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The Drosophila *easily shocked* Gene: A Mutation in a Phospholipid Synthetic Pathway Causes Seizure, Neuronal Failure, and Paralysis

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Summary

We have characterized *easily shocked* (*eas*), a Drosophila "bang-sensitive" paralytic mutant. Electrophysiological recordings from flight muscles in the giant fiber pathway of adult *eas* flies reveal that induction of paralysis with electrical stimulation results in a brief seizure, followed by a failure of the muscles to respond to giant fiber stimulation. Molecular cloning, germline transformation, and biochemical experiments show that *eas* mutants are defective in the gene for ethanolamine kinase, which is required for a pathway of phosphatidylethanolamine synthesis. Assays of phospholipid composition reveal that total phosphatidylethanolamine is decreased in *eas* mutants. The data suggest that *eas* bang sensitivity is due to an excitability defect caused by altered membrane phospholipid composition.

Introduction

A feature of all cells is the presence of bilayer membranes of a complex lipid composition. However, the role of lipid composition in biological membrane function is not well understood. Best known is the role of specific lipid-derived molecules, such as inositol phosphates and diacylglycerol, as messengers in transmembrane signal transduction (Exton, 1994). Recently, it has become increasingly clear that the regulation of lipid composition and metabolism is important to the maintenance of membrane traffic and organelle integrity (Kahn et al., 1993). Possibly related to these issues is the potential for lipid composition to affect membrane curvature, fluidity, and fusion (Sheetz and Singer, 1974; Sundler, 1984). Additionally, many membrane proteins studied in vitro are sensitive to lipid environment or the presence of specific lipid cofactors (Yeagle, 1989). These considerations make it likely that lipid composition is an important factor in regulating the function of biological membranes, though demonstrations of this are few.

The relative lack of information on the roles of lipid composition in membrane function is in part due to the difficulty of manipulating it in vivo. In yeast, a genetic approach has revealed a link between phospholipid metabolism and competence for membrane traffic in the Golgi apparatus (Cleves et al., 1991; McGee et al., 1994), and in Paramecium a mutant with an altered membrane lipid composition has abnormal ion channel function (Forte et al., 1981). These studies show that mutants affecting phospholipid metabolism can provide an entry point into studying the physiological roles of lipids (McGee et al., 1993). In the current work, we present evidence for a surprising relationship among phospholipid metabolism, nervous system function, and behavior in Drosophila melanogaster.

In Drosophila, behavioral genetics has been used to study genes required for proper nervous system function (Hall, 1985). One class of behavioral mutants that has not been extensively studied is the "bang-sensitive" paralytic class. Bang sensitives are distinguished by their unusual phenotype, transient paralysis following a brief mechanical shock (Benzer, 1971). Several lines of evidence indicate that the bang-sensitive phenotype involves altered neuronal excitability. All bang sensitives are suppressed at permissive temperatures by *mle*^{napts} (*maleless–no action potential–temperature sensitive*) (Ganetzky and Wu, 1982), which decreases membrane excitability (Wu and Ganetzky, 1980). One bang-sensitive mutant, *bang-senseless* (*bss*), has several presynaptic defects at the larval neuromuscular junction, including reduced spontaneous transmitter release, abnormally rapidly developing facilitation, and nerve hyperexcitability (Jan and Jan, 1978; Ganetzky and Wu, 1982). Another interesting feature of the bang-sensitive phenotype is a refractory period following recovery from paralysis during which the paralytic response to mechanical stimulation cannot be elicited (Ganetzky and Wu, 1982).

Hypotheses put forward to explain this data have included defects in the Na^+–K^+ ATPase or a sodium-sensitive site (Jan and Jan, 1978) and ion channel defects (Ganetzky and Wu, 1982). Since the known Drosophila mutations in ion channels do not cause bang-sensitive phenotypes (Wu and Ganetzky, 1992), the bang-sensitive mutations might represent a different type of excitability defect. Recently, a Drosophila Na^+–K^+ ATPase mutant has been isolated that has a weak mechanical shock–sensitive phenotype that bears some similarity to the bang-sensitive defect (Schubiger et al., 1994). Only one of the original bang-sensitive genes, *technical knockout* (*tko*), has been cloned (Royden et al., 1987). It is proposed to encode a mitochondrial ribosomal protein, suggesting defects in ATP synthesis or calcium buffering (Royden et al., 1987). However, the cause of paralysis in any bang sensitive is still unknown.

In this paper we present findings from two lines of study on *easily shocked* (*eas*), a typical member of the bang-sensitive class. First, we report the results of electrophysiological experiments on adult *eas* flies, which show that paralysis can be induced by a brief, intense electrical stimulus. The stimulus triggers a short period of intense sei-

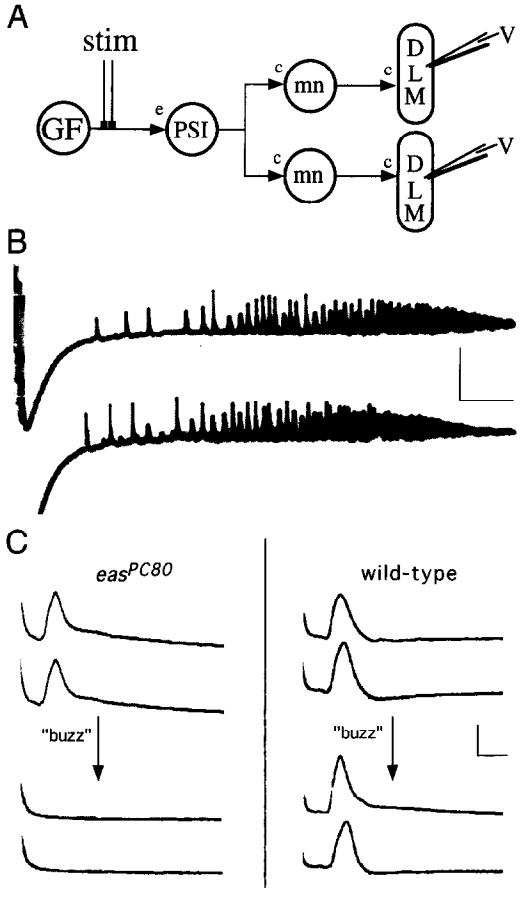

Figure 1. GF System Transmission Defects in eas^{PC80} Adults

(A) Schematic of the pathway used in this study. One GF pathway of two is diagrammed. The GF is an interneuron that drives the initial escape response in adult Drosophila. It has a cell body in the brain and major synaptic outputs to jump and flight motor muscle pathways in the thoracic ganglion, and it receives sensory input from the visual system (Tanouye and Wyman, 1980). An electrical synapse (e) joins the GF with the peripherally synapsing interneuron (PSI), which makes chemical synapses (c) with all five DLM motor neurons (mn). Stimulation with electrodes in the head evokes large synchronous EJPs in all six DLMs on each side of the animal, two of which we recorded from in these experiments. The GF system has a number of additional motor outputs that are not indicated here. This figure incorporates data from several sources, particularly Tanouye and Wyman (1980), King and Wyman (1980), Ikeda et al. (1980), and Gorczyca and Hall (1984).

(B) Seizure-like activity following a buzz in eas^{PC80}. The large stimulus artifact from the buzz is visible at the beginning of the traces. Note the increase in the frequency and the decrease in amplitude of the activity as the seizure progresses. Vertical calibration, 20 mV; horizontal calibration, 200 ms.

