COVER SHEET

TITLE: **A ts-paralytic neurodegeneration mutant in *Drosophila* reveals differential requirement for the glycolytic gene *Aldolase* in neurons and glia**

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A ts-paralytic neurodegeneration mutant in *Drosophila* reveals differential requirement for the glycolytic gene *Aldolase* in neurons and glia

ABSTRACT

Several neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease have been linked to metabolic disruption in neurons. This suggests that neurons are particularly sensitive to problems in energy metabolism. However, a controversy currently exists regarding the role of glycolysis in neurons. One hypothesis, the astrocyte-neuron lactate shuttle (ANLS) model, proposes that neurons do not perform their own glycolysis but depend on lactate provided by glial cells as an energy substrate for ATP production. A neurodegeneration mutant in *Drosophila melanogaster*, known as *M4*, has been identified and mapped to a small region containing the gene that encodes aldolase. Aldolase is a vital glycolytic enzyme, and a mutation in its coding sequence would likely cause energy deprivation, possibly leading to degeneration of neural tissue and early death. These *Drosophila* mutants provide a model system to investigate the energy requirements for neuronal viability. To test the ANLS hypothesis, tissue-specific rescue experiments were performed, in which aldolase function was selectively restored to mutants in their neurons and glia individually. The results have shown that, contrary to the ANLS model, neuronal glycolysis is required for viability.

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A ts-paralytic neurodegeneration mutant in *Drosophila* reveals differential requirement for the glycolytic gene *Aldolase* in neurons and glia

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Abstract

Several neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease have been linked to metabolic disruption in neurons. This suggests that neurons are particularly sensitive to problems in energy metabolism. However, a controversy currently exists regarding the role of glycolysis in neurons. One hypothesis, the astrocyte-neuron lactate shuttle (ANLS) model, proposes that neurons do not perform their own glycolysis but depend on lactate provided by glial cells as an energy substrate for ATP production. A neurodegeneration mutant in *Drosophila melanogaster*, known as *M4*, has been identified and mapped to a small region containing the gene that encodes aldolase. Aldolase is a vital glycolytic enzyme, and a mutation in its coding sequence would likely cause energy deprivation, possibly leading to degeneration of neural tissue and early death. These *Drosophila* mutants provide a model system to investigate the energy requirements for neuronal viability. To test the ANLS hypothesis, tissue-specific rescue experiments were performed, in which aldolase function was selectively restored to mutants in their neurons and glia individually. The results have shown that, contrary to the ANLS model, neuronal glycolysis is required for viability.

Introduction

Neurodegeneration is the cause of several diseases in humans, such as Huntington's, Parkinson's, and Alzheimer's diseases, which have been linked to genetic mutations. *Drosophila melanogaster* has served as a useful model for the identification and study of mutations that lead to these conditions.[i,ii,iii] The Ganetzky lab performed a forward genetic screen to isolate genes required to protect neurons from premature death in *Drosophila*. One of the mutants isolated, *M4*, exhibits several notable phenotypes: temperature sensitive (TS) paralysis, shortened lifespan, and progressive degeneration of brain tissue. Through deletion mapping and sequencing, the *M4* phenotypes were attributed to a mutation in the gene encoding *Aldolase*, an enzyme that

functions in glycolysis to catalyze the breakdown of fructose-1,6-biphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

The overall goal of this study was to investigate the requirement for glycolysis for neuronal viability. Importantly, many neurodegenerative diseases are associated with mitochondrial dysfunction leading to impaired energy metabolism.[iv] We hypothesize that a mutation in the *Aldolase* gene causes an energy deficit that affects neurons more than other tissues. Since neurons are highly metabolic, a mutation in *Aldolase* could have a strong effect on these cells. Metabolic mutations in a model organism that result in neurodegeneration allow us to ask if neurons are especially sensitive to glycolytic disruption by restoring aldolase function to neurons in mutant flies and determining the effect on lifespan.

In addition, though disruption of mitochondrial metabolism has been clearly linked to neuronal dysfunction,[iv] the role of glycolysis in neurons is a matter of some disagreement. An ongoing question concerns the division of metabolic processes between neurons and glial cells. One model, the astrocyte-neuron lactate shuttle (ANLS), proposes that neurons rely on external lactate, provided by glycolysis in glial cells, as a substrate for ATP production.[v] However, other studies show that neurons use lactate only under very specific conditions, and under physiological conditions, their main energy substrate is glucose.[vi] Aldolase mutants in *Drosophila* will allow us to address the issue of energy production in brain tissue directly and *in vivo*, by testing whether selectively restoring glycolytic function to neurons or glia suppresses the phenotypes of *M4*.

Results

Complementation testing placed *M4* in a genomic region spanning *Aldolase* and *CG6154*

Previous deficiency mapping had restricted the location of the *M4* mutation to the right arm of the third chromosome (D.L. Miller, personal communication, 2008). To narrow this region further, *M4* mutants were crossed stocks carrying chromosomal deficiencies in the *M4* region. The deficiencies used were Tl-P (stock 1910), BSC496 (stock 25000), BSC512 (stock 25016), and Espl3 (stock 5601).

The F_1 progeny were tested for complementation of the *M4* TS-paralysis phenotype. When *M4* homozygotes are placed at 37°C, they begin to become immobilized within approximately four minutes, with complete paralysis by eight minutes. TS tests for the deficiency crosses revealed that *Espl3/M4* flies did not exhibit the TS phenotype, while *BSC512/M4, BSC469/M4*, and *Tl-P/M4* were temperature sensitive (Figure 1A). This indicated that the *M4* mutation was located in the region of overlap between BSC512, BSC469, and Tl-P. This region contains two genes: *Aldolase*, which encodes a glycolytic enzyme; and *CG6154*, an uncharacterized gene.

Lifespan analysis revealed that *M4* flies exhibited a significantly shorter lifespan than wild-type (CS) flies and *M4/+* flies. With respect to the deficiency stocks crossed to *M4*, aging experiments showed that deficiency Espl3, when crossed to *M4* (*Espl3/M4*), had a lifespan more similar to wild-type than to *M4* (Figure 1B). Flies carrying deficiencies BSC512, BSC469, and Tl-P crossed to *M4* exhibited lifespan patterns more similar to those of *M4* flies (Figure 1B), supporting the TS complementation mapping.

Genomic rescue confirms deficiency mapping

Transgenic flies carrying a genomic segment spanning the *CG6154-Ald* region were crossed into an *M4* background (Figure 2A). This transgene partially corrected lifespan defects and TS-paralysis in *M4* homozygotes, further confirming the accuracy of the deficiency mapping. (Figure 2C) Curiously, more complete rescue was observed when the transgene was expressed in an *M4*, deficiency double heterozygous background. TS testing showed rescue of paralysis for

both *M4* and *M4/def.* (Figure 2B) The chromosomal deficiency Def(3R)BSC512 was used in these experiments.

