondrial energy production in these mutants. Electrophysiological analysis of mutants affecting mitochondrial proteins revealed an increased likelihood for a specific pattern of seizure activity. Our data implicate cellular metabolism in regulating seizure susceptibility and suggest that differential sensitivity of neuronal subtypes to metabolic changes underlies distinct types of seizure activity.

STUDIES in Drosophila over the past 25 years have led to the discovery of mutants exhibiting stress-sensitive seizures and paralysis (Benzer 1971; Grigliatti *et al.* 1973; Homyk *et al.* 1980; Ganetzky and Wu 1982; Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000). Mutants that exhibit seizures and paralysis upon mechanical stimulation ("banging"), are termed bang-sensitive paralytics. Suppression of bang-sensitive mutant phenotypes following the disruption of voltage-gated Na^+ channels demonstrated that seizure behavior likely involves neuronal hyperexcitability (Ganetzky and Wu 1982). Simultaneous electrophysiological and behavioral characterization of these mutants demonstrated that seizure behavior was paralleled by distinct states of neuronal activity at the electrophysiological level (Lee and Wu 2002). Electrical stimulation can elicit seizures in both wild-type and mutant animals, although the required stimulation voltage is much less in bang-sensitive mutants, demonstrating their increased seizure susceptibility (Kuebler and Tanouye 2000). Additionally, administration of valproate, phenytoin, gabapentin, and potassium bromide, all of which act as anticonvulsants in humans, suppresses seizures in bang-sensitive mutants (Kuebler and Tanouye 2002; Reynolds *et al.* 2004; Tan *et al.* 2004).

The affected loci of several of the bang-sensitive mutations have been identified, including two mitochondrial proteins, a mitochondrial ribosomal protein (*technical knockout, tko*), and an adenine nucleotide translocase (*stress-sensitive B*, *sesB*) (Royden *et al.* 1987; Zhang *et al.* 1999). Additionally, mutations in an ethanolamine kinase (*easily shocked*, *eas*) and an aminopeptidase (*slamdance*, *sda*) also result in bang sensitivity (Table 1) (Pavlidis *et al.* 1994; Zhang *et al.* 2002). Although molecular identification of two mutants with mitochondrial defects suggests that impaired aerobic metabolism may generate this seizure phenotype, the physiological mechanism remains unclear (Jan and Jan 1978; Ganetzky and Wu 1982; Royden *et al.* 1987; Schubiger *et al.* 1994; Zhang *et al.* 1999).

To further characterize the mechanisms underlying bang sensitivity in Drosophila and to test the hypothesis that these seizure phenotypes are due to metabolic disruption, we have characterized a new bang-sensitive mutant and performed additional analysis of previously studied mutants. We molecularly identified *knockdown* (*kdn*) and found that it encodes citrate synthase, an essential metabolic protein. Measurements of ATP levels revealed reductions in *kdn* as well as in several other bang-sensitive mutants. Electrophysiological analysis revealed alterations in neuronal firing patterns in the giant fiber (GF) pathway of these mutants. Our results demonstrate that metabolic perturbations can alter membrane excitability and increase seizure susceptibility.

MATERIALS AND METHODS

Fly strains: Flies were cultured on cornmeal–molasses agar medium at 22°–29°. Bang-sensitive mutant strains used in this study include kdn^1, tko^{25t}, eas^1, bas^1, bss^1, and $sesB^1$. Genomic deficiency lines in the 5D–F region—*Df(1)dx81*, *Df(1)5D*,

[1]*Corresponding author:* Laboratory of Genetics, Genetics/Biotechnology Bldg., 425-G Henry Mall, University of Wisconsin, Madison, WI 53706-1580. E-mail: ganetzky@wisc.edu

TABLE 1

Summary of phenotypes and gene products among known bang-sensitive Drosophila mutants

Locus/genotype	Growth	BS	Type I	Type II	Affected gene product	Reference
bang senseless (*bss*)	Normal	++	−	+	Unknown	Ganetzky and Wu (1982)
slamdance (*sda*)	Normal	++	−	+	Aminopeptidase	Zhang *et al.* (2002)
easily shocked (*eas*)	Normal	++	+	+	Ethanolamine kinase	Pavlidis *et al.* (1994)
bang sensitive (*bas*)	Normal	++	+	−	Unknown	Grigliatti *et al.* (1973)
technical knockout (*tko*)	Delayed	+/++	+	−	Mitochondrial riboprotein	Royden *et al.* (1987)
stress-sensitive B (*sesB*)	Delayed	+	+	−	Mitochondrial ATP translocase	Zhang *et al.* (1999)
knockdown (*kdn*)	Delayed	+	+	−	Mitochondrial citrate synthase	This report

Mutations affecting mitochondrial proteins result in delayed development. Bang-sensitive (BS) testing resulted in a range of behaviors, including no sensitivity to mechanical stimulation (−; as in wild-type controls) to mild (+) and moderate (++) bang sensitivity. Type I and II seizure activity is denoted as commonly observed (+; ≥50%) and rarely or never seen (−; <5%).

Df(1)G4e[l]H24i[R], and *Df(1)JF5* (diagrammed in Figure 1A)—were utilized for complementation analysis and mapping of *kdn*. *P*-element lines disrupting the *kdn* locus (CG3861) were isolated elsewhere [*PG129* (Bourbon *et al.* 2002) and *KG04873* (Berkeley Drosophila Genome Project)]. Aconitase lines *p07054* and *Df(2L)TW1* were used for genetic interaction experiments. Wild type and control refer to Canton-S, unless otherwise stated.

Behavioral tests: Flies were collected under CO_2 at 0–3 days after eclosion and kept at 36 animals/vial for 1–2 days before behavioral analysis. Vials were mechanically stimulated by placement in a bench-top vortex for 15 sec at the maximum setting. The time for each fly to right itself after vortexing was recorded (Ganetzky and Wu 1982).

