Journal of Visualized Experiments

In vivo forward genetic screen to identify novel neuroprotective genes in Drosophila melanogaster

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59720R3
Full Title:	In vivo forward genetic screen to identify novel neuroprotective genes in Drosophila melanogaster
Keywords:	Drosophila; neurodegeneration; forward genetic screen; climbing assay; histology; gene mapping, DNA sequencing
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

In Vivo Forward Genetic Screen to Identify Novel Neuroprotective Genes in *Drosophila melanogaster*

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KEYWORDS:

Drosophila melanogaster, neurodegeneration, forward genetic screen, climbing assay, histology, gene mapping, DNA sequencing

SUMMARY:

We present a protocol using a forward genetic approach to screen for mutants exhibiting neurodegeneration in *Drosophila melanogaster*. It incorporates a climbing assay, histology analysis, gene mapping and DNA sequencing to ultimately identify novel genes related to the process of neuroprotection.

ABSTRACT:

There is much to understand about the onset and progression of neurodegenerative diseases, including the underlying genes responsible. Forward genetic screening using chemical mutagens is a useful strategy for mapping mutant phenotypes to genes among *Drosophila* and other model organisms that share conserved cellular pathways with humans. If the mutated gene of interest is not lethal in early developmental stages of flies, a climbing assay can be conducted to screen for phenotypic indicators of decreased brain functioning, such as low climbing rates. Subsequently, secondary histological analysis of brain tissue can be performed in order to verify the neuroprotective function of the gene by scoring neurodegeneration phenotypes. Gene

mapping strategies include meiotic and deficiency mapping that rely on these same assays can be followed by DNA sequencing to identify possible nucleotide changes in the gene of interest.

INTRODUCTION:

Neurons are for the most part post-mitotic and incapable of dividing[1,2]. In most animals, neuroprotective mechanisms exist to maintain these cells throughout the organism's lifespan, especially at old age when neurons are most vulnerable to damage. Genes underlying these mechanisms can be identified in mutants exhibiting neurodegeneration, a phenotypic indicator for the loss of neuroprotection, using a forward genetic protocol. Forward genetic screens using chemical mutagens such as ethyl methanesulfonate (EMS) or N-ethyl-N-nitrosourea (ENU) are particularly useful due to the random point mutations they induce, resulting in an inherently unbiased approach that has shed light on numerous gene functions in eukaryotic model organisms[3-5] (in contrast, X-ray mutagenesis creates DNA breaks and can result in rearrangement rather than point mutations[6]).

The common fruit fly *Drosophila melanogaster* is an ideal subject for these screens due to its high quality, well annotated genome sequence, its long history as a model organism with highly developed genetic tools, and most significantly, its shared evolutionary history with humans[7,8]. A limiting factor in the applicability of this protocol is early lethality caused by the mutated genes, which would prevent testing at old age[9]. However, for non-lethal mutations, a climbing assay, which takes advantage of negative geotaxis, is a simple, although extensive, method of quantifying impaired motor functioning [10]. To exhibit sufficient locomotor reactivity, flies depend on neural functions to determine direction, sense its position, and coordinate movement. The inability of flies to sufficiently climb in response to stimuli can therefore indicate neurological defects[11]. Once a particular defective climbing phenotype is identified, further testing using a secondary screen such as histological analysis of brain tissue, can be used to identify neurodegeneration in climbing-defective flies. Subsequent gene mapping can then be used to reveal the genomic region on the chromosome carrying the defective neuroprotective gene of interest. To narrow down the chromosomal region of interest, meiotic mapping using mutant fly lines carrying dominant marker genes with known locations on the chromosome can be performed. The marker genes serve as a reference point for the mutation as the frequency of recombination between two *loci* provides a measurable distance that can be used to map the approximate location of a gene. Finally, crossing the mutant lines with lines carrying balanced deficiencies on the meiotically mapped chromosomal region of interest creates a complementation test in which the gene of interest can be verified if its known phenotype is expressed [5]. Polymorphic nucleotide sequences in the identified gene, possibly resulting in an altered amino acid sequences, can be evaluated by sequencing the gene and comparing it to the Drosophila genome sequence. Subsequent characterization of the gene of interest can include testing of additional mutant alleles, mutation rescue experiments and examination of additional phenotypes.

PROTOCOL:

1. Preparation and aging of flies

1.1 Obtain or generate[6] a collection of *Drosophila* mutants that will be used for the genetic screen. Here, ENU-mutagenized lines mapped to the second chromosome and balanced over CyO are used.

1.2. Amplify experimental genotype lines in an incubator set at 25 °C, 12 h light/dark cycle on cornmeal-molasses medium.

1.3. Collect around 20 homozygous progeny between 0-2 days of adult eclosion from each experimental genotype. Eliminate sex bias by consistently collecting either male or female flies.

1.4. Age the flies on cornmeal-molasses medium as desired at 29 °C, flipping the vials every 2-3 days in order to maintain the flies on fresh food as they age. In the screen presented here, flies were aged for 10-12 days.

1.5. Assemble a climbing assay testing chamber using two vials and tape as previously described[10]. The vials should be connected at both open ends. Use a ruler to mark a 5 cm line on one end of the chamber.

2. Protocol 1: Climbing assay (adapted from Ali et al.**[10])**

2.1. Validate the climbing assay. In this study, test the climbing assay based on previously described methodology by Ali et al.[10], using *Elav^{C155}>UAS-Tau^{R406W}* (exhibiting low climbing pass rates over the 5 cm line) and *Elav^{C155}>UAS-GFP* (control) and *Elav^{C155}>UAS-mCherry* (control) flies.

2.2. Flip the flies into a testing chamber, one line at a time (do not use CO_2 anesthesia) and allow them to recover for 5 min. If testing is done on different days, perform the assay at the same time of the day. In this study, all climbing assays were performed in the afternoon to control for circadian effects on locomotion[12].

NOTE: Avoid exposing the flies to direct sunlight when testing; this will interfere with their ability to climb.

2.3. Forcibly tap the chamber (marked side down) onto a mouse pad placed on a solid surface 3 times so all flies begin the assay at the bottom. Observe fly locomotion over a period of 10 s.

2.4. Record the number of flies that reach or pass the 5 cm line within this time, as well as the total number of flies tested. Repeat the protocol, waiting 1 min between each trial, for a minimum of 3 trial replicates per line.

NOTE: In the present study, each vial contained between 2 and 19 flies for the initial screening (male flies) and between 6 and 21 flies for the mutant deficiency analysis of crossed female lines (Section 5.3.).

3. Protocol 2: Selection of *Drosophila* strains for further analysis

3.1. Enter the climbing assay data into Microsoft Excel or similar software and calculate the climbing pass rate for each line over all replicates as a percentage.

3.2. Perform statistical analysis to detect mutant lines that deviate significantly from the mean climbing percent value of all lines using the R software package[13].

3.2.1. Read in data files curated for R (.csv or .txt files with a column for each variable or factor, and rows representing the specific values).

NOTE: Here, data for males (initial assay) and females (line 867 crossed to deficiency lines) were entered into separate .xlsx sheets as two columns, one where the rows of the column identify the mutant line and how many times the assay was replicated and the other where rows represent the mean climbing success (as a percentage) for each replicate vial of flies.

3.2.2. Read the data into R using the following code:

```
mali<-read.csv("../male_init.csv")
femdef<-read.csv("../fem_def_cross.csv")
```

NOTE: The ".." in the path directories are absolute paths from the root directory of the user's computer and would need to be specified by the individual user for their computer's directory paths.

3.2.3. Perform linear modeling

3.2.3.1. Perform dummy variable regression with the **lm** function in R. For the initial analysis of mutant lines, evaluate the significance of factor level effects (mutant lines) on percent climbing success against the grand mean for a zero mean centered response variable (percent success).

NOTE: The "-1" at the end of the **lm** function call in section 3.2.3.2. removes the intercept and allows us to test all factor levels against the zero mean centered mean of percent climbing pass rate.

3.2.3.2. Print the results to the screen using the R **summary** function:

```
mali_anova<-lm(scale(mali$P_Success)~mali$Mutant_line -1)
summary(mali_anova)
```

3.2.3.3. Use control lines (if present) as the baseline to test factor level effects against using the following R code:

```
x<-relevel(femdef$Mutant_line, ref="a867_plus")
femdef_anova<-lm(femdef$P_success~x)
summary(femdef_anova)
```

NOTE: The first line of code reorders the factor levels so that the negative control line becomes

the baseline intercept to which all other factor levels (mutant lines) are tested against with the built-in *t*-tests in the **lm** function.

3.3. Choose a threshold for candidate lines depending on age at the time of testing. To determine the threshold, perform a preliminary test at the desired age using known mutants that exhibit neurodegeneration and low climbing pass rates in comparison to wild type, control flies[10].

NOTE: A passing rate below 50% may be appropriate for 10-days-old flies as previously reported for *Drosophila* lines overexpressing the human neurodegeneration-related gene *Tau* in the nervous system[10]. Older flies should have a higher passing rate threshold since they are expected to exhibit decreased locomotion, thus, increased neurodegeneration.

4. Protocol 3: Histology analysis

4.1. Wearing gloves, sever fly heads with a surgical blade following the climbing assay and use a paintbrush to gently place them in 1 mL of Carnoy's fixative (6:3:1 of 100% EtOH: chloroform: glacial acetic acid) in a 1.5 mL micro centrifuge tube O/N at 4 °C. Make sure the heads sink in the fixative solution.

4.2. Replace the fixative solution the following day with 1 mL of 70% EtOH and maintain the samples still at 4 °C for future analysis.

NOTE: The protocol may be paused at this step.