(C) Failure of DLM responses in eas^{PC80} flies following a buzz. *eas* responses are on the left; wild type, on the right. The top traces show normal responses to test pulses before delivering a buzz. In the bottom traces, a 50 ms buzz causes a transient loss of DLM responses to GF stimulation in eas^{PC80} but has no effect on the wild-type fly (the seizure that occurred in the eas^{PC80} fly is not shown). The failure in the mutant is not due to an increase in GF threshold, as increasing test stimulus voltage does not bring back the DLM response (data not shown). Vertical calibration is 20 mV; horizontal calibration, 2 ms.

zure-like activity followed by a failure of transmission through the giant fiber (GF) pathway. Molecular and biochemical analyses show that *eas* flies have a defect in the structural gene for ethanolamine kinase, which is required for one pathway of synthesis of phosphatidylethanolamine (PE). Correspondingly, PE content is decreased in *eas* mutants. These results provide evidence for the importance of phospholipid metabolism to neuronal excitability.

Results

The *eas* Behavior

Undisturbed eas^{PC80} flies do not show any obvious behavioral defects until subjected to a moderately strong mechanical stimulus. When long mechanical stimuli are used, such as the 10 s of vortexing used in the assay of Ganetzky and Wu (1982), all eas^{PC80} flies become paralyzed, while wild-type flies are unaffected. A very brief bang (such as a single tap of the culture vial on a bench top), while not consistently paralyzing all *eas* flies, allows examination of the events immediately following the stimulus. In affected flies, a brief bang initiates a period of hyperactivity lasting 1–2 s, during which the flies fall over and vigorously flap their wings, shake and bend their legs, and flex their abdomens. This activity decays rapidly, ending in a completely paralyzed state, although occasionally flies that become hyperactive recover without paralysis. When long stimuli (i.e., 10 s of vortexing) are used, the preparalysis hyperactivity is not observed, apparently because the flies are affected at the beginning of the stimulus and the hyperactivity is over before the stimulus ends.

Paralysis is characterized by a relaxed posture of the wings, legs, body, and proboscis. After 20–30 s of complete paralysis (this duration was highly reproducible among flies), a period we refer to as the postparalysis hyperactive phase begins, characterized by massive uncoordinated motor activity similar to that observed before the commencement of paralysis. Finally, flies right themselves (often with difficulty), and normal behavior begins soon afterward. The median time for recovery from the bang is ~110 s (n = 147 flies). Subsequent to recovery, eas^{PC80} flies cannot be paralyzed again for a refractory period spanning 3–5 min.

Seizure and Failure in the GF Pathway of *eas* Adults

We investigated the electrophysiological basis of *eas* paralysis using a modification of a standard method for stimulating and recording from the adult fly GF pathway (Figure 1A). We found that delivery of a 200 Hz train of stimuli with a relatively high voltage for 50–100 ms (which we refer to as a buzz) to the head of an eas^{PC80} fly causes a striking abnormal response in the GF pathway. Following the buzz, a brief (~1–2 s) period of very intense, seizure-like activity occurs in the dorsal longitudinal muscles (DLMs) (Figure 1B). The DLM activity during the "seizure" increases in frequency to over 100 Hz while decreasing in amplitude (Figure 1B). Following the seizure, no evoked or spontaneous DLM responses occur for 29 ± 6 s (27 trials for 12 flies) (Figure 1C). Normal evoked responses did not return for an average of 101 ± 57 s (n = 41 trials for 18 flies). In control experiments with wild-type flies, DLM responses were not affected by short buzzes (Figure 1C). However, very long buzz durations (500–1000 ms)

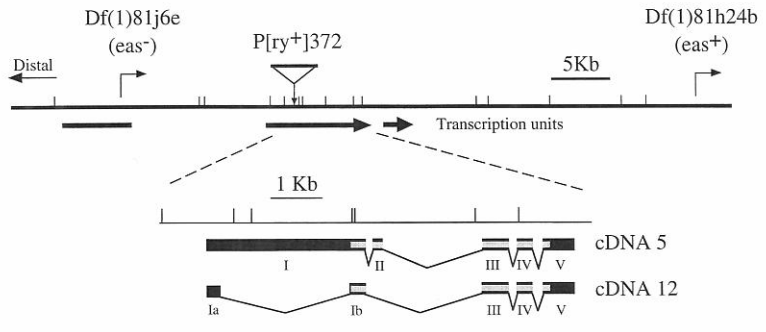

Figure 2. Physical Map of the *eas* Region and *eas* cDNAs

EcoRI sites are denoted by vertical ticks on both the large scale and expanded scale maps. The locations of the deletion breakpoints of *Df(1)81j6e* and *Df(1)81h24b*, which define the *eas* locus, are shown with arrows indicating the direction of the deleted DNA (Jones and Rubin, 1990; Heilig et al., 1991). The lethal P element (eas^{P372}) that failed to complement the *eas* phenotype was mapped to the location indicated by the method of plasmid rescue of flanking genomic DNA and by genomic Southern blotting. Three transcription units from the *81j6e–81h24b* region were identified by Northern analysis of genomic clones spanning the region at a minimum resolution of 10 kb. The direction of transcription, if known, is indicated by arrows; differentiating the two closely spaced transcription units on the right was done at a resolution of 200 bp. The center transcription unit contains the eas^{P372} insertion site, indicated near the 5′ end of this unit. The genomic organization of two cDNAs derived from this transcription unit are shown schematically on the expanded portion of the map. Roman numerals designate exons. The stippled bars within the cDNAs represent coding sequences.

sometimes caused evoked responses to fail (data not shown). This failure was much briefer than that observed in the mutant (31 ± 8 s; n = 17 for five flies) and was never preceded by seizure-like activity (data not shown).

The buzz appears to mimic the effects of a mechanical bang on eas^{PC80} flies. The seizure and failure apparently reflect the preparalysis hyperactivity and paralysis phases of the behavioral response. This is supported by direct observation of eas^{PC80} flies during recordings, which display first a burst of motor activity and then a complete cessation of voluntary movements following a buzz (data not shown). Quantitatively, the physiological effects of a buzz on the GF pathway follows a very similar time course to the behavioral effects of a bang: brief hyperactivity (seizure) followed by paralysis (failure) for 30 s, with recovery at roughly 100 s. We have also observed what is apparently an electrophysiological correlate of the postparalysis hyperactive phase (P. P. and M. A. T., unpublished data). The correlations between the electrophysiological and behavioral phenotypes strongly suggest that *eas* bang-sensitive paralysis is due to failure occurring in many parts of the nervous system. The cause of this failure is as yet unclear, but might involve a failure of action potential propagation or of synaptic transmission.

In tests of the general properties of the GF pathway, *eas* flies responded normally. Stimulation of *eas* GFs at 0.8 Hz produces DLM responses that are normal in threshold (12–20 V), appearance (Figure 1C), and latency (1.3 ms). When tested with twin pulses, *eas* DLMs followed GF stimuli separated by a minimum of 8–10 ms, similar to wild-type flies (Tanouye and Wyman, 1980). Thus, under conditions of mild stimulation, the GF–DLM responses of *eas* are normal, but when the flies are subjected to a brief and relatively intense stimulus, seizure, failure, and paralysis result.