Molecular analysis: Deficiency chromosomes were made heterozygous with GFP-tagged balancer chromosomes and homozygous deficiency embryos were identified as lacking GFP expression. GFP-expressing embryos from these stocks served as positive controls. Genomic DNA was isolated from 10 embryos of each genotype and all experiments were done in duplicate. Breakpoint mapping primers for deficiency analysis in Figure 1 were as follows: set (A) 5′-TGACTCGACTGC TCCGCTTGACTG-3′ and 5′-GACGGCGAGGGCTGCTACTA CCAC-3′, (B) 5′-GCGCTGCCCACCACTCACCTAT-3′ and 5′-AAAGCGGCAAATGTTCCTAT-3′, (C) 5′-AAAATGCGCAAAC CACCGACAACC-3′ and 5′-TAGGCCAGACCCAGCACCCGTA TG-3′, (D) 5′-CCCCGCCCCCAGCATTAGGT-3′ and 5′-CCGTTC GTTCAGGGCACTCAGGT-3′, (E) 5′-GCCGTCGGTTGTCAA GGTGGAGTC-3′ and 5′-AGATGATGGCGTGCTGCGGTGAA-3′, and (F) 5′-CGTGCTCAACTGCCCGTCGTA-3′ and 5′-CTGGC CGCCCCTTTTTGTAGC-3′.

Analysis of candidate gene expression in homozygous *P*-element mutants was performed using RNA collected from non-GFP expressing embryos (as above). RNA from embryos homogenized in 3 M LiCl/6 M urea was ultimately DNAse treated and purified through further phenol chloroform extraction. Reverse transcriptase reactions (Sigma-Aldrich, St. Louis) were performed on RNA from each genotype using a polyT primer. Subsequent PCR analysis of generated cDNA was performed using primers specific to the gene under study and primer pairs were from different constitutive exons where possible. To generate internal controls for these RT–PCR reactions, we introduced concentrated rp49 primers (32 μM) at the start of PCR cycle 15. Samples from PCR reactions were extracted at the end of cycles 30, 35, and 40.

ATP assays: ATP content was determined by using a Harta MicrolumiXS Microplate Luminometer with Lumiterm II acquisition software (Harta Instruments, Gaithersburg, MD) and an ATP determination kit A-22066 (Invitrogen, Carlsbad, CA). ATP extraction was performed using bodies from 15 adult females/genotype, collected at room temperature, and transferred to 1.5-ml Eppendorf tubes on ice containing 200 μl of 6 M guanidine hydrochloride. All samples were generated in duplicate. The preparations were homogenized and placed at 95° for 5 min. After incubation, samples were centrifuged at 14,000 rpm for 5 min and 120 μl of the supernatant was transferred into a new 1.5-ml Eppendorf tube. This extract was mixed, diluted 1000-fold with TE buffer at pH 8, and 20 μl was placed in triplicate in a 96-well, white-walled, white-bottom plate. Standard reaction solution (180 μl) was simultaneously added to each well and mixed using a multipetter. High-gain 1-sec exposure "glow" reads were obtained in triplicate at ~15 min after reaction initiation. Sample values were corrected for protein content using DC protein assays (Bio-Rad Laboratories) and ATP concentrations were normalized as a percentage of wild-type control samples.

Electrophysiological analysis: Recordings from the GF escape circuit were performed as previously described (Kuebler and Tanouye 2000; Lee and Wu 2002). Briefly, the head and thorax of animals were tethered to a micropipette using nail polish under CO_2 anesthesia and kept in a humidified chamber for at least 20 min to allow recovery. Glass microelectrodes were used to record potentials from the dorsal longitudinal indirect flight muscles (DLMs) and the reference electrode was placed in the abdomen. A sampling rate of 200 μsec was used with an Axopatch1D amplifier in current clamp = 0 configuration. An uninsulated electrolytically sharpened tungsten electrode was placed in each eye for GF circuit stimulation. Using a stimulation protocol optimized for eliciting initial discharge seizure activity (Lee and Wu 2002), we applied high-frequency electroconvulsive stimulation consisting of 0.2-msec pulses given at 200 Hz over a 300-msec train duration. Stimulation voltage was increased beyond the GF threshold until seizures were elicited. An interstimulus interval of at least 5 min was utilized to avoid the refractory state.

RESULTS

Mapping of *kdn*: *kdn* homozygotes are developmentally delayed; time to eclosion is 4–7 days longer than wild type at room temperature. This delay is similar to that reported for *sesB* and *tko* as well as that for other mutants with deficits in cellular metabolism, including *maggie*, *sluggishA*, *arginase*, and the *Minute* mutants. Bang-sensitive paralysis in *kdn* homozygotes shows incomplete penetrance and variable expressivity that is