_M4_ is a missense mutation in a conserved region of _Aldolase_

Sequencing the region implicated by deficiency mapping revealed a missense mutation in the *Aldolase* coding region. This mutation changes a highly conserved arginine near the enzyme's active site to a histidine (Figure 3).

_M4_ mutants exhibit an expected reduction in ATP levels

The mutation in *Aldolase,* a gene encoding a glycolytic enzyme, suggested that *M4* mutants might have been dying prematurely due to insufficient ATP production by glycolysis. To determine whether the glycolytic function of the aldolase protein was disrupted in *M4* mutants, we performed quantitative ATP assays. *M4* homozygous and *M4/def* flies were shown to have decreased ATP levels relative to wild-type and heterozygous controls (Figure 4). This supported the hypothesis that glycolysis was disrupted in the mutants.

Tissue-specific _Aldolase_ rescue

To investigate the role of glycolysis in neurons and glia, we expressed an *Aldolase* cDNA in *M4* mutants using the UAS/Gal4 system. The effects of exogenous *Aldolase* expression were tested for the temperature sensitivity, shortened lifespan, and neurodegenerative phenotypes of *M4* mutants. Expressing *Aldolase* ubiquitously using Tubulin-Gal4 rescued the TS phenotype (Figure 5A) and neurodegeneration (Figure 7A), which was examined by horizontal sections of adult brains. Lifespan was also extended, but it was not restored to a wild-type lifespan (Figure 6A). Expression in muscle with 24B-Gal4 showed little to no rescue of lifespan (Figure 6B) or TS-paralysis (Figure 5B), though it did appear to rescue neurodegeneration when age-matched to *24B-Gal4, M4* (no transgene) flies had reached the 50% point of their lifespan (Figure 7B).

Driving *Aldolase* expression in neurons using C155-Gal4 restored lifespan to a level comparable to that achieved by driving with Tubulin-Gal4 (Figure 6C). However, no strong rescue of temperature sensitive paralysis was achieved by neuronal expression of *Aldolase* (Figure 5C). Neurodegeneration appeared to be rescued by *UAS-Ald* expression when flies expressing the driver alone reached the 50% point of their lifespan. However, at later time points, neurodegeneration was seen in the presence of the *Aldolase* rescue construct to a degree similar to what was seen in the driver alone (Figure 7C). This degeneration, however, may have been due to other age-related issues, as it was seen in most of the controls at late stages of life as well.

Expressing *Aldolase* in glia using MIB-Gal4 was insufficient to rescue shortened lifespan (Figure 6D), but partial rescue of temperature-sensitive paralysis was achieved (Figure 5D). The effects on neurodegeneration were similar to those seen in neuronal expression: when *M4* reached the half-point of its lifespan, the *Aldolase* construct appeared to rescue degeneration; at a later time point, degeneration was evident even when *Aldolase* was expressed (Figure 7D). We therefore determined that both neuronal and glial expression of *Aldolase* were sufficient to partially rescue neurodegeneration.

Tissue specific loss of *Aldolase* by RNAi

In parallel to determining the effects of restoring *Aldolase* to *M4* mutants, we investigated the effects of knocking down *Aldolase* in a wild-type background. We expressed an *Aldolase* RNAi using the same tissue-specific Gal4 drivers as were used with the rescue construct. Expressing the RNAi ubiquitously with Tubulin-Gal4 was lethal. Surprisingly, we were also found that muscle expression with 24B-Gal4 was lethal.

Driving RNAi expression in neurons and glia both caused a dramatic decrease in lifespan (Figure 8). However, loss of *Aldolase* in neurons caused no visible degeneration (Figure 9A), while its loss in glia resulted in a severe neurodegenerative phenotype (Figure 9B).

Discussion

Incomplete lifespan rescue suggests additional defect

One of the most surprising results attained in this study was that the genomic rescue construct restored lifespan more fully to *M4/def* flies than to *M4* homozygotes. Interestingly, we also found that the Gal4 drivers that rescued lifespan when expressing the *UAS-Ald* rescue construct (Tub-Gal4 and C155-Gal4) also failed to restore lifespan to a wild-type level. This suggests that there may be another defect on the *M4* chromosome in addition to the *Aldolase* mutation. This secondary defect would presumably lie within the span of the BSC512 deficiency, as the heterozygous absence of this chromosomal segment allows full rescue of lifespan by genomic *Aldolase.* A *BSC512, M4* recombinant stock has been generated in order to repeat rescue experiments in a deficiency background.

Counter to ANLS, neurons are dependent on glycolysis

The astrocyte-neuron lactate shuttle (ANLS) model would predict that decreasing glycolytic integrity in neurons would have milder effects than in glia. However, the RNAi experiments performed here reveal that neuronal and glial loss of aldolase have nearly identical effects on lifespan. This indicates that glycolysis function may be equally necessary in neurons and glia. Additionally, restoring aldolase to neurons in *M4* mutants was sufficient to rescue lifespan. No rescue of lifespan was achieved by restoring aldolase to glia. These results contradict the ANLS hypothesis, suggesting that neurons have a requirement for their own glycolysis and are not dependent on glia for energetic substrates.

Loss of Aldolase reduces lifespan independent of neurodegeneration

Unexpectedly, aldolase seems to be differentially required for normal lifespan and protection against neurodegeneration. For instance, RNAi knockdown shows that neuronal

aldolase is required for normal lifespan, but not for protection against overt neurodegeneration, and restoring aldolase to neurons rescues lifespan while only slightly improving *M4* neurodegeneration. Also, knockdown of aldolase in glia induces widespread neuronal tissue loss, without further reducing lifespan, while glial expression of aldolase has little to no effect on lifespan and neurodegeneration of *M4* mutants. This suggests that the widespread neurodegeneration and lifespan defects caused by aldolase deficiency are concomitant, yet independent outcomes of glycolytic dysfunction.

Materials and Methods

Fly Stocks

M4 mutants were generated by ethylmethane sulfonate mutagenesis and were maintained in the laboratory's TS-paralytic mutant collection. Wild-type flies were Canton-S (CS) from Bloomington Stock Center. All stocks were maintained at room temperature (approximately 22°C).

Temperature sensitivity testing

Flies were collected one to two days after eclosion and aged for 10 days at room temperature (approximately 22°C). They were then transferred to vials in a 37.5°C water bath, and the percent climbing, walking, and paralyzed was recorded at two-minute intervals.

Lifespan analysis

Flies collected one to two days after eclosion were shifted to 28°C, and transferred to fresh vials every three days. The number left alive for each genotype was recorded daily. Multiple collections were taken for each genotype, and groups from each collection were aged independently under identical conditions. The final numbers were then combined to generate a cumulative lifespan curve.

Histology

Analysis of neurodegeneration was performed on a subset of brains taken from flies in the aging experiments. Four to eight heads from each genotype were cut off at different points in the genotypes' lifespans (50% or end point) and fixed overnight at 4°C in a fixative of 60% ethanol, 30% chloroform, and 10% acetic acid. They were then washed in 70% ethanol and processed and embedded into paraffin. The paraffin blocks were frontally sectioned in 5-μm slices. Slides were stained with hematoxylin and eosin and examined under a light microscope.