4.3. Place the heads from step 4.2. at a maximum of five per microbiopsy cassette. Immediately place the cassettes into a container filled with 70% EtOH. Store the container at 4 °C prior to shipping to a histology facility for processing and paraffin embedding of the heads. If equipment for tissue processing and embedding is available, follow step 4.4.

4.4. Embed the heads in paraffin for microtome sectioning:

4.4.1. Follow the program settings for an automated tissue-processing machine in the order presented in **Table 1** to dehydrate, clear and infiltrate with paraffin the heads.

4.4.2. Transfer the samples into metal base molds that fit the cassette using paraffin heated at 60 °C using a paraffin embedding station.

4.4.3. Orient the heads (antero-posterior orientation facing the top) in the base mold using fine forceps to allow proper visualization of the brain tissue following sectioning. This can be done by reheating the paraffin at 60 °C and repositioning the heads if necessary.

NOTE: Metal molds can be replaced by disposable base molds.

4.4.4. Position the cassette on the top of the corresponding base mold and gently move these to

the refrigerated side of the paraffin embedding station (4 °C). Wait until the block hardens prior removing it from the station.

4.4.5. Remove the block form the mold by gently dissociating the mold from the cassette.

4.5. Trim each paraffin block prior sectioning to minimize the surface that will be sectioned.

4.6. Set the microtome to cut 5 µm sections of each block.

4.7. Place sectioned paraffin ribbon containing the tissue in a heated water bath (35 °C) for a maximum of 5 min.

4.8. Immerse a poly-lysine coated slide (helps to retain the tissue) in the heated water bath, placing the sectioned ribbon on the slide using wood applicators. Let the slides air dry O/N at room temperature.

4.9. Heat the slides for 15 min at 63 °C using a slide warmer.

4.10. Place the slides on a slide staining rack and stain with hematoxylin and eosin (H&E) under a fume hood by respecting the following steps.

4.10.1. Place the rack in Histochoice (non-toxic replacement of Xylene, which helps to remove the paraffin from the sample) for 5 min.

4.10.2. Transfer the rack in a container filled with Histochoice for 5 min.

4.10.3. Transfer the rack in a container filled with 100% EtOH 1 for 5 min.

4.10.4. Transfer the rack in a container filled with 100% EtOH 2 for 5 min.

4.10.5. Transfer the rack in a container filled with 95% EtOH for 3 min.

4.10.6. Transfer the rack in a container filled with 70% EtOH for 3 min.

4.10.7. Transfer the rack in a container filled with Distilled H_2O for 5 min.

4.10.8. Transfer the rack in a container filled with Harris Hematoxylin for 2-5 min.

4.10.9. Take the rack out of the Hematoxylin solution and place it under running tap water for 10 min.

4.10.10. Transfer the rack in a container filled with distilled water by doing a short dunk.

4.10.11. Transfer the rack in a container filled with Eosin for 1-2 min.

4.10.12. Transfer the rack in a container filled with 95% EtOH by doing a short dunk.

4.10.13. Transfer the rack in a container filled with 95% EtOH for 5 min.

4.10.14. Transfer the rack in a container filled with 100% EtOH for 5 min.

4.10.15. Transfer the rack in a container filled with 100% EtOH for 5 min.

4.10.16. Transfer the rack in a container filled with Histochoice (or Xylene) for 5-10 min until all slides are mounted.

4.10.17. Mount the slide and coverslip (24 x 60 mm of size). Using forceps, take the slide out of the Histochoice or Xylene solution and make a thin strip of mounting medium on the top of it. Gently place the coverslip on the top, trying to avoid the formation of bubbles.

4.10.18. Let the mounting medium on mounted slides harden O/N under the fume hood prior to analysis.

4.10.19. Store stained slides in a box at room temperature.

4.11. Quantify neurodegeneration by looking at all serial sections on the slide at 20X magnification under a light microscope. Examine the entire brain, taking qualitative note of size and number of vacuoles that appear in three consecutive sections.

4.12. Obtain images of representative brain sections at approximately mid-brain using a light microscope equipped with imaging software.

5. Protocol 4: Gene mapping of recessive mutant phenotype

5.1. Outcross the candidate line to a wild type *CantonS* (BL_ 9517) or another wild type or isogenized strain (e.g., *OregonR* (BL_ 2376), w^{1118} (BL_5905) or *yw* (BL_ 6599) to determine whether the phenotype is dominant or recessive.

NOTE: Results from this step will guide the next steps of the mapping. If the phenotype is recessive, then deficiency mapping can be performed.

5.1.1 Using a paintbrush, make a cross by placing in a food-containing vial CO_2-anesthetized flies of the mutant line of interest (5 males) and the *CantonS* line (10-15 virgin females). Place the vial at 25 °C.

5.1.2. Collect heterozygous F1 progeny and repeat climbing and histology experiments.

5.1.3. Compare the obtained results to the control line alone and to the homozygous mutants. A

good indication of recessive phenotype will be if heterozygous mutants exhibit similar phenotypes as wild type flies.

5.2. Perform meiotic mapping to roughly estimate the location of the mutation on the second chromosome using the *wg*$^{Sp-1}$ *J*^{1} *L*^{2} *Pin*^{1}*/CyO* (BL_3227) or an equivalent strain with associated dominant markers at known cytological location. In this case, the mutation was previously known to be located on the second chromosome.

5.2.1. Using a paintbrush, make a cross by placing in a food-containing vial CO_2-anesthetized flies of the mutant line of interest (5 males) and the *wg*$^{Sp-1}$ *J*^{1} *L*^{2} *Pin*^{1}*/CyO* line (10 females). Place the vial at 25 °C.

NOTE: The goal of this cross is to generate heterozygous females carrying the chromosome with the new mutation and the marker chromosome, which will recombine during meiosis[6,14]. Female F1 progeny are chosen rather than males since recombination does not occur in males.

5.2.2. Collect at least 15 virgin female progeny carrying the chromosome with the new mutation and the marker chromosome and cross them to 5 males from a balanced line that carries another visible dominant marker (e.g., *CyO/sna*^{Sco} (BL_2555) or equivalent).

5.2.3. Collect heterozygous male progeny carrying either *Sco* or *CyO* and the potentially recombined chromosome from the cross in step 5.2.2. and individually mate them to virgin females from the stock carrying the mutated chromosome.

5.2.4. Collect the progeny from final cross described in step 5.2.3. and age the flies at 29 °C (in this study flies were aged for 10-14 days).

5.2.5. Collect heads, perform histology analysis as described in Protocol 3 and look at the slides with brain sections to determine the degree of neuropathology as described in step 4.11. Histology analysis is performed rather than locomotion analysis due to phenotypes that may affect the climbing assay, such as *Jammed (J)*, which causes fluid buildup in the wings[15].

5.3. Perform deficiency mapping on narrowed region of the chromosome to refine the location of the recessive mutation using the climbing assay. Each deficiency line has a deletion of different region of the chromosomes, allowing them to be used for complementation testing.

5.3.1. Start with large deficiencies that span the region identified in the meiotic mapping, which can then be further narrowed down by the use of smaller deficiencies. If the deficiency analysis results in more than one candidate gene, the use of RNA interference (RNAi) stocks are recommended to target them.

5.3.2. Cross lines from the *Drosophila* deficiency kit[16] for the second chromosome (DK2L in this study) and look for non-complementation of the phenotype of interest (in this study: climbing pass rate of 8.5% which corresponds to the pass rate of homozygous flies from line 867 used as

positive control).

5.3.3. Confirm the neurodegeneration phenotype using histology verification as described in Section 4 (Protocol 3).

6. Protocol 5: DNA sequencing and analysis

NOTE: Keep in mind that ENU mutagenesis introduces point mutations[11].

6.1. Design forward and reverse primer sequences flanking the exon-coding region of the *brat* gene for amplification by Polymerase Chain Reaction (PCR) of the region of interest (in the case of line 867 a DNA fragment of the size of 3,247 bp is amplified for sequencing[17]).

6.2. Extract DNA from a single fly[18] :

6.2.1. Gently squish the fly in a 0.5 mL micro centrifuge tube containing 50 µL of Squishing buffer (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl, and 200 µg/mL Proteinase K; the enzyme has to be diluted fresh from a frozen stock prior each use) using a P100 or P200 pipet tip.

6.2.2. Incubate for 30 min at 37 °C

6.2.3. Deactivate the Proteinase K by placing the tube at 95 °C for 2 min.

NOTE: The extracted DNA can be stored at 4 °C for several months.

6.3. Perform the PCR reaction using a high-fidelity DNA Taq Polymerase (reagents and thermal cycling conditions used in this study are shown in **Tables 2** and **Table 3**, respectively) by respecting manufacturer's instructions and run the PCR product on a 1% agarose gel containing Invitrogen SYBR Safe DNA Gel Stain, which is a non-toxic alternative of Ethidium bromide.

6.4. Excise the band of the correct size from the gel with a scalpel and purify the PCR product using the Wizard SV Gel and PCR Clean-Up System (Promega) or equivalent kit, by respecting manufacturer's instructions.

6.5. Design forward and reverse primers that will be used for DNA sequencing in order to cover the entire region of interest, if possible. Sequence the gel-purified DNA from the previous step to identify point mutations. High accuracy in automated fluorescent Sanger sequencing could be achieved for up to 700 bp.

NOTE: The Ex Taq polymerase (TaKaRa) can be used to amplify PCR products from genomic DNA templates up to 20 kb of size.

6.6. Analyze obtained DNA sequences using the A plasmid Editor (ApE, by M. Wayne Davis) or similar software by aligning them and comparing them to the sequences obtained following

sequencing of the same genomic region of a reference strain (e.g., originally mutagenized line).