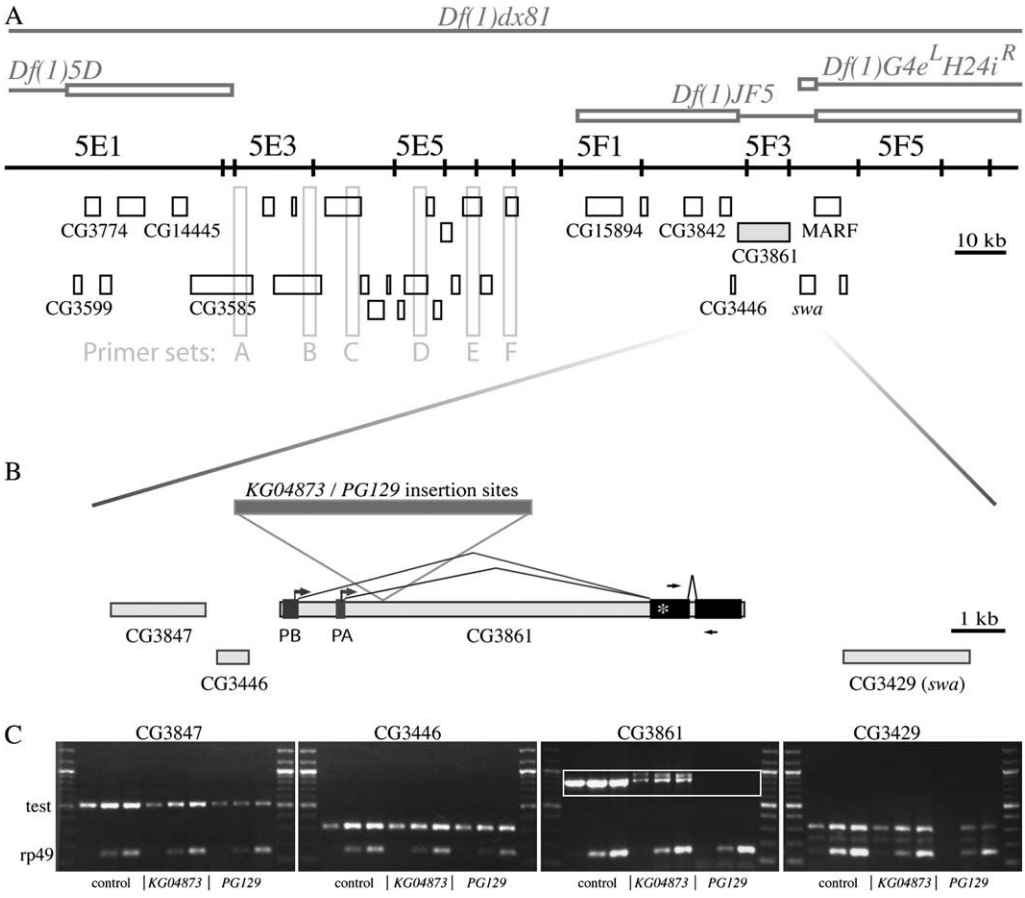

FIGURE 1.—Genetic mapping of the *kdn* locus. (A) Complementation testing of *kdn* bang sensitivity using X chromosome deficiencies in the 5A–6A region resulted in failure of complementation by two deficiencies, *Df(1)dx81* and *Df(1)JF5*. Several of these deficiencies were described as having breakpoints in the 5E3–E5 region. However, PCR analysis of genomic DNA from homozygous deficiency animals revealed that this region was intact in all deficiencies except *Df(1)dx81* (primer sets A–F). Empirically defined breakpoint limits based on our PCR analysis are depicted as boxes and deleted segments are represented as solid lines. The results of these experiments placed the *kdn* locus cytologically at 5F1–4 and between CG15894 and CG3861. (B) The failure of two independently generated *P*-element insertion lines, *PG129* and *KG04873*, to complement the *kdn* bang-sensitive phenotype implicates gene CG3861 as the affected gene. Two unique transcripts of CG3861 have been identified and suggest two alternative initial exons. Both *P*-element insertion sites map to within 20 bp of each other and are located in between the alternative starting exons and the constitutive remaining exons. * denotes the location of the only observed sequence alteration in this region of the original *kdn* chromosome and is in the first constitutive exon of CG3861. (C) Semiquantitative RT–PCR gene expression studies revealed a reduction only in the CG3861 transcript. cDNA reverse transcribed from isolated homozygous *P*-element and control animal RNA was used as PCR template (see MATERIALS AND METHODS). Bands shown are samples taken from the PCR reactions at cycles 30, 35, and 40. The bottom band is an internal control for RNA and the top band is a product generated from experimental intron-spanning primers specific to the gene under study. The specific reduction of CG3861 transcript in *KG04873* and *PG129* homozygous lines supports the specific disruption of CG3861 expression due to the element insertions. The larger band in *KG04873* corresponds to the genomic size PCR product amplified from remaining genomic DNA and the absence of cDNA template. The DNA ladder in all gels is 100 bp.

independent of differences in age, rearing density, time of eclosion, or time of day.

On the basis of previous recombinational mapping (GANETZKY and WU 1982), we performed deficiency mapping of *kdn* in the region between cytological positions 5A and 9F on the X chromosome. *Df(1)dx81* failed to complement *kdn*, whereas *Df(1)C149*, *Df(1)N73*, *Df(1)5D*, and *Df(1)G4e[L]H24i[R]* did complement *kdn*, placing the gene in the region 5E–F. Cytological analysis of *Df(1)5D* by R. KREBER (personal communication) revealed breakpoints at 5D2–3;5E4–6, which defined a distal limit for *kdn*. Failure of complementation by *Df(1)JF5*, with reported breakpoints of 5E5;5E8, further refined the location of *kdn*. Bang-sensitive paralysis was stronger and less variable when *kdn* was heterozygous with a noncomplementing deficiency than in *kdn* homozygotes. Females carrying a single wild-type allele of *kdn* (*Df/+*) were completely wild type as were *kdn/Dp(1;Y)dx+1* males, which carry X-chromosome region 5A;6D inserted into the Y chromosome.

Attempts to align the cytological map with the genomic sequence in the 5E interval indicated that the alignment given in FlyBase is incorrect. Primer pairs designed to cover cytological regions 5E2 through 5E8 (domains A–F in Figure 1A) gave positive PCR products for templates isolated from embryos homozygous for each of the deficiencies examined except *Df(1)dx81*. Since homozygous deficiency embryos should have completely lacked one or more of the genomic regions examined in these PCR experiments according to the previously assigned breakpoints, it was necessary to ascertain the correct breakpoints for these deficiencies at the molecular level. These corrected breakpoints are indicated diagrammatically in Figure 1A.

Two independently generated *P*-element insertion lines, *PG129* and *KG04873*, fail to complement *kdn*. Both lines

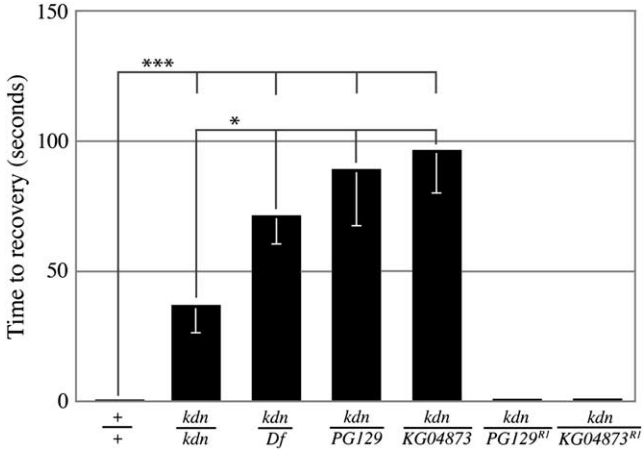

Figure 2.—Behavioral analysis of various *kdn* genotypes. Animals of the genotype indicated were observed for their time to recover following 15 sec of mechanical stimulation ($n > 10$ for each sample; error bars represent SEM). Wild-type and control animals right themselves instantly, while bang-sensitive mutants display different durations of paralysis or immobility prior to delayed discharge spasms and recovery. Flies containing the *kdn* mutation over a more severe allele, such as a deletion or *P*-element insertion, require significantly longer periods to recover from paralysis. Animals heterozygous for *kdn* and a revertant that precisely excises the *P* element (*PG129*^{RI} and *KG04873*^{RI}) exhibit no sensitivity to mechanical stress. Nonparametric Mann–Whitney *U* analyses were performed, with * and *** representing statistical significance of $P < 0.05$ and $P < 0.001$, respectively.

