Neuropathology in *Drosophila* Mutants With Increased Seizure Susceptibility

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ABSTRACT

Genetic factors are known to contribute to seizure susceptibility, although the long-term effects of these predisposing factors on neuronal viability remain unclear. To examine the consequences of genetic factors conferring increased seizure susceptibility, we surveyed a class of *Drosophila* mutants that exhibit seizures and paralysis following mechanical stimulation. These bang-sensitive seizure mutants exhibit shortened life spans and age-dependent neurodegeneration. Because the increased seizure susceptibility in these mutants likely results from altered metabolism and since the Na⁺/K⁺ ATPase consumes the majority of ATP in neurons, we examined the effect of *ATPα* mutations in combination with bang-sensitive mutations. We found that double mutants exhibit strikingly reduced life spans and age-dependent uncoordination and inactivity. These results emphasize the importance of proper cellular metabolism in maintaining both the activity and viability of neurons.

THE epilepsies and neurodegenerative disorders represent the majority of neurological diseases and they have many points of overlap. For example, neurodegeneration disrupts nervous system function and neuronal death has been observed following seizures (SUTULA 2004; CENDES 2005). Moreover, antiepileptic drugs are used to prevent neuronal loss associated with seizures (PITKÄNEN 2002; SUTULA 2002), although some antiepileptic drugs have also been found to induce apoptotic neurodegeneration (BITTIGAU *et al.* 2002). Such links suggest that among the genetic components underlying epilepsy and neurodegeneration, certain risk factors may be shared.

These neurological diseases have been extensively modeled and studied in the genetically tractable system of *Drosophila*. In addition to the availability of numerous seizure mutants, many mutants have been isolated, exhibiting pronounced age-dependent neurodegeneration (reviewed by BILEN and BONINI 2005; CELOTTO and PALLADINO 2005; KRETZSCHMAR 2005). Bang-sensitive mutants exhibit behavioral seizures and paralysis following mechanical stimulation that usually become more severe with age. It was proposed that these mutants were associated with defects leading to increased membrane excitability (BENZER 1971; GANETZKY and WU 1982). This idea is further supported by the reduced

threshold for electrically induced seizures in these mutants (KUEBLER and TANOUYE 2000). Molecular identification of several of these mutants has suggested that they result from defects in mitochondrial metabolism (ROYDEN *et al.* 1987; ZHANG *et al.* 1999; FERGESTAD *et al.* 2006a), similar to metabolically linked seizures in humans (reviewed in SIMON and JOHNS 1999; DE VIVO 2002; VAN GELDER and SHERWIN 2003; PATEL 2004). Seizures exhibited by these mutants respond to anti-convulsant drugs used in vertebrates (KUEBLER and TANOUYE 2002; REYNOLDS *et al.* 2004; TAN *et al.* 2004), further demonstrating that many of the molecular mechanisms mediating neuronal activity are conserved.

To examine the long-term effects of genetic perturbations conferring increased seizure susceptibility, we performed aging and histological analyses on these seizure mutants alone and in combination with *ATPα* mutations. Our studies of bang-sensitive seizure mutants revealed reduced life spans and appearance of age-dependent spongiform-like degeneration in the nervous system. Strong genetic interactions were identified between bang-sensitive mutations and dominant mutations affecting *ATPα*, the Na⁺/K⁺ ATPase α-subunit, known to cause conditional seizures, neurodegeneration, and early death, supporting a model in which metabolic perturbations result in both altered neuronal activity and neurodegeneration.

We dedicate this article to the memory of Seymour Benzer, mentor, friend, and father of our field.

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MATERIALS AND METHODS

Fly strains: Fly stocks were cultured on cornmeal–molasses agar medium at 22–25°. Strains used in this study include *tho*^{25t},

eat¹, *eat²*, *kdn^{PC64}* (referred to elsewhere as *kdn¹*), *bas¹*, *bss^{MW1}*, *slgA¹*, *sesB¹*, *sesB Df(1)HC133*, and *sesB^{orgi}*, as well as *ATPα* alleles *DTS1*, *DTS2*, *2206*, and *DTS1R1*. Unless otherwise stated, wild type refers to Canton-S (C-S), which is the original background for many of these mutants.

