Temperature-Sensitive Paralytic Mutants Are Enriched For Those Causing Neurodegeneration in Drosophila

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ABSTRACT

Age-dependent neurodegeneration is a pathological condition found in many metazoans. Despite the biological and medical significance of this condition, the cellular and molecular mechanisms underlying neurodegeneration are poorly understood. The availability of a large collection of mutants exhibiting neurodegeneration will provide a valuable resource to elucidate these mechanisms. We have developed an effective screen for isolating neurodegeneration mutants in Drosophila. This screen is based on the observation that neuronal dysfunction, which leads to observable behavioral phenotypes, is often associated with neurodegeneration. Thus, we used a secondary histological screen to examine a collection of mutants originally isolated on the basis of conditional paralytic phenotypes. Using this strategy, we have identified 15 mutations affecting at least nine loci that cause gross neurodegenerative pathology. Here, we present a genetic, behavioral, and anatomical analysis of *vacuous* (*vacu*), the first of these mutants to be characterized, and an overview of other mutants isolated in the screen. *vacu* is a recessive mutation located cytologically at 85D-E that causes locomotor defects in both larvae and adults as well as neuronal hyperactivity. In addition, *vacu* exhibits extensive age-dependent neurodegeneration throughout the central nervous system. We also identified mutations in at least eight other loci that showed significant levels of neurodegeneration with a diverse array of neuropathological phenotypes. These results demonstrate the effectiveness of our screen in identifying mutations causing neurodegeneration. Further studies of *vacu* and the other neurodegenerative mutants isolated should ultimately help dissect the biochemical pathways leading to neurodegeneration.

NEURODEGENERATION is a pathological condition known to occur in metazoans from worms to humans. Many studies in worms, flies, mice, and humans have demonstrated that the pathology is largely genetic in origin and often the result of single gene defects (reviewed in FEANY 2000; FORMAN *et al.* 2000; FORTINI and BONINI 2000; HEINTZ and ZOGHBI 2000; GOEDERT 2001; MACCIONI *et al.* 2001; SIPIONE and CATTANEO 2001). Despite the widespread occurrence of neurodegeneration and its increasing medical significance, present understanding of the molecular pathogenesis of these diseases is still quite incomplete. Specifically, knowledge of what insults are capable of causing the primary neuropathology and how these cause the secondary neurodegeneration that ensues are limited (LEIST and NICOTERA 1998). A large collection of neurodegeneration mutants in a tractable model organism, such as Drosophila, will be extremely valuable for further genetic, molecular, and pharmacological studies to identify the key proteins and to dissect the biochemical pathways responsible for neuropathology. The information and tools derived from such studies could also facilitate the development of novel therapeutic agents for treatment of human neurodegenerative disorders.

Previous studies have shown that Drosophila is an excellent system for modeling human neurodegenerative conditions (MUTSUDDI and NAMBU 1998; FORTINI and BONINI 2000). For example, expression of human α-synuclein and tau proteins in the Drosophila nervous system results in neurodegenerative syndromes that share many of the phenotypic features associated with Parkinson's and Alzheimer's diseases, respectively (FEANY and BENDER 2000; WITTMANN *et al.* 2001). Similarly, expression of human Huntingtin containing a polyglutamine tract encoded by an expanded trinucleotide repeat causes neuropathological defects in the fly reminiscent of the human disease (JACKSON *et al.* 1998; WARRICK *et al.* 1998). Use of this experimental model of Huntington's disease has enabled the isolation of various suppressors, including the molecular chaperone HSP70 (WARRICK *et al.* 1999) and mutations in endogenous chaperonin-like molecules (KAZEMI-ESFARJANI and BENZER 2000). The striking phenotypic similarities between Drosophila and humans for various neurodegenerative disorders suggests that Drosophila will be an incisive tool for uncovering key mechanisms of general significance.

In addition to modeling neurodegenerative disorders by expression of human genes in Drosophila, screens have been carried out in Drosophila for single-gene mutations that cause neurodegeneration. These mutants, including *drop dead, swiss cheese, eggroll, spongecake,* and *bubblegum* (BUCHANAN and BENZER 1993; KRETZSCHMAR *et al.* 1997;

Min and Benzer 1997, 1999), typically have moderate to markedly reduced life spans associated with manifestation of neuropathology. The neuropathological phenotypes of these mutants, including vacuolization and the accumulation of MCBs, are similar to those seen in Tay-Sachs and Creutzfeldt-Jakob patients.

Mutants such as *weaver* (*wv*), *lurcher* (*Lc*), and *tottering* (*tg*) that exhibit neurodegeneration have also been identified in mice. These mutants were all originally discovered on the basis of defects in locomotor behavior and have been found to affect genes encoding ion channels and neurotransmitter receptors. Studies of these mutants have demonstrated an important connection between aberrant neuronal signaling properties and neurodegeneration (Murtomaki *et al.* 1995; Norman *et al.* 1995; Fletcher *et al.* 1996; Zuo *et al.* 1997). In the worm sensory system, the connection between proper ion channel function and neurodegeneration has also been established (Hall *et al.* 1997). An indirect connection between ion channels and neurodegeneration has also been made in Drosophila from analysis of *dADAR* mutants. This gene encodes an RNA editing enzyme required for adenosine-to-inosine type RNA editing of pre-mRNAs encoding several different ion channels in Drosophila. Notably, *dADAR* null mutants have been found to undergo extensive neurodegeneration (Palladino *et al.* 2000).

Although these studies in flies and mice have been very informative, the number of known mutants exhibiting neurodegeneration is still rather small. Our goal is to obtain a large collection of neurodegeneration mutants in Drosophila for further investigation of the relevant mechanisms. The connection between neuronal dysfunction and neurodegeneration revealed by studies of mutants such as *wv*, *Lc*, and *tg* in mice and *dADAR* in flies suggested an approach to circumvent the inherent difficulties of a direct histological screen for additional neurodegeneration mutants. We reasoned that mutants causing neurodegeneration should be enriched among a collection of mutants isolated on the basis of aberrant behaviors likely to be associated with altered or impaired neural function. We isolated a large collection of temperature-sensitive paralytic mutants, among which we have identified many genes encoding ion channels and proteins required for synaptic transmission (Loughney *et al.* 1989; Atkinson *et al.* 1991; Pallanck *et al.* 1995; Titus *et al.* 1997; Littleton *et al.* 1998). By performing a secondary histological screen on these mutants, we have now identified 15 independent mutations encompassing at least nine loci that are associated with neurodegeneration. Here, we provide details of our screen, an overview of the mutants isolated, and a characterization of *vacuous* (*vacu*), the first of these mutants to be analyzed.

Our results demonstrate that behavioral mutants should be a fertile source of new neurodegeneration mutants and that our two-step procedure is an effective way of obtaining a larger collection of such mutants. Detailed phenotypic, molecular, and genetic characterization of these mutants will ultimately help unravel the critical pathways required for maintenance of neuronal viability and have broad mechanistic and therapeutic implications for natural senescence and neurodegenerative diseases.