are lethal when homozygous and when heterozygous with *Df(1)JF5*. Flies heterozygous for *kdn* and either of these *P* elements displayed the same increase in severity of the bang-sensitive phenotype as in *kdn/Df* flies, suggesting that the insertions are *kdn* null alleles. Transposase-mediated precise excision of each *P* element resulted in viable revertants that complemented *kdn* (Figure 2). The *P*-element insertion sites of both *PG129* and *KG04873* are within the same large intron of CG3861 (Figure 1B), identifying it as the *kdn* locus. Consistent with this, RT–PCR analysis revealed that of the four genes neighboring these elements, only CG3861 showed a marked reduction in expression in embryos homozygous for the *P*-element insertions (Figure 1C).

Molecular identification of *kdn*: CG3861 encodes citrate synthase, the first enzyme in the Krebs cycle. Sequence analysis of the original *kdn* mutant revealed an R95H substitution in the encoded protein (Figure 3). This change was not found in the wild-type background in which *kdn* was generated, nor were any sequence changes found in the neighboring gene, CG3446. The arginine residue altered in *kdn* is highly conserved in all species from yeast to humans (Figure 3) and corresponds to R94 in the human protein. This residue has not been implicated previously in enzymatic function (Wiegand and Remington 1986).

Genetic interactions with other mutants: Our results suggest that altered cellular metabolism may underlie bang sensitivity in Drosophila. To further examine this possibility, we examined available adult viable mutants with affected metabolism, including *Glycerol 3 phosphate dehydrogenase*, *arginase*, and *sluggishA*, which encodes a proline oxidase, to determine if bang-sensitive phenotypes could be observed in mutants not previously analyzed for seizure behavior. None of the mutants in this limited sample exhibited bang sensitivity (data not shown). A genetic analysis to test the connection between metabolism and seizure behavior was performed by combining the *kdn* mutation, as well as other bang-sensitive mutations, with a deletion that removes the gene encoding aconitase, the enzyme directly following citrate synthase in the Krebs cycle. No significant alteration in the seizure phenotype was observed in animals heterozygous for this deletion and hemizygous for *kdn*, *tko*, *eas*, *bas*, or *bss*. Flies doubly heterozygous for either *kdn* or *tko* and the aconitase deletion also showed normal responses to mechanical shock. Similarly, heterozygotes for two or three of the bang-sensitive mutations (*kdn*, *sesB*, *tko*, and *eas*) appeared normal (Table 2), although increased bouts of flight-like wing activity were observed. These results indicate that partial reductions in these metabolic proteins do not have sufficient additive phenotypic effects to cause a bang-sensitive phenotype. However, both *bss* and *sda* mutations confer a partially dominant increase in seizure susceptibility (Ganetzky and Wu 1982; Kuebler and Tanouye 2000), suggesting that the cellular processes affected by these mutations are sensitive to gene dosage. We also tested for summation of seizure susceptibility in homozygous double mutant combinations. *kdn sesB* and *sesB eas* double mutants are small, weak, developmentally delayed, and exhibit dramatically increased sensitivity to mechanical stimulation (Table 2). The summation of bang sensitivity is similar to that reported

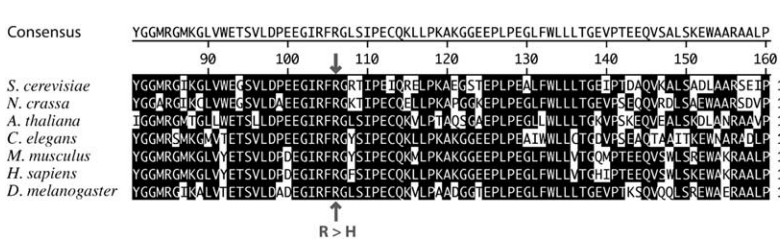

Figure 3.—Sequence alignment of citrate synthase in the region of the *kdn* mutation. This region is highly conserved in all known eukaryotic citrate synthase sequences. The arginine 95 residue is mutated to a histidine in *kdn* as the result of a G-to-A base pair change. This residue is completely conserved in all sequences examined, from yeast to human.

TABLE 2

Genetic interactions among combinations of various bang-sensitive mutations

Locus/genotype	Development	BS
bss bas	Normal	+++
bss bas/+ +	Normal	+
kdn sesB	Delayed	+++
kdn sesB/+ +	Normal	−
sesB eas	Delayed	+++
sesB eas/+ +	Normal	−
+ *kdn sesB*/*tko*^{25t} + +	Normal	−

Bang-sensitive (BS) testing resulted in a range of behaviors, including no sensitivity to mechanical stimulation (−) to a strong bang-sensitive phenotype (+++).