Life-span analysis: Life spans were measured at 29° according to standard protocols as described previously (KRETZSCHMAR *et al.* 1997; LIN *et al.* 1998; MIN and BENZER 1999; PALLADINO *et al.* 2002; PALLADINO *et al.* 2003; FERGESTAD *et al.* 2006b). In brief, newly eclosed animals were collected, separated by sex, placed in vials (up to 20 per vial), and transferred to fresh vials daily, and survivorship was recorded for each vial. Animals removed for analysis were subtracted from the total population in calculations. To determine 50% survivorship, each vial was scored individually and the time at which ≤50% flies remained alive was noted. These values were determined from at least 8 vials of each genotype and then averaged to calculate mean 50% survivorship and standard deviation (Tables 1 and 2). The Mann–Whitney test was used to compare mean 50% survivorship among different genotypes. For life-span plots (Figures 1, 3, 5, and 6) the average survivorship for all vials of a given genotype was calculated daily and plotted as a function of time. Note that because of the way mean 50% survivorships are calculated, these values do not correspond precisely with the midpoint of the life-span plots.

Histology: Histological analyses were performed as previously described (PALLADINO *et al.* 2002, 2003). Briefly, heads and bodies from adult flies were dissected and placed in Carnoy's fixative at room temperature for 1–2 days and then washed with 70% ethanol and processed into paraffin. Heads and bodies were embedded to obtain frontal and sagittal sections, respectively. Serial 4-μm sections were stained with hematoxylin and eosin and examined under a light microscope (*n* > 20 for each genotype). Neurodegeneration for each genotype was scored as previously described (FERGESTAD *et al.* 2006b). Briefly, each genotype was assigned a score from 0 to 5 on the basis of the frequency and severity of vacuolar pathology observed in brain serial sections as follows. A score of 0 was assigned to brains exhibiting no gross neuropathology or a single small vacuolar lesion (<12 μm in diameter). A score of 1 was assigned to brains exhibiting sporadic small individual vacuolar lesions (<12 μm in diameter) in multiple sections. If small individual vacuolar lesions (<12 μm in diameter) occurred more frequently and appeared in most sections of each brain, a score of 2 was assigned. Brains exhibiting widespread small vacuolar lesions (<15 μm in diameter) affecting the majority of sections or large or clustering vacuolar lesions (15–20 μm in diameter) were assigned a value of 3. Brains with numerous (>100) vacuolar lesions (10–20 μm in diameter) in individual sections and/or clustering vacuolar lesions affecting an area >500 μm² were given a score of 4. Brains exhibiting severe and extensive lesions resulting in loss of >40% of the brain tissue were assigned a score of 5.

Behavioral testing: Flies were collected under CO₂ at 0–2 days after eclosion and kept at 3–10 animals per vial for 1–2 days before behavioral analysis. Vials were mechanically stimulated by placement in a benchtop vortex for 15 sec at the maximum setting. The time for each fly to right itself after vortexing was recorded (GANETZKY and WU 1982).

RESULTS

Bang-sensitive mutants exhibit progressive neuronal loss and early death: Although the various mechanisms mediating neurodegeneration remain unclear, evidence suggests that factors conferring increased seizure susceptibility may predispose neurons to degeneration. To inves-

FIGURE 1.—Bang-sensitive mutants exhibit reduced life spans. *eas* and *tko* display mild reduction in life span compared with wild type (C-S). More severe reduction in life span was observed in *sesB*, *bas*, *bss*, and *kdn*. Points represent mean survivorship determined daily for multiple individual vials of each genotype as described in MATERIALS AND METHODS. See Table 1 for summary.

tigate the link between seizures and neurodegeneration in more detail, we examined bang-sensitive seizure mutants for effects on life span and neuronal survival. We found significantly reduced life spans in five of the six bang-sensitive mutant lines examined: *stress-sensitive B* (*sesB*), *knockdown* (*kdn*), *bang-sensitive* (*bas*), *bang senseless* (*bss*), and *technical knockout* (*tko*) all show significant reductions in life span, whereas *easily shocked* (*eas¹*) did not (Figure 1, Table 1).