MATERIALS AND METHODS

Fly strains: Flies were cultured on standard medium at room temperature (21–23°). The collection of temperature-sensitive paralytic mutants examined in this study was isolated primarily in screens of all of the major chromosomes following mutagenesis with ethyl methanesulfonate (EMS). Mutants that became paralyzed or that showed severe motor impairment within 5 min after being placed at 38° were saved for further analysis. *vacuous* (*vacu*) was generated on an *st*-marked third chromosome and was originally isolated on the basis of a larval paralytic phenotype at 38°. Wild-type controls were from the Canton-S strain.

Life-span analysis: Flies were raised to adulthood at 23° and newly eclosed flies were placed in vials at low density (10–20 flies per vial) and incubated at 28°. Males and females were kept in separate vials. Flies were transferred daily into fresh vials to minimize deaths caused by bacterial infection or becoming stuck in the medium. The number of surviving flies was recorded daily. Survival curves were generated by calculating the percentage of surviving flies and plotting this as a function of time in days. Surviving flies were removed for histological analysis and flies that suffered incidental deaths were excluded from the calculation. Statistical analyses were performed using Student's *t*-tests. For the survey of life spans (Figure 8) the identical procedure was followed with the exception that the flies were transferred onto fresh food and counted every 3 days. This analysis includes all of the temperature-sensitive (TS) neurodegeneration mutants identified in our screen ($n = 15$), 41 TS mutants found to have no apparent neurodegenerative phenotype in our screen ($n = 41$), and wild-type controls ($n = 6$).

Behavioral assays: *Collecting samples:* Four groups of flies, corresponding to "young" and "aged" populations of wild-type and *vacu* flies, were tested in the behavioral assays. Ten samples of 20 flies each ($n = 200$ for each group) were collected for each of these four groups. Young flies were collected within 24 hr of eclosion and aged for 24–48 hr at 28° before testing. Aged flies were also collected within 24 hr of eclosion and maintained at 28° for 11 days. Each sample of flies was subjected to the same set of behavioral assays in the same order to test running, climbing, and flight abilities.

Running ability: Eight flies were randomly selected from each sample and placed in a 10-ml glass pipette; both ends of the pipette were sealed with wax film to prevent escape ($n = 80$ animals per group). One end of the pipette (20 cm) was darkened by placing it inside a dark-colored foam block. With the pipette secured horizontally in the foam, light from a fiber-optic lamp was shined directly into the exposed tip of the pipette. The lamp was placed as close to the tip of the pipette as possible. For each trial, the flies were knocked to the foam-covered end of the pipette and then returned to the horizontal test position. The time required for the first six flies of each sample to enter the light-exposed portion of the pipette was recorded. Four trials were completed for each sample. Performance coefficients were calculated for each sample by assigning numerical scores for the time in seconds required by each fly to run the tube according to the following

scale: 1, $\leq$30 sec; 2, 31–60 sec; 3, 61–90 sec; 4, 91–120 sec; 5, 121–150 sec; 6, 151–180 sec; 7, 181–210 sec; 8, 211–240 sec; 9, 241–270 sec; 10, 271–300 sec; 11, $\geq$301 sec. Each individual fly was given a score and these scores were then averaged for each sample for statistical analysis using Student's t-test. Data are presented as mean $\pm$ SEM.

Climbing ability: Ten flies were randomly selected from each sample and placed in a 250-ml glass graduated cylinder that was sealed at the top with wax film to prevent escape (n = 100 animals per group). A fiber-optic lamp illuminated the cylinder from the top. The flies were gently knocked to the bottom of the cylinder and the time required for 50% of the flies to cross the 150-ml line (17.5 cm) was recorded. Four trials were completed for each sample. Times for each sample were averaged for statistical analysis using Student's t-test. Data are presented as mean $\pm$ SEM.

Flight assay: Flight assays were performed essentially as in BENZER (1973) and ELKINS *et al.* (1986). Flies were dropped into the top of a 500-ml glass graduated cylinder through a glass funnel whose end reached the 500-ml mark (n = 200 per group). The inside surface of the cylinder was coated with paraffin oil, causing flies to become stuck where they strike the wall. The strongest fliers initiate flight immediately and become stuck near the top of the cylinder. The weaker fliers fall farther and become stuck near the bottom of the cylinder. The vertical distribution of each group of flies over the length of the cylinder was determined to measure flying ability. Performance coefficients were calculated by assigning numerical scores for the distance fallen by each fly before becoming stuck according to the following scale: 1, <3 cm; 2, 3–6 cm; 3, 6–9 cm; 4, 9–12 cm; 5, 12–15 cm; 6, 15–18 cm, 7, 18–21 cm; 8, 21–24 cm; 9, 24–27 cm; 10, 27–30 cm. These scores were then averaged for each group of flies for statistical analysis using Student's t-test. Data are presented as mean $\pm$ SEM.

Histological examination: Heads or bodies from adult flies of wild-type and mutant flies were dissected and fixed in freshly prepared Carnoy's at room temperature for 4–12 hr, washed in 70% ethanol, and processed into paraffin using standard histological procedures. Heads were embedded to obtain frontal sections and the bodies were embedded to obtain sagittal sections. Serial 4-μm sections were obtained, stained with hematoxylin and eosin, and examined under a light microscope (n > 40, each genotype). Occurrence of neurodegeneration was indicated by the vacuolar appearance of neural tissues of the brain or ganglia. Young animals were collected within 24 hr of eclosion, aged for 24–48 hr at 28° and processed as above. Aged animals were collected within 24 hr of eclosion, aged at 28°, and screened for gross pathology at the age of 50% survivorship for that population (approximately posteclosion days 18–20 and 39–42 for *vacu* and wild type, respectively). For histological examination of larvae, the entire central nervous system (CNS) was removed, fixed in Bouin's, and processed into JB-4 embedding medium (Polysciences, Warrington, PA). Horizontal 3-μm sections were obtained and stained with hematoxylin and eosin and then examined under a microscope (n > 12, each genotype). Wild type was Canton-S in all comparisons. Additionally, aged w, y, cnbw, st, and bwst stocks were examined histologically as controls for strain differences that may exist in the collection of mutants screened.

Electrophysiology: Electroretinograms (ERGs) were recorded essentially as described (HOTTA and BENZER 1969; PAK *et al.* 1969). Briefly, flies were anesthetized with CO_2, their wings and anterior legs were surgically removed, and the flies were immobilized in plasticine and allowed to recover for 15 min. A temperature-controlled stage was used with a temperature probe inserted into the plasticine adjacent to the animal. Glass recording and reference electrodes filled with 3 M KCl were placed in the cornea and thorax, respectively. Following

dark adaptation (5 min), photoreceptor responses to brief light exposures were recorded. Traces were amplified using an Axopatch 1-D amplifier in current clamp mode (clamping at zero) and recorded using Clampex 6.0.3 software (Axon Instruments, Foster City, CA). Current traces were filtered at 1 kHz and consecutive traces are reported from representative animals (n > 6 for each genotype).