Cloning and Characterization of *eas*

Cloning *eas* was facilitated by previous molecular studies of the 14B chromosomal region (Jones and Rubin, 1990; Surdej et al., 1990; Heilig et al., 1991) and a series of deletion breakpoints spanning the area (Falk et al., 1984). We mapped *eas* to a region defined by two of these closely spaced deletion breakpoints, *81j6e* and *81h24b* (Figure 2). Northern blot analysis revealed three transcription units in the region. Initial identification of the putative *eas* transcript was made possible by the isolation of a lethal P element–induced allele of *eas*, eas^{P372} (Figure 2). Genomic Southern blotting and plasmid rescue of flanking genomic DNA identified the eas^{P372} insertion site as being within the center transcription unit in the *81j6e–81h24b* region (Figure 2).

We isolated cDNAs from an embryonic library (Zinn et al., 1988) with a probe that hybridized to all the transcripts detected in the putative *eas* transcription unit on Northern blots. We analyzed two cDNAs in detail. One, designated clone 12, was representative in structure of all but one of the cDNAs we isolated. Clone 12 hybridizes to all the transcripts detected from this unit on a developmental Northern blot (Figure 3A). The most abundant transcript (2.4 kb) detected with both genomic and cDNA probes corresponds closely in size to clone 12. A single cDNA, clone 5 (4.6 kb), is a splicing variant that includes the first intron, which is removed from clone 12, and contains an additional short exon (exon II) (see Figure 2). A clone 5–specific probe hybridizes to the two larger weakly expressed transcripts on a Northern blot (Figure 3B), indicating that this variant cDNA is not a cloning artifact. The smaller of the two transcripts is close in size to clone 5, ~5 kb (Figure 3B).

Sequence analysis of clone 12 revealed a long open reading frame with a hypothetical translation product of 495 amino acids (Figure 4). The splice variant clone 5 has an altered predicted protein product, inserting 22 amino acids into the protein sequence owing to the inclusion of the 66 bp exon II. The additional intron sequences between exons Ia and Ib do not affect the open reading frame in clone 5, as the splice site falls in the 5′ noncoding region of the cDNA (Figures 2 and 4).

Comparisons of the deduced proteins with sequence data bases revealed significant similarity to previously isolated choline/ethanolamine kinases from human (Hosaka et al., 1992), rat (Uchida and Yamashita, 1992), and yeast (Hosaka et al., 1989) (Figure 5). The region of homology extends from amino acid residues 204 to 489 and includes

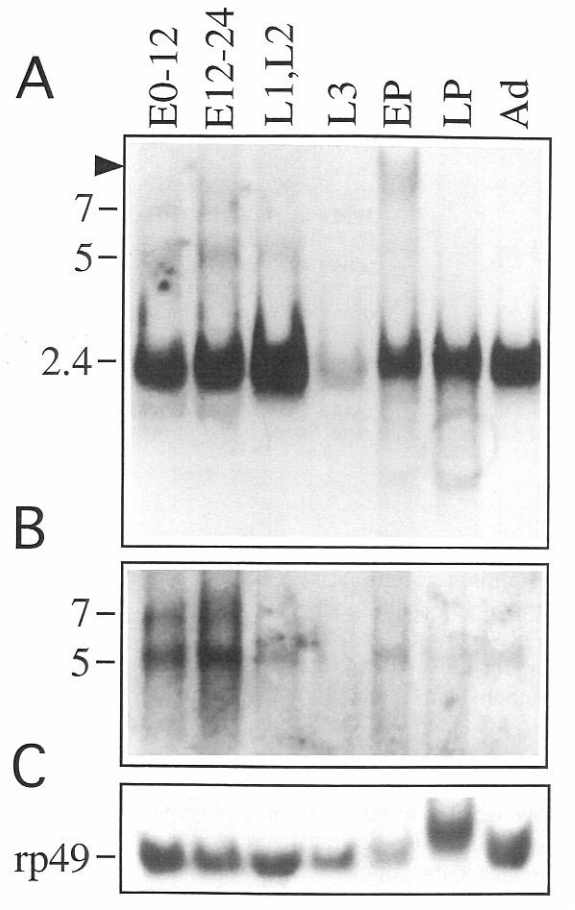

Figure 3. Developmental Northern Blot Analysis of *eas* cDNAs

The stages represented are 0–12 hr (0–12E) and 12–24 hr (12–24E) embryos, first and second larval instars (L1, L2), third instar (L3), early (EP) and late (LP) pupae, and adults (Ad).

(A) Probed with clone 12, corresponding to the common region of the isolated cDNAs. At least three transcripts are detected, indicated by ticks, with sizes in kilobases. The arrowhead indicates an artifactual band that we often observed with different probes. The most prominent transcript (2.4 kb), expressed in all stages, corresponds approximately in size to clone 12. A similar hybridization pattern was obtained with genomic probes from the *eas* locus.

(B) A probe specific for clone 5 (a 1 kb BsmI fragment from exon I) hybridizes to an ~5 kb transcript detectable in all stages except third instar. This probe also hybridizes to a larger (~7 kb) transcript detectable only in embryonic stages.

(C) Ribosomal protein 49 probe (rp49) (O'Connell and Rosbash, 1984), included as a loading control.

a putative kinase domain (Brenner, 1987). The amino terminus of the protein is not conserved among the yeast, fly, and mammalian sequences. The additional 22 amino acids predicted in clone 5 lie in this unconserved region. Choline/ethanolamine kinases are cytoplasmic enzymes that catalyze the first step of the synthesis of phosphatidylcholine (PC) and PE via the Kennedy cycle (cytidine diphosphate [CDP] pathway) (Kennedy, 1957). This suggests that *eas* mutants are defective in the synthesis of membrane phospholipids.

Molecular Basis of *eas* Mutations

We determined the molecular basis of the known *eas* alleles: the original isolate (eas^{PC80}) and eas^{P372}, acquired in the course of this work. The exact insertion site of the P element in eas^{P372} was determined by sequencing a section of the plasmid-rescued genomic DNA with a primer targeting the end of the P element construct. This analysis showed that eas^{P372} had inserted at nucleotide position 27, in the 5′ noncoding sequences of the gene (see Figure 4). We determined the molecular basis for the ethyl methanesulfonate (EMS)-induced eas^{PC80} mutation by comparing the sequences of polymerase chain reaction (PCR)-amplified *eas* sequences from mutant and wild-type flies. This analysis revealed a 2 bp deletion in eas^{PC80} at nucleotide position 1004–1005 (Figure 6). This deletion causes a frameshift in the open reading frame, bringing a stop codon in-frame at nucleotide position 1078. The protein product of this altered transcript is predicted to contain only the first 260 amino acids of the full-length gene product. This peptide would have only a portion of the conserved sequences in the protein and would completely lack the highly conserved kinase domain presumably required for enzymatic activity.

The molecular bases of both of the *eas* alleles are consistent with the cDNAs we identified as representing the *eas* gene. It is, however, somewhat surprising that eas^{PC80} is a frameshift mutation expected to destroy all enzymatic activity, since it is not the most severe *eas* allele (eas^{P372} being lethal). A possible explanation for this is that eas^{P372} affects another nearby locus as well as *eas*. Alternatively, the amino-terminal peptide predicted in eas^{PC80} might serve some vital function that is not provided by eas^{P372}.