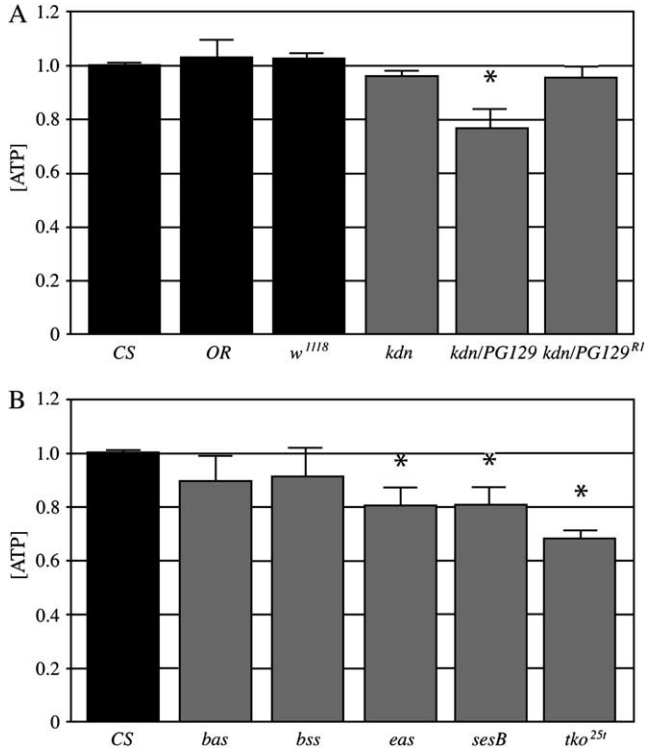

FIGURE 4.—ATP levels are reduced in bang-sensitive mutants. (A) Wild-type strains Canton-S and Oregon-R, as well as w^{1118} controls, exhibit no significant change in ATP levels, while *kdn* mutants exhibit a reduction in ATP. *kdn/PG129* heterozygotes with a strong bang-sensitive phenotype exhibited a significant reduction in ATP levels, whereas ATP levels in *kdn* homozygotes with mild and variable bang sensitivity were not significantly reduced. Similarly, *kdn/PG129*^{R1} animals, which are not bang sensitive, displayed normal ATP levels. (B) Other bang-sensitive mutants had significant reductions in ATP levels. Animals homozygous for *eas*, *sesB*, and *tko* all exhibited significantly reduced levels of ATP. Reductions in ATP levels were observed in *bas* and *bss* mutants, although these reductions were not quite significant. *N* values ≥6 were used for each genotype. *U*-test analyses revealing significant changes in ATP levels are denoted with * ($P < 0.01$).

previously for *bss bas* double mutants (LEE and WU 2002) and suggests that all bang-sensitive mutations may ultimately impair the same neuronal mechanism.

Decreased ATP levels in bang-sensitive mutants: To determine the effects of these mutations on energy levels, we performed quantitative ATP assays. ATP levels in control lines, including Canton-S, Oregon-R, and our w^{1118}, were not significantly different from each other (Figure 4A). Homozygous *kdn* mutants had a mild reduction in ATP levels but this was not significant ($P = 0.1$, $n = 8$). Heterozygous *kdn/PG129* animals displayed a significant reduction in ATP ($P < 0.001$, $n = 5$), which correlates with their significantly enhanced bang sensitivity relative to *kdn* homozygotes (Figure 2). Conversely, ATP levels were normal ($P > 0.2$; Figure 4A) in *kdn/PG129*^{R1} flies.

Other bang-sensitive mutants had similar decreases in ATP levels (Figure 4B). Although ATP levels were reduced in *bas* and *bss* mutants, the difference was not significant compared with wild type ($P = 0.07$ and $P = 0.18$, respectively). ATP levels were significantly reduced in *eas*, *tko*, and *sesB* mutants ($P < 0.01$ for each; Figure 4B). These results support the idea that bang-sensitive mutations are associated with defects in cellular metabolism.

Neurons in the giant fiber pathway have differential sensitivities to seizure induction in metabolic mutants: The GF pathway in Drosophila is a neural circuit that mediates an escape reflex by initiating flight behavior in response to various sensory stimuli (TANOUYE and WYMAN 1980). By recording intracellularly from the DLMs, electrical activity in the GF pathway can be assayed. In bang-sensitive mutants this circuit exhibits physiological seizure activity that correlates with the behavioral seizure phenotype (LEE and WU 2002). Physiological and behavioral seizures can be elicited in wild-type as well as in mutant animals by high-frequency electrical brain stimulation, but stimuli of longer duration or greater intensity are required in the former (Figure 5A) (KUEBLER and TANOUYE 2000; LEE and WU 2002). Absence of seizure activity recorded from outputs of the GF pathway other than DLMs suggests that the aberrant activity originates in the peripherally synapsing interneuron (PSI) and/or DLM motor neuron rather than in the giant neuron itself (PAVLIDIS and TANOUYE 1995; LEE and WU 2002) (Figure 5G). This seizure activity begins with an initial neuronal burst or discharge, two classes of which have been distinguished in bang-sensitive mutants (PAVLIDIS and TANOUYE 1995). Type I seizures are exemplified by trains at ~10–30 Hz lacking any clear firing pattern that terminate abruptly (Figure 5A). Type II seizures exhibit increasing firing frequency with a concomitant decrease in transmission amplitude. Type II seizures predominate in *bss* mutants (Figure 5B) and most likely result from motor neuron dysfunction because reductions in amplitude of DLM responses evoked by action potentials should not result from defects in a neuron upstream of the motor neuron. *kdn* and *tko* display