sesB was originally identified as a recessive conditional seizure mutant sensitive to mechanical shock (HOMYK and SHEPPARD 1977) and later found to encode the mitochondrial ADP/ATP translocase (ZHANG *et al.* 1999). As impairment of this gene should have critical effects on cellular ATP levels, we examined an allelic series of *sesB* mutant lines for behavioral and histological pathologies. The *sesB¹* allele displays significantly reduced life span (Figure 1, Table 1), consistent with other reports (ZHIMULEV *et al.* 1987; CELOTTO *et al.* 2006). Mean age for 50% survivorship at 29° was 26.2 ± 1.8 days for *sesB¹*, 7.5 ± 0.6 days for *sesB^{orgi}*, 23.7 ± 1.5 days for *sesB¹/sesB^{orgi}*, and 10.5 ± 1.3 days for *sesB¹/Df* compared with 38 ± 4.4 days for wild-type controls. Histological analyses of *sesB¹* animals at 50% survivorship revealed age-dependent neurodegeneration (Figure 2B, Table 2). Although less pathology was observed in *sesB¹/Df* animals than in *sesB¹* homozygotes at their respective time points for 50% survivorship (10 days *vs.* 26 days), similar levels of neurodegeneration were seen in both genotypes when age matched at 10 days old (data not shown). These results indicate that the allelic differences in life span are not due solely to differences in neuronal loss and that manifestation of neurodegeneration may also involve a component that depends on absolute time. *sesB* mutants also exhibit age-dependent

TABLE 1
Life spans of *Drosophila* bang-sensitive mutants

Mutant	BS	50% $\pm$ SD	Affected gene product	Reference
Canton-S (C-S)	—	38 $\pm$ 4.4		
<i>bang senseless</i> (<i>bss</i>)	++	23 $\pm$ 5.7	Unknown	GANETZKY and WU (1982)
<i>easily shocked</i> (<i>eas</i>)	++	35 $\pm$ 2.8	Ethanolamine kinase	PAVLIDIS <i>et al.</i> (1994)
<i>bang sensitive</i> (<i>bas</i>)	++	23 $\pm$ 3.5	Unknown	GRIGLIATTI <i>et al.</i> (1973)
<i>technical knockout</i> (<i>tko</i>)	+ / ++	31 $\pm$ 2.3	Mitochondrial ribosomal protein	ROYDEN <i>et al.</i> (1987)
<i>stress-sensitive B</i> (<i>sesB</i>)	+	26 $\pm$ 1.8	Mitochondrial ATP translocase	ZHANG <i>et al.</i> (1999)
<i>knockdown</i> (<i>kdn</i>)	+	18 $\pm$ 1.7	Mitochondrial citrate synthase	FERGESTAD <i>et al.</i> (2006a)

Bang-sensitive (BS) behavior is scored following a standard mechanical stimulus based on the severity of the seizures and the time required to recover from paralysis. Mutants are arbitrarily divided into two categories, those requiring an average recovery time of <30 sec ("mild" bang-sensitivity, denoted "+") and those requiring >30 sec ("strong" bang-sensitivity, denoted as "++"). Wild-type flies are not affected by the mechanical stimulus and are scored as "—". Life span for each genotype is represented as the mean age in days (at 29°) at which only 50% of the original population was still surviving $\pm$ standard deviation.

pathology in the thoracic ganglion as well as in muscle (CELOTTO *et al.* 2006).

We also observed varying degrees of age-dependent neurodegeneration in each of the other bang-sensitive mutants examined at their respective time points for 50% survival (Figure 2, Table 2). On the basis of evoked electrical seizure activity in the giant fiber pathway, two loosely defined classes of bang-sensitive mutants have been distinguished (PAVLIDIS and TANOUYE 1995; FERGESTAD *et al.* 2006a). Type I seizures, which are thought to originate upstream of flight muscle motor neurons, are characterized by 10- to 30-Hz spike trains that lack any clear pattern and terminate abruptly. Type II seizures, which appear to originate in the motor neurons, exhibit increasing firing frequency with a concomitant decrease in transmission amplitude. Type II seizures predominate in *bss* mutants and most likely result from motor neuron dysfunction because reductions in amplitude of dorsal longitudinal muscle responses evoked by action potentials should not result from defects in a neuron upstream of the motor neuron. *kdn*, *sesB*, and *tko* display predominantly type I initial discharge seizures, whereas *eas* exhibits both types of seizure activity (PAVLIDIS and TANOUYE 1995; FERGESTAD *et al.* 2006a). These phenotypes fall along a continuum and most likely represent quantitative rather than qualitative differences among the various bang-sensitive mutants. In general, however, type I seizures are observed in bang-sensitive mutants with weaker behavioral phenotypes and type II seizures in those with stronger behavioral phenotypes.