6.6.1. Open sequencing files with ApE. As reference, use an ApE file containing the genomic consensus sequence of the *brat* gene downloaded in a FASTA format from Flybase, or a file containing results for sequence of genetic background control (e.g., originally mutagenized *Drosophila* line).

6.6.2. Go to **Tools**, then **Align sequences**. Using the computer's mouse, select all sequences to compare. Remember to indicate the reference sequence used for this alignment in the drop menu.

6.6.3. Inspect the sequenced region for nucleotide changes in comparison with the reference sequence. If desired, save the alignment on the computer in .rtf format by clicking on **Text**, then **Save**.

NOTE: If no mutations were identified, the DNA sequencing and analysis protocol would be repeated with primers designed to include non-coding regions of the gene.

7. Protocol 6: Further analysis of candidate gene function.

7.1. Perform rescue experiments in neuroblasts to determine whether the gene *brat* is responsible for the neurodegeneration phenotype.

7.1.1. Double balance a *UAS-brat* [19] and a neuroblast-specific *worniu-Gal4* (*wor-G4*) stocks carrying the constructs on the third chromosome, as well as the 867 line, which is mutant for the gene *brat* located on the second chromosome.

7.1.1.1. Make three separate crosses by placing in three different food-containing vials 5 males from *UAS-Brat*, *wor-G4* and *867* lines and add to each set of males 10-15 virgin females from a line carrying markers and balancers for the second and third chromosomes (*Sp/CyO; Dr/Tm3,sb*; BL_59967).

7.1.1.2. Collect 10-15 virgin females from the F1 of each cross with the following genotypes: *+/CyO; UAS-brat/Tm3,sb*, *+/CyO; wor-G4/Tm3,sb* and *867/CyO;+/Tm3,sb* and 5 males of each of the following genotypes: *+/Sp;UAS-brat/Tm3,sb, +/Sp; wor-G4/Tm3,sb* and *867/CyO; +/Dr*.

7.1.1.3. Cross colelcted *+/CyO; UAS-brat/Tm3,sb* virgin females to *+/Sp; UAS-brat/Tm3,sb* males; in a separate vial make a second cross with *+/CyO; wor-G4 /Tm3,sb* virgin females to *+/Sp; wor-G4/Tm3,sb* males *and then in a third vial cross 867/CyO;+/Tm3,sb* virgin females and *867/CyO; +/Dr* males.

7.1.1.4. From the F1 of each of these crosses, collect both males and females of the following genotypes: *Sp/CyO; UAS-brat/Tm3,sb* from the first cross, *Sp/CyO; wor-G4/Tm3,sb* from the second cross and *867/CyO; Dr/Tm3,sb* from the second cross.

7.1.1.5. Perform a final cross between brothers and sisters collected from each F1 progeny to establish permanent double balanced stocks for all three lines.

7.1.2. Combine *UAS-brat* and *wor-G4* with the *867* mutation.

7.1.2.1. Collect sufficient number (~20-30 flies) of virgin females from the *867/CyO; Dr/Tm3,sb* double balanced stock to perform two crosses.

7.1.2.2. Place 10-15 virgin females with ~5 males from the *Sp/CyO; UAS-brat/Tm3,sb* (cross #1) and in a second vial 10-15 virgin females with ~5 *Sp/CyO; wor-G4/Tm3,sb* males.

7.1.2.3. Collect brothers and sisters from the F1 of each cross with the following genotypes: *867/CyO; UAS-brat/Tm3,sb* from cross #1 and *867/CyO;wor-G4/Tm3,sb* from cross #2. Use collected F1 progeny from each cross to establish permanent stocks by crossing brothers and sisters between them.

NOTE: Following this final step, stocks for *867/CyO; UAS-brat/Tm3,sb* and *867/CyO; wor-G4/Tm3,sb* should be generated.

7.1.3. Perform the rescue experiment.

7.1.3.1. Collect ~15-20 virgin females from the *867/CyO; wor-G4/Tm3,sb* stock and cross them to 5-10 males from the stock *867/CyO; UAS-brat/Tm3,sb*. As control, cross ~15-20 virgin females from the *867/CyO; wor-G4/Tm3,sb* and ~15-20 virgin females from the *867/CyO; UAS-brat/Tm3,sb* line to the *867/CyO* line alone.

NOTE: *867* flies carry a temperature-sensitive allele of *brat* and the rescue cross should be performed at 29 °C.

7.1.3.2. Collect the following F1 progeny from each cross by carefully selecting away marked balancers: *867/867; wor-G4/UAS-brat* (rescue), *867/867; wor-G4/+* (control) and *867/867; UAS-brat/+* (control).

7.1.3.3. Age the flies as desired at 29 °C and perform histology analysis on brains to look for neurodegeneration by using the protocol described in Section 4 (Protocol 3).

7.2. Perform age-dependent analysis of neurodegeneration in 867 mutants.

7.2.1. Collect *867;pcna-GFP* and *yw* control flies, which carry a reporter gene (*pcna-GFP*) and are viable at 25°C and exhibit both higher penetrance and higher expressivity of the neurodegeneration phenotype than 867 alone at this temperature [17].

7.2.2. Age the flies up to 5, 15 and 25 as described in Section 1.4 and perform subsequent

histology analysis by following the protocol in Section 4 (Protocol 3).

REPRESENTATIVE RESULTS:

In this aerticle, we present the steps used to identify the gene *brain tumor* (*brat*) as playing a role in maintenance of neuronal integrity (e.g., neuroprotection) in adult flies[17]; a methodology that can be used to identify genes involved in neuroprotection. We used a forward genetic approach (the strategy is outlined in **Figure 1A**) to screen through a collection of chemically mutagenized flies using a climbing assay (the apparatus used for this assay is shown in **Figure 1B**). Among 235 homozygous lines, about 37% of tested lines exhibited a climbing pass rate below 50% when tested at the age of 10-12 days (**Figure 1C**). 42% of tested lines exhibited significantly different climbing behavior when their percent climbing pass rate (CPR) was compared to the mean climbing percent value of all tested lines using one-way ANOVA (**Figure 1D**). Subsequent histological screen on 51 of the lines exhibiting the lowest climbing pass rate revealed that 29 of these lines showed visible appearance of holes in the brain neuropil (ranging from mild to severe) indicative of neurodegeneration[20] (**Figure 2**). Among the lines showing defective climbing behavior and severe neurodegeneration, we choose to map the mutation underlying the phenotype in line 867 (0% CPR and P value= 1.36e-14 based on one-way ANOVA). The neurodegeneration phenotype in 867 flies is recessive because the brains of heterozygous 867 flies that have been outcrossed to a wild type strain are comparable to these of controls (**Figure 3A**). Using histology, meiotic mapping situated the mutation in the 31-51 cytological locations, between the phenotypic markers *J* (*Jammed*) and *L* (*Lobe*) on the second *Drosophila* chromosome. Using the climbing assay, deficiency mapping between cytological locations 31 and 51 with lines from the *Drosophila* Deficiency Kit for chromosome 2L (DK2L)[16] mapped the mutation in a region encompassing 118 genes (**Figure 3B**; where 867/+ serves as control for the statistical analysis). Examining the brain phenotype of 867 mutants crossed to additional deficiency lines (**Figure 3C**) confirmed that the mutation is contained in a region of the genome that includes the gene *brat*. A complete list of all additional genes contained within these deletions can be found in Flybase by clicking on the link associated with the deleted segment (e.g., for Df(2L)ED1272 the deleted segment is 37C5—38A2). Based on previous observations that *brat* mutants have supernumerary cells in their brains[21], a phenotype also observed in 867 mutants, *brat* was selected for DNA sequencing analysis. Sanger sequencing of the *brat* gene containing the exon-coding region identified G/A nucleotide change at position 37,739 of the gene (**Figure 4A**), leading to glycine to glutamic acid (G/E) change at position 470 of the Brat protein[17] (**Figure 4B**). Rescue experiments confirm that Brat plays a neuroprotective role, as the overexpression of the functional gene in the 867 line suppresses the neurodegeneration phenotype (**Figure 5A**). Moreover, the phenotype in 867 mutants worsens over time, suggesting that *brat* plays an age-dependent role in neuroprotection[17] (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Forward genetic screen to identify novel neuroprotective genes. (A). Screen strategy. **(B).** Climbing assay testing apparatus. **(C).** Climbing assay results for males from 235 homozygous ENU-mutagenized lines. Pie chart represents the proportions of tested fly lines that fall into 4 groups of climbing pass rates: 0% , 1-20% , 21-50% , and 51-100% . **(D).** Results from 1 way ANOVA statistical analysis in which mutant lines were compared to the mean climbing percent value of

all tested lines. Represented is the number for two P value categories: P<0.05 (significant change) and P>0.05 (not significant change).

Figure 2. Secondary histology screen. H&E-stained mid-brain sections illustrating, from left to right, no neurodegeneration, mild neurodegeneration, and confirmed neurodegeneration. A total of 51 homozygous lines were retested by histology after identification of candidates using the climbing assay. Arrows indicate the holes in the brain indicative of neurodegeneration. The region circled by the dotted lines shows the severe neurodegenration observed in line 867.

Figure 3. Identification of the neuroprotective gene *brat* following deficiency mapping using the climbing assay. (A). Shown are representative images of H&E-stained mid-brain sections of 18-20 days old w^{1118} (Control), heterozygous 867 (*867/+*) and homozygous *867* mutant flies. (**B**). The deficiency line Df(2L)ED1272 (BL_24116) does not complement the 867 mutant phenotype (black histogram) based on the climbing assay. Histological verification shows that Df(2L)ED1272 line doesn't complement the 867 neurodegeneration phenotype, whereas Df(2L)ED1315 (BL_9269) does. Graph represents mean and standard deviation of 5 climbing trials for each line. Asterisks indicate significance based on one-way ANOVA, in which the mean climbing percent value of each line was compared to the mean climbing percent value of line *867/+* (green histogram). * P<0.05, ** P<0.01 and ***P<0.001. (**C).** Schematic representation of additional deficiency lines showing non-complementation of the 867 phenotype by histology. This figure has been modified from Loewen et al.[17]; an article published under the Creative Commons Attribution 4.0 International License.