RESULTS

A collection of paralytic mutants as a source of neurodegeneration mutants: Previous studies have established a connection between neuronal dysfunction and neurodegeneration. Therefore, we hypothesized that neurodegeneration mutants would be enriched among a collection of mutants isolated on the basis of behavioral phenotypes indicative of altered or impaired neural activity. A large collection of such mutants has been assembled in our laboratory over a 20-year period for studies of neuronal signaling (WU *et al.* 1978; LITTLETON *et al.* 1998). This collection consists primarily of independent, chemically induced, TS paralytic mutations. These mutants become paralyzed, ataxic, or severely uncoordinated in <5 min when placed at 37°–38°. The behavioral phenotypes are reversible and the flies recover normal or nearly normal locomotor activity with a time course that varies from a few seconds to several hours depending on the mutant strain.

Electrophysiological and molecular characterization of a subset of these mutations has revealed that many of them affect genes encoding ion channels, components of the synaptic machinery, and other proteins required for the proper generation and transmission of electrical signals in the nervous system (WU and GANETZKY 1992; LITTLETON *et al.* 1999). Thus, this collection seemed like an excellent potential source of neurodegeneration mutants and we investigated this possibility by direct histological examination. Flies from each mutant strain were aged until the midpoints of their respective survival curves, at which point heads were removed and processed for histology. Serial frontal sections of 20–35 animals of each genotype were examined for gross neuropathology. Among ~70 different mutant lines examined, we identified 15 (ND1–15) that exhibit extensive CNS neurodegeneration. Additionally we examined six control strains to determine the frequency and types of pathology that appear in aged wild-type animals. In these strains lesions appearing as an apparent hole in the tissue were always rare, extremely small, and never clustered. Thus, normal senescence in flies does not appear to be associated with significant pathology. The high frequency of neurodegeneration mutants found in our screen demonstrates the utility of this approach.

Among the mutants obtained in our screen, we found three alleles each of *para* (ND1–3) and *comatose* (ND4–6) in addition to a number of mutations that appear to represent novel loci. Recombination mapping using the temperature-sensitive paralytic phenotype was performed

on all of the neurodegeneration mutants discovered in our screen. The recombination data yielded the following map positions with error given as standard deviation: 3-70.4 ± 0.5 (ND7, 8), 3-15 ± 1.0 (ND9, 10), 3-27 ± 1.7 (ND11), 3-48 ± 0.5 (ND12), 3-43 ± 1.5 (ND13), 3-100 ± 3 (ND14), and 2-55 ± 3.0 (ND15). ND2, ND7, ND8, and ND15 are dominant paralytic mutants; all the others are recessive. These mutations represent at least nine distinct loci. Below, we describe in more detail ND12 (*vacuous*), the first of these neurodegeneration mutants to be characterized.

Isolation and mapping of *vacu*: *vacuous* (*vacu*) is a recessive third chromosome mutation that was originally identified on the basis of larval paralysis at 38°. Mutant larvae placed on a heated agar slab immediately cease crawling, unlike wild-type larvae that crawl vigorously at this temperature. Paralysis of *vacu* larvae is rapidly reversed upon return to 21°. Although *vacu* adults do show behavioral defects that become more apparent as the flies age (see below), younger adults do not show overt TS paralysis.

We mapped the larval paralytic phenotype of *vacu* recombinationally between *st* (3-44.0) and *Sb* (3-58.2). Among 80 recombinants recovered between *st* and *Sb*, 24 were between *st* and *vacu*, placing *vacu* approximately 4.3 map units to the right of *st*. To refine this mapping, we used existing deletions in the relevant interval to determine the cytological location of *vacu*. $Df(3R)by^{10}$ (85D8-12; 85E7-F1), $Df(3R)by^{62}$ (85D11-14; 85F6), and $Df(3R)by^{77}$ (85D8-12; 86B4) all uncovered the *vacu* paralytic phenotype, whereas $Df(3R)by^{416}$ (85D10-12; 85E1-2) did not. These results suggest that the *vacu* mutation is either in the 85D8-85D10 interval or in the interval between 85E1-3 and 85E7-F1. No other deletions are currently available to resolve these two possibilities.

***vacu* adults have reduced life span and abnormal behavior:** Although newly eclosed *vacu* adults did not show any overt behavioral abnormalities either at 21° or at 38°, closer examination revealed that as they age, *vacu* adults differ markedly from wild type in viability and locomotor activity.

Survival curves reveal that the life span of *vacu* at 28° is only about half that of controls (Figure 1). The midpoint of the survival curve is ∼20 days *vs.* 42 days for controls. In addition to wild-type controls, we also examined the life span of *para^ts1^*, a previously characterized TS paralytic mutant. The life span of *para^ts1^* is essentially the same as that of wild type, indicating that the reduced life span of *vacu* is characteristic of this mutant and not a phenotype shared by all TS paralytic mutants.

In addition, *vacu* adults appeared to have generally sluggish locomotor activity at 20°–22°, which became more pronounced as the flies aged. We used several different assays to quantify these differences from wild-type flies. In one assay, flies were placed at one end of a darkened, horizontal tube and allowed to run toward a light source at the other end. The time required for flies to run to the lighted end was determined. Young

wild-type adults typically required <30 sec (Figure 2A). Young *vacu* adults were somewhat slower than the controls although they still required only ∼45 sec to run the length of the tube. Both wild-type and *vacu* adults showed an age-dependent decrement in this behavior, but the decline was much more severe for *vacu* (Figure 2A). Thus, aged wild-type adults ran the tube in ∼150 sec, whereas aged *vacu* adults required ∼300 sec.

In a second behavioral assay, flies climbed up a vertical tube against gravity toward a light source and the time required for 50% of the flies to cross a line 17.5 cm from the bottom of the tube was determined. Young wild-type adults reached this point within 5–10 sec (Figure 2B). Aged wild-type adults were slower, requiring about 30–35 sec to cross the line. In contrast, *vacu* adults, both young and old, performed very poorly in this assay. Neither group of *vacu* adults completed the task within the total 5-min period allotted (Figure 2B). Because even young *vacu* adults performed so poorly in this assay, it was not possible to assess whether vertical climbing ability declined further with age.

We also measured flight ability using the procedure originally described by Benzer (1973). Flies were dropped into a 500-ml graduated cylinder whose inside wall was covered with paraffin oil. When released into the top of the tube, strong fliers immediately initiate flight, striking the wall and becoming stuck in the oil near the top of the cylinder. Weak fliers drop farther before becoming stuck. Thus, the vertical distribution of the flies over the length of the cylinder is a measure of their flying ability. Wild-type flies showed a decline in flight ability with age (Figure 3, A and B). Both young and old *vacu* adults exhibited considerably reduced flight ability compared with the age-matched wild-type controls. Moreover, the age-dependent decrease in this ability was even more marked in *vacu* adults (Figure 3, A and B).