Rescue of *eas* Paralysis by Germline Transformation

We attempted to rescue the *eas* behavioral phenotype by germline transformation with a P element construct (P[w^+ *hs-c12*]) containing clone 12 under the control of a heat shock–inducible promoter (Pirrotta, 1988). Four independent w^+ transformants were obtained and crossed to *w* eas^{PC80} f mutant flies. Before heat shocks, all male *w* eas^{PC80} f progeny carrying the P[w^+ *hs-c12*] transposon were paralyzed by 10 s of vortexing, but after activation of the transgene, only 24% were bang sensitive (Table 1). As expected, all control *w* eas^{PC80} f flies were still bang sensitive after the heat shocks (Table 1). In many flies, the rescue of the phenotype was stable for days after heat shocking (data not shown). These data confirm our identification of the *eas* locus. The fact that some flies are not rescued by the heat shocks may reflect differences between the levels and patterns of expression of the minigene as compared with *eas* expressed from its endogenous promoter. For example, we have not yet explored the possibility that there may be some requirement for the gene during development that could affect the penetrance of the behavioral phenotype.

An attempt to rescue the lethality of eas^{P372} by delivering heat shocks during development to the progeny of a cross between eas^{P372}/*FM6; ry* females and males carrying the

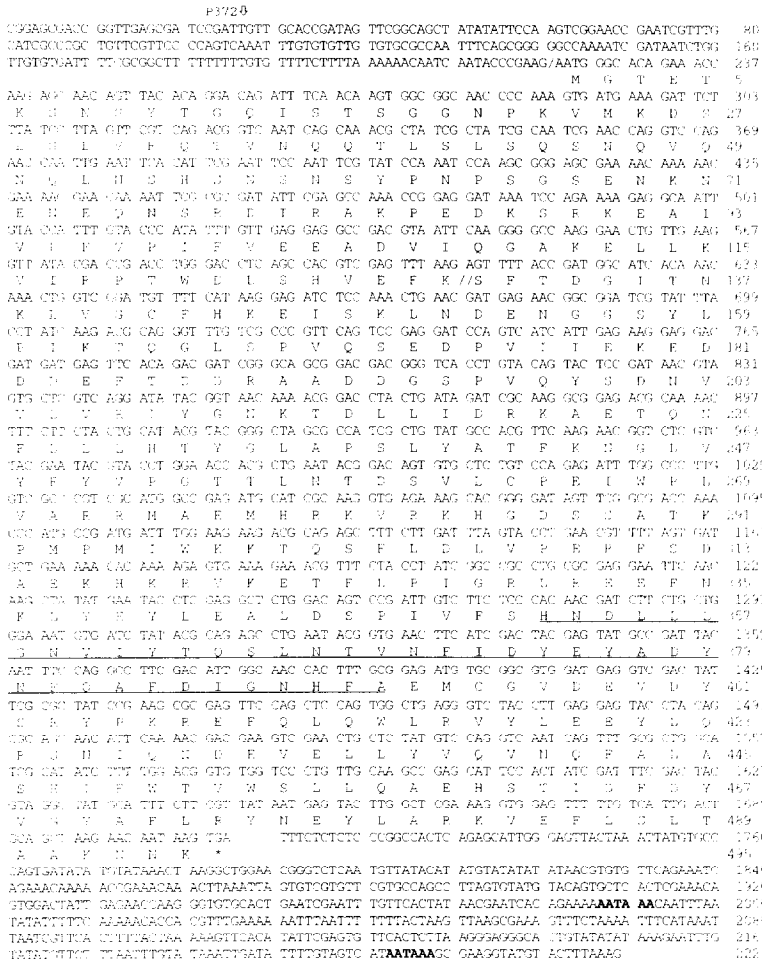

Figure 4. Sequence of an *eas* cDNA

Shown is the complete sequence of cDNA clone 12 with its hypothetical 495 amino acid translation product. The longest cDNA isolated, clone 5, contains an additional 2362 bp in the 5′ noncoding region (inserted at the shill at nucleotide 221) and an additional 22 amino acids (IRVVPQIEDRVSGPKCDGDDDA) inserted after residue 129 (indicated by the double shill). The location of the *eas*^{P372} insertion is indicated (arrow at nucleotide 27), as are two possible polyadenylation signal sequences (shown in bold face); the region with homology to the canonical kinase domain is underlined (Brenner, 1987).

eas transgene failed to yield viable *eas*^{P372}; P[*w*$^{+}$ *hs-c12*] males. While this result could be reconciled with a lethal effect of *eas*^{P372} on another locus, as suggested by our molecular analysis of the *eas*^{P372} and *eas*^{PC80} mutations, it is also possible that the heat shock protocol we used did not provide sufficient or properly timed *eas* expression.

eas Is Associated with an Ethanolamine Kinase Activity

Previous work on insect ethanolamine and choline kinase enzyme activities indicates that dipterans may have separate ethanolamine and choline kinases (Shelley and Hodgson, 1971; Ramabrahman and Subrahmanyam, 1981), suggesting that eas might be specific for one of these two substrates. Furthermore, we expected *eas* mutants to be deficient in the corresponding activity. Analysis of choline and ethanolamine kinase activities in crude homogenates of mutant and wild-type (Canton S) flies showed that *eas*^{PC80} is deficient in ethanolamine kinase activity, while choline kinase activity was not affected (Figure 7A). The severity of the defect suggests that eas is the major, if not the only, ethanolamine kinase in the fly, while the specific-

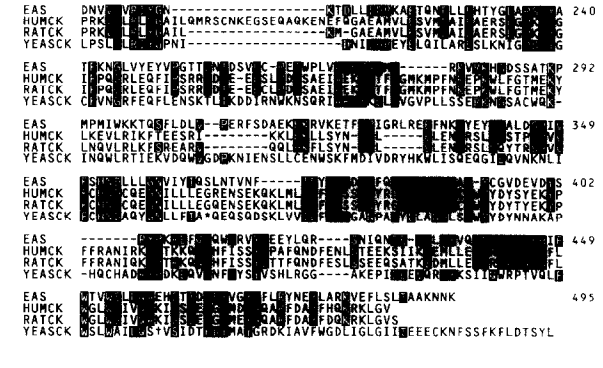

Figure 5. Sequence Homology of the Predicted *eas* Gene Product

Sequence homology of the predicted *eas* gene product with previously isolated choline/ethanolamine kinase family members from human (Hosaka et al., 1992), rat (Uchida and Yamashita, 1992), and yeast (Hosaka et al., 1989). Only the carboxy-terminal 295 amino acids are shown, as the amino terminus is not well conserved. Amino acid identities are indicated by black boxes. In the region shown, eas is 30% identical to the human sequence (51% conserved). The asterisk and dagger in the yeast sequence signify sites from which 41 and 47 unconserved residues, respectively, were removed to improve the alignments.