Figure 4. Point mutation in the *brat* gene leads to an amino acid change in the Coiled-coil domain of the Brat protein. (A). *brat* gene model and primers used for the amplification and sequencing of the coding region. (**B).** DNA sequencing of the *brat* locus identifies a nucleotide change that leads to an amino acid change in the Coiled Coil domain of the Brat protein. This figure has been modified from Loewen et al.[17]; an article published under the Creative Commons Attribution 4.0 International License.

Figure 5. Further analysis of *brat* mutants confirms its neuroprotective role in *Drosophila*. (**A).** Using the Gal-4>UAS system[22] , neuroblast-specific expression of the functional *brat* gene rescues the neurodegeneration phenotype in 867 mutants. (**B).** Age-dependent neurodegeneration is observed in 867 mutants carrying a reporter gene *pcna-GFP*. $brat^{chs}$ corresponds to the name of the *brat* allele in line 867, which was named *cheesehead* (*chs*). Shown are representative H&E-stained midbrain sections of *yw* (control) and homozygous $brat^{chs}$*; pcna-GFP* flies of the indicated ages. This figure has been modified from Loewen et al.[17]; an article published under the Creative Commons Attribution 4.0 International License.

Table 1. Recommended program settings for automated tissue processor. The steps in the order listed here can be used with an automated tissue processor for fixed heads.

Table 2. Reagents used for PCR reaction. Reagents and respective volumes used in the PCR reaction to amplify the *brat*-coding region.

Table 3. Thermal cycling conditions used for PCR. The steps in the order listed here can be used to program a thermal cycler using the reaction mix shown in **Table 2**.

DISCUSSION:

Forward genetic screens in *Drosophila* have been an effective approach to identify genes involved in different biological processes, including age-dependent neuroprotection[5,23-25]. Using this strategy, we were successful in identifying *brat* as a novel neuroprotective gene[17].

One critical step in this protocol involves the proper orientation of heads (as described in Section 4.4.3.) for histology analysis. Additionally, markers of the line used for the meiotic mapping should not interfere with the phenotype of the new mutation (in this study: neurodegeneration). We recommended running an initial test to confirm that the chosen markers do not cause neurodegeneration of their own. For instance, *Jammed (J)* causes wing blisters that could possibly interfere with climbing as is the case for amyloid precursor protein (APP)-overexpressing flies that also have blistered wings [26]. *Lobe (L)* leads to reduction in eye size; however, we do not observe central brain degeneration and this marker can be used to map central brain neurodegeneration. *Pin* and *Sternoplural (Sp)* are also appropriate to use, as brains of flies carrying these markers are comparable to brains of wild type controls.

One disadvantage of the forward genetic methodology in flies is the length of time required to narrow regions of DNA to the gene of interest[3,5]. The use of improved mapping strategies[27] along with the availability of next generation sequencing technologies [28] should allow even easier identification of mutations associated with phenotypes of interest. Forward genetic screens can be labor-intensive. For instance, histology confirmation, which assays directly for neurodegeneration in the brain, alone requires a considerable amount of time. However, this approach will be much more difficult and expensive to perform in mammalian models, not necessarily allowing extensive genetic studies. Chemical mutagenesis screens are advantageous because the identification of point mutations could provide critical information about the function of affected genes' products. The identification of a point mutation causing an amino acid change in a conserved domain of the Brat protein (**Figure 4B**) illustrates the importance of the coiled-coil domain of this TRIM-NHL protein in neuroprotection[18]. The climbing assay, which measures locomotor behavior, is certainly advantageous by allowing rapid testing of large numbers of flies for defects in mobility, also often seen in human neurodegeneration[25]. This assay could also be performed in another screen setting such as genome-wide in vivo RNA interference (RNAi) screen that could be used to identify neuroprotective genes. Although we decreased the distance between the bottom of the test chamber and the passing line from 8 cm [10] to 5 cm to set a stricter passing rate in the present study, low climbing rates did not mirror neurodegeneration for large number of lines. Neurodegeneration may become apparent after the onset of behavioral defects, meaning that flies may need to be aged for longer before vacuoles appear. Another possibility is that the locomotor defects we observe in certain lines are not due to defective neurological functioning but rather to muscle weakness or more general unfitness of the flies. Additionally, it is possible that we are missing potential candidates, in which climbing defects do not manifest at younger age (e.g., 10-days old) but rather become prominent

later in life. This screening strategy could certainly be applied to identify age-dependent genes, thus eliminating genes associated with potential defects of neural development. The protocol presented here can be adjusted depending on the desired probability of candidate lines to contain the mutated gene involved in neuroprotection. Lowering the passing rate threshold directly is another means of achieving the same result. These candidates would theoretically exhibit greater neurodegeneration, which could save time and resources during confirmation and mapping.

In general, the protocol describes a straightforward way to screen for neurodegeneration and subsequently identify neuroprotective gene candidates. This genetic approach is not limited to the behavior and histological assays described here, and may also be used to screen for other phenotypes like temperature-sensitive (ts) paralysis, egg laying or fertility phenotypes, just to cite a few. For instance, *Drosophila* ts-paralytic mutants are known to be enriched for neurodegeneration[29] and could represent an additional source for the identification of neuroprotective genes.

ACKNOWLEDGMENTS:

We are particularly grateful to Dr. Barry Ganetzky, in who's lab the genetic screen was performed, allowing the identification and characterization of *brat* as a neuroprotective gene. We thank Dr. Steven Robinow for kindly providing the collection of ENU-mutagenized flies used in the genetic screen presented in this article. We thank Drs. Grace Boekhoff-Falk and David Wassarman for helpful discussions throughout the duration of this project, Ling Ling Ho and Bob Kreber for technical assistance, Dr. Aki Ikeda for the use of his microtome facility at the University of Wisconsin and Dr. Kim Lackey and the Optical Analysis Facility at the University of Alabama.

DISCLOSURES:

The authors declare no conflicts of interest.

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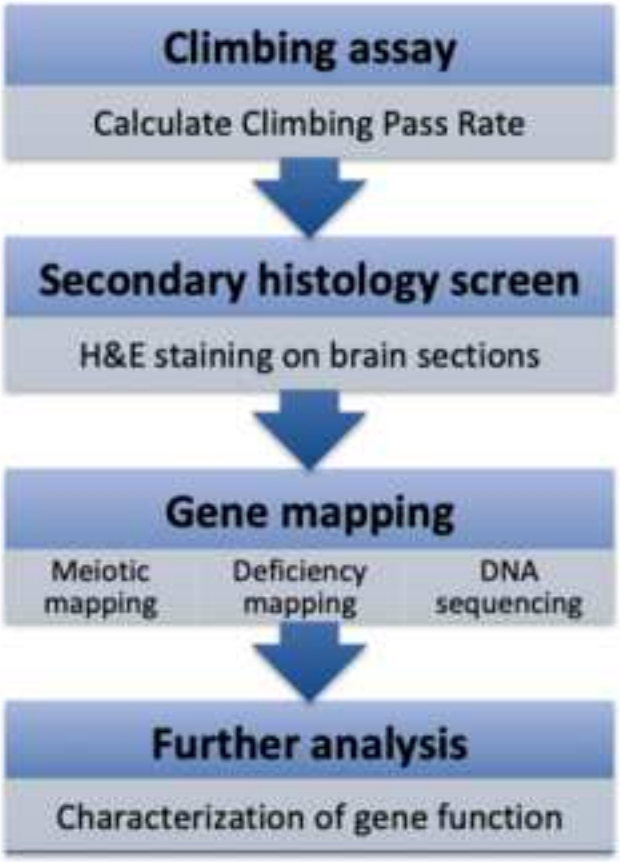
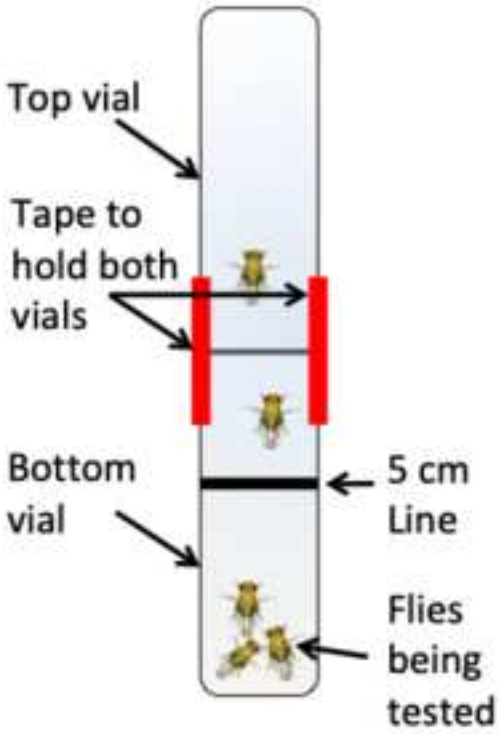
Figure 1

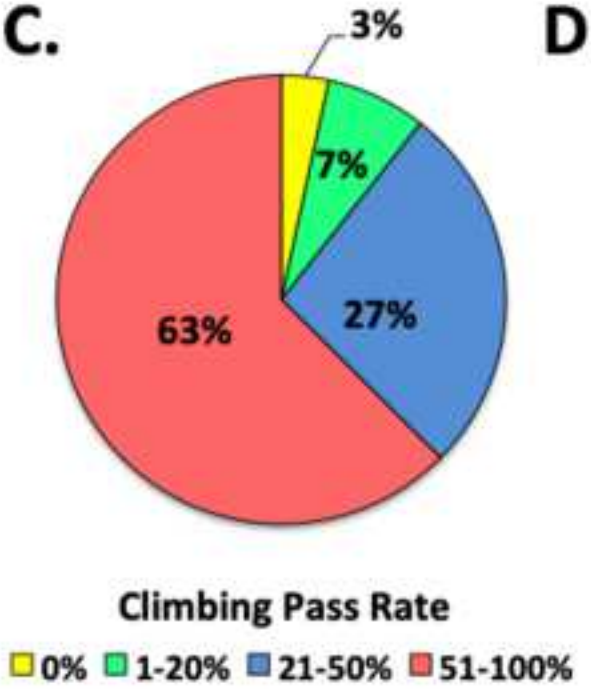
A.



