Astrocyte-like glia-specific gene *deathstar* is crucial for normal development, adult locomotion and life span of male *Drosophila*

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ABSTRACT

Drosophila melanogaster is a proper model organism for studying the development and function of the nervous system. The Drosophila nervous system consists of distinct cell types with significant homologies to various cell types of more advanced organisms, including human. Among all cell types of the nervous system, astrocyte-like glia (ALG) have conserved functions to mammals and are essential for normal physiology and behaviours of the fly.

In this study, we exploited the gene expression profile of single cells in *Drosophila* optic lobe to identify the genes with specific expression pattern in each cell type. Through a bioinformatical analysis of the data, a novel ALG-specific gene (here assigned as *deathstar, dea*) was identified. Immunostaining of *deathstar* in the central nervous system (CNS) showed its presence in specific regions of *Drosophila* ventral nerve cord, which previously has been characterized as ALG cells. Consistent with the bioinformatical analysis, *deathstar*-related signals were overlapped with the signals of the previously reported ALG marker, *Eaat1*, supporting its specific expression in ALG cells.

At the physiological level, RNAi-mediated suppression of *deathstar* gene impeded the normal development of male flies without any effects on females. Cell type-specific expression of *deathstar* RNAi showed that *deathstar* gene affects locomotion behaviour and lifespan of *D. melanogaster*, in an ALG-specific manner.

Taken together, we showed that bioinformatical analysis of a previously reported expression data of *Drosophila* optic lobe successfully predicted the ALG-specific expression pattern of *deathstar* gene. Moreover, it was consistent with the ALG-specific effects of this gene on locomotion and lifespan of *D. melanogaster*, *in vivo*.

Keywords: Astrocyte-like glia, *CG11000* gene, Cell type, Male development, *Drosophila melanogaster*, Single cell RNA-seq, Specific marker, *deathstar*

INTRODUCTION

The function of the central nervous system (CNS) depends on a complex network of several neuronal and glial cell types [1, 2]. Due to the feasibility of genetic manipulation and behavioural analysis, *Drosophila melanogaster* has emerged as a powerful model organism for studying the development and function of the nervous system [3, 4]. There is a functional homology between the cell types in the nervous system of *D. melanogaster* and those of more advanced organisms, including human [5]. Glial cells constitute an essential part of CNS [5] , and until now, several distinct types of these cells were also identified in the *Drosophila* nervous system [6, 7]. Each cell types have a distinct profile of gene expression [8] and have their specific functions matched to their physical territories or physiological states [9]. Based on the differences in gene expression profile, each cell type of *Drosophila* nervous system can be distinguished by a set of genes, which are known as molecular markers for each cell type [6].
In addition to the existed molecular markers for each cell type, there may be some uncharacterized genes in *D. melanogaster*, which are capable of discerning a specific cell type in the nervous system. Moreover, a specific cell type likely differs only in the expression of the limited number of genes which confer a new function to it [10, 11]. In this study, we used a publicly available expression profile of 55,000 single cells of *Drosophila* optic lobe in order to find novel molecular markers for specific cell types, following by experimental confirmation of the predicted results.

MATERIALS AND METHODS

Dataset

A single-cell RNA-sequencing (RNA-seq) data of a recently published paper [12] was selected from the data repository NCBI Gene Expression Omnibus (GEO) (accession number: GSE103771). It contained the expression profile of 17272 *Drosophila* genes in a population of 55000 single cells from the optic lobe of *Drosophila* brain. Also, the data of expression profile for the whole brain of *D. melanogaster* across four different developmental stages (days 5, 10, 20 and 40) were downloaded from NCBI GEO (accession number: GSE107049).

Bioinformatics analyses

We analyzed the expression matrix of the single-cell RNA-seq data of *Drosophila* optic lobe (accession number: GSE103771) based on the expression level of known markers previously reported for each cell type. For each cell type, six single cells were selected according to the expression level of the markers. In total, forty two cells comprising seven cell types of *Drosophila* nervous system (assigned as perineural glia (PNG), subperineural glia (SPG), cortex glia (CG), neuropil ensheathing glia (EGN), tract ensheathing glia (EGT), astrocyte-like glia (ALG) and neurons (Neu)) were selected for downstream analyses. For confirmation of this cell type classification, hierarchical clustering was performed for the selected forty-two cells based on their similarities in transcriptome and presented as a heatmap with colour codes for the Pearson correlation coefficient. The clusters with at least three cells (of all six cells) were validated as unique cell type. To identify the genes enriched in a specific cell type, we first identified differentially expressed genes (DEGs) between the examined cell types and all other cells by the R package limma [13]. Then, after applying adjusted *P*-values (Benjamini–Hochberg

correction) of < 0.05 and $\log_2 FC \geq 1.5$, the specificity of the candidate genes with highest degree of enrichment was determined by analysis of their expression across all the examined single cells (unpaired t-test; *P*-value < 0.05).