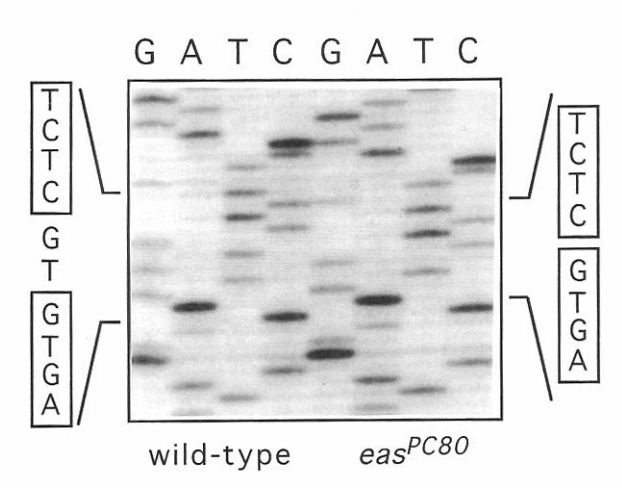

Figure 6. *eas*^{PC80} Is a Frameshift Mutation

Sequence analysis of cDNA amplified from the *eas* locus in *eas*^{PC80} and wild-type flies. Bottom is 5′. Two bases (1004 and 1005, indicated by unboxed TG) are deleted in *eas*^{PC80} as compared with sequences amplified from wild-type flies. Identical results were obtained from material amplified from genomic DNA (data not shown).

Table 1. Rescue of *eas* Paralysis by Germline Transformation

Line	Percent Paralyzed (n)	
	Transformed	Control
D1-8	28 (134)	100 (122)
J2-16	0 (49)	100 (46)
K1-13	3 (35)	100 (22)
D-23	59 (41)	100 (33)
Total	24 (259)	100 (223)

The results of experiments testing the ability of the P[*w*$^+$ *hs-c12*] element to rescue the *eas* behavioral phenotype. The four transformant lines (representing independent transposition events) are listed on the left. For rescue of the behavioral phenotype, we compared male *w eas*^{PC80} *f* flies carrying P[*w*$^+$ *hs-c12*] (transformed) with their male *w eas*^{PC80} *f* siblings lacking the P element (control). The percent of flies that were bang sensitive after heat shocks is listed, with the number of flies tested in parentheses.

ity of the defect suggests that eas is specific for ethanolamine.

Additional experiments confirmed that eas is highly specific for ethanolamine. First, the ethanolamine kinase activity of wild-type homogenates could not be inhibited by adding choline (tested up to 10 mM choline versus 2 mM ethanolamine; data not shown); if *eas* had a single binding site for ethanolamine and choline, choline would be expected to behave as a competitive inhibitor of ethanolamine kinase activity. We also found that under the conditions tested, the choline and ethanolamine kinase activities had different optimum ATP concentrations (see Experimental Procedures), further suggesting that these activities are separate. The strongest evidence for the substrate specificity of eas for ethanolamine is that increasing the gene dose of *eas* is accompanied by an increase in ethanolamine kinase activity, with no change in choline kinase activity (Figure 7B). This experiment also strengthens the conclusion that eas is the major ethanolamine kinase in the fly, as the gene dosage activity relationship predicts very little activity at a gene dose of zero (legend to Figure 7B). However, we cannot rule out the possibility of trace levels of activity (presumably such activity would reside in another protein, as *eas*^{PC80} is a frameshift mutation).

We also tested the second allele of *eas*, *eas*^{P372}, for ethanolamine kinase activity. Because *eas*^{P372} is lethal, we assayed activity in flies heterozygous for this mutation. *eas*^{P372}*/FM6* flies had 47.6% ± 5.4% of the activity in control flies. The severity of this mutation in terms of activity is comparable to *eas*^{PC80}; when combined in trans with *eas*^{PC80}, ethanolamine kinase activity is extremely low and not distinguishable from the activity in *eas*^{PC80} (data not shown). Finally, two other bang-sensitive mutants, *bss* and *bang-sensitive*, have normal levels of ethanolamine and choline kinase activities (data not shown), indicating that the ethanolamine kinase defect is specific for *eas* and not a general property of bang-sensitive mutants.

Altered Phospholipid Composition in *eas* Flies

As an initial test of the effects of the ethanolamine kinase defect on the structure of *eas* mutant membranes, we quantified the major phospholipid species in lipid extracts of whole *eas*^{PC80} and *eas*$^+$ flies. As in most other dipterans (Fast, 1966), PE was the major phospholipid (59%) in wild-type flies, in contrast with most other eukaryotic organisms where PC is the most abundant phospholipid. We found that in *eas*^{PC80} flies, the fraction of PE is slightly, but significantly, depressed (Table 2). This decrease in PE was accompanied by an increase in the proportion of PC present, while the proportions of the other major phospholipids were not significantly altered (Table 2). The net effect of these changes is a reduction of the PE/PC ratio from ~2.8 to 2.3 (Table 2). The same results were obtained in *eas* mutants with a different genetic background (*w eas f*; PE/PC = 2.4 ± 0.02, two duplicate determinations on one extract); similarly, wild-type values were stable in a different *eas*$^+$ background (*w*; PE/PC = 2.8 ± 0.14, determined as for *w eas f*). This indicates that the change in the PE/PC ratio is due to the *eas*^{PC80} mutation and not another genetic locus. Thus, in whole flies, there are measurable changes in the phospholipid content of *eas* mutants. It will be of interest to determine whether these changes are distributed uniformly between tissues and within cells and whether there are changes in minor lipid species or in fatty acid composition.

Discussion

eas Encodes Ethanolamine Kinase

eas is the first member of the choline/ethanolamine kinase family to be cloned that apparently lacks significant choline kinase activity. We showed this specificity by gene dosage experiments in which an increase in the dosage of *eas* does not cause a change in total choline kinase activity and an attempt to inhibit ethanolamine kinase activity with choline. However, without studying the protein in isolation,

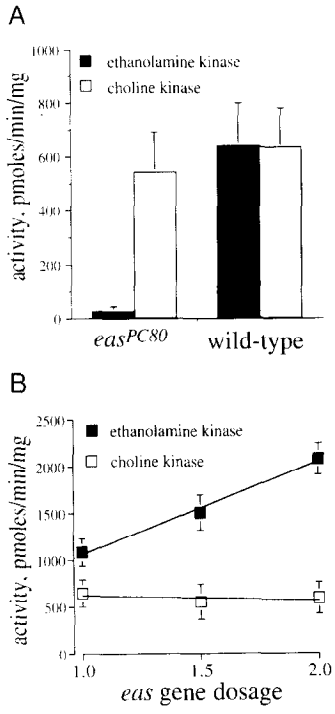

Figure 7. *eas* Is Associated with an Ethanolamine Kinase Activity

(A) Choline and ethanolamine kinase activities in eas^{PC80} and wild-type flies. eas^{PC80} flies have greatly reduced ethanolamine kinase activity, but no significant change in choline kinase activity ($P > 0.3$ for choline kinase, Student's t test). The ethanolamine kinase activity in eas^{PC80} is not distinguishable from blank values ($P > 0.2$). Since the origin of eas^{PC80} is uncertain, we did not attempt to control for genetic background effects; however, similar results were also obtained using mutant and wild-type flies of slightly different genetic backgrounds. Error bars indicate standard deviation.

(B) Relationship between ethanolamine kinase activity and gene dosage of *eas*. Male and female flies (2 and 1.5 doses, respectively) carrying a duplication (*l(1) f; Dp(1;4) f⁺r⁺ Df(1) 81h24b/+* and *C(1) y w f; Dp(1;4) f⁺r⁺ Df(1) 81h24b/+*) that is *eas*⁺ were compared with flies carrying a duplication (*l(1) f; Dp(1;4) f⁺r⁺ Df(1)81j6e/+*) that is *eas* (1 dose). The lines represent least-square fits calculated for the data; R^2 values were 0.99 for ethanolamine kinase and 0.34 for choline kinase. The activity axis intercept for the calculated ethanolamine kinase line is only 61 pmol/mg per minute, consistent with eas being the major or only ethanolamine kinase present in the fly.

it is difficult to be sure that eas does not have any choline kinase activity, though it would be a very small fraction of the total in the fly.