B.



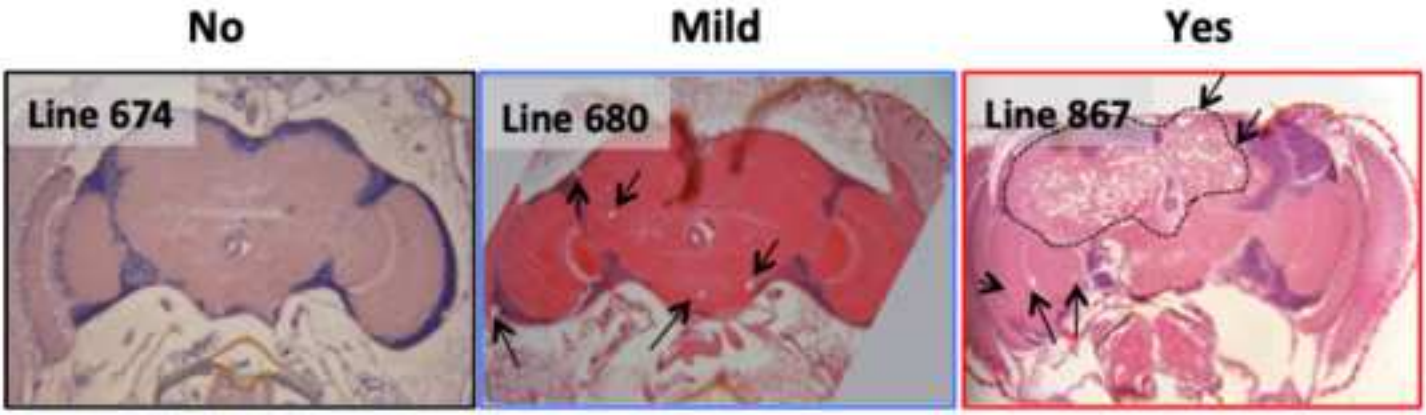
C.



D.

	Non-significant (P>0.05)	Significant (P<0.05)
Number of lines	99	136

Figure 2



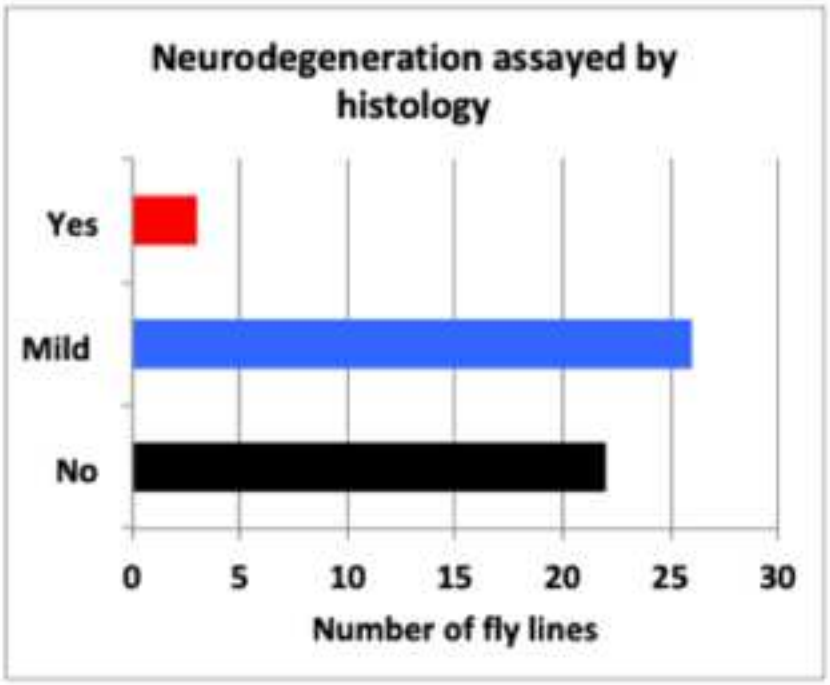


Figure 3

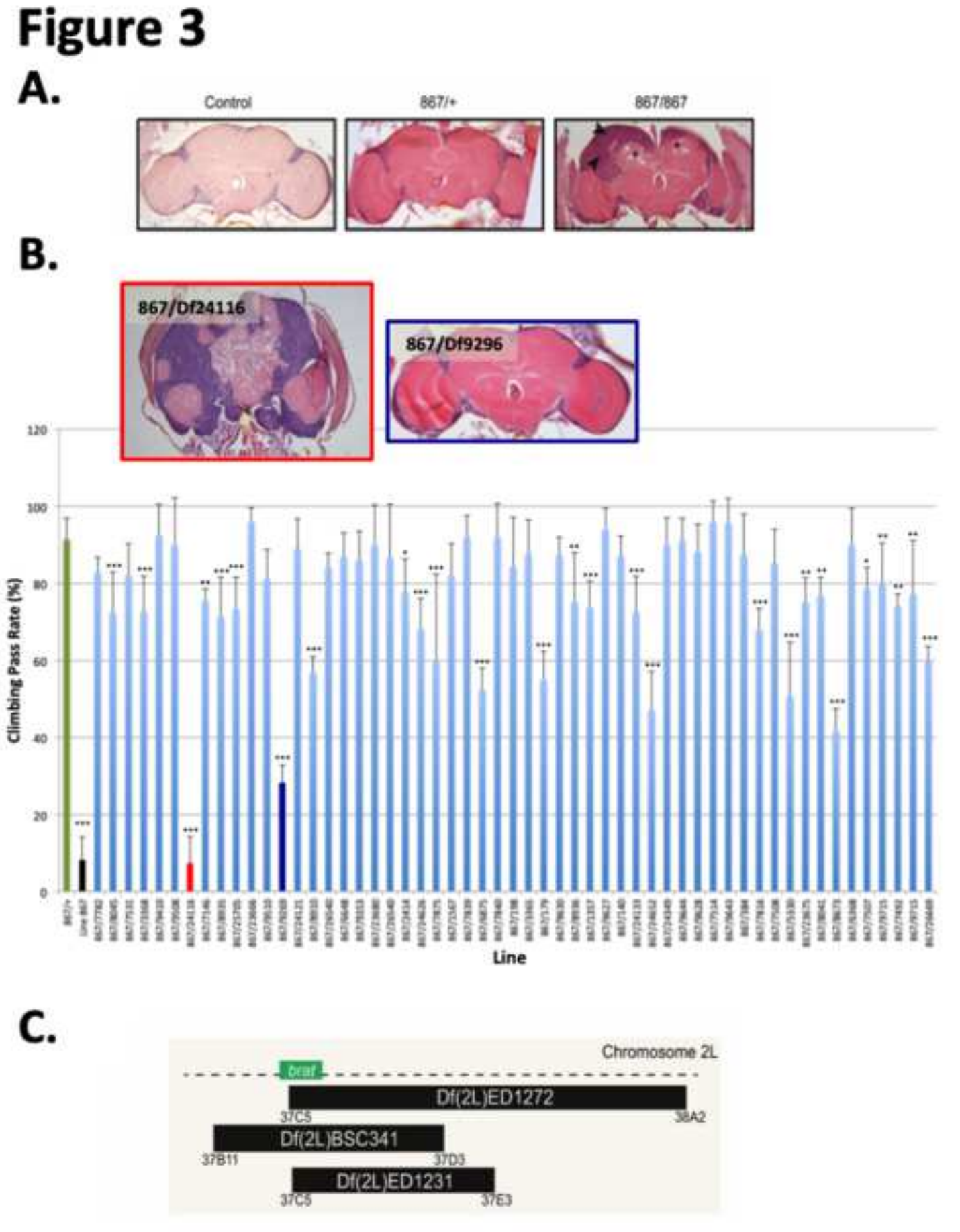
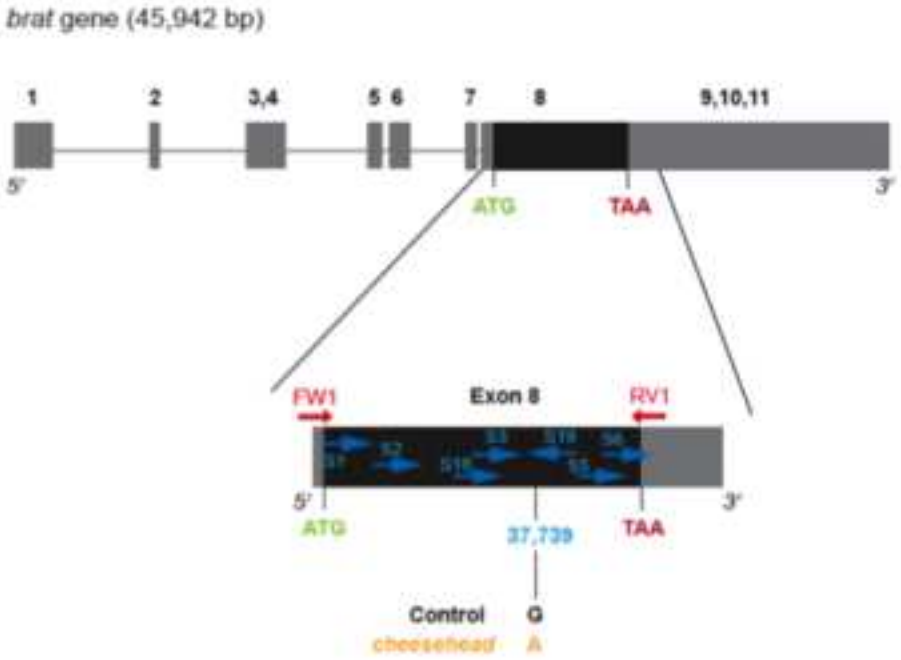


Figure 4

A.