***vacu* adults have electrophysiological defects:** To determine whether the behavioral defects of *vacu* adults were paralleled by disruption of synaptic transmission, we performed ERG recordings at various temperatures. The ERG is an extracellular recording from the compound eye that measures light-induced depolarization of photoreceptors as well as the synaptic-mediated responses of second-order neurons in the visual system (Hotta and Benzer 1969; Pak *et al.* 1969). The ERG consists of a component maintained during the entire light flash that corresponds to the light-dependent depolarization of the photoreceptor cells as well as on- and off-transients that appear at the beginning and end of a light flash, respectively. The on- and off-transients represent responses from the second-order neurons in the lamina. If synaptic transmission between photoreceptor cells and laminar neurons is blocked, the on- and off-transients are preferentially lost. Thus, ERG recordings can be used as an assay of synaptic function in the visual pathway.
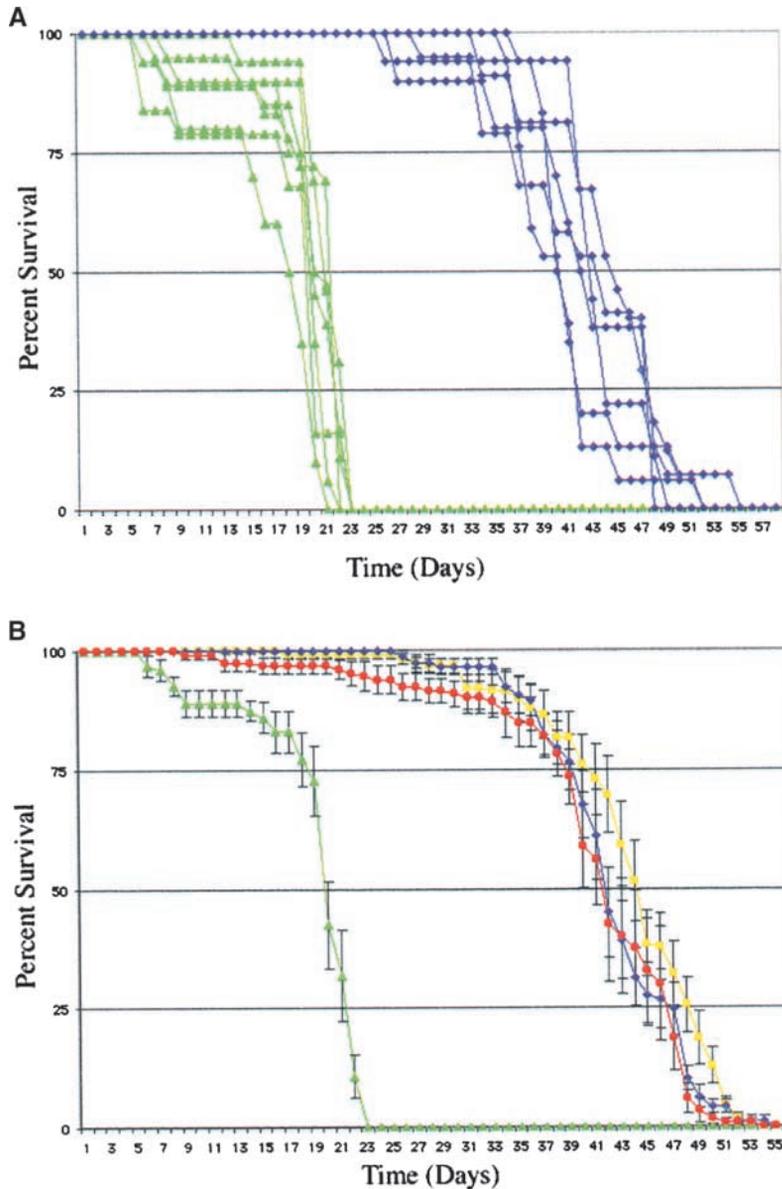
Despite the behavioral deficits in *vacu*, the ERGs of

FIGURE 1.—Reduced life span in *vacuous* mutants. Survival as a function of age was determined for populations of flies of various genotypes at 28°. (A) Survival curves for six independent populations of *vacu* (green) and wild type (blue) are shown. (B) Pooled data from multiple independent populations were used to produce single survival curves for *vacu* (green), wild type (blue), y^1 (yellow), and *para^{ts1}* (red). Each point represents mean survival ±SEM. Both 50% and maximal age are reduced in *vacu* animals (19.3 and 41.5 days) *vs.* wild type (43.6 and 50.7; $P < 0.001$). In contrast, y^1 and *para^{ts1}* do not differ appreciably from wild type ($P > 0.05$).

adults appeared relatively normal both at 20° and at 37° (Figure 4). However, these recordings did uncover anomalous electrical activity in the mutant, suggestive of dysfunction in the nervous system (Figure 4). This anomalous activity is apparent even at 20° as recurrent spikes in the ERG trace at 20° before, during, and after the light exposure. When the temperature is raised to 34°–37°, more prominent sustained bursts of apparent activity are observed in *vacu* adults both preceding and following the light flash (Figure 4). Upon return to 20°, the more extreme bursting activity disappears but the small recurrent spikes persist. We believe that this excessive activity originates in the ventral ganglion and is being detected by the ERG ground electrode, which is inserted into the thorax. Similar bursting activity has been previously observed in mutants such as *seizure* (*sei*), which are known to cause neuronal membrane hyperexcitability (ELKINS and GANETZKY 1990; TITUS *et al.* 1997).

These results suggest that loss of *vacu* function may result in neuronal hyperexcitability. Elucidation of the precise nature of this defect will require further investigation.