Sequencing

The *Aldolase* gene region was amplified from wild-type and *M4* genomic DNA by PCR, using primers specific to exons 1-3, 3-7, and 8-10. These PCR products were sequenced with the PCR primers as well as additional sequencing primers.

Genomic Rescue

Three BAC inserts spanning overlapping segments of the *CG6154-Ald* region were purified from DH10B *E. coli* and transformed into EPI300 electrocompetent *E. coli.* DNA from these bacteria was then isolated using a QIAGEN-Large Construct Kit. Only one of the BAC segments, which spanned both genes, was successfully purified and injected into embryos. This genomic fragment integrated on the 2nd chromosome in these embryos, and transgenic flies were then crossed to *M4* to produce a stock with the genomic fragment in an *M4* mutant background.

ATP Assays

Ten to twenty female flies of each genotype *w1118, M4/+; M4, def/+,* and *M4/def* were collected at approximately two days post-eclosion. These were split into two groups: the first remained at room temperature, and the second was shifted to 28°C. After two days, they were placed into pre-chilled tubes on dry ice and stored at -80°C. After freezing, whole-body extract was produced for ATP assays using Molecular Probes ATP Determination Kit.

UAS-Ald – Tissue-Specific Rescue

Aldolase cDNAs in a chloramphenicol-resistant vector were digested with EcoRI and XhoI and ligated into a pUAS vector. The resulting constructs were injected into *Drosophila* embryos, and transgenic lines were established. The UAS-Ald construct integrated onto the second chromosome. Transgenic flies were crossed to *M4* mutants to attain *UAS-Ald/CyO; M4/Tb* flies. These were crossed to flies expressing tissue-specific Gal-4 drivers (*C155-Gal4, MIB-Gal-4, Tub-Gal4*, and *24B-Gal4*). The resulting progeny carried a Gal-4 driver and the *Aldolase* transgene in an *M4* mutant background (*X-Gal4; UAS-Ald; M4*).

RNAi – Tissue-Specific Knockdown

Fly stocks expressing RNAi against *Aldolase* were ordered from the Vienna *Drosophila* RNAi Center. These flies (Stock no. 47667) were crossed to tissue-specific Gal-4 drivers, and lifespan and neurodegeneration were analyzed in the progeny.

*Partial funding for this study was provided by the Cargill-Benevega fund

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[ii] Iijima-Ando, K.; Hearn, S.A.; Shenton, C; Gatt, A.; Zhao, L.; Iijima, K. Mitochondrial mislocalization underlies Abeta42-induced neuronal dysfunction in a Drosophila model of Alzheimer's disease. *PLoS One.* 4(12): e8310 2009.

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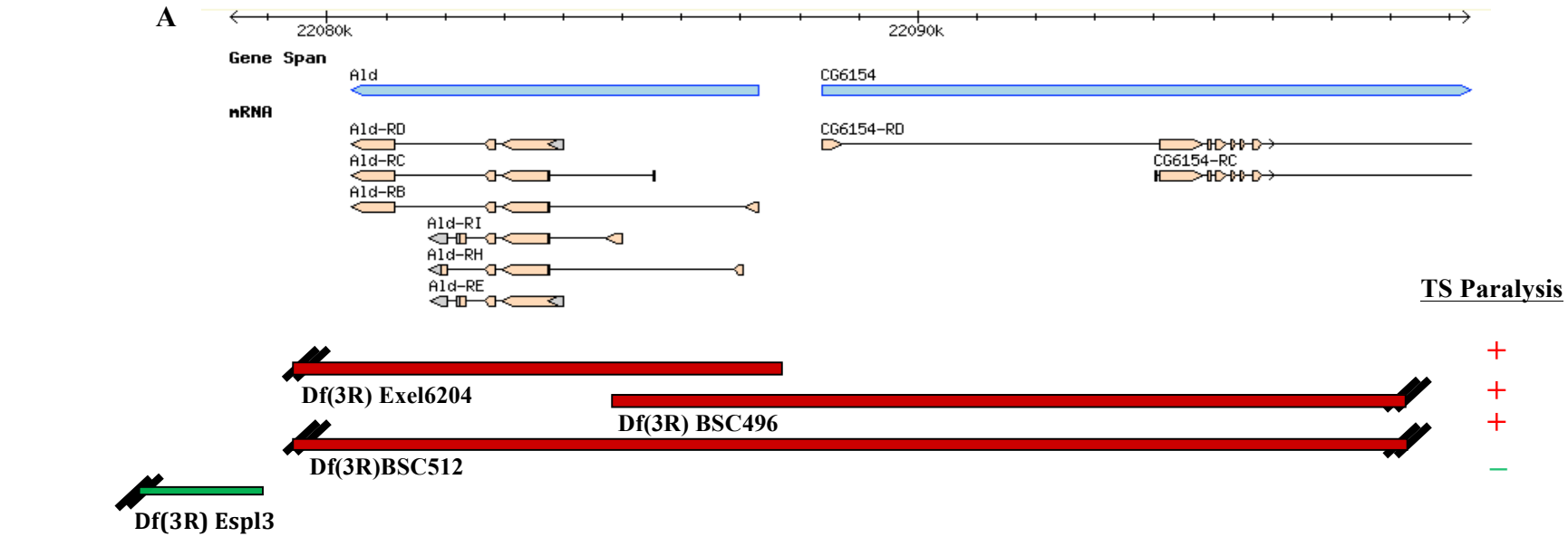
FIGURES