Primers used to amplify 3,247 bp DNA fragment for sequencing (5'-->3')

FW1: AATCCCTCCACTTGTTCT
RV1: CAGATCCTCCTCCTTTCC

Primers used for sequencing (5'-->3')

FW1: AATCCCTCCACTTGTTCT
S1 FW: CAAACAATGGCGGCAACT
S2 FW: AAATTCCCAGGAGCAACA
S18 FW: AATCCCTCCACTTGTTCT
S3 FW: ACCGAATTGCTCAAGGAA
S19 RV: GTCTGGTGATGATTGTGGTGGCC
S5 FW: CTATCTATCCGCCCAAGT
S6 FW: CAAGTTCGGGTGCTCTAA
RV1: CAGATCCTCCTCCTTTCC

B.

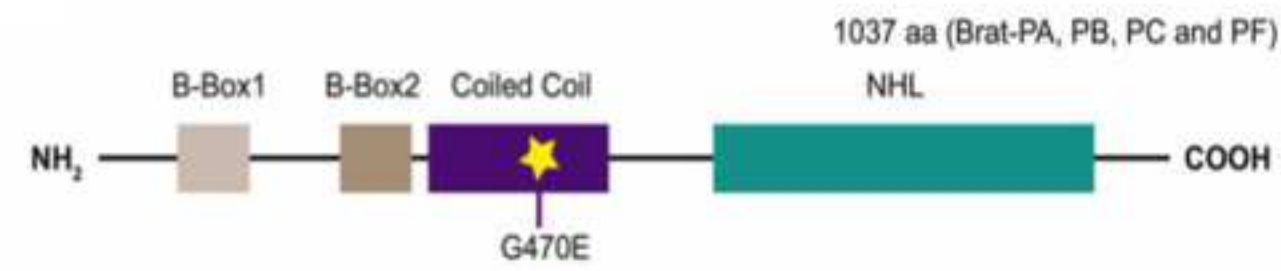


Figure 5

A.

B.

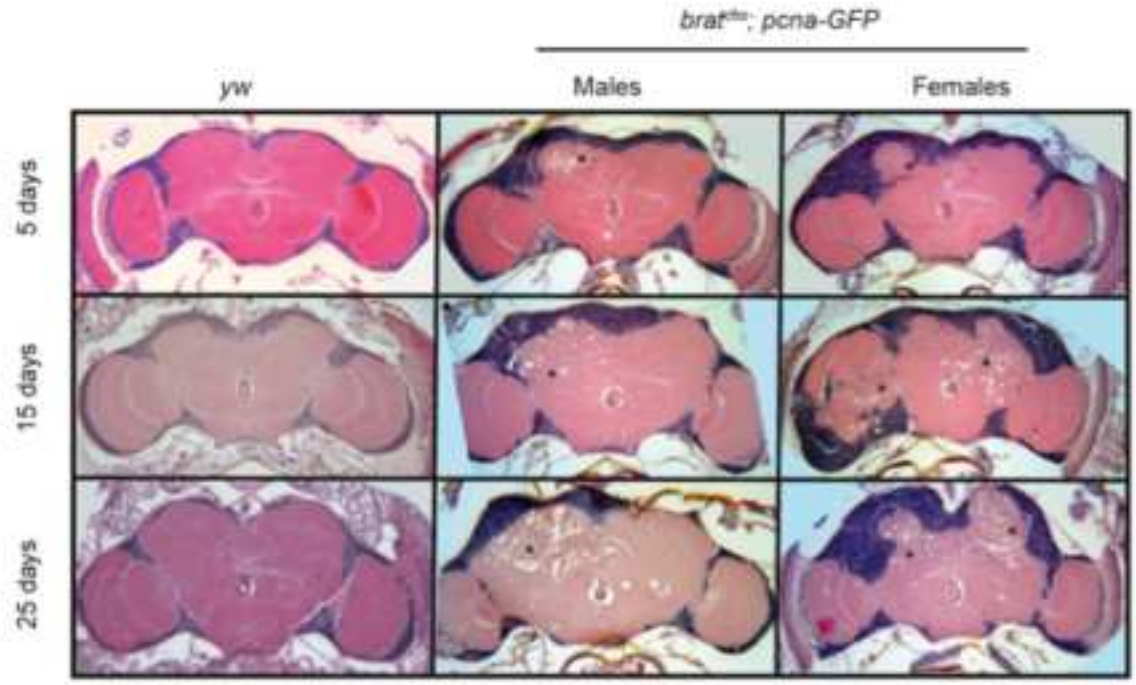


Table 1. Recommended program settings for automated tissue processor

Station	Time	Temperature	Vacuum/ Pressure	Solution
1		**NO DELAY-QUICK START**		
2	OFF	OFF	OFF	
3	: 15	37°C	ON/ON	80%EtOH
4	: 15	37°C	ON/ON	95% EtOH
5	: 15	37°C	ON/ON	100% EtOH
6	: 15	37°C	ON/ON	100% EtOH
7	: 15	37°C	ON/ON	100% EtOH
8	: 15	37°C	ON/ON	Xylenes
9	: 15	37°C	ON/ON	Xylenes
10	: 15	37°C	ON/ON	Xylenes
11	: 30	58°C	ON/ON	Paraffin
12	: 15	58°C	ON/ON	Paraffin
13	OFF	58°C	OFF	Paraffin
14	: 15	58°C	ON/ON	Paraffin

Table 2. Reagents used for PCR reaction

Reagent	Volume (µL)
10x ExTaq Buffer (Mg2+ plus) (20mM)	2.5
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
2.5mM dNTPs	2
Template DNA	2
TaKaRa Ex Tq (5 U/µL)	0.25
Nuclease-free water	13.25
Total volume	25

Table 3. Thermal cycling conditions used for PCR

Step	Temperature	Time
35 cycles	95°C	15 s
	55°C	45 s
	72°C	3 min 10 s
Hold	4°C	∞

Table of Materials

Click here to access/download;Table of Materials;Materials table-3.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Major equipment			
Fume hood for histology			
Light Microscope	Nikon	Eclipe E100	Preferred objective for imaging is X20
Imaging software	Nikon		
Microscope Camera	Nikon		
Thermal cycler	Eppendorf		
Fly pushing and climbing assay			
VWR® Drosophila Vial, Narrow	VWR	75813-160	
VWR® General-Purpose Laboratory Labeling Tape	VWR	89097-912	
Standard mouse pad			
Stereoscope	Motic		Model SMZ-168
CO2 anesthesia station (Blowgun, foot valve, Ultimate Flypad)	Genesee Scientific	54-104, 59-121, 59-172	Doesn't iinclude CO2 tank
Fine-Tip Brushes	SOLO HORTON BRUSHES, INC.		
Drosophila Incubator	VWR	89510-750	
Gene mapping			
CantonS	Bloomington Drosophila Stock Center	9517	
w^{1118}	Bloomington Drosophila Stock Center	5905	
yw	Bloomington Drosophila Stock Center	6599	
Drosophila line used for recombination mapping	Bloomington Drosophila Stock Center	3227	Genotype: wg[Sp-1] J[1] L[2] Pin[1]/CyO, P{ry[+t7.2]=ftz/lacB}E3

CyO/sno[Sco]	Bloomington Drosophila Stock Center	2555	Drosophila balancer line used for recombination mapping
Deficiency Kit for chromosome 2L	Bloomington Drosophila Stock Center	DK2L	Cook *et al* ., 2012

Histology analysis

Ethanol, (100%)	Thermo Fischer Scientific	A4094	
Chloroform	Thermo Fischer Scientific	C298-500	
Glacial Acetic Acid	Thermo Fischer Scientific	A38-500	
Fisherbrand™ Premium Microcentrifuge Tubes: 1.5mL	Thermo Fischer Scientific	05-408-129	
Histochoice clearing agent 1X	VWR Life Sciences	97060-934	
Harris Hematoxylin	VWR	95057-858	
Eosin	VWR	95057-848	
Thermo Scientific™ Richard-Allan Scientific™ Mounting Medium	Thermo Scientific™ 4112	22-110-610	
Unifrost Poly-L-Lysine microscope slides, 75x25x1mm, EverMark Select Plus	Azer Scientific		
Fisherbrand™ Cover Glasses: Rectangles	Fisherbrand	12-545M	Dimensions: 24x60 mm
Traceable timer	VWR		
Slide Warmer	Barnstead International		model no. 26025
Slide tray and racks	DWK Life Sciences		Rack to hold 20 slides
Fisherbrand™ General-Purpose Extra-Long Forceps	Fisherbrand	10-316A	
Kimwipes™	Kimberly-Clark™ Professional		
6 inch Puritan applicators	Hardwood Products Company, Guilford, Maine	807-12	
VWR® Razor Blades	VWR	55411-050	

Tupperware or glass containers for histology liquids			16 + 1 for running water
High Profile Coated Microtome Blades	VWR	95057-834	
Corning™ Round Ice Bucket with Lid, 4L Beaker	Corning™		Or other container for ice water and cassettes
Tissue Bath	Precision Scientific Company	66630	
Microtome	Leica Biosystems		

Molecular analysis

Wizard® SV Gel and PCR Clean-Up System	Promega	A9282	
Ex Taq DNA polymerase	TaKaRa		5 U/µl
Invitrogen™ SYBR™ Safe™ DNA Gel Stain	Invitrogen™		
UltraPure™ Agarose	Invitrogen™		
1 Kb Plus DNA Ladder	Invitrogen™		
ApE-A plasmid Editor software			Available for free download

Statistical analysis

R software package

Further analysis

y[1] w[*]; wg[Sp-1]/CyO; Dr[1]/TM3, Sb[	Bloomington Drosophila Stock Center	59967

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Author(s): Olivia Grevedon, Harris Bolus, Shu Hui Lye, Keaton Schmitz, Jesualdo Fuentes-Gonzalez, Kathryn Hatchell, Lyndsey Bley, Jason Pienaar, Carla Loewen, Stanislava Chtarbanova

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Editorial comments:

The manuscript has been modified and the updated manuscript, **59720_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
This point has been addressed.