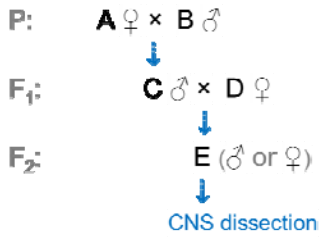
Fly stocks and crosses

All the *Drosophila melanogaster* lines in this study were prepared from Bloomington Drosophila Stock Center (BDSC). After receiving the stocks they were cultured in standard conditions (standard cornmeal-agar yeast fly food at 25° C on a 12-h light:12-h dark condition) as previously described [14]. CO_2 was used as an anesthetic. For functional analysis of the genes, the GAL4/UAS-RNAi system [15] was used to deplete their functions in a cell-type specific manner. The fly stocks together with their crosses for functional analyses of *deathstar* gene are summarized in table 1. After crossing the virgin females of UAS-*deathstar*-RNAi line with males of different GAL4 driver lines (x-GAL4), ensuring expression of RNAi in cell type-specific manner, the transgenic progenies (x-GAL4 > UAS-RNAi) were collected based on their genotypes and assessed for their behaviors (Table 1). For the crosses of fluorescent labeling experiment, the two binary systems of GAL4/UAS-GFP and LexA/LexAoP-RFP were used simultaneously in an individual male and female flies. The crossing scheme for this experiment is summarized in table 2.

Parents for crosses		Progenies
UAS- RNAi lines (♀)	GAL4 lines (♂)	
w^- ; +/+ ; *deathstar*-RNAi (# 51918)	Canton-S(#)	CS

w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; PNG-GAL4/ TM3(#)	Non-TM3
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; SPG-GAL4/ TM3(#)	Non-TM3
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; CG-GAL4/ TM3(#)	Non-TM3
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; EGN-GAL4/ TM3(#)	Non-TM3
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; EGT-GAL4/ TM3(#)	Non-TM3
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; ALG-GAL4/ TM6B(#)	Non-TM6B
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; +/+ ; Neuron-GAL4/ TM6B(#)	Non-TM6B
w⁻ ; +/+ ; *deathstar* -RNAi	+ ; repo-GAL80/CyO ; ALG-GAL4 /TM6B (#)	Non-CyO, Non-TM6B

Table 1. Summary of crosses and experimental genotypes for behavioral analyses. In order to generate the transgenic flies with expression of *deathstar*-RNAi in cell type-specific manner, the male flies of different GAL4 driver lines were crossed with the females harboring UAS-*deathstar*-dsRNAi in their genome (BDSC#: 51918). All the females (♀) for these crosses were virgin. Among the progenies of these crosses, the flies with proper genotypes were selected based on the absence of balancer chromosomes (CyO, TM3 and TM6B).

Lines	Fly stocks (Genotypes and Bloomington ID)	Cross scheme
A	UAS-RFP, lexAop-GFP ; Sp/CyO ; TM2/TM6B (# 32229)	
B	w- ; ALG-LexA/CyO ; TM2/TM6B (Made by us)	
C	UAS-RFP, lexAop-GFP ; ALG-LexA/CyO ; TM2/TM6B	
D	w- ; +/+ ; *deathstar* -GAL4 (23416)	
E	UAS-RFP, lexAop-mCD8GFP ; ALG-LexA/+ ;	

	TM6B/ *deathstar* -GAL4 (Made by us)	

Table 2. Crossing scheme for imaging experiments of CNS in *Drosophila*. The line A contains RFP and GFP reporter genes under regulation of UAS and LexAop sequences, respectively. Line B expresses LexA protein in an ALG-specific manner. By crossing the lines A and B (Parents), the fly line C (F_1 generation) was produced and then crossed with *deathstar* -GAL4 line (D) to produce F_2 generation. Among the flies of F_2, the males and females that lack balancer chromosomes but contain the components of the binary systems UAS/GAL4 and LexA/LexAop were selected for central nervous system (CNS) dissection. The females (♀) of all crosses were virgin.

Immunostaining and imaging

As described before [16], brains dissected from adults 5 days after eclosion were fixed in 4% formaldehyde for 30 min at room temperature, washed with 1% PBT three times (30 min each) and blocked in 5% normal donkey serum for 30 min. The brains were then incubated with primary anti-bodies in 1% PBT at 4° C overnight followed with fluorophore-conjugated secondary antibodies for 1 hour at room temperature. Brains were mounted with antifade mounting solution (Invitrogen, catalog #S2828) on slides for imaging.