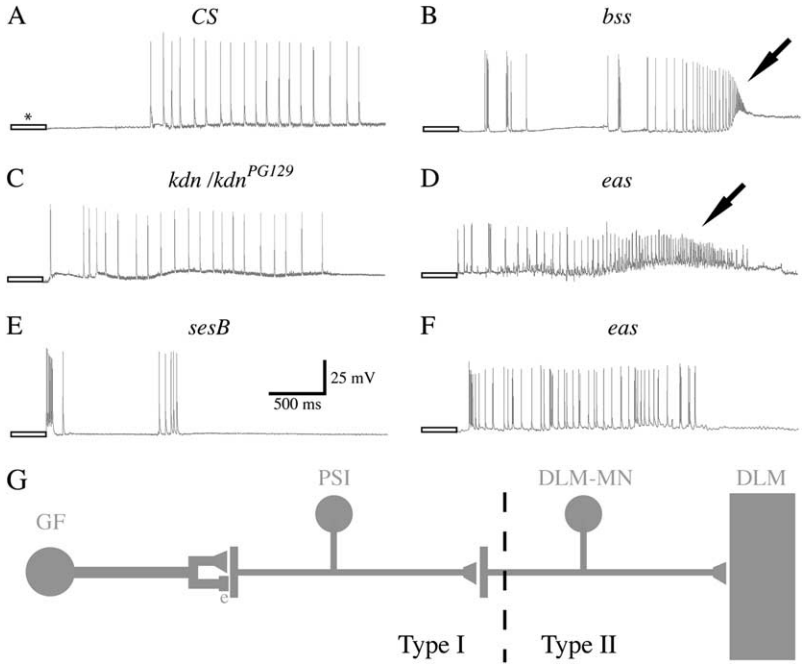

FIGURE 5.—Representative traces of distinct types of initial seizure activity in the GF-DLM neuronal circuit from bang-sensitive mutants. (A) Application of high-frequency electrical stimulation to the brain in Canton-S elicits a behavioral seizure with corresponding bursts of activity in DLMs. Inducing the seizure phenotypes in wild-type animals requires that the voltage of the pulses be increased (denoted with an *) during the stimulation train (depicted as open boxes). (B) Following light brain stimulation, *bss* mutants exhibit bursts of spike activity in DLMs that ultimately increase in frequency and decrease in amplitude (arrows) indicative of type II seizures. (C and E) *kdn* and *sesB* exhibit initial DLM spiking with constant amplitude and variable firing rates typical of type I seizures. (D and F) *eas* mutants predominately exhibit type II seizure activity, although type I activity was also commonly observed. (G) A diagram of the giant fiber (GF) to dorsal longitudinal muscle (DLM) escape reflex circuit (KING and WYMAN 1980). Some of the *en passant* chemical and electrical (e) synaptic outputs of the giant fiber neuron directly stimulate the PSI interneuron. The PSI neuron in turn innervates multiple DLM motor neurons that synapse onto distinct DLMs. The increasing frequency and decreasing amplitude of DLM spikes seen in type II seizure activity reflect electrical activity of the motor neuron because attenuation in synaptic output from the PSI should evoke "all-or-nothing" action potentials in the motor neuron with corresponding DLM activity.

predominantly type I initial discharge seizures (PAVLIDIS and TANOUYE 1995) (Figure 5C). *sesB* mutants also have type I initial discharge seizures (Figure 5E). Type II activity is most common in *eas*, although type I seizures were frequently observed as well (Figure 5, D and F) (PAVLIDIS and TANOUYE 1995). Thus, it appears that type I seizures, which are most likely to originate upstream of the motor neuron, are the most readily evoked since they predominate in the mutants showing the weakest bang-sensitive behavior (Table 1). Conversely, type II seizures, which involve uncontrolled motor neuron activity, are most often observed in mutants with the strongest bang-sensitive behavioral phenotype, suggesting that the motor neuron may be able to tolerate metabolic perturbations better than other neurons (Figure 5G).

DISCUSSION

Mutations that cause seizures in response to mechanical stimulation include defects in genes encoding a mitochondrial ribosomal protein (ROYDEN *et al.* 1987), an ethanolamine kinase (PAVLIDIS *et al.* 1994), the ADP/ATP translocase (ZHANG *et al.* 1999), and an aminopeptidase (ZHANG *et al.* 2002). The identification of another bang-sensitive mutation in a gene encoding a key component of the Krebs cycle supports the hypothesis that cellular energy, the primary product of mitochondrial function, is closely connected with regulation of seizure susceptibility. In fact, *kdn* is the third Drosophila seizure mutation associated with a mitochondrial protein (Figure 6). Following molecular identification of the Drosophila *tko* gene, the first human epilepsy-associated gene to be identified was also found to encode a gene involved in mitochondrial protein synthesis (SHOFFNER *et al.* 1990), suggesting the existence of conserved mechanisms. Unlike *tko* and *sesB* mutations, which have potentially broad effects on mitochondria and cellular function, disruption of citrate synthase by the *kdn* mutation most likely results in a specific impairment of aerobic metabolism. This finding provides a direct link between citrate synthase and seizure susceptibility.

Previous evidence links metabolic disruption with seizure susceptibility. These disruptions may be transient, as in hypoglycemia and ischemia, or constant, as caused by mutations that disrupt mitochondrial function (DIMAURO *et al.* 2002; NORDLI and DE VIVO 2002; VIGEVANO and BARTULI 2002). Mutations affecting the two enzymes upstream of citrate synthase in the Krebs cycle, pyruvate carboxylase and pyruvate dehydrogenase, as well as less common mutations perturbing various steps in the TCA cycle, have been linked with human seizure disorders (DE VIVO 1993; DE MEIRLEIR 2002). The phenotypes observed in the Drosophila mutants investigated here are strikingly similar to those seen in the related human syndromes. These similarities include developmental delay, inactivity, reduced viability, decreased life span, and seizures. Although mutation of a glycolytic enzyme in Drosophila can confer seizures and paralysis upon exposure to elevated temperature (WANG *et al.* 2004), it does not exhibit bang-sensitive seizures. Thus, bang-sensitive seizure susceptibility may

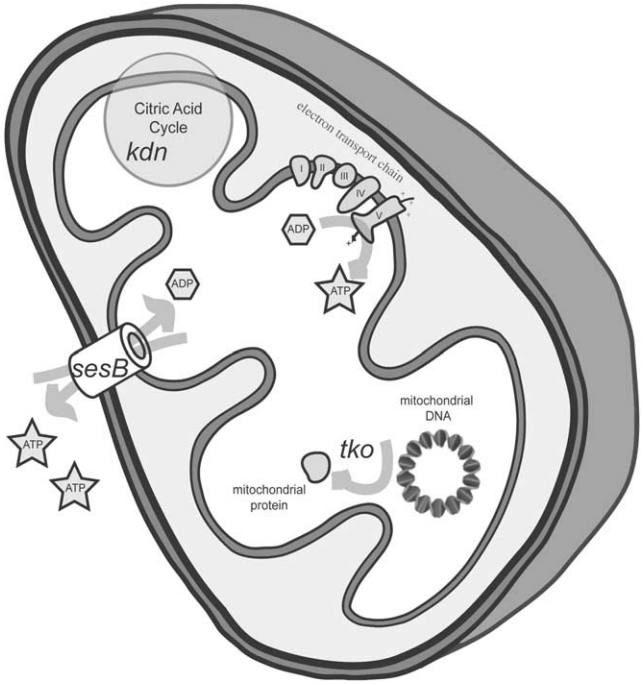

FIGURE 6.—Sites of mitochondrial disruption in bang-sensitive mutants. Mutations in *technical knockout* (*tko*) are thought to disrupt translation of mitochondrial proteins. Mutations in *stress-sensitive B* (*sesB*), which encodes the ADP/ATP translocase, may disrupt nucleotide transport and signaling in mitochondria. Mutations in the *knockdown* (*kdn*) gene disrupt an enzyme implicated in metabolism. All of these mutations are associated with reduction in ATP levels suggesting that metabolic disruption may be a general mechanism underlying bang-sensitive seizures in Drosophila.