There is a precedent for a separation of ethanolamine and choline kinases in some organisms and in dipterans in particular. The two activities are separable in mosquito (Ramabrahman and Subrahmanyam, 1981) and are at least partially separable in the blowfly (Shelley and Hodgson, 1971). In budding yeast, there is evidence for a minor ethanolamine-specific kinase as well as a combined choline/ethanolamine kinase (Hosaka et al., 1989). In contrast, the two activities are associated with the same molecule in most other organisms, though this has been a point of some controversy (Porter and Kent, 1990). On the basis of the sequence homology of *eas* to the mammalian and yeast choline/ethanolamine kinases, we cannot determine

Table 2. Comparison of Relative Amounts of Major Phospholipids in *eas* Mutant and Wild-Type Flies

Parameter	Wild Type	eas^{PC80}	P Value
% PC	21.1 ± 0.7	24.3 ± 0.6	0.012
% PI + PS	12.2 ± 1.9	11.7 ± 0.7	0.478
% PE	59.1 ± 2.1	56.1 ± 1.5	0.019
% PA + DPG	8.3 ± 0.7	8.1 ± 0.9	0.161
PE/PC	2.80 ± 0.01	2.31 ± 0.09	0.001
(PI + PS)/PC	0.58 ± 0.10	0.47 ± 0.03	0.086

Major phospholipids were quantified in lipid extracts of whole eas^{PC80} and wild-type (Canton S) flies. Abbreviations not listed in the text: PI + PS, phosphatidylinositol plus PS; PA + DPG, phosphatidic acid and diphosphatidylglycerol. The identification of phosphatidic acid and diphosphatidylglycerol (cardiolipin) is tentative. Values are mean ± SD of four assays of mutant and wild-type lipids. Mutant and wild-type values from each experiment were paired in determining P values (Student's t test).

which regions of the protein are responsible for the difference in substrate specificity.

A Role for Ethanolamine Kinase in Nervous System Function

The ethanolamine kinase defect in *eas* has a dramatic effect on nervous system excitability. The combination of extreme hyperactivity (what we have termed seizure) and failure suggests a model for the phenotype in which the intense activity of the seizure, triggered by a brief electrical buzz or mechanical bang, results in inactivation of a labile site in the nervous system. Specifically, the hyperactivity phase of the phenotype might inactivate action potential ion channels or deplete readily releasable neurotransmitter stores, blocking transmission until recovery occurs.

How does a buzz or bang trigger a seizure? One possibility is that *eas* neurons are inherently hyperexcitable, but in a way that is not apparent until "set off" by a relatively strong stimulus. Alternatively, the seizure might result from an imbalance between excitatory and inhibitory pathways following the buzz. The latter possibility is suggested by the observation that pharmacological antagonists of inhibitory transmitters cause convulsions in many organisms, including insects (Olsen et al., 1976), and that decreased inhibition is thought to contribute to the formation of seizures in epilepsy (Martin, 1993). Such an imbalance could result from abnormal lability of *eas* neurons, rather than increased excitability; such lability might also result in the inactivation of excitatory pathways by the seizure.

Despite the uncertain etiology of the seizure, data from other studies are currently more supportive of a hyperexcitability defect in *eas*. First, a recently isolated Na^+–K^+ ATPase mutant (Schubiger et al., 1994), which suffers from behavioral hyperexcitability (J. Palka, personal communication; P. P., unpublished data), can be briefly paralyzed by mechanical stimuli (Schubiger et al., 1994). Second, the *bss* larval electrophysiological phenotype is characterized by nerve hyperexcitability and abnormal facilitation (Jan and Jan, 1978; Ganetzky and Wu, 1982). Third, the bang-sensitive defect of *eas* is suppressed by

mle^{napts} (Ganetzky and Wu, 1982), which also suppresses the hyperexcitability defects in *Shaker* and *seizure* mutants (Ganetzky and Wu, 1982; Jackson et al., 1985). Fourth, the *tko* mutation is proposed to affect mitochondrial function (Royden et al., 1987). This could cause hyperexcitability by decreasing ATP synthesis (and therefore ion pump activity) or perhaps by a failure of the mitochondria to buffer calcium in presynaptic terminals, resulting in increased transmitter release (Alnaes and Rahamimoff, 1975). Taken together with our data, these studies suggest that *eas* paralysis is due to a hyperexcitability defect that results in failure in the nervous system.

A determination of the cause of the seizure will be critical in testing this hypothesis. It will also be of interest to determine the site of failure. It has been proposed that synaptic transmission is affected in bang-sensitive mutants (Jan and Jan, 1978; Royden et al., 1987). The decrease in the amplitude of the DLM activity during the seizure (Figure 1C) is consistent with the failure of the DLM motor neuron synapses, as opposed to the all-or-none failure that might be expected if action potential propagation were affected. We are currently exploring this and other possible explanations for this defect.

Ethanolamine Kinase and Phospholipid Metabolism

Ethanolamine kinase is required for the synthesis of PE via the CDP–ethanolamine pathway. Ethanolamine kinase phosphorylates ethanolamine, which is further modified by CTP–phosphoethanolamine cytidylyltransferase before it is incorporated into PE (Kennedy, 1957). Consistent with its role in this pathway, *eas* mutants have decreased levels of PE. However, the deviations from wild type we found in whole flies were small, and it is unknown whether these changes, if uniformly distributed in the fly, could explain the excitability defects. It is possible that the small overall change in PC and PE levels reflects larger changes within certain cell types or subcellular membrane compartments. Also, there may be changes in the levels of other lipids that we did not assay. Regardless, it is clear that *eas*^{PC80} does not have a gross inability to synthesize PE. This is interesting because the CDP–ethanolamine pathway is thought normally to be the major source of PE in most animals, including insects (Downer, 1985). Although we cannot rule out the possibility of trace ethanolamine kinase activity in *eas* mutants, it seems likely that other pathways (e.g., decarboxylation of phosphatidylserine [PS]) are capable of providing most of the PE in flies. It would be interesting to see how the *eas* mutation affects the activity of other lipid synthetic pathways.

Phospholipids and Excitability in *eas*

The altered membrane phospholipid composition in *eas* suggests that the *eas* behavioral defects arise from the effects of the mutation on lipid metabolism. There are several ways in which this might generate changes in excitability. First, the generation of lipid-derived second messenger signals might be impaired or altered in *eas* flies. This could lead to altered ion channel or neurotransmitter receptor function via changes in phosphorylation state, for example (Swope et al., 1992; Li et al., 1993). Although we consider it relatively unlikely that second messenger generation plays a direct role in the rapid events following a bang, there might be chronic effects of *eas* on membrane excitability or synaptic function that result in a susceptibility to paralysis. Another general way in which *eas* might cause excitability defects is by altering the activity of membrane proteins required for normal excitability. Lipid environment has been shown to affect the activity of many membrane proteins in vitro (Yeagle, 1989). This mechanism has been proposed for the alteration of ion channel function in a Paramecium mutant with an abnormal membrane composition (Forte et al., 1981). A third mechanism is suggested by recent findings showing that the regulation of lipid metabolism and composition is important to membrane and protein traffic in cells (Herman et al., 1992; Brown et al., 1993; McGee et al., 1994). Additionally, lipid composition has significant effects on processes such as membrane fusion in vitro (Sundler, 1984). Thus, the *eas* mutation might result in altered organelle integrity, membrane protein traffic, or neurotransmitter secretion.