Consistent with this classification, the various bang-sensitive mutants displayed a range of neuropathology that corresponded with the severity of the seizure phenotypes (GANETZKY and WU 1982; PAVLIDIS and TANOUYE 1995; FERGESTAD *et al.* 2006a). In each case, the onset of neuropathology was age dependent as newly eclosed flies looked normal (data not shown). *kdn* exhibits the mildest seizure phenotype and the least neurodegeneration. When *kdn* is heterozygous with a deletion that uncovers the gene, we observe a more striking and consistent bang-

sensitive phenotype (FERGESTAD *et al.* 2006a), significantly shorter life spans (14 days, $P < 0.05$), and more prominent neurodegeneration (data not shown). The extent of neurodegeneration observed in each bang-sensitive mutant was consistent and highly penetrant for that particular genotype. Micrographs shown in Figure 2 are typical for each genotype. These results demonstrate a strong correlation between increased seizure susceptibility and age-dependent neurodegeneration in bang-sensitive mutants.

Synergistic pathological phenotypes in bang-sensitive mutants: Although age-dependent neuropathology is observed in bang-sensitive seizure mutants, these effects may result from cellular impairments independent of the mechanisms conferring increased seizure susceptibility. To determine if the mechanisms underlying increased seizure susceptibility correlate with the observed pathologies, we combined bang-sensitive mutations that exhibit strong (*eas*) and weak (*kdn* and *sesB*) behavioral phenotypes (Table 1). When *sesB* was recombined either with *kdn*, one of the least susceptible bang-sensitive mutants, or with *eas*, one of the most susceptible, the double mutants showed pronounced behavioral seizures even in response to mild stimulation. The *kdn sesB* and *sesB eas* double-mutant animals are inactive and show a pronounced reduction in life span (Figure 3A) with 50% survivorship at day 7.5 ± 1.0 and 7.25 ± 0.95 , respectively. Histological examination of double-mutant animals at 50% survivorship revealed similar marked levels of degeneration in the brain (Figure 3, C and E) whereas single-mutant age-matched controls show minimal neurodegeneration (Figure 3, B, D, and F). These results suggest that bang-sensitive mutations ultimately disrupt a shared mechanism mediating seizure susceptibility. Moreover, the increase in neurodegeneration associated with increased seizure susceptibility in double-mutant combinations indicates a strong correlation between the severity of the behavioral and neurodegenerative phenotypes.

Bang-sensitive and dominant *ATP α* mutations interact: Molecular identification of bang-sensitive mutants

FIGURE 2.—Neurodegeneration in bang-sensitive mutants. Frontal sections at approximately midbrain from adults of the indicated mutants aged to their respective median life spans. The large hole in the middle of each section is the esophagus. Wild-type controls (C-S) exhibit little or no pathology whereas the bang-sensitive mutants exhibit neurodegeneration. The figures shown are representative of each genotype; the degree of neurodegeneration seen in each mutant is highly penetrant and similar amounts of neurodegeneration are observed in multiple individuals of each genotype. Magnified views of the boxed region of the brain are shown to the right. See Table 1 for summary. Bar, 50 μ m.

has revealed that mitochondrial proteins are often affected. Moreover, we have recently observed reduced ATP levels in several bang-sensitive mutants (FERGESTAD *et al.* 2006a), suggesting that mitochondrial metabolism is defective in these mutants. Because the Na^+/K^+ ATPase is the major consumer of ATP in neurons, it is possible that defective metabolism in these mutants

directly alters pump function. We examined bang-sensitive and *ATP α* mutations for phenotypic interactions in double mutants to determine if the neuropathology observed in bang-sensitive mutants might result from metabolic disruption of the Na^+/K^+ ATPase. For this purpose, we used *ATP α ^{DTS1}*, which acts in a dominant negative manner to impair pump activity, although the precise molecular mechanism is still unknown (PALLADINO *et al.* 2003). Although *ATP α ^{DTS1}* in combination with a bang-sensitive mutation resulted in only moderate increases in recovery time following mechanical shock (Figure 4) (PALLADINO *et al.* 2003; TROTTA *et al.* 2004), the double mutants exhibited severe locomotor defects and inactivity within a few days of eclosion as well as a striking reduction in life span (Figure 5A, Table 2). Surprisingly, combination of the shortest-lived bang-sensitive mutant, *kdn*, with *ATP α ^{DTS1}* resulted in only a slight further reduction in life span, suggesting that these altered life spans are not simply the summation of reduced viability. Consistent with this interpretation, although mild neurodegeneration was observed in *eas*; *ATP α ^{DTS1}* / + males, histological analysis of other bang-sensitive mutant animals at 50% survivorship revealed no obvious neurodegeneration when combined with *ATP α ^{DTS1}* (Figure 5, B–E), suggesting that severe neuronal dysfunction may result in early death before the appearance of gross histological pathology.