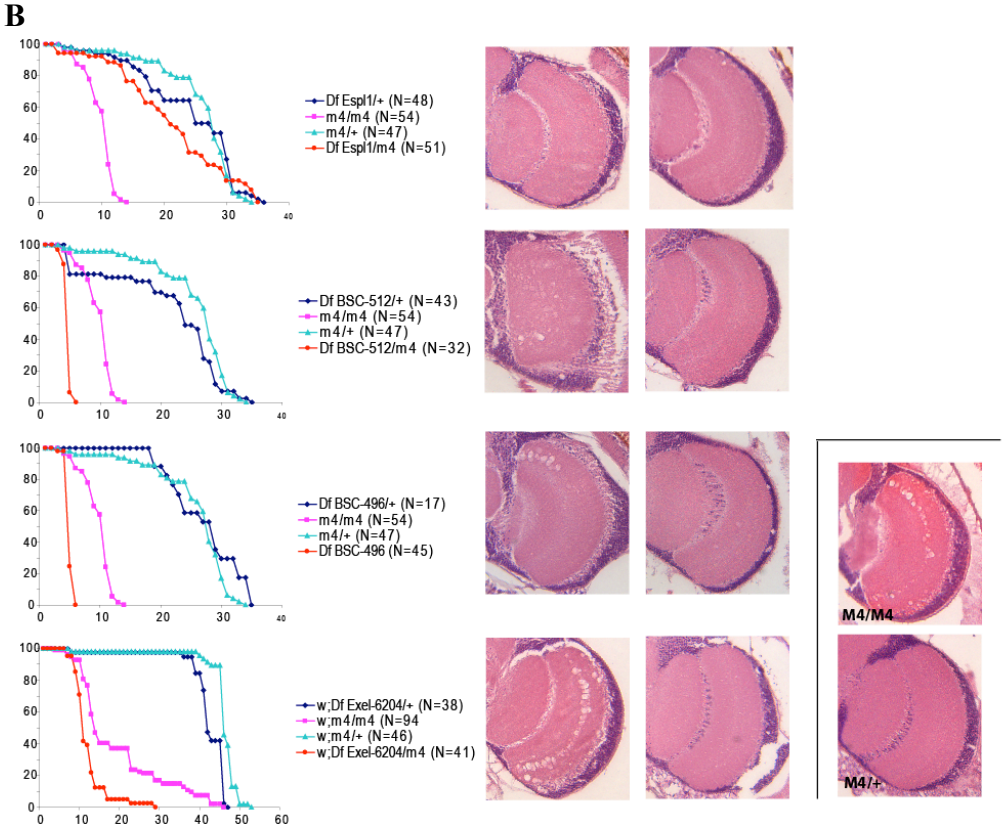


Figure 1. Complementation testing places *M4* in a region containing *Aldolase* and *CG6154*.
A) Map of the *M4* region on chromosome 3 and local deficiencies. Espl3 complements TS paralysis, while Exel6204, BSC496, and BSC512 do not. Complementation of B) lifespan and C) neurodegeneration by Espl3 correlate with TS-paralysis mapping.

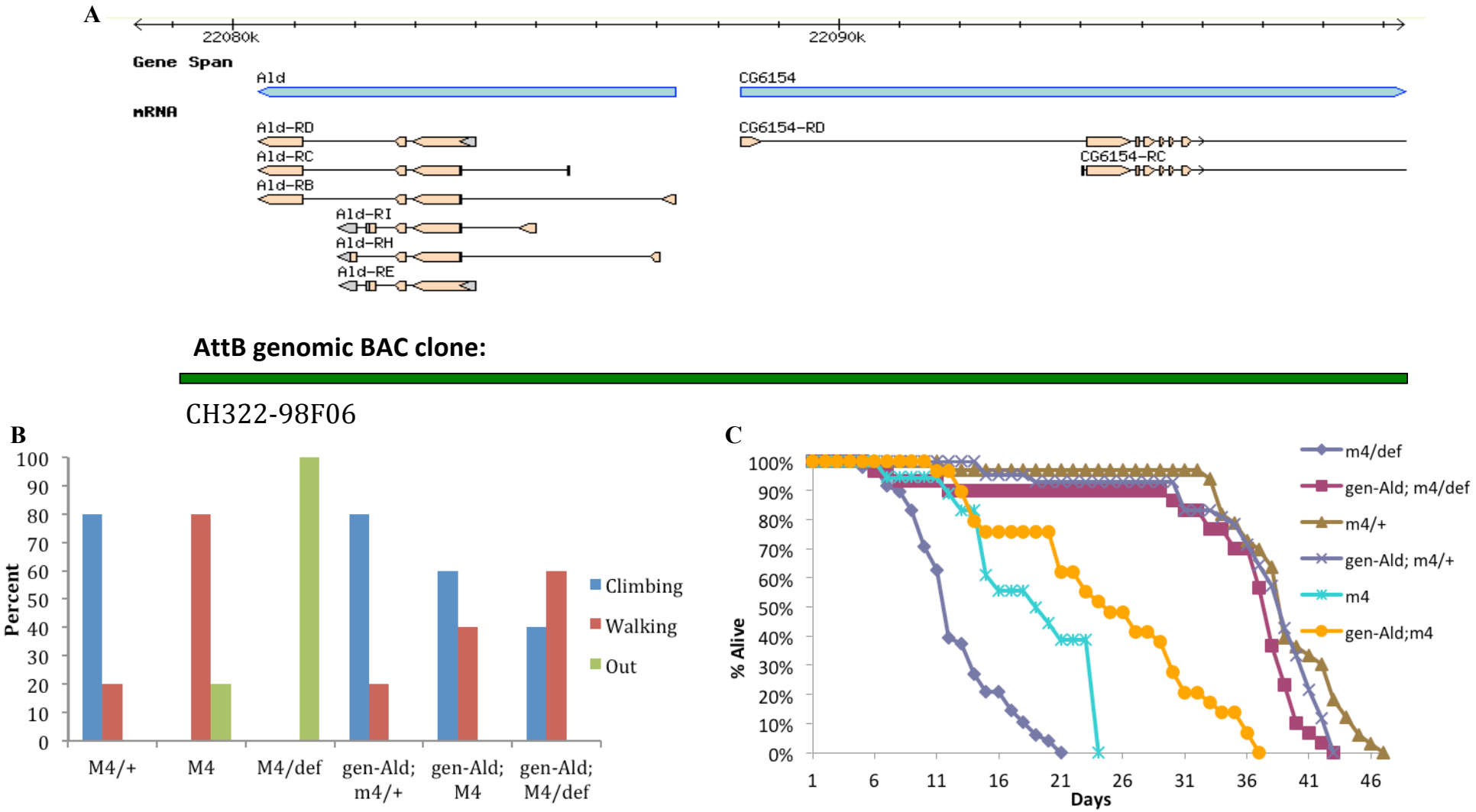


Figure 2. Genomic rescue of *Aldolase* corrects *M4* TS and lifespan defects. A) Position of BAC clone CH322-98F06 in the genome, spanning both the *Aldolase* and *CG6154* genes. B) The genomic fragment prevents TS-paralysis in *M4* and *M4/def* flies. C) The genomic fragment extends lifespan in *M4* flies, and extends *M4/def* lifespan to a wild-type length. Deficiency used was BSC512.