***vacu* causes age-dependent neurodegeneration:** In accord with the profound age-dependent phenotypes, shortened life spans, and the electrophysiological result suggesting neuronal hyperexcitability described above, *vacu* animals were found to be incurring extensive neuropathology. Histological analysis of frontal sections of heads from *vacu* adults revealed a readily observable pattern of neurodegeneration (Figure 5B). There appears to be a widespread loss of neural tissue in the neuropil of the central brain and in the optic lobes (evident as the appearance of apparent vacuolization throughout these regions). Neuropathology is highly penetrant in aged *vacu* animals: every animal tested exhibited significant gross pathology ($n > 60$ animals).
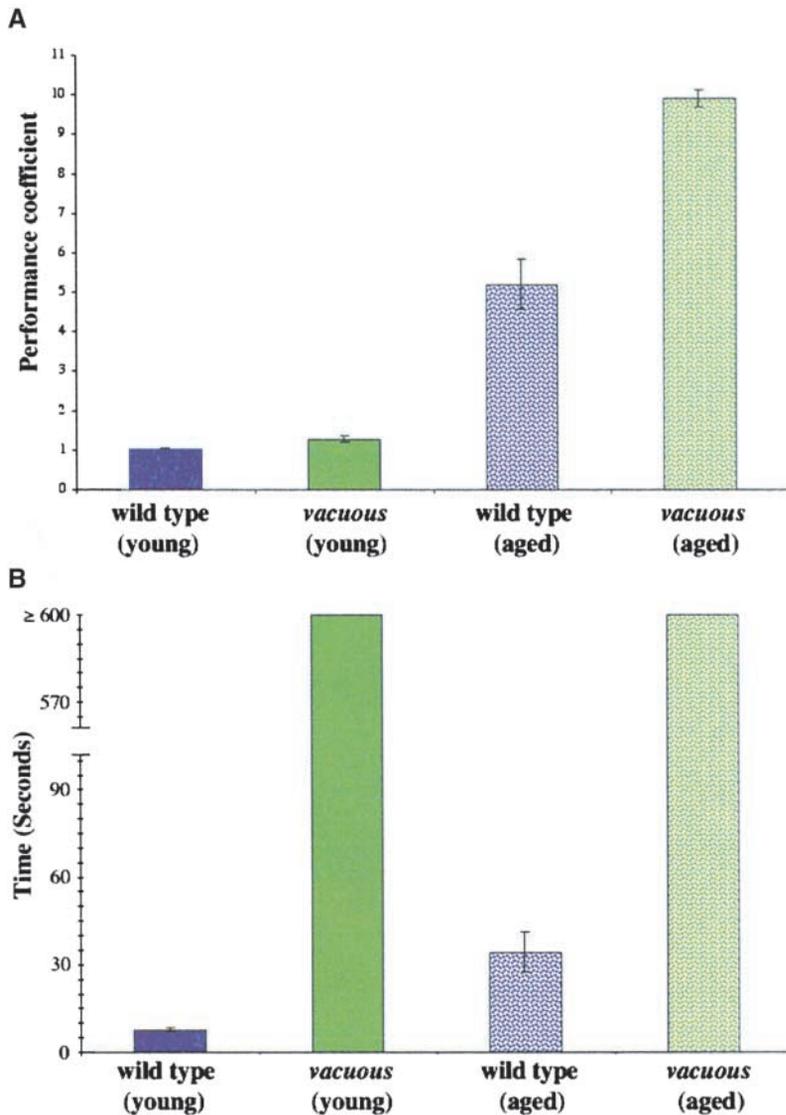
A

B

FIGURE 2.—Locomotor defects in *vacuous* adults. (A) Test of running ability. The time required for flies to run the length of a horizontal tube toward a light source was measured in seconds and assigned a numerical score as described in MATERIALS AND METHODS. A higher performance index indicates longer time was required to run the tube. *vacu* and wild-type animals that were either young or aged were tested. Young and aged flies were 24–72 hr and 11–12 days posteclosion, respectively. Both *vacu* and wild type showed a significant age-dependent decline in performance ($P < 0.001$). Age-dependent decrement was more severe in *vacu* than in wild type (7.5- *vs.* 5.1-fold increase). Aged wild type performed significantly better than *vacu* animals ($P < 0.001$). (B) Test of climbing ability. The time required for 50% of the flies to run up a vertical cylinder past a line at 17.5 cm was measured. Wild-type flies showed a small age-dependent decrease in behavior ($P < 0.001$). Both young and old *vacu* flies performed extremely poorly in this assay; none of the mutant flies completed the test within the total time allotted (5 min).

Aged control animals never exhibited significant gross pathology and even a single vacuolar clearing was seldom observed ($n > 50$ animals). Neurodegeneration in *vacu* adults is not limited to the brain but can also be readily observed in sagittal sections of the thoracic ganglion (Figure 5D). The thoracic ganglion pathology is also highly penetrant in *vacu* animals. The neuropathology observed in the brain and thoracic ganglion was similar in appearance, resulting in apparent massive tissue losses. In contrast with what was observed in neural tissues, no apparent degeneration was found in sections of the flight muscles in aged *vacu* mutants (data not shown).

To determine whether the onset of neurodegeneration varied as a function of developmental stage and age, we also performed a histological analysis of third instar larvae and newly eclosed adults. Despite the fact that *vacu* larvae show a strong behavioral defect, we found no obvious evidence of neurodegeneration in the larval CNS (Figure 6, A and B). Additionally, young

vacu adults, within 36 hr after eclosion, also did not exhibit neurodegenerative phenotypes (Figure 6, C and D), demonstrating that *vacu* neurodegeneration is progressive.

Considering the massive degeneration seen in the optic system, we hypothesized that some of the degeneration might be light dependent. To test this hypothesis, we dark-reared *vacu* animals from early pupae through adulthood. Aged *vacu* animals were examined for pathology, as above. Both the dark-reared and light-reared control *vacu* animals demonstrated neuropathology that was not overtly different, suggesting that the process is light independent (data not shown).

Survey of other neurodegeneration mutants in the collection: In addition to *vacu*, we identified mutations in at least eight other loci that showed significant levels of neurodegeneration. The patterns of neurodegeneration displayed by these mutants represent a diverse array of neuropathological phenotypes, which can be grouped into several broad categories (Figure 7). In addition to
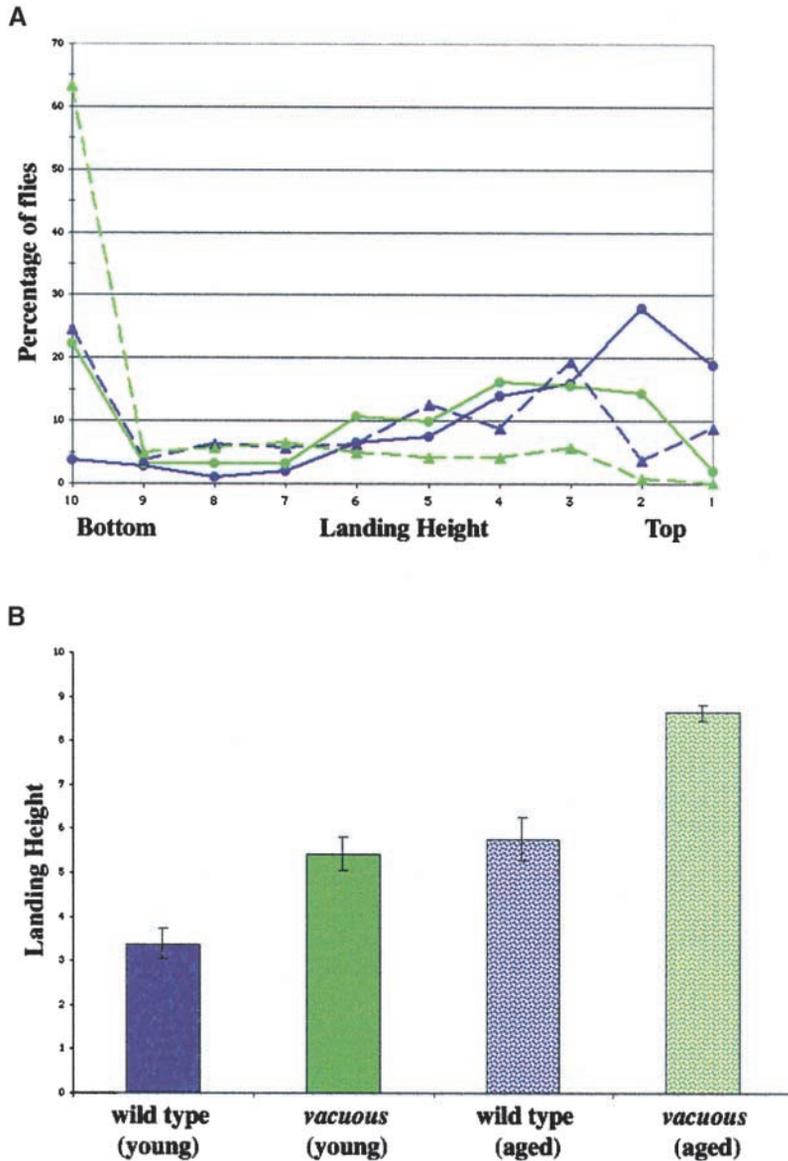
Figure 3.—Flight defect in *vacuous* mutants. The distribution of *vacu* and wild-type animals indicates flight performance. A numerical score, depending on where the fly landed, was assigned to each fly as described in MATERIALS AND METHODS. Flies becoming stuck near the top (1) are strong fliers, whereas flies that fall to the bottom (10) before becoming stuck are weak fliers. (A) Plot of landing height scores for young (solid line) and aged (broken lines) populations of *vacu* (green) and wild-type (blue) flies. (B) The results from A are plotted as bar graphs to depict the average performance of each group. Both wild-type and *vacu* flies show an age-dependent decrease in flight ability but *vacu* flies of both age groups perform markedly worse than wild type and the decline in flight ability with age is steeper for *vacu*.