Although heterozygosity for any of the bang-sensitive mutants did not cause a reduction in life span (Table 2), a significant further reduction in life span was observed for *eas*, *tko*, and *bss* heterozygotes in an *ATP α ^{DTS1}* / + background (Table 2 and Figure 6A). No significant genetic interactions were observed between any bang-sensitive mutants and the less severe *ATP α* alleles, *2206* and *DTS1R1*. Although *ATP α ^{DTS1}* enhanced the severity of the behavioral and life-span phenotypes of flies heterozygous for some bang-sensitive mutations, the neurodegeneration observed in these flies was comparable to that seen in *ATP α ^{DTS1}* / + age-matched controls (Figure 6, B–E). Thus, although the severity of neurodegeneration was not strikingly increased, these animals exhibited altered behavior and significantly reduced life spans, consistent with a model in which mild reductions in metabolism may further aggravate the cellular mechanisms perturbed in *ATP α ^{DTS1}* mutants leading to loss of organismal viability but not enhanced neuronal death.

The life span of *eas*/Y; *ATP α ^{DTS1}* / + flies ($T_{50\%}$ = 4 days) is much shorter than expected if *eas* ($T_{50\%}$ = 35 days) and the *ATP α ^{DTS1}* ($T_{50\%}$ = 14 days) were acting in a simple additive fashion. One possibility is that the metabolic defect in *eas* exacerbates the defect in Na^+/K^+ pump activity caused by *ATP α ^{DTS1}*, resulting in synergistic effects. To test whether other available metabolic mutations might interact in a similar fashion with *ATP α ^{DTS1}*, we examined the effect of *shuggishA* (*slgA*), which perturbs proline oxidase but is not bang sensitive (MARKOW and MERRIAM 1977; HAYWARD *et al.* 1993). *slgA* mutants alone

TABLE 2
Life span and neuropathology of double mutants with $ATP\alpha^{DTS1}$

Genotype	50% $\pm$ SD +/+	Neuropathology +/+	50% $\pm$ SD $DTS1/+$	Neuropathology $DTS1/+$
+ /Y	38 $\pm$ 4.4	0–1	14 $\pm$ 0.8	4
<i>eas</i> /Y	35 $\pm$ 2.8	2	4 $\pm$ 0.8	2
<i>tko</i> /Y	31 $\pm$ 2.3	1–3	3 $\pm$ 0.6	NA
<i>sesB</i> /Y	26 $\pm$ 1.8	3	7 $\pm$ 0.4	2
<i>bss</i> /Y	23 $\pm$ 5.7	0–1	5 $\pm$ 0.6	0–1
<i>bas</i> /Y	23 $\pm$ 3.5	1–2	4 $\pm$ 0.5	0
<i>kdn</i> /Y	18 $\pm$ 1.7	0–1	11 $\pm$ 2.8	0–1
+ /+	38 $\pm$ 4.4	0–1	23 $\pm$ 3.4	4
<i>eas</i> /+	44 $\pm$ 3.1	1	14 $\pm$ 6.0	4
<i>tko</i> /+	48 $\pm$ 4.6	1	16 $\pm$ 6.4	3–4
<i>sesB</i> /+	43 $\pm$ 7.8	2	27 $\pm$ 3.2	3–4
<i>bss</i> /+	45 $\pm$ 1.0	1	15 $\pm$ 3.8	3–4
<i>bas</i> /+	46 $\pm$ 5.3	0	24 $\pm$ 7.4	3
<i>kdn</i> /+	44 $\pm$ 3.5	0–1	20 $\pm$ 6.2	2–4