2. The highlighted protocol steps are over the 2.75 page limit (including heading and spacing). Please highlight fewer steps for filming.
This point has been addressed.

3. Please number all figures in the order of their appearance in the manuscript. For example, figure 3 was mentioned after figure 1 and before figure 2, so it should be numbered as figure 2.
This point has been addressed. All Figure citations have been removed from the Protocol section and appear now in the right order in the text of the Representative results section. The text in the Representative results section has been updated accordingly.

4. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
This point has been addressed in the Protocol sections.

5. Figure 4: Please add a title for the whole figure in figure legend.
This point has been addressed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Chtarbanova et al present a nice overview of the use of a forward genetic screen in Drosophila melanogaster to identify genes that lead to neurodegeneration when dysregualted. They describe a method where they test strains that have been mutagenized by exposure to N-ethyl-N-nitrosurea (ENU) for a defect in negative geotaxis in adult flies aged at 29oC for 10-12 days. The flies with a negative geotaxis defect are decapitated and the heads are fixed and the integrity of the brain is examined using standard histology and microscopy techniques. Flies with neurodegenerative vacuoles in the brain are selected as candidates for gene mapping which they do by meiotic recombination, deficiency mapping and DNA sequencing. They present data of a gene called brat that they identified as being mutated and causative of the negative geotaxis defect and brain degeneration, they prove this by expressing the normal form of this gene in the mutant background and observe rescue of brain degeneration.

Overall, this article is interesting and useful but needs further work before it is ready to replicate as a method which is a requirement for publication in JoVe. I also do not agree with the title "In vivo forward genetic screen to identify novel neuroprotective genes in Drosophila melanogaster" the screen identifies genes that when dysregulated lead to neurodegeneration not neuroprotection. I think one of the strengths of this paper is the elegant use of complicated Drosophila genetics. These genetic approaches were established decades ago, and nowadays they receive little attention, as a result students and post docs lack a critical understanding of this type of Drosophila genetics. I think this will be a great resource if the authors generate a video which also includes a description and discussion of the genetics on a white board. I have detailed my suggestions for the protocol and places where I think a video would be extremely helpful in the major comments section.

We thank the reviewer for the thoughtful comments, which we think helped to improve the quality of our manuscript. In response to the reviewer's comment regarding the article's title, we would like to explain our reasoning behind the term "neuroprotective genes". In our opinion, there are mechanisms that maintain central nervous system integrity throughout life, preventing brain cells' death. We call genes encoding factors involved in such processes "neuroprotective". We reason that deregulation of neuroprotective genes as result of mutations could lead to improper functioning of the factors they encode resulting in neurodegenerative phenotypes that reflect the loss of neuroprotection.

Major Concerns:
Introduction
A. You should state that EMS and ENU mutagenesis introduce point mutations whereas X-rays introduce DNA breaks and as a result sometimes DNA rearrangements.
We thank the reviewer for pointing this out. This point has been addressed and a reference provided (see lines 56-59 of revised version).

Methods:
The protocol section:
Preparation
A. The preparation section describes collecting and aging flies and assembling the negative geotaxis apparatus. The title should be changed to reflect this, "Preparation and aging of flies???".
The title of this section has been changed as per reviewer's recommendation.

B. Had the ENU flies been previously mapped to a specific chromosome? If so or if not that should be described.
Indeed, the collection of flies used in this study has been previously mapped to the second chromosome. This information has been now included in the revised manuscript (line 92).

C. Are the ENU flies heterozygous over a balancer, or homozygous? If they are over a balancer is the balancer present in the aging flies, or have they been outcrossed, or are you using homozygous flies? Is the mutation/phenotype you are looking for dominant?
The ENU-mutagenized flies were balanced over CyO with homozygous flies present in most stocks. Although we have tested both, homozygous flies and flies carrying the balancer, we have now incorporated data for homozygous flies only and have updated figures and text accordingly (see lines 93 and 98 and Figures 1C, 1D, and Figure 2 of the revised manuscript). Among these lines, homozygous flies from line 867 exhibited defective climbing phenotype. We therefore looked for recessive mutations/phenotypes.

D. The light conditions in the incubator should also be described; was it a 12 hr light/dark cycle or a 24 hr light cycle?
This point has been addressed (line 95 in the revised manuscript).

E. What was the food that the flies were reared and aged on?
This point has been addressed (lines 96 and 101 in the revised manuscript).

F. A whiteboard video should be generated showing these ENU flies and whether they were outcrossed.
As only data for homozygous flies is presented in the revised manuscript, we

decided not to incorporate this information at this step. The homozygous ENU lines will be still shown in the white board video, in which crossing schemes for recombination and deficiency mapping will be outlined.

Protocol 1: Climbing assay
A. A video of the flies being put into the chamber, put aside, and then tapped down 3 times for the assay would be beneficial, as would doing it side by side with normal flies and flies with a negative geotaxis defect. The person should explain during this time in the video what negative geotaxis is.
We thank the reviewer for making this recommendation. We will present the apparatus in the video and explain what the negative geotaxis is.

B. There is no mention of a positive control. What was the fly strain used as a control for normal geotaxis? This should be detailed in this description and video.
Prior initiation of the screen, the efficiency of the climbing assay was tested by determining climbing pass rates for 10-days-old flies in which Tau^{R406W}-, GFP-(control) and mCherry (control) are overexpressed in the brain using the pan neuronal $Elav^{C155}$-Gal4 driver. As previously reported, 10-days-old Tau-overexpressing flies exhibited climbing defects in comparison with GFP- and mCherry-overexpressing flies (Ali et al., 2011). We have included a new section (Section 2.1) describing the use of this control experiment in the revised manuscript (see lines 111-114).

C. Line 99: "about 3 times" does this really change between repeats? Or is it 3 times?
We have corrected the text to 3 times (line 125).

D. The time of day that the negative geotaxis assay was performed should be described as flies behave differently at different times of the day.
All climbing assays were performed in the afternoon. This information along with a reference has now been included in the text (lines 117-119).

E. Decapitation should be in the histology section and not in this section.
This has been changed according to the reviewer's recommendation (see lines 319-322 of revised text).

Protocol 2: Select candidates
A. This should read "Selection of Drosophila strains for further analysis"
The title of the section has been changed (line 145).

B. In general, I do not think this section is clear or correct. To determine which flies strains have a negative geotaxis defect the mutant strains should be compared to an age-matched control genotype. The percentage of flies that pass the line (2cm/5cm/8cm) should be calculated and plotted in excel or a statistics

program such as prism. Statistics should be used to determine if a mutant line is different to the control strain. A T test for comparing 2 lines, a one-way ANOVA (plus post-hoc test) for comparing three or more strains, and two-way ANOVA (plus post-hoc test) for comparing two or more lines at two or more time points (e.g. 10 days vs 20 days). This should be incorporated into this section.
We thank the reviewer for making this point. We agree that a statistical test would be appropriate to perform and for the 235 ENU lines we have done the analysis by comparing the mean climbing percent value of all lines (which corresponds to 59%) to each individual mutant line using one-way ANOVA. We have incorporated a table in Figure 1 (Figure 1D), which shows the number of lines showing significant P-Values. The information for line 867 has been updated in the Representative results section of the text (Line 849).
Similarly, we analyzed using one-way ANOVA the data for the deficiency mapping and have now added error bars corresponding to standard deviations and additional age-matched controls (867/+ and 867/867) in the same graph. Additionally, we have incorporated a detailed protocol on how the statistical analysis was performed using the R software package (see section 3.2. in the revised manuscript, lines 150-151).

Protocol 3: Histology analysis
A. The decapitation and fixation of fly heads should be in this section, and a video of the decapitation and placing the flies in the tube of fixative solution is needed.
We will present this in the video file (text has been highlighted in the text accordingly).

B. Do you add any tissue to push heads down into the fixative?
Using a paintbrush heads are gently placed in the tube containing the fixative, without touching the liquid. As per reviewer's recommendation, we will show this step in the video. This information has been added to the text (Line 206).

C. You need to describe how the tubes are stored, are the still or rotating?
Tubes for both, fixative and EtOH70% are still; we have incorporated this information in the revised text (lines 207 and 211).

D. You need to describe the embedding procedure, the chemical used, the time of incubation, the temperature and whether a vacuum was used on any of the incubations.
We have incorporated a table that includes the conditions and chemical reagents used for the automated processor as well as a new section detailing the embedding procedure (Section 4.4., lines 220-244).