Primary antibodies used here are chicken anti-GFP (Aves Labs, 1:1000), rabbit anti-DsRed express (Clontech, 1:250) and mouse anti-Bruchpilot (nc82) (DSHB, 1:50). Fluorophore-conjugated secondary antibodies used here are Alexa Fluor 488-conjugated goat anti-chicken (Invitrogen, 1:100). Alexa Fluor 488-conjugated donkey anti-rabbit (Invitrogen, 1:100), RRX-conjugated donkey anti-rabbit (Jackson Laboratory, 1:100), and Dylight 405-conjugated donkey anti-mouse (Jackson Laboratory, 1:100).

Images were collected using a Zeiss LSM 7 MP microscope scanning confocal microscope. Projection of the confocal stacks, adjusting the brightness and contrast of the images were performed by NIH ImageJ [17], then presented by Adobe Illustrator CC 2019 for windows.

Developmental assays

To assess the possible function of *deathstar* gene in the development of *D. melanogaster* from pupae to adulthood stage, the newly eclosed *D. melanogaster* adults of the crosses in table 1, were collected and counted based on their genotypes. After counting, data were presented as the percentage of male and female flies with *deathstar* RNAi to the total number of eclosed flies.

Lifespan assay

Lifespan was measured at room temperature according to standard protocols. In brief, newly ecloded flies (0 to 3 days) were collected (50 per genotype) and then placed in vials (25 flies per vial) and transferred to fresh vials every three days. Survival was recorded for each genotype (two vials). We scored flies stacked in the food as death events in all the vials analyzed. We created survival curves with Prism version 6 (GraphPad Software, San Diego, CA, USA) using the method of Kaplan and Meier.

Climbing assay

For the climbing assay, flies were separated into groups of 25 adults per each vial and kept in 29° C. At the time of experiment, the groups of 25 flies were placed in an empty climbing vial and then tapped down to the bottom. They were allowed to climb from the bottom to top of the vial (8 cm) for 20 seconds. In order to evaluate the climbing pattern of each genotype, the climbing

height achieved by each genotype was calculated in every 5 seconds after the tapping and initiating negative geotaxis. The results of climbing assay were obtained by video recording of climbing flies then processed by ImageJ software. Data was presented as graphs using Prism version 6 (GraphPad Software, San Diego, CA, USA). Flies with Canton-S genotype were used as control group.

Statistical analysis

Statistical analysis and graphic representations were performed using Prism 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Unpaired t test or one-way ANOVA (using the Bonferroni's multiple comparison) were applied depending on the measurements analyzed in the corresponding experiment. In all cases averages with SEM are plotted. *P*-values were calculated by two-tailed unpaired Student's t-test unless otherwise specified, using GraphPad Prism 6 software. All data are presented as the mean ± SEM. $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

RESULTS

Cell type identification of the single cells of *Drosophila* optic lobe based on their specific markers

To identify the cells corresponding to distinct subtypes of glia and neurons, the expression profile of the sequenced single cells by Konstantinides et al. [12] was analyzed based on their previously-reported marker genes (Fig. 1A). According to the expression profile of 55000 sequenced single cells, the cells with the highest expression of each cell type-specific marker were selected and classified. A total of forty-two cells were identified and classified into seven cell types. The heatmap of gene expression for these selected cells confirmed the unique expression pattern of each marker in the corresponding cell types (Fig. 1B). *CG4797* and *gem* are differentially expressed in PNG, and SPG were distinguished by the three markers *Mdr65*, *Moody* and *Gli*. Other glial cells weåre successfully distinguished by single marker; *Cyp4g15*, *CG9657*, *CG34340* and *Eaat1*. Moreover, these genes could individually separate CG, EGN, EGT and ALG, respectively. Neuronal cells were determined based on the highest expression of *nSyb* and *elav* genes (Fig. 1B).