In considering all these possible mechanisms, we stress the specificity of the *eas* defect. Under most conditions, *eas* behavior and electrophysiology are apparently largely normal, as are development and viability. This suggests a certain level of specificity of the *eas* defect at the cellular level. This may mean that the relevant effect of *eas* on cell function is restricted to particular cells or parts of cells or to specific proteins.

Conclusion

The work presented here provides evidence for an important role of phospholipid metabolism in nervous system function. We have demonstrated a striking electrophysiological defect in *eas* and showed that *eas* mutants are defective in the structural gene for ethanolamine kinase. This suggests that the behavioral phenotype is caused by a membrane lipid composition defect, a hypothesis supported by the altered phospholipid composition in *eas* flies. Identification of the exact process disrupted in *eas* mutants should provide insight into the function of normal neurons as well as helping us understand the defect underlying other bang-senstive mutants.

Experimental Procedures

Behavioral Testing

Flies were maintained on standard cornmeal agar medium at room temperature (21°C–23°C). Before testing, flies were generally aged at least 3 days (the duration of paralysis is decreased in young *eas* flies [P. P., unpublished data]), and flies were allowed to rest for at least 2 hr between tests or after exposure to anesthesia. To test for the presence of the *eas* phenotype and to quantitate the duration of paralysis and recovery time, we vortexed small groups of flies (usually 10) in a clean fly vial for 10 s (Ganetzky and Wu, 1982). The number of flies standing was noted at 10 s intervals until all flies were standing or for 4 min, whichever was shortest. To observe the preparalysis hyperactivity, we tapped the vial firmly one to three times on the bench top or vortexed it momentarily.

Electrophysiology

We used a modification of basic intracellular DLM recording conditions that have been described by Tanouye and Wyman (1980). Flies were reared and studied at 24°C, and *eas* flies were first tested for the

bang-sensitive phenotype before use in electrophysiology. An ether-anesthetized fly is glued to an insect pin with cyanoacrylate adhesive. This pin is placed across the cervical connective in a position that does not interfere with wing or leg movements, while immobilizing the head and thorax. The pin is then used to attach the fly to the recording stage. We used uninsulated tungsten electrodes to record simultaneously from two ipsilateral DLMs. Stimulation with a Dagan S-900 stimulator is delivered to the head of the fly with two uninsulated tungsten electrodes, and a ground electrode is inserted into the abdomen. After mounting and insertion of electrodes, the fly is allowed to recover for 15 min, by which time voluntary movements are observed and flight activity can be initiated by puffing air at the fly. A digital storage oscilloscope (Hitachi) was used to record responses that were then photographed. The stimulation protocol to test the effects of a buzz was as follows. The fly was given three to five test pulses at 0.8 Hz to check the DLM response to GF stimulation. Threshold was typically 15–20 V with a 0.5 ms stimulus duration. An electrical buzz was then delivered with a second stimulator, in the form of a short train of relatively high voltage stimuli. Typically, we used a 50–100 ms train of 50 V 200 Hz stimuli with a 0.5 ms pulse duration. During the buzz we were unable to record DLM responses owing to the large stimulus artifacts generated. Test pulses were then delivered at 0.8 Hz until normal responses were observed, defined here by two successive test pulses giving rise to normal latency responses in both DLMs. This protocol could be repeated many times with a given fly; flies were rested 15 min between testing in this manner. Following frequency was determined by delivering paired pulses of varying interpulse intervals; following frequency was defined as the minimum interpulse interval at which both DLMs can respond to both stimuli.

Deletion Mapping of *eas*

eas^{PC80} and eas^{RH11} (originally isolated in the laboratory of S. Benzer [Benzer, 1971]) were obtained from the collection of C.-F. Wu (University of Iowa, Iowa City). Additional copies of these stocks were obtained from the collection of L. Hall (State University of New York at Buffalo). The *eas* locus has been mapped by recombination to ~54.1 on the X chromosome (14B) (Ganetzky and Wu, 1982). Complementation mapping was accomplished by crossing *eas f* males to *C(1) y w f* virgin females carrying deletions in the *Dp(1;4)r⁺f⁺* chromosome (Falk et al., 1984). The deletions from this series we used were *81j6e*, *81l12h*, and *81h24b* (Falk et al., 1984). Progeny (aged 3–7 days) were tested for bang sensitivity, and testing was usually repeated once or twice on successive days to confirm the phenotypes.

Isolation of a P Element Insertion in *eas*

The P element–induced allele of *eas* was isolated by mobilizing a P element inserted in the *disconnected* locus, *rL219* (*ry⁺*) (Heilig et al., 1991), using a Δ2-3 transposase source (Robertson et al., 1988) as follows: *rL219; ry* virgins were crossed to *Sb Δ2-3 ry/TM6 ry* males. *Sb⁻* virgin progeny were then crossed to eas^{PC80} f; ry males. A total of 5232 *Sb⁺ ry⁺* males and virgin female progeny were tested for *eas* behavior. Positive-testing (bang-sensitive) flies were crossed to *eas; ry* males, and progeny were again tested for the bang-sensitive phenotype. eas^{P372} was isolated as a bang-sensitive female and is hemizygous and homozygous lethal. The Δ2-3 chromosome was reintroduced into eas^{P372} to remobilize the P element. Revertant lines were isolated that complemented eas^{PC80} and were hemi- and homozygous viable and were not bang sensitive, indicating that the eas^{P372} insertion is wholly responsible for uncovering the *eas* phenotype. The insertion site within the *81j6e–81h24b* region was determined by genomic Southern blot analysis.

Molecular Techniques

Standard techniques were employed (e.g., Sambrook et al., 1989) for the manipulation of RNA and DNA. cDNA sequence information was obtained primarily by using a γδ transposon insertion-based technique (Strathmann et al., 1991) to generate priming sites within pBluescript KS II(+) subclones (Stratagene), followed by double-stranded PCR cycle sequencing with ^{32}P end-labeled primers (GIBCO BRL). Specific primers were used to close single-stranded gaps. Both strands were completely sequenced for all sequences obtained. Sequences were compared with sequence data bases using BLAST (Altschul et al., 1993) and Intelligenetics software.

For the molecular analysis of the *eas* genomic region, we made use of published information and genomic clones isolated by other workers (Jones and Rubin, 1990; Surdej et al., 1990; Heilig et al., 1991). We isolated additional genomic clones from phage and cosmid libraries. Restriction fragments from these clones were used to probe Northern blots of RNA from different developmental stages. The intron–exon boundaries of the *eas* gene were determined by sequencing portions of genomic fragments predicted to include introns based on Southern blotting and PCR data.

Plasmid rescue of P element–flanking genomic DNA (Wilson et al., 1989) was performed by digesting 2–5 μg of genomic DNA from the PZ (Mlodzic and Hiromi, 1992)-containing eas^{P372}/FM6 with XhoI alone or in combination with NheI or SpeI, followed by ligation and transformation into JH5α electrocompetent cells (Bio-Rad). Transformants were selected on kanamycin (10 μg/ml) plates plus X-Gal and IPTG. DNA from putative positive (*kan^R*, *lacZ⁺*) clones were characterized by restriction mapping and Southern blotting.