vacu, we found other mutants that show a somewhat similar pattern of fine vacuolar pathology widely distributed throughout the neuropil of the central brain and optic lobes. A second category includes mutants with larger clusters of vacuolar structures scattered throughout the neuropil (Figure 7A). Another group consists of mutants with similar large clusters that are predominantly localized to specific regions of the brain (Figure 7B). The final category, consisting of a single mutant, causes neuropathology that is limited to visual regions of the brain (Figure 7C).

These neurodegeneration mutants cannot be grouped into a single category on the basis of life span or behavior, which is not surprising given their diverse neuropathological phenotypes. Although TS paralytic mutants with markedly reduced life span are highly enriched for those associated with neurodegeneration, a majority of our neurodegeneration mutants had life spans in the normal range (Figure 8). Thus, we would not be able to identify all the mutants in our collection causing neurodegeneration solely on the basis of life-span analysis. Similarly, we were not able to find a single behavioral phenotype, such as early loss of adult locomotor activity, that was common among all the neurodegeneration mutants or that allowed us to distinguish these mutants from the bulk of the collection that did not cause neurodegeneration.

DISCUSSION

Human neurodegenerative illnesses, such as amyotrophic lateral sclerosis and Huntington's, Parkinson's, and Alzheimer's diseases, are characterized by progressive behavioral deficits, premature death, and, in some cases, profound cognitive impairment. Onset of the symptoms of these diseases correlates with the appearance of neuropathology. Mutations that underlie some forms of these diseases are known, but the molecular mechanisms that
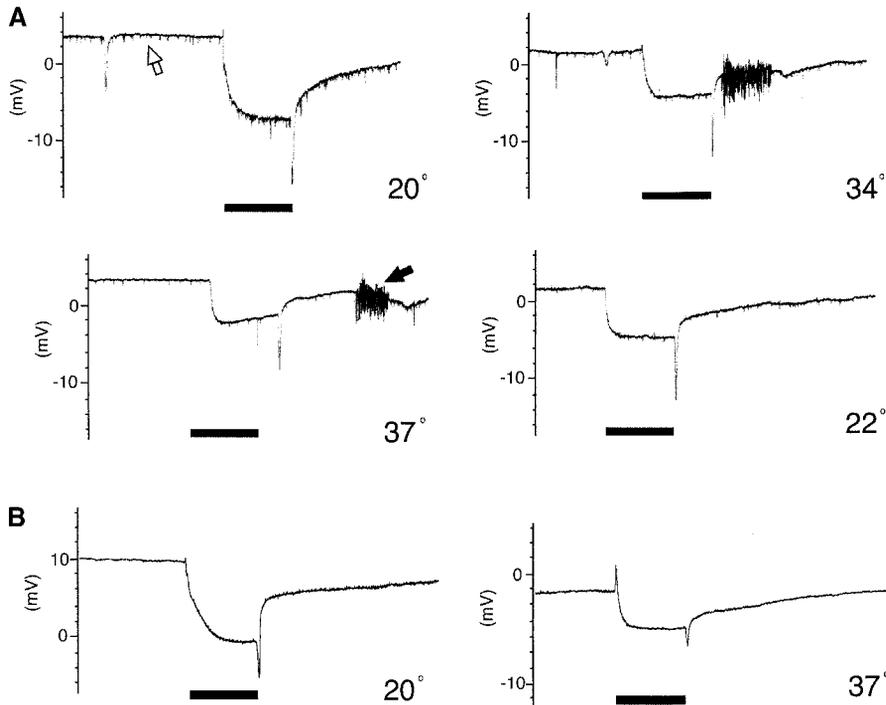
FIGURE 4.—Electrophysiological dysfunction in *vacuous* mutants. Electroretinograms were performed on *vacu* (A) and wild-type animals (B). *vacu* and wild type demonstrate a normal ERG response with on- and off-transient recording (arrows) coincident with the beginning and end of the light period (solid bar). Recordings were taken at temperatures between 20° and 37° (as indicated for each trace) in the following order: 20°, 34°, 37°, and 22°. Sporadic depolarization events can be seen at all temperatures in *vacu* animals (open arrow). Bursts of apparent electrical activity that did not occur in wild-type animals (solid arrow) are present in *vacu* animals at 34° and 37°.