The life span of males hemizygous for a bang-sensitive mutation (top) in combination with $DTS1$ is very reduced in comparison with heterozygous bang-sensitive females (bottom), which may not allow sufficient time for neurodegeneration to appear grossly as vacuolar brain lesions. Longevity (at 29°) is presented as the age in days at which 50% of the flies of the indicated genotype have died. Third chromosome genotype is indicated along the top. $DTS1$ is $ATP\alpha^{DTS1}$, a dominant temperature-sensitive $ATP\alpha$ allele. Neuropathology was scored 0 (none) to 5 (marked) at the median age. NA, not available.

showed mild neurodegeneration but no reduction in life span. Similar to *eas*, hemizygous *slgA* males and heterozygous females showed a significant further reduction in life span in an $ATP\alpha^{DTS1}$ heterozygous background (*slgA*/Y; $DTS1$ /+, 9.2 $\pm$ 3.6 days; *slgA*/+; $DTS1$ /+, 14.3 $\pm$ 1.5 days, $P < 0.05$ for both). This result supports a model in which the interactions with $ATP\alpha^{DTS1}$ described here are mediated, at least in part, by general disruption of cellular metabolism further aggravating Na^+/K^+ ATPase dysfunction and are independent of changes in seizure susceptibility. Thus, the lack of seizure-like behaviors in *slgA* mutants suggests that not all metabolic changes confer increased seizure susceptibility while general metabolic impairment affects neuronal viability.

DISCUSSION

Bang-sensitive mutants exhibit progressive neuron loss and early death: Bang-sensitive mutants are associated with a progressive increase in seizure susceptibility. With age, seizures become more easily triggered and the subsequent paralysis lasts longer (GANETZKY and WU 1982). Furthermore, our data show that these mutants also display varying degrees of age-dependent neurodegeneration that is independent of seizure induction. Previous analysis of other bang-sensitive mutants also reported reduced viability for both *sesA* and *sesE* (HOMYK *et al.* 1980; HOMYK *et al.* 1986).

All bang-sensitive mutations examined to date display some degree of neuropathology indicating that the genes and proteins affected by these mutations normally

provide some cellular protection. Moreover, the protective roles of the affected proteins appear to be conserved. For example, in humans disruption of adenine nucleotide translocase, encoded by *sesB* in flies, causes myopathy (BAKKER *et al.* 1993) and has been genetically linked to progressive external ophthalmoplegia (KAUKONEN *et al.* 2000; NAPOLI *et al.* 2001; KOMAKI *et al.* 2002). Mutations in *tko* correlate with mutations in human myoclonus epilepsy with “ragged red fibers” (MERRF) (BERKOVIC *et al.* 1989; SHOFFNER *et al.* 1990; CANAFOGLIA *et al.* 2001). Citrate synthase, disrupted in *kdn* mutants (FERGESTAD *et al.* 2006a), is blocked by MPTP (VILLA *et al.* 1994), which causes Parkinson’s disease in humans. Moreover, the phenotypes of bang-sensitive mutants parallel human diseases that have been linked to mitochondrial impairment, such as epilepsies, encephalopathies, and myopathies (LEONARD and SCHAPIRA 2000a,b; KUNZ 2002).

Double mutants of *sesB* with either *eas* or *kdn* had nearly identical phenotypes. Both double-mutant strains exhibited extreme bang-sensitive paralysis, severely reduced viability, and early neurodegeneration. This is consistent with summation of bang sensitivity previously reported for *bas* and *bss* mutations (ENGEL and WU 1994; LEE and WU 2002) and suggests that bang-sensitive mutations may ultimately impinge on the same processes mediating neuronal excitability and viability.

Because bang-sensitive mutants cause increased seizure activity as well as neurodegeneration, it is reasonable to ask whether aberrant neuronal activity is required for the observed neurodegeneration or if the

FIGURE 3.—Life-span reduction and neuropathology are more extreme in bang-sensitive double mutants. (A) Life span of *sesB eas* and *kdn sesB* double mutants is severely reduced compared with corresponding single mutants. (B–F) Representative brain frontal sections from bang-sensitive double mutants at the midpoint of their survival curves compared with single mutants of the same age. At one week after eclosion, double mutants show widespread neuropathology including vacuolar-like lesions throughout the neuropil and loss of cell bodies. This pathology is more extensive and severe than that seen in single mutants. Bar, 50 μ m.