Analysis of Mutant Sequences

The insertion site of the P element in eas^{P372} was determined by sequencing the end of the genomic rescued fragment flanking the insertion site using a primer complementary to a site near the end of the P element sequence (ATACTTCGGTAAGCTTCGGC). To examine the sequences of the eas^{PC80} mutation, we designed PCR primers to amplify the coding region of the gene in four overlapping sections. The predicted products corresponded to the following nucleotide positions (as numbered in Figure 3): primer set 1, 241–946; primer set 2, 862–1230; primer set 3, 1127–1458; primer set 4, 1400–1750. These primers were used to amplify *eas* sequences from either randomly primed reverse-transcribed adult total RNA (Kawasaki, 1990) or genomic DNA. Genomic fragments were only amplified using primer sets 2 and 3, as the other regions contained introns that interfered with analysis or production of PCR products. PCR products were cloned into Bluescript and sequenced on both strands. The 2 bp deletion in eas^{PC80} (contained within the product of PCR with primer set 2) was confirmed by sequencing at least three different subclones from both RT–PCR and genomic DNA–PCR. Other regions were sequenced at least twice. We note that what was supposed to be a second EMS-induced allele, eas^{RH11}, had an identical deletion to eas^{PC80} (data not shown); additional eas^{RH11} and eas^{PC80} strains from different stock collections all contained the same lesion, indicating that there is actually probably only one EMS allele of *eas*.

Germline Transformation

For construction of P[*w⁺* hs-c12], a pBluescript KS II subclone of cDNA clone 12 was digested with HindIII and NotI. NotI linkers (Promega) were ligated onto the purified and Klenow-treated insert fragment. This fragment was subcloned into the NotI site of pCaSpeRhs (Pirrotta, 1988). We used this minigene construct for transformation because a small second transcription unit is closely apposed (<600 bp) to the *eas* locus, which made isolation of a suitable genomic fragment difficult.

The P[*w⁺ hs-c12*] construct was coinjected with the pπ25.1wc-turbo helper plasmid (Karess and Rubin, 1984) into w^{1118} embryos, as described (Spradling, 1986). The products of four independent transposition events were isolated, and male transformants (w^{1118}; P[*w⁺ hs-c12*]) were crossed to *w* eas^{PC80} *f* virgins. Male progeny (both *w* eas^{PC80} f and *w* eas^{PC80} *f*; P[*w⁺ hs-c12*] siblings) were tested for bang sensitivity before as well as after the administration of heat shocks. With a single 2 hr heat shock, rescue was not evident until at least 4 hr after shifting back to room temperature and had a relatively low penetrance. Best results were obtained when the flies were heat shocked at 37°C for 2 hr on three successive days and then tested for bang sensitivity the day after the last shock.

To test for rescue of the lethal phenotype of eas^{P372}, we crossed male transformants to eas^{P372}/*FM6; ry* virgins. The progeny of these crosses were given daily heat shocks (29°C for 0.5 hr followed by 1 hr at 37°C) during development, starting at either 0, 2, 5, or 10 days after egg laying; an additional group was not given any heat shocks during development. Adults were examined for the appearance of *B⁺* males, which would only be viable if eas^{P372}; P[*w⁺ hs-c12*] males were rescued by the transgene.

Choline Kinase and Ethanolamine Kinase Assays

Standard methods were adapted to assay choline kinase activity (Burt and Brody, 1975) and ethanolamine kinase activity (Ulane, 1982). In brief, 7- to 10-day-old flies (50–200 mg) were homogenized in ice-cold 50 mM Tris (pH 9.0) with either a high speed rotary homogenizer (Tissue Tearor, Biospec Products) or a glass homogenizer. The homogenate was centrifuged for 10 min at 15,000 × g at 4°C to remove debris (this unwashed pellet contained less than 5% of the total choline kinase or ethanolamine activity in wild-type flies). In a typical assay, duplicate 50 μl aliquots of the supernatant were assayed in a 100 μl reaction mixture containing 0.05 M Tris (pH 9), 2 mM [^{14}C]methyl choline chloride (1 mCi/mmol; ICN Biomedicals) or 2 mM [^{14}C]ethanolamine (1 mCi/mmol, purified by ion exchange chromatography before use [Weinhold and Rethy, 1974]), 5 mM ATP (10 mM for ethanolamine kinase; choline kinase activity was inhibited by ATP concentrations above 5 mM), 10 mM $MgCl_2$, and 150 mM KCl. The reactions were incubated at 30°C for 5 min and stopped by immersion of the tubes in boiling water for 2 min or, in the case of ethanolamine kinase, by the addition of 10 μl of glacial acetic acid. Unreacted choline was removed from choline kinase reactions by four extractions with 250 μl of 30 mg/ml tetraphenylboron in butyronitrile, and radioactivity in the aqueous phase was determined by scintillation counting (Burt and Brody, 1975). Unreacted ethanolamine was removed from ethanolamine kinase reactions by paper chromatography of an aliquot of the reaction mixture, and the radioactivity in the origin was determined (Ulane, 1982). For calculation of specific activity, values for blank samples (an aliquot of wild-type homogenate that was stopped before incubation at 30°C) were subtracted that, together with the amount of protein in the sample (Bio-Rad; BSA standard), yielded a value for picomoles of substrate phosphorylated per minute per milligram of protein.

Phospholipid Analysis

Lipids were extracted from whole adult *eas* and wild-type flies using the method of Folch et al. (1957). Wet weight of 200 mg of whole flies yielded enough phospholipids for one or two analysis in duplicate. Lipids were separated by thin layer chromatography on silica gel H (Alltech), utilizing a prewash run (acetone:petroleum ether, 1:3) to elute neutral lipids followed by chromatography in chloroform:methanol: acetic acid:water (100:65:12:5) to resolve individual phospholipids classes (Skipski and Barclay, 1969). To visualize the separated lipids, the TLC plates were stained in iodine vapor. Individual lipids (PC, phosphatidylinositol, PS, PE) were identified by comparison of R_f values with phospholipid standards (Sigma or Fluka); in addition, the identity of the amines PS and PE were confirmed by staining with ninhydrin (Skipski and Barclay, 1969). Phosphatidylinositol and PS were not well resolved and were thus combined for quantitative analysis. A fraction that migrated with the second solvent front was also analyzed as it contained a significant proportion (~8%) of the total phospholipids; this spot primarily contains phosphatidic acid and diphosphatidylglycerol (Skipski and Barclay, 1969). Other minor phospholipid species, representing a total of ~5% of the total phospholipids, were not quantified. The spots were scraped from the TLC plates for analysis of phosphate by the method of Bartlett (1959). The ratios of phosphate in the samples were taken as representing the molar ratios of the different phosphate-containing lipids (this assumption yields an underestimate of the relative amounts of some minor lipids, such as diphosphatidylglycerol, but is unimportant for the interpretation of our results). Mutant and wild-type extracts were assayed in parallel in a given experiment. Each experiment was a duplicate determination on a single set of wild-type and mutant extracts that were prepared in parallel.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L35603 (cDNA clone 5) and L35604 (cDNA clone 12).