C. A description of the embedding of fly heads into the paraffin block is needed by video on a white board to show the orientation of the head when embedding and

in relation to the blade when cutting, and also of the person embedding the head, and any tricks you use to make sure it's orientated.
As per reviewer's recommendation, we will show this step in the video.

D. The trimming of the block should be in the video as should the sectioning, floating out and mounting the wax ribbon onto slides.
As per reviewer's recommendation, we will show these steps in the video.

E. Hematoxylin and eosin stain is fine in the text; this can also be videoed (start with putting slides in first trough, then explain the next steps with voice over and text, video the slides in running tap water so that the reader/viewer can see where the water hits in relation to the slides, then voice over with the next steps and video the slides being mounted.
This is a very helpful suggestion. We will show the relevant steps in the video.

F. Quantification of neurodegeneration needs more explanation. Do you look at the whole brain, a specific region, is it qualitative or are you counting vacuoles bigger than a certain size? You should also mention the statistics used here.
This point has been addressed (see Section 4.11 and lines 306-308 in the revised text).

Protocol 4: Gene mapping
A. Overall the genetics in this section could be made clearer. For example, an introduction paragraph (few sentences) describing the two approaches and why you would use them in the order that you do them in, same for the video.
We have incorporated relevant information in the introduction (lines 72-81).

B. Did you know the affected chromosome before starting these crosses?
Please see response to comment "Preparations: B" above.

C. I think at least you need both sets of crosses (meiotic mapping and deficiency crosses) in a figure but ideally, you should write out the crosses one by one on a white board and video yourself talking through the cross. For meiotic recombination you should give an idea of what ratios you would expect in your progeny, maybe give an example where the mutation is close to one of the markers and a second where it is far away.
We thank the reviewer for making the recommendation to write the crosses on the white board. We agree that this will be helpful for the audience to better visualize the mapping strategies. We will show these crossing schemes in the video.

D. Line 174: You state that you should use markers that should not induce neurodegeneration in the meiotic mapping, you need to make a table with Drosophila genotypes that are good for this purpose and those that are not good

for this purpose. Or at least of those that you would recommend to use or avoid. This can also go in the video.
Because of limitations in text length in protocol sections and notes, we have included information about the use of markers in the Discussion section of the revised manuscript (see lines 633-641). We hope the reviewer finds them satisfactory.

E. Line 183: Why in the meiotic mapping did you perform histology analysis and not locomotor analysis? You should explain this or describe that you could do both.
The mapping line had the jammed wings, which supposedly could interfere with the climbing ability, as reported for flies with blistered wings (Peng et al., 2015) This point has been addressed in the revised text (see lines 358-359 and 636-638).

F. You also need to state what you are looking for in 5.1.5 and what this means in respect to the mutation's position on the chromosome.
This information has now been added to the text (lines 685-686).

G. You have not described what a deficiency line is. This needs to be made clear for people to understand as should the break size and the number of genes that are missing from the deficiency line.
A deficiency line has been defined in the revised text (see lines 356-357).

H. Complementation should be described as should the mutant phenotype that you have is it recessive or dominant, what you are looking for in the progeny would change depending of this. If it is dominant "a deficiency line that lacks the region that is mutated will not rescue the locomotor defect, while deficiency lines that harbor the normal form of the mutated gene will rescue the dominant mutant phenotype" and explain what this means to the position of the mutation."
This is an excellent point and we appreciate the reviewer's comment. We have changed the title of the paragraph to "Gene mapping of recessive mutant phenotype" (Line 313) and included an additional step 5.1 that directs the reader how to test whether the phenotype of the line of interest is dominant or recessive.

I. Did you do an initial test with a large deficiency line and then narrow down further with additional deficiency lines? If so this should be described.
We used smaller deficiencies that spanned (or not) the brat locus to confirm the phenotype and the spanning ones are showing in Figure 3C. In addition to the neurodegeneration phenotype in 867 flies, we also observed a known supernumerary cell phenotype for the brat gene; this is what directed us to focus on the brat and test additional deficiency lines. This point is discussed in the Representative results section.

J. It would be beneficial to also describe what else you would do should the analysis with the deficiency lines give you more than one candidate gene e.g. RNAi lines to those target genes.
This point has been addressed as suggested by the reviewer. Refer to text in Section 5.3.1 (lines 366-368).

Protocol 5: DNA sequencing
A. You need to reiterate that ENU mutagenesis introduces point mutations.
This has been addressed by including a note (line 380).

B. Did you only sequence coding regions of the gene?
Indeed, in this study we did sequence the coding region of the brat gene. A new figure (Figure 4A) has been included, showing the region sequenced and providing the sequences of the primers used for both amplification of the fragment and for sequencing. Additionally, we have incorporated technical information about the DNA extraction, reagents and conditions needed to perform the PCR reaction as well as the steps needed to align the sequences using the ApE software (please, see Section 6.1. and lines 420-435).

C. What would you have done if there was no point mutation in the coding sequence?
We have added a note addressing this point (lines 437-438)

Protocol 6: is missing
A. You should describe the work you did to prove that brat was the gene i.e. the UAS-brat experiments.
We thank the reviewer for pointing this out. We have included a new section 7: Protocol 6: Further analysis of candidate gene function (lines 440-521). This section details the crosses needed to generate rescue stock lines, the analysis done including rescue experiments and characterization of age-dependent phenotypes. Additionally, a new figure (Figure 5) accompanies this section.

Discussion:
A. Line 277: You state that neurodegeneration often doesn't mirror a behavior defect. You should mention that this could be because the behavioral defect comes before the loss of brain integrity and flies may have to be aged for longer before vacuoles appear.
This point has been addressed as suggested by the reviewer (lines 665-667).

B. You should also discus that an additional means to narrow down candidate strains could be to identify those that have no defect in negative geotaxis at young ages but develop the defect at older ages. In this way, you remove the strains that are born with a defect i.e a developmental effect and select those

with an adult and age-dependent effect, or vice versa.
This point has been addressed as suggested by the reviewer (lines 1009-1013).

C. You can discuss that this genetic approach is applicable to other behaviors and phenotypes e.g. egg laying, fertility etc.
We have addressed this point by adding examples of other phenotypes as recommended by the reviewer (lines 669-673).

Figure 1:
A. A photograph or cartoon of the vials used in the negative geotaxis assay is needed.
We have added a cartoon to visualize the testing apparatus as part of Figure 1 (Figure 1B).

Figure 2:
A. In panel A you maybe want to put line 674 first and title it NO/normal? and then the worst degeneration last.
We thank the reviewer for making this suggestion. We have exchanged the images as recommended.

Figure 3:
A. The graph in panel does not have a label on its X axis.
This has been fixed. Thanks for pointing this out.

B. There should be standard deviation bars to represent the three repeats, and statistics.
We have addressed this point by adding error bars as recommended by the reviewer.

C. Panel B indicate where the other genes are in relation to brat and the other deficiencies.
As 118 genes is a substantial number to add to the figure, to address this point, we have included a sentence in the representative results section thet indicates how to obtain the information regarding the genes located in the covered region by consulting Flybase (lines 547-549).

Figure 4
A. Panel D should also have a normal brain. Add CS from paper
To address this point we have included a figure in Figure 3A to show brain neurodegeneration phenotype in controls, as well as in heterozygous 867 flies.

Minor Concerns:
Line 62 and 63: Although I think it is important to that you discuss the limitations of your technique you should change words like tedious/laborious to extensive,

for example. Maybe in the discussion you should say that although this approach involves extensive fly genetics these studies would be more difficult in a mammalian setting. And talk about the benefit of doing this over RNAi screens, e.g. point mutations may tell us critical things about the protein/enzymes function, and vice versa the benefit of an RNAi screen that could also be used with the same negative geotaxis response and histology.
We appreciate these comments. They have been addressed in the discussion.

Reviewer #2:
Manuscript Summary:
The manuscript describes a method to identify neuroprotective genes in a classical forward genetic screen. The protocol is based on established methods but has been assembled for the purpose of identifying such genes. However, there are two major concerns that should be addressed before publication.

Major Concerns:
1) In the discussion the authors mention that most of the lines selected from the climbing assay did not show neurodegeneration in the histology. They propose that this could be solved by lowering the passing rate threshold. However, a climbing phenotype could be due to changes in muscles or general unfitness of the flies and is not necessarily due to neurodegeneration. This major limitation of using the climbing assay should be discussed
We thank the reviewer for making this recommendation. We have edited the discussion and hope the reviewer will find it satisfactory.

2) Although the brat flies do show neurodegeneration there is a very prominent phenotype of supernumerary cells suggesting that there are major defects in brain development. Therefore this is not necessarily a neuroprotective gene that maintains neurons during aging but a gene that affects brain development and thereby causes cell death. Especially because no age is mentioned in the figure legends the degeneration could occur early on; so is there a difference in young versus old mutants?
We thank the reviewer for bringing this point up. Brat's function has been extensively studied during development of the nervous system and a role for this protein in neural stem cell division has been previously established. Additionally, we also see supernumerary cells in the brains of 867 flies. Although to date we haven't been able to uncouple the supernumerary phenotype from the neurodegenerative phenotype in these mutants, we have observed that 867 mutants exhibit age-dependent worsening of the neurodegenerative phenotype. To address the reviewer's point we have included a new figure (Figure 5), which illustrates this age-dependent phenotype.

Minor Concerns:
The authors should include references for performing the screen. So even if they

obtained the lines form another laboratory, other labs might have to perform the mutagenesis and therefore references how to do this should be included.
This point has been addressed by adding a reference on line 91 (Section 1.1).