involve a specific block in mitochondrial metabolism. Furthermore, the existence of lethal alleles in every bang-sensitive gene identified in Drosophila suggests that reduction in metabolism to a particular degree increases seizure susceptibility, but further disruption of metabolism is not compatible with life.

Quantitative analysis confirmed that bang-sensitive mutants reduce ATP levels with the reduction greatest in mutants with defects in mitochondrial proteins. Although comparisons between *kdn/kdn* and *kdn/PG129* animals show a good correlation between the strength of the bang-sensitivity phenotype and the reduction in ATP levels, this correlation does not hold for the other bang-sensitive mutants examined here. Thus, it is possible that increased seizure susceptibility of some bang-sensitive mutants involves some factor(s) in addition to or instead of metabolic perturbations. Nonetheless, the enrichment for mutations affecting mitochondrial proteins with resultant decreases in ATP levels among bang-sensitive mutants and the identification of *kdn* as yet another mutation in this category strongly suggest that there is an important link between seizure susceptibility and metabolic perturbations.

Although we cannot rule out secondary developmental defects in the nervous system, it appears that there is a threshold for metabolic function below which increased seizure susceptibility occurs. Application of mitochondrial toxins, such as 3-nitropropionic acid, results in seizures in vertebrate models (LUDOLPH *et al.* 1992; URBANSKA *et al.* 1998), suggesting that some of the key underlying mechanisms are conserved. Such summation was also observed between *sesB* and *eas* mutants, which exhibit different types of seizure activity, further suggesting that both mutations ultimately impair the same processes. Summation of seizure susceptibility in bang-sensitive double mutants may help explain why manifestation of various epilepsies in humans varies with the background genotype.

Initial seizure activity observed in bang-sensitive mutants following mechanical or electrical stimulation may reflect unchecked neuronal firing due to prolonged membrane depolarization. Such depolarization may be enhanced in cells lacking energy since it has been estimated that 75–90% of neuronal ATP is expended solely to maintain ionic gradients across the cell membrane (BEAL *et al.* 1993; LEES 1993; ATTWELL and LAUGHLIN 2001). Although specific mutations in the largest consumer of neuronal ATP, the Na^+/K^+ ATPase, result in temperature-sensitive paralysis and mild bang sensitivity, no mutations affecting the ATPase are known to cause the stereotypic behavioral and electrophysiological phenotypes seen in the class of bang-sensitive mutants (SCHUBIGER *et al.* 1994; PAVLIDIS and TANOUYE 1995). Thus, it remains unclear if reduced Na^+/K^+ pump function is closely connected with increased seizure susceptibility in bang-sensitive mutants. Alternative mechanisms, such as defects in Ca^{2+} buffering and/or synaptic function due to mitochondrial impairment, may exist (ROYDEN *et al.* 1987; PAVLIDIS *et al.* 1994; TROTTA *et al.* 2004).

Independent of the cellular mechanism, our results suggest that the initial seizure-like neurotransmission observed at the DLM in these convulsive mutants results from aberrant activity of either the PSI interneuron or the DLM motor neuron. The occurrence of type II seizure activity in the more extreme bang-sensitive mutants most likely results from uncontrolled motor neuron firing. In addition to the ethanolamine kinase mutant *eas*, the only other identified bang-sensitive mutant exhibiting type II seizure activity is *sda*, which encodes an aminopeptidase (ZHANG *et al.* 2002). The mechanistic link, if any, between these two gene products is unknown. Isolation and characterization of additional mutations in this class may prove informative. We suggest that type I seizures prevail in animals exhibiting milder seizure susceptibility because the PSI is more sensitive than the motor neuron to metabolic perturbations.

These results link increased seizure susceptibility with animals defective in cellular metabolism. Moreover, these results suggest that neurons have differential susceptibilities to seizures following metabolic impairment. It

will be informative to identify alterations in the most critical human metabolic pathways and investigate their correlation with predisposition toward epilepsy. Current studies are aimed at detailed dissection of the neuronal circuits mediating the bang-sensitive phenotype in flies and physiological analysis of seizure onset. It will also ultimately be useful to compare metabolic profiles of these neurons to determine which factors are associated with increased seizure susceptibility.

We thank Seymour Benzer, Marie-Laure Samson, and the Centre for Biologie du Developpement in Toulouse for fly stocks; Dan Kuebler and Mark Tanouye for technical advice; Robert Kreber for polytene chromosome analysis; Julie Simpson for helpful comments; and Al Laughon for luminometer use. This research was supported by NIH grants NS-44722 to T.F. and NS-15390 to B.G. This is paper no. 3628 from the Laboratory of Genetics.

LITERATURE CITED

Attwell, D., and S. B. Laughlin, 2001 An energy budget for signaling in the grey matter of the brain. J. Cereb. Blood Flow Metab. **21:** 1133–1145.

Beal, M. F., B. T. Hyman and W. Koroshetz, 1993 Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? Trends Neurosci. **16:** 125–131.

Benzer, S., 1971 From the gene to behavior. JAMA **218:** 1015–1022.

Bourbon, H. M., G. Gonzy-Treboul, F. Peronnet, M. F. Alin, C. Ardourel *et al.*, 2002 A P-insertion screen identifying novel X-linked essential genes in *Drosophila*. Mech. Dev. **110:** 71–83.

De Meirleir, L., 2002 Defects of pyruvate metabolism and the Krebs cycle. J. Child Neurol. **17**(Suppl. 3): 3S26–33.