are required for maintenance of neuronal viability and whose disruption leads to these disorders are not understood. Just as other complex biological processes such as regulation of the cell cycle and pattern formation during embryonic development have yielded to intensive genetic analysis, a similar approach will help elucidate the mechanisms that underlie neurodegeneration. A large and comprehensive set of mutations that cause neurodegeneration would be an extremely valuable resource in pursuing this strategy. Here we describe a screen in Drosophila that has led to the identification of a number of new mutations that exhibit neurodegeneration. These mutants define at least nine genes and exhibit diverse patterns of neuropathology ranging from widespread loss of neural tissue throughout the central brain to highly localized defects in particular regions of the brain. These mutants substantially enlarge the set of known neurodegeneration mutants in Drosophila and should therefore prove to be an extremely valuable resource in the genetic dissection of neurodegeneration.
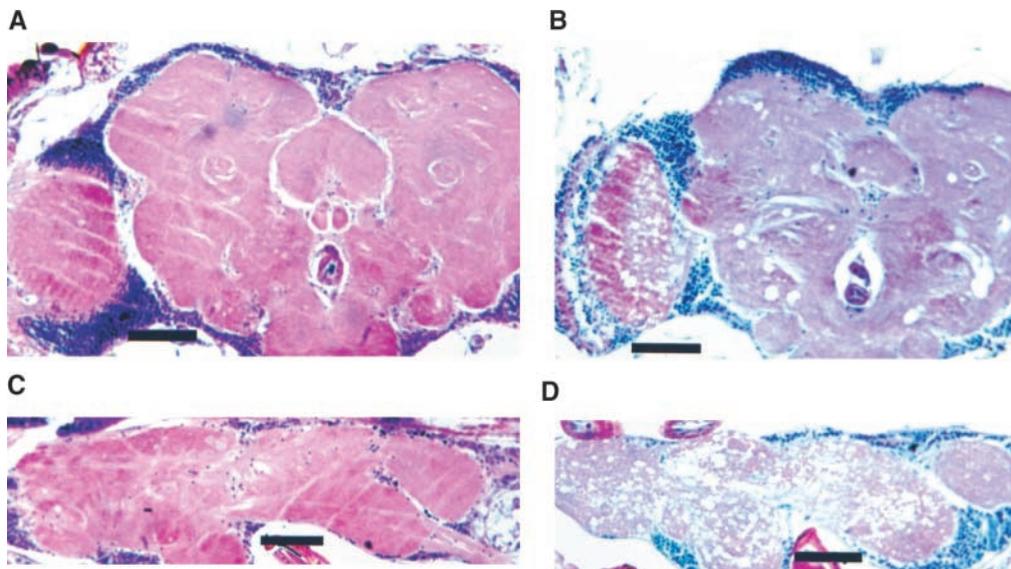

FIGURE 5.—Neurodegeneration in the central nervous system of *vacuous* mutants. Frontal sections of brains from aged wild-type (A) and *vacu* (B) flies. Neurodegeneration, marked by extensive vacuolization, is especially prominent in the optic lobes of *vacu* flies but extends into the central brain as well. Sagittal sections of thoracic ganglia from wild-type (C) and *vacu* (D) flies. Extensive vacuolization is evident throughout the entire ganglion of *vacu* flies. The large hole just below the center of the brain is the esophagus. Tissues from both wild-type and *vacu* flies were obtained from individuals aged to ∼50% survivorship, 39 days for wild type and 18–19 days for *vacu*. Bar, 50 μm.
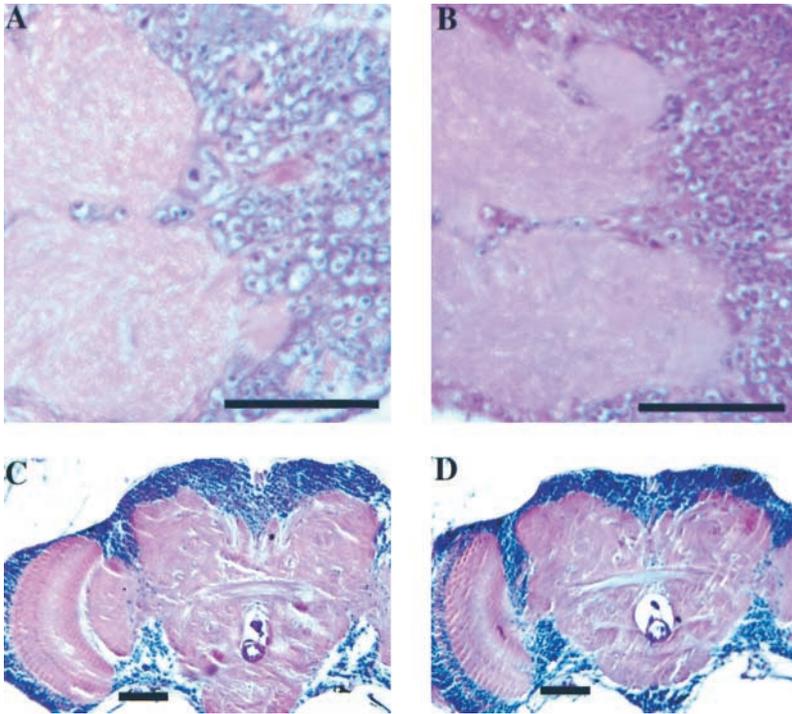
FIGURE 6.—Onset of neurodegeneration in *vacuous* mutants is age dependent. Horizontal sections of the larval central nervous system from wild-type (A) and *vacu* (B) third instar larvae do not reveal any obvious neuropathology. Frontal sections of young wild-type (C) and *vacuous* (D) adults are shown. The extensive vacuolization seen in older *vacu* flies is not evident in young *vacu* flies, which do not differ noticeably from wild-type controls. Bar, 50 μm.

One of these mutants, *vacuous*, exhibits extensive age-dependent neurodegeneration throughout the central nervous system that correlates with an age-dependent decrement in locomotor ability. In addition, the life span of *vacu* mutants is reduced by about half. An electrophysiological defect resulting in excessive neuronal
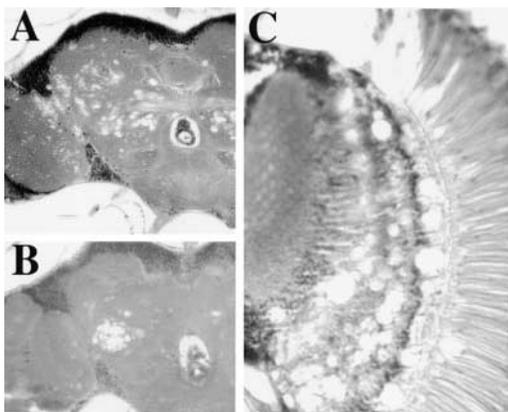

FIGURE 7.—Examples of other neurodegenerative phenotypes observed among mutants identified in this screen. The mutants we identified as causing neurodegeneration encompass a broad spectrum of distinct phenotypes. Shown are frontal sections of brains from aged individuals of different mutants that are representative of the distinct phenotypes we observed. (A) Example of mutants exhibiting clusters of vacuolization that are distributed widely throughout the brain (ND7). (B) Example of mutants showing clusters of vacuolization that are localized focally in specific regions of the brain (ND8). (C) A mutant showing extensive neuropathology that is limited to the eye (ND13).

activity, which may contribute to neurodegeneration, was also found in *vacu* mutants.

An effective screen for neurodegeneration mutants: Although a direct histological screen for neurodegeneration mutants has the advantage of being completely unbiased, the expense and labor required to perform such a screen make it impractical. Neurodegeneration mutants have previously been isolated in Drosophila in screens that first selected flies on the basis of a defect in phototaxis or a reduced life span (HEISENBERG and BOHL 1979; COOMBE and HEISENBERG 1986; BUCHANAN and BENZER 1993; KRETZSCHMAR *et al.* 1997; MIN and BENZER 1997). Our approach to identifying neurodegeneration mutants by histological analysis of mutants preselected on the basis of TS paralytic phenotypes has proven to be a useful and productive one that is complementary to those that have been reported previously.