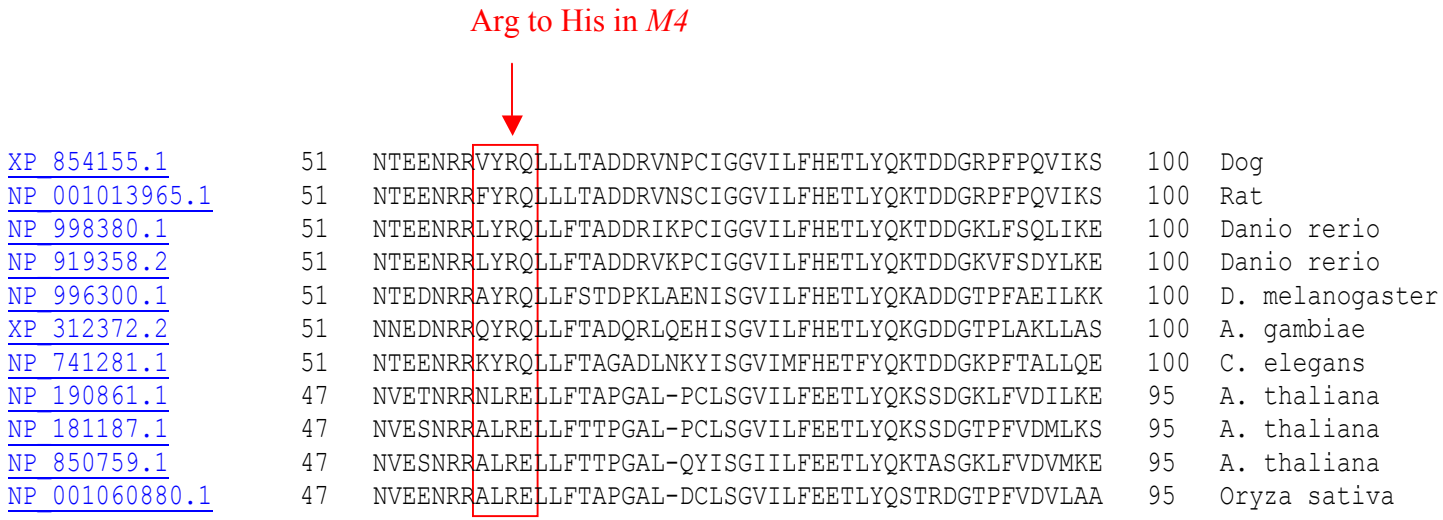


Figure 3. *M4* has a missense mutation in a highly conserved region of *Aldolase*.

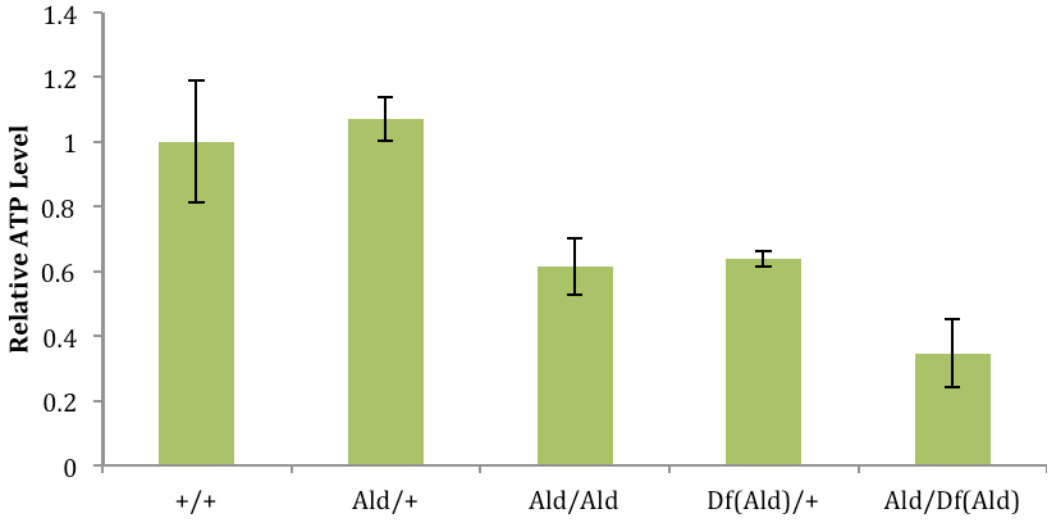


Figure 4. ATP levels are significantly reduced in *Aldolase* mutants. Flies were aged for 5 days at 28°C . Triplicate measurements were made from two independent samples of whole body extracts from each genotype.

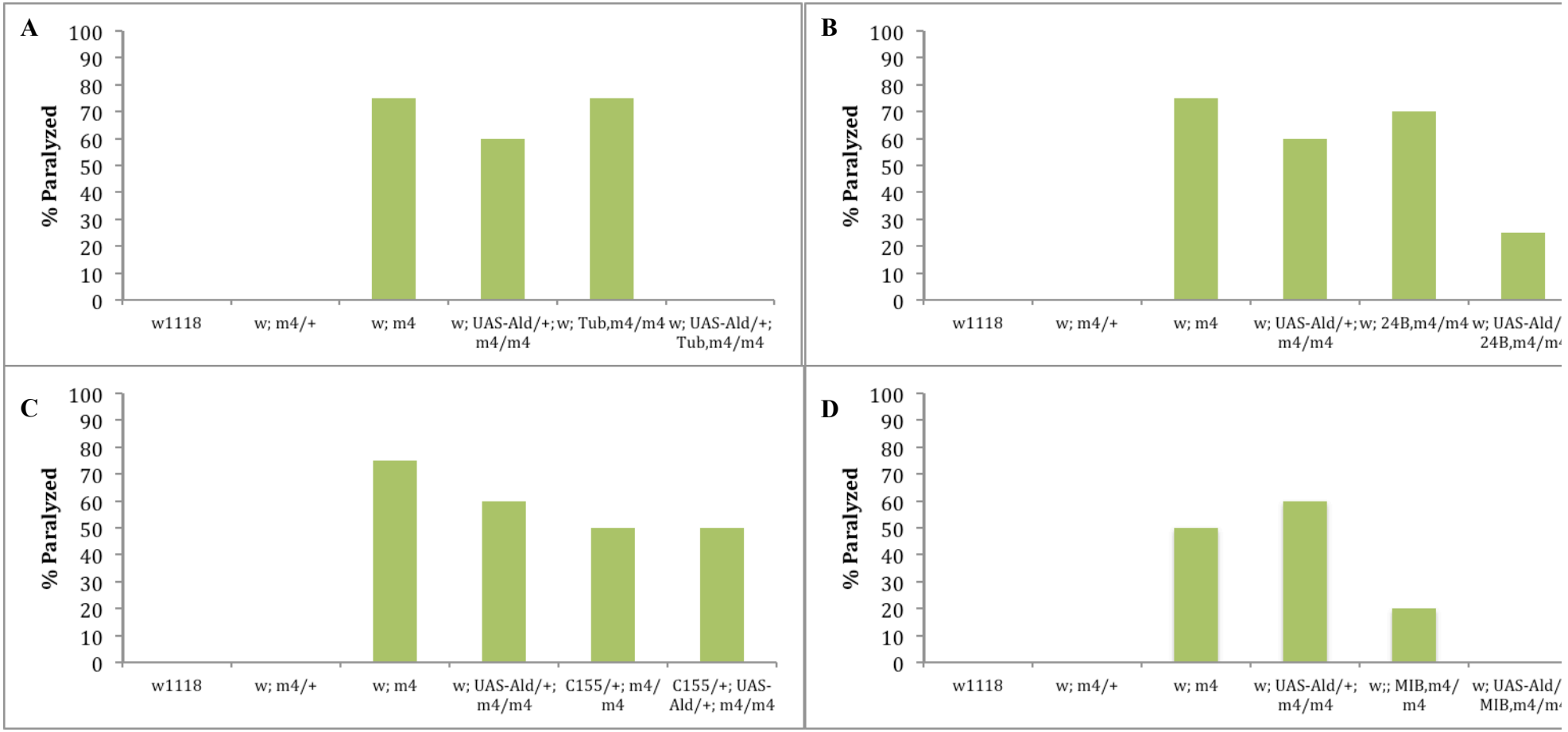


Figure 5. An *Aldolase* transgene rescues temperature sensitive paralysis most effectively when expressed in ubiquitously or in glia. Percent of flies paralyzed after 7 minutes at 37°C. Flies were shifted to 37° after 10 days at 28°. A) Ubiquitous expression with Tubulin-Gal4. B) Expression in muscle with 24B-Gal4. C). Expression in neurons with C155-Gal4. D) Expression in glia with MIB-Gal4