De Vivo, D. C., 1993 The expanding clinical spectrum of mitochondrial diseases. Brain Dev. **15:** 1–22.

DiMauro, S., A. L. Andreu and D. C. De Vivo, 2002 Mitochondrial disorders. J. Child Neurol. **17**(Suppl. 3): 3S35–45.

Ganetzky, B., and C. F. Wu, 1982 Indirect suppression involving behavioral mutants with altered nerve excitability in *Drosophila melanogaster*. Genetics **100:** 597–614.

Grigliatti, T. A., L. Hall, R. Rosenbluth and D. T. Suzuki, 1973 Temperature-sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults. Mol. Gen. Genet. **120:** 107–114.

Homyk, T., Jr., J. Szidonya and D. T. Suzuki, 1980 Behavioral mutants of *Drosophila melanogaster*. III. Isolation and mapping of mutations by direct visual observations of behavioral phenotypes. Mol. Gen. Genet. **177:** 553–565.

Jan, Y. N., and L. Y. Jan, 1978 Genetic dissection of short-term and long-term facilitation at the *Drosophila* neuromuscular junction. Proc. Natl. Acad. Sci. USA **75:** 515–519.

King, D. G., and R. J. Wyman, 1980 Anatomy of the giant fibre pathway in *Drosophila*. I. Three thoracic components of the pathway. J. Neurocytol. **9:** 753–770.

Kuebler, D., and M. A. Tanouye, 2000 Modifications of seizure susceptibility in *Drosophila*. J. Neurophysiol. **83:** 998–1009.

Kuebler, D., and M. Tanouye, 2002 Anticonvulsant valproate reduces seizure-susceptibility in mutant *Drosophila*. Brain Res. **958:** 36–42.

Lee, J., and C. F. Wu, 2002 Electroconvulsive seizure behavior in *Drosophila*: analysis of the physiological repertoire underlying a stereotyped action pattern in bang-sensitive mutants. J. Neurosci. **22:** 11065–11079.

Lees, G. J., 1993 Contributory mechanisms in the causation of neurodegenerative disorders. Neuroscience **54:** 287–322.

Ludolph, A. C., M. Seelig, A. G. Ludolph, M. I. Sabri and P. S. Spencer, 1992 ATP deficits and neuronal degeneration induced by 3-nitropropionic acid. Ann. NY Acad. Sci. **648:** 300–302.

Nordli, D. R., Jr., and D. C. De Vivo, 2002 Classification of infantile seizures: implications for identification and treatment of inborn errors of metabolism. J Child Neurol. **17**(Suppl. 3): 3S3–7.

Pavlidis, P., and M. A. Tanouye, 1995 Seizures and failures in the giant fiber pathway of *Drosophila* bang-sensitive paralytic mutants. J. Neurosci. **15:** 5810–5819.

Pavlidis, P., M. Ramaswami and M. A. Tanouye, 1994 The *Drosophila* easily shocked gene: a mutation in a phospholipid synthetic pathway causes seizure, neuronal failure, and paralysis. Cell **79:** 23–33.

Reynolds, E. R., E. A. Stauffer, L. Feeney, E. Rojahn, B. Jacobs *et al.*, 2004 Treatment with the antiepileptic drugs phenytoin and gabapentin ameliorates seizure and paralysis of *Drosophila* bang-sensitive mutants. J. Neurobiol. **58:** 503–513.

Royden, C. S., V. Pirrotta and L. Y. Jan, 1987 The tko locus, site of a behavioral mutation in *D. melanogaster*, codes for a protein homologous to prokaryotic ribosomal protein S12. Cell **51:** 165–173.

Schubiger, M., Y. Feng, D. M. Fambrough and J. Palka, 1994 A mutation of the *Drosophila* sodium pump alpha subunit gene results in bang-sensitive paralysis. Neuron **12:** 373–381.

Shoffner, J. M., M. T. Lott, A. M. Lezza, P. Seibel, S. W. Ballinger *et al.*, 1990 Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell **61:** 931–937.

Tan, J. S., F. Lin and M. A. Tanouye, 2004 Potassium bromide, an anticonvulsant, is effective at alleviating seizures in the *Drosophila* bang-sensitive mutant bang senseless. Brain Res. **1020:** 45–52.

Tanouye, M. A., and R. J. Wyman, 1980 Motor outputs of giant nerve fiber in *Drosophila*. J. Neurophysiol. **44:** 405–421.

Trotta, N., C. K. Rodesch, T. Fergestad and K. Broadie, 2004 Cellular bases of activity-dependent paralysis in *Drosophila* stress-sensitive mutants. J. Neurobiol. **60:** 328–347.

Urbanska, E. M., P. Blaszczak, T. Saran, Z. Kleinrok and W. A. Turski, 1998 Mitochondrial toxin 3-nitropropionic acid evokes seizures in mice. Eur. J. Pharmacol. **359:** 55–58.

Vigevano, F., and A. Bartuli, 2002 Infantile epileptic syndromes and metabolic etiologies. J. Child Neurol. **17**(Suppl. 3): 3S9–13.

Wang, P., S. Saraswati, Z. Guan, C. J. Watkins, R. J. Wurtman *et al.*, 2004 A *Drosophila* temperature-sensitive seizure mutant in phosphoglycerate kinase disrupts ATP generation and alters synaptic function. J. Neurosci. **24:** 4518–4529.

Wiegand, G., and S. J. Remington, 1986 Citrate synthase: structure, control, and mechanism. Annu. Rev. Biophys. Biophys. Chem. **15:** 97–117.

Zhang, Y. Q., J. Roote, S. Brogna, A. W. Davis, D. A. Barbash *et al.*, 1999 *stress sensitive B* encodes an adenine nucleotide translocase in *Drosophila melanogaster*. Genetics **153:** 891–903.

Zhang, H., J. Tan, E. Reynolds, D. Kuebler, S. Faulhaber *et al.*, 2002 The Drosophila slamdance gene: a mutation in an aminopeptidase can cause seizure, paralysis and neuronal failure. Genetics **162:** 1283–1299.

Communicating editor: R. S. Hawley