phenotypes are independent consequences of the same underlying defect. A number of studies in mammals have suggested that even brief seizures can result in long-term neuronal damage (reviewed by SUTULA and PITKÄNEN 2002; SUTULA *et al.* 2003). The correlation we observe between seizure susceptibility in the various

bang-sensitive mutants and the degree of neurodegeneration they exhibit, would seem to support a direct connection between these phenotypes. This model is consistent with our finding that both bang sensitivity and neuropathology become more severe as *kdn* or *sesB* function are further decreased. However, the correla-

and *tko*, respectively). Paralysis was scored up to 3 min. Error bars represent standard deviation and asterisks indicate significant differences with $P < 0.001$ for all.

FIGURE 4.—The $ATP\alpha^{DTS1}$ mutation increases time to recovery following mechanical stimulation. Two to three days following eclosion, animals were examined at room temperature for time to recovery from bang-induced paralysis. $ATP\alpha^{DTS1}$ mutants exhibit mild or no bang sensitivity when reared at room temperature (PALLADINO *et al.* 2003). Only several $ATP\alpha^{DTS1}$ mutant males exhibited brief paralysis following mechanical shock, although this was significantly different from wild-type controls (~10-fold increase in mean recovery time). Introduction of the $ATP\alpha^{DTS1}$ mutation into hemizygous bang-sensitive males resulted in significant increases in the period of paralysis following mechanical stimulation (~3-fold and 2-fold increases for *eas* and *tko*, respectively).

FIGURE 5.—Hemizygous bang-sensitive mutants show synergistic interactions with $ATP\alpha^{DTS1}$. (A) Life spans of males heterozygous for $ATP\alpha^{DTS1}$ are shown in combination with various bang-sensitive mutants. These combinations result in a striking reduction in life span with most double-mutant animals surviving only several days (controls live ~2 weeks, open circles). (B–E) Representative frontal brain sections from double-mutant and control animals at midpoints of corresponding survival curves. Despite substantial reduction in life span of double mutants, no corresponding enhancement of neuropathology is observed. See Table 2 for summary. Bar, 50 μ m.

FIGURE 6.—Heterozygotes for some bang-sensitive mutations show strong reductions in life span in combination with $ATP\alpha^{DTS1}/+$ but no corresponding increase in neuropathology. (A) Animals heterozygous for *eas*, *tko*, or *bss* mutations have significant further reductions in life span in an $ATP\alpha^{DTS1}/+$ background whereas *sesB*, *bas*, and *kdn* do not (see Table 2). (B–E) Representative brain frontal sections from the indicated genotypes at median survivorship compared with age-matched $ATP\alpha^{DTS1}/+$ controls. Magnified views of the boxed region of the brain are shown to the right. Comparable degrees of neuropathology are observed in all genotypes shown. See Table 2 for summary. Bar, 50 μ m.

tion could also reflect the degree of metabolic impairment resulting from specific mutations. To distinguish these possibilities in the future it will be necessary to specifically block seizures without alleviating the metabolic defect.

Interactions between dominant $ATP\alpha$ and bang-sensitive mutations: Developmental time for *tko*, *kdn*, and *sesB* is significantly increased compared with wild-type flies as has been reported for other metabolically impaired mutants (MIKLOS *et al.* 1987; SAEBOE-LARSEN

et al. 1998). These bang-sensitive mutants also exhibit reduced levels of ATP (FERGESTAD *et al.* 2006a). As the overwhelming majority of cellular energy is expended on the Na^+/K^+ ATPase to maintain ionic gradients in excitable cells (ERECINSKA and DAGANI 1990; BEAL *et al.* 1993; LEES 1993; THERIEN and BLOSTEIN 2000; ATTWELL and LAUGHLIN 2001), disruption of mitochondrial metabolism may perturb membrane excitability through reduced ATP availability for Na^+/K^+ pump function. Because Na^+/K^+ ATPase activity is tightly regulated by ATP concentration (LOPINA 2000), disruption of ATP levels in these mutants should directly impair pump function and ionic homeostasis. Although some mutations in *ATP α* have been reported to confer mild bang sensitivity (SCHUBIGER *et al.* 1994; PALLADINO *et al.* 2003), these mutants lack the CNS-derived seizure-like behavioral and physiological activity characteristic of the canonical bang-sensitive class (PAVLIDIS and TANOUYE 1995). Furthermore, *sesB* and *ATP α* mutants exhibit distinct defects in neurotransmission (TROTTA *et al.* 2004). These results indicate that the increased seizure susceptibility in bang-sensitive mutants is not due simply to disruption of the Na^+/K^+ gradient. Although the *DTSI* mutation prolonged the time for recovery from bang-sensitive paralysis in combination with the various mutations examined, it did not significantly alter the threshold of mechanical stimulation for initial seizure induction, suggesting that different molecular mechanisms underlie seizure susceptibility and recovery from paralysis. Na^+/K^+ pump activity apparently plays a more significant role in recovery of neurons from seizure-induced paralysis than in the initial induction of seizure activity.