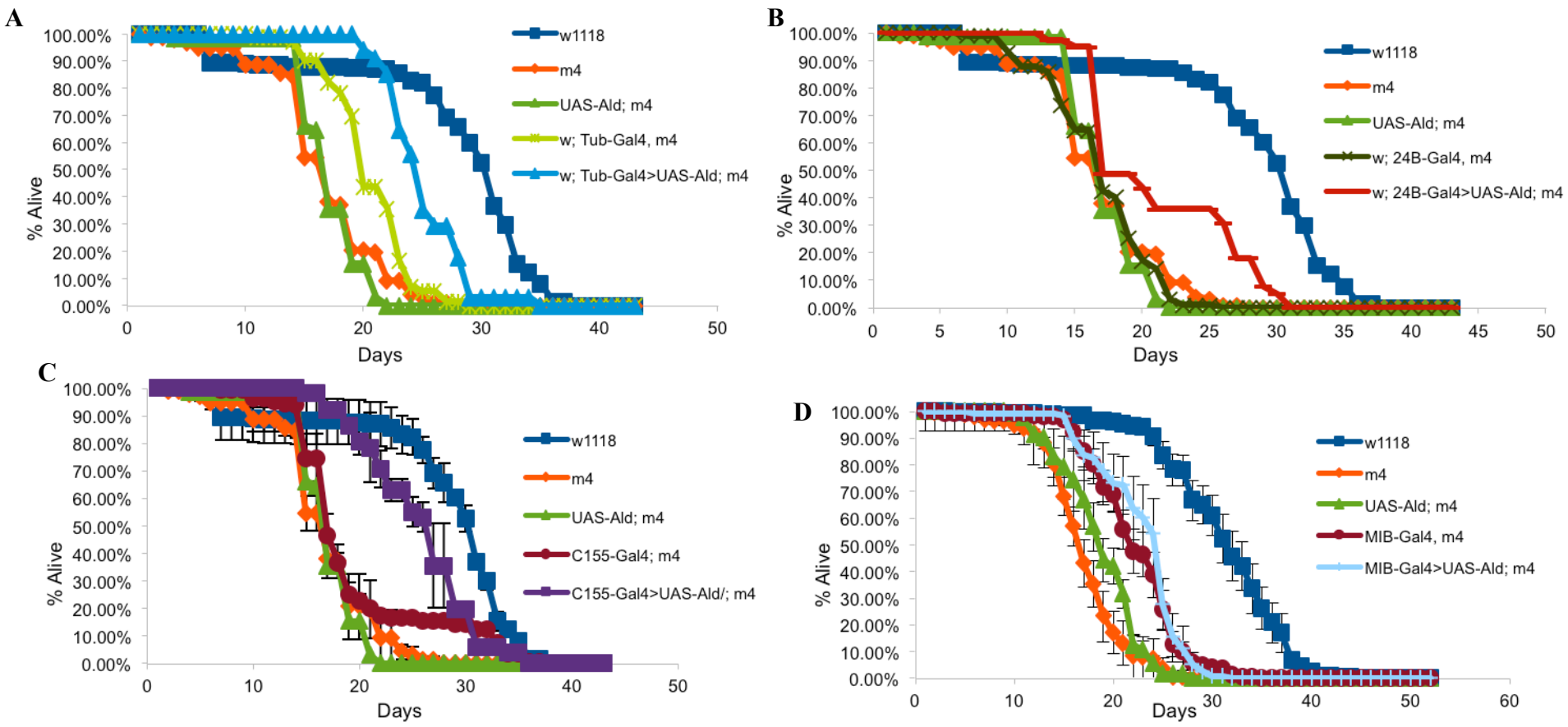
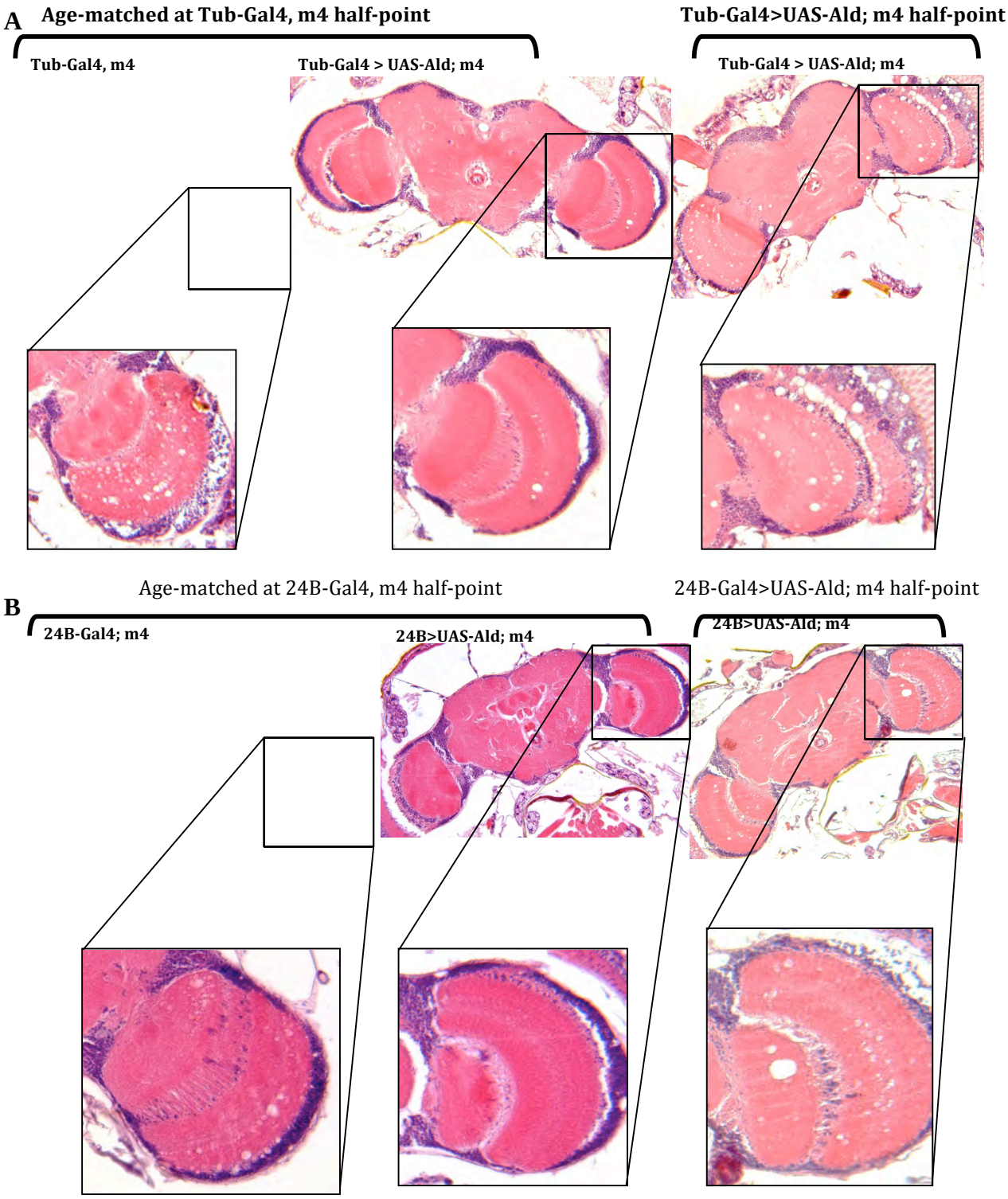