In another large-scale screen, 60 mutant lines (1.2%) with reduced life span were isolated from 5000 mutagenized lines examined (MIN and BENZER 1997). Of these, 2 were reported to cause neurodegeneration (3.3 or 0.04% of the mutagenized lines). By comparison, we have identified TS paralytic mutants at a frequency of 0.1–0.2%. In our secondary histological screen, we found extensive neurodegeneration in ∼20% of the lines we examined (0.02–0.04% of the mutagenized lines). Thus, our overall yield of neurodegeneration mutants is comparable to that described by MIN and BENZER (1997). However, because it is much easier to score paralysis than life span and because the incidence of neurodegeneration appears to be higher among paralytic mutants
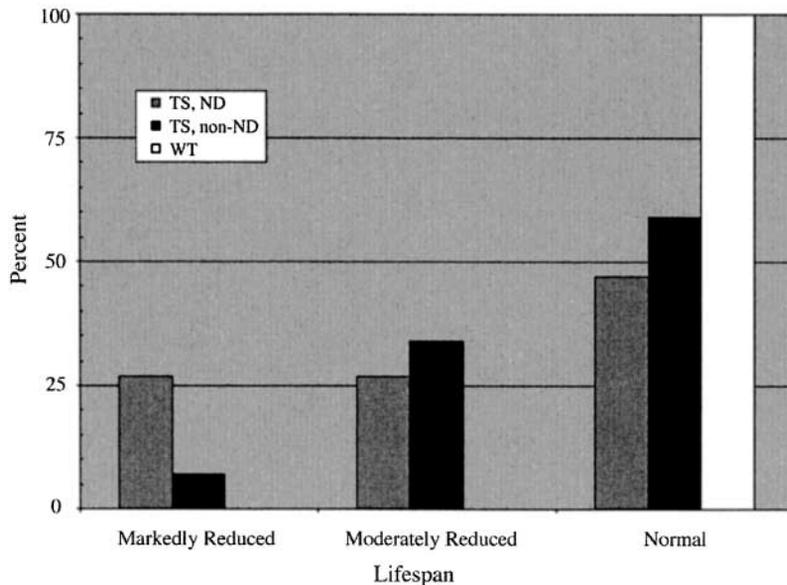
FIGURE 8.—Neurodegeneration mutants identified in this screen have varying effects on life span. Survival as a function of age was determined for populations of flies of each genotype at 28°. The time required for each population to be reduced to 50% survivorship was used to compare life spans of the various strains. For each group, the percentage of lines with markedly reduced life spans (≤8 days), moderately reduced life spans (9–16 days), and normal life spans (≥17 days) was determined. Neurodegeneration mutants showing markedly reduced life span include ND4–6 and ND14. Those showing moderately reduced life spans include ND2, ND7, ND9, and *vacuous*. All others had normal life spans. TS, ND (shaded): TS paralytic mutants exhibiting neurodegeneration. TS, non-ND (solid): TS paralytic mutants not exhibiting neurodegeneration. WT (open): wild-type controls.

than among those with reduced life span, our approach may simplify the isolation of a large set of neurodegeneration mutants.

As shown in Figure 8, approximately half of the neurodegeneration mutants we identified had essentially normal life spans. These mutants would presumably be missed in screens using life span as the primary criterion for identifying neurodegeneration mutants. Conversely, our screens did not identify alleles of any of the neurodegeneration mutants described by Benzer nor have any of these mutants been reported to cause TS paralysis. Thus, it appears that the two screens have resulted in the isolation of nonoverlapping sets of mutants.

On the basis of existing information, it is hard to estimate the size of the total set of neurodegeneration mutants in Drosophila. The vast majority of paralytic mutants in our collection are represented by single alleles. Similarly, most of the neurodegeneration mutants we have identified among this collection are represented by single alleles. In those cases where we have multiple alleles of a particular neurodegeneration gene, they do not represent independent isolates. Instead, once we discovered that mutations of a particular gene caused neurodegeneration, we deliberately examined other alleles of the same gene, if they were available, to determine if different alleles shared this phenotype. What can be concluded from these observations is that it does not appear that either the set of paralytic mutants or the set of neurodegeneration mutants is near saturation.

One premise behind our screening strategy was the idea that perturbation of neuronal signaling pathways could be an important contributing factor to neurodegeneration. This premise is supported by the high incidence of neurodegeneration mutants we found among our collection of TS paralytic mutants. An unknown in our screen is what fraction of neurodegeneration mutants were missed because they did not display the

kind of overt locomotor defects we originally searched for. The effectiveness of our screen improves with the extent of overlap between neurodegeneration mutants and paralytic mutants. We believe that our approach will have only limited bias because it is reasonable to expect that most mutants with significant brain neurodegeneration will manifest some behavioral deficit. Moreover, this bias can be further limited in future screens by expanding the array of behaviors examined in our primary screen. In any case, as exemplified by the isolation of *vacu*, our screen has been very useful in expanding the number of known neurodegeneration mutants in Drosophila.

Neurodegeneration in *vacu* mutants: Similar to what is seen in progressive neurodegenerative diseases in humans, *vacu* shows an age-dependent decline in behavior that correlates with the onset of observable neuropathology. Although we do not know what initiates the onset of neurodegeneration in *vacu*, it is of interest to ask whether the neuropathology is responsible for the behavioral phenotypes. We believe this is unlikely because *vacu* larvae show TS paralysis but not obvious neuropathology. Moreover, young *vacu* adults show severely impaired climbing ability before the apparent onset of neurodegeneration. These data, along with the observed electrophysiological defect in *vacu*, suggest that the primary defect in *vacu* is a perturbation of neural function that precedes and ultimately leads to the observed neuropathology.

An electrophysiological defect in *vacu* was uncovered in our ERG recordings. This defect was manifested as bursts of neural activity that appeared to originate in the thoracic ganglion. Similar bursting phenotypes have been observed in *seizure* mutants as well as in some bang-sensitive mutants and have been interpreted as neural hyperactivity and physiological seizures (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000;

KUEBLER *et al.* 2001). As excitotoxicity is one known cause of neurodegeneration, it will be of interest to determine whether there is a direct causal connection between the electrophysiological and neuropathological phenotypes of *vacu*.

To address this question and to elucidate the *in vivo* physiological function of *vacu*, it is essential to identify the encoded protein. Formally, identification of the affected protein and transgenic rescue will be required to ascribe all of the observed phenotypes to defects in *vacu*. Because these mutants are associated with a striking loss of neural tissue in the brain and thoracic ganglion, regardless of what the affected protein proves to be, it will offer new insights into the molecular pathways that impinge on maintenance of neuronal viability. Moreover, the *vacu* phenotypes of larval TS paralysis, lack of adult climbing activity, and reduced adult life span lend themselves very well to suppressor screens to further dissect the relevant pathways responsible for neurodegeneration in this mutant. Ultimately, *vacu* and the other mutants identified in our screen should provide incisive tools for understanding the molecular mechanisms that underlie neural senescence and neurodegenerative diseases.

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