More strikingly, the life span of several bang-sensitive mutants was significantly reduced in double-mutant combinations with *ATP α ^{DTSI}*. Despite the reduction in life span, there was no corresponding enhancement of neurodegeneration in these flies—perhaps because they died so soon for other reasons that overt histological pathology did not have time to develop and progress. The early death observed in these double mutants may therefore result from severe neuronal dysfunction due to impaired metabolism. We conclude that increased seizure susceptibility in bang-sensitive mutants likely results from metabolic defects that disrupt neuronal signaling mechanisms in addition to any effects on the Na^+/K^+ pump. The life-span data suggest that impaired metabolism further disrupts Na^+/K^+ pump activity resulting in increased cell dysfunction and death.

Reduced ATP production by mitochondria can induce apoptosis in neurons or increase their sensitivity to apoptosis (GORMAN *et al.* 2000). It has also been suggested that cellular ATP levels are a determinant for apoptosis (RICHTER *et al.* 1996), most likely through the Na^+/K^+ ATPase (WANG *et al.* 2003). Furthermore, insufficient Na^+/K^+ ATPase activity to maintain ionic balances in response to episodes of ischemia, hypogly-

cemia, and epilepsy contributes to the neuropathy observed in those disorders (LEES 1991). In fact, reduced Na^+/K^+ ATPase function is probably a common event in a number of neurodegenerative and metabolic disorders (BEAL *et al.* 1993; YU 2003). Impaired metabolism in bang-sensitive mutants likely augments neuronal dysfunction in Na^+/K^+ pump mutants with increasing demands on cellular metabolism. Small changes in available ATP have been reported to alter Na^+/K^+ ATPase activity and efficient mitochondrial oxidative phosphorylation may be required to provide enough ATP to support sodium pump function during active neural signaling (ERECINSKA and DAGANI 1990). The neurodegeneration observed in bang-sensitive mutants described here most likely results from defects in metabolism and ionic homeostasis. Metabolic disruption can impact distinct pathways initiating cell death (LEES 1993; BEAL 2000). This, combined with the loss of K^+ and Ca^{2+} homeostasis due to Na^+/K^+ ATPase failure (KUNZ 2002; YU 2003), present several possible degenerative mechanisms in these mutants.

Most metabolic mutants in *Drosophila* do not exhibit bang-sensitive paralysis or seizures, suggesting that a specific metabolic disruption or degree of metabolic impairment may be required to cause increased seizure susceptibility as well as neurodegeneration. For example, dominant mutations in *Gpdh* impair flight but do not cause seizures (KOTARSKI *et al.* 1983), likely because the glycerol phosphate shuttle is a more significant metabolic pathway in flight muscle than in neurons. Further dissection of the specific regions and pathways by which metabolic defects increase seizure susceptibility and trigger cell death will have major implications for understanding how these processes interact in neurological diseases.

Although occurrence of seizures in various experimental systems is not always associated with strong reductions in life span, it is now clear that single or repeated brief seizures can produce neuronal death (SUTULA *et al.* 2003; CENDES 2005). Consistent with the results we have observed for the metabolic mutants investigated here, it appears that distinct metabolic changes can result both in increased seizure susceptibility and neurodegeneration (KUNZ 2002) and that impaired neuronal viability may be independent of actual seizures. These results further link seizures and neurodegeneration and suggest that in human patients affected by certain seizure disorders effective therapeutic intervention may require amelioration of the metabolic deficit in addition to controlling seizures (LADO *et al.* 2000; PITKÄNEN 2002; SUTULA 2002; TROJNAR *et al.* 2002).

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