Figure 6. *Aldolase* transgene rescues most effectively when expressed ubiquitously or in neurons. Flies were collected within two days of eclosion and aged at 28°C. A) Ubiquitous expression with Tubulin-Gal4. B) Expression in muscle with 24B-Gal4. C) Expression in neurons with C155-Gal4. D) Expression in glia with MIB-Gal4.


A
Age-matched at Tub-Gal4, m4 half-point
Tub-Gal4>UAS-Ald; m4 half-point
Tub-Gal4, m4
Tub-Gal4 > UAS-Ald; m4
Tub-Gal4 > UAS-Ald; m4
B
Age-matched at 24B-Gal4, m4 half-point
24B-Gal4>UAS-Ald; m4 half-point
24B-Gal4; m4
24B>UAS-Ald; m4
24B>UAS-Ald; m4

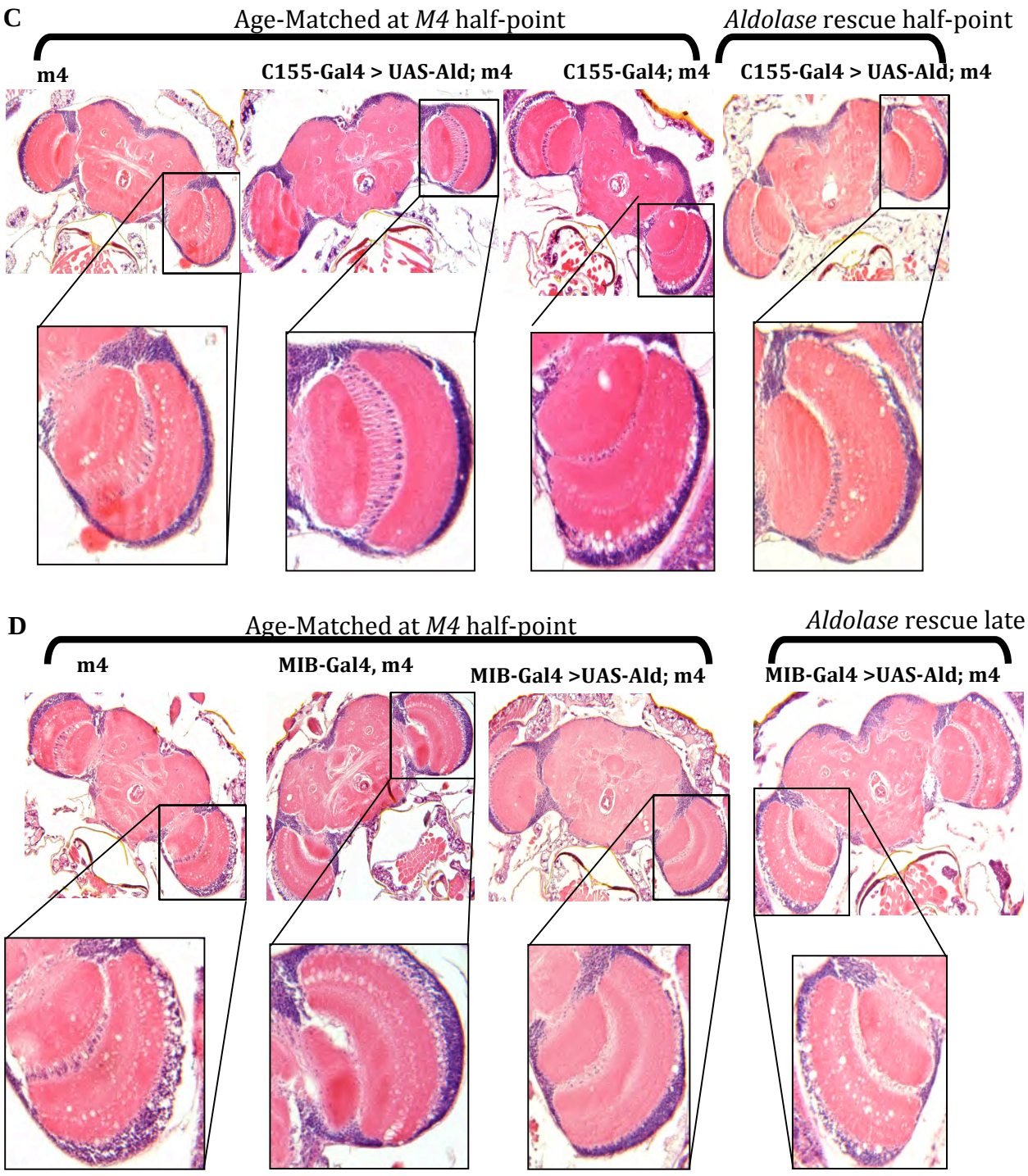
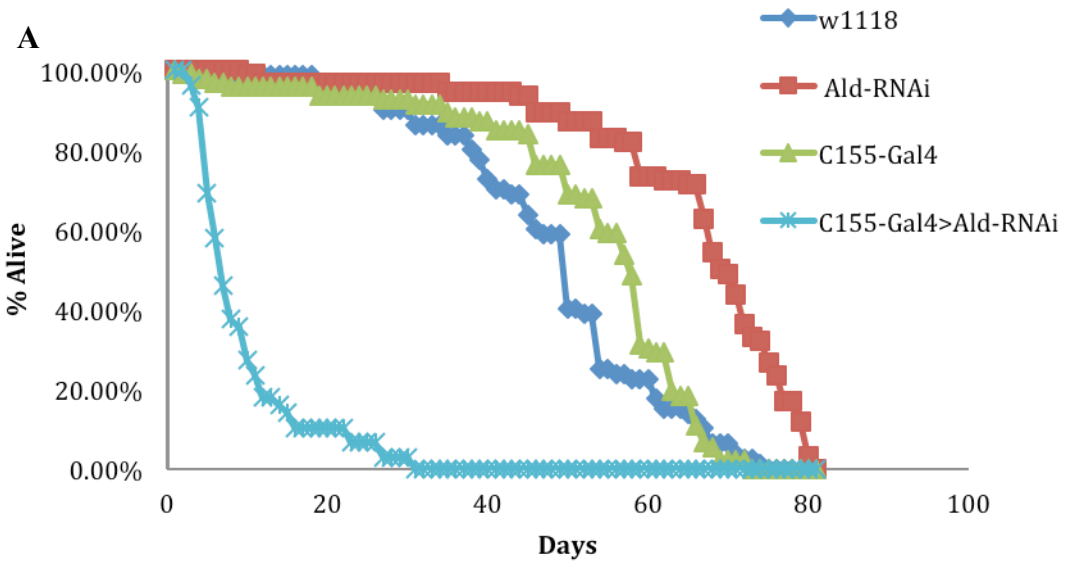


Figure 7. Expression of *Aldolase* in neurons or glia partially rescues neurodegeneration. Flies were aged at 28°C and heads were collected when *M4* or the UAS driver with *M4* reached the half-point of their lifespan. A) Ubiquitous expression with Tubulin-Gal4. B) Expression in muscle with 24B-Gal4. C) Expression in neurons with C155-Gal4. D) Expression in glia with MIB-Gal4.



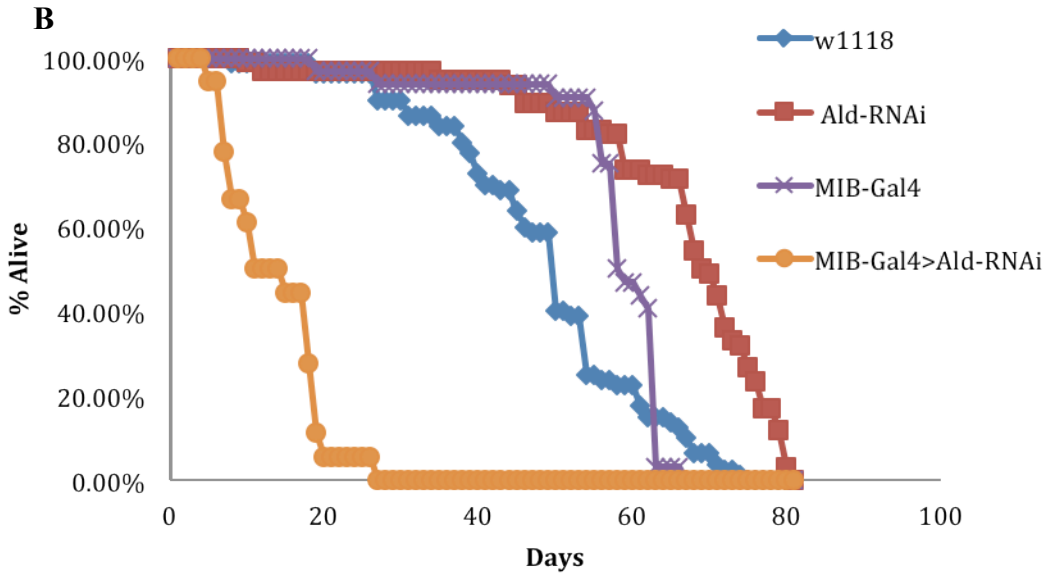


Figure 8. *Aldolase* RNAi results in significantly shortened lifespan when expressed in either neurons or glia. Flies were aged at 28°C. A) Loss of Aldolase in neurons, driving RNAi expression with C155-Gal4. B) Loss of Aldolase in glia, driving RNAi with MIB-Gal4.

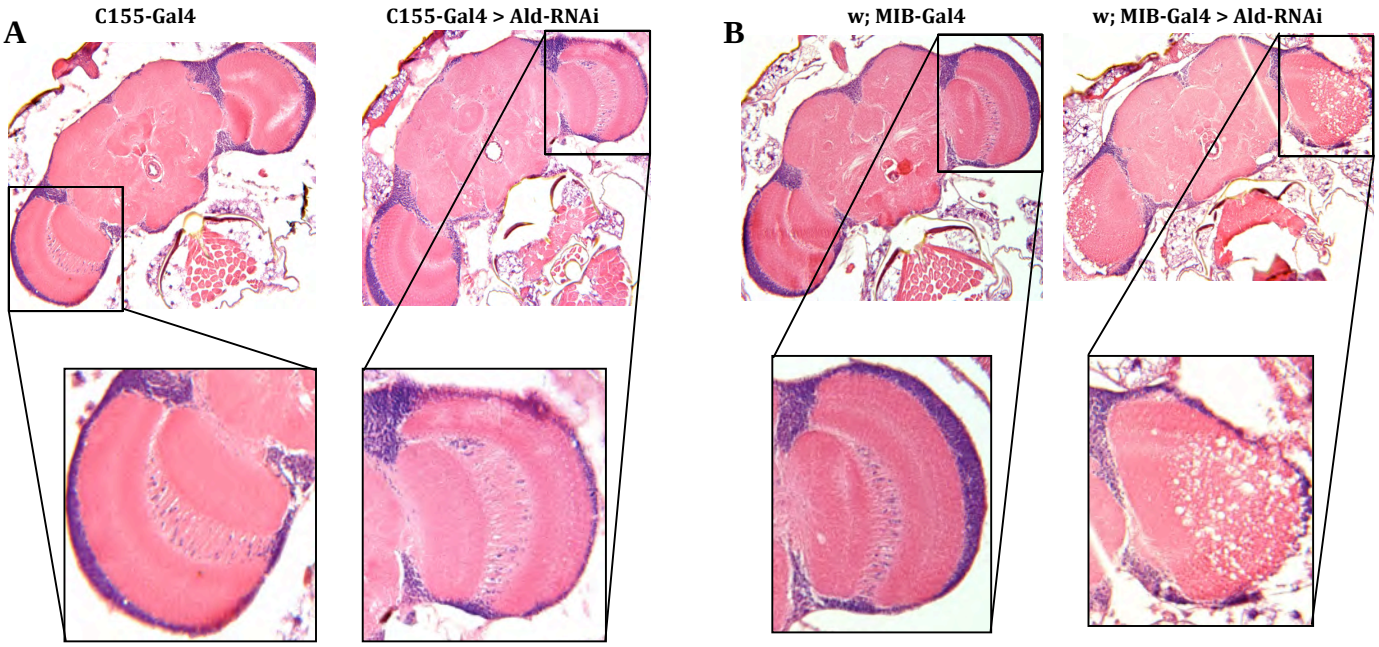


Figure 9. Loss of Aldolase in glia, but not neurons, causes neurodegeneration. Flies were aged at 28°C and heads were collected when 75% of the Driver>Ald-RNAi flies had died. A) Expression of Aldolase RNAi in neurons with C155-Gal4. B) Expression of Aldolase RNAi in glia with MIB-Gal4.