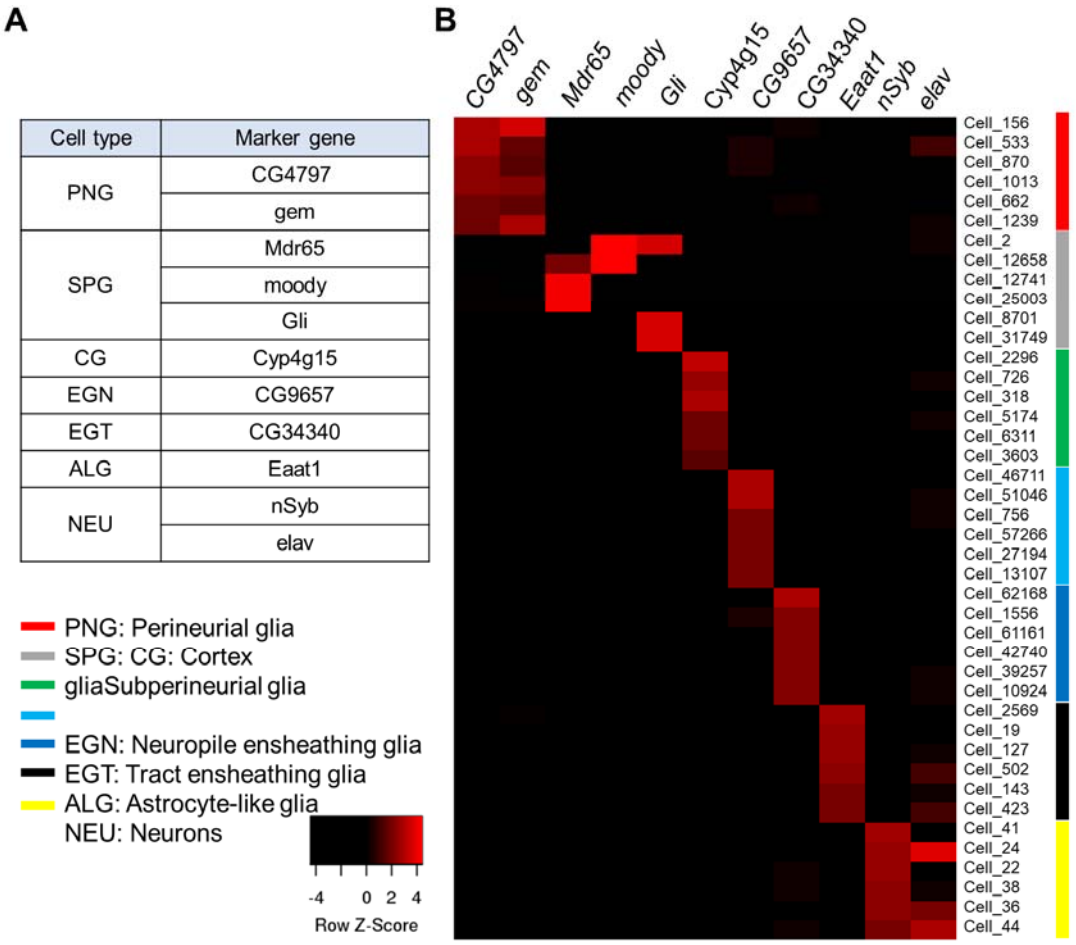


Figure 1. Identification of *Drosophila* neural cell types through analysis of their specific marker genes. A) Specific marker genes, previously reported as markers for each cell type of *Drosophila* glial cells. B) Heatmap of gene expression for 42 selected cells from 55000 single sequenced cells, in which, six different cells were identified as a unique cell type (denoted by different colors). Cells' numbers correspond to the numbering method of Konstantinides et al. Red and black colors indicate the marker-positive and marker-negative cells, respectively. Color intensities in the heatmap denote the level of gene expression based on RPKM per single cell.

Hierarchical clustering of *Drosophila* neural cell subtypes based on their similarities in transcriptome

As shown above, different types of *Drosophila* neural cells could be classified based on some specific marker genes (Fig. 1). In order to know whether the classified cells in each category have the same expression profile or not, correlation analysis between the selected cells was carried out based on their transcriptome analysis. Results showed that most of the cells expressing the same genetic marker(s) tend to be clustered together, suggesting their similar transcriptome and accuracy of the identification of cell types (Fig. 2A). However, the cells with the highest expression of SPG markers and some other cells could not be classified in a single cluster suggesting their transcriptome dissimilarity or partial insufficiency of the available genetic markers for clustering (e.g. expression heterogeneity of the SPG markers: *Mdr65*, *Moody* and *Gli*).

With at least 3 of 6 similar cells in each category, differential expression analysis was performed in order to find the genes with specific expression in each cell type. While the identified PNG were positive for the expression of their two known genetic markers (*CG4797* and *gem*) (Fig. 1B), there were two sub-clusters of PNG (PNG-α and PNG-β) in our transcriptome analysis which suggests the presence of a novel subtype for PNG (Fig. 2A). The list of differentially expressed genes for each cell type in addition to differentially expressed genes between the two subtypes of PNG is presented in supplemental file S1.

Using the transcriptome data of the studied cell types and their clustering results, we assigned the degree of differences between them as a dendrogram (Fig. 2B). Accordingly, the Venn diagram

was used to illustrate the number of unique and shared differentially-expressed gene for each cell type. The extent of unique differentially expressed genes for each cell type is as follows: 98.33% (59 of 60) for PNG, 100% (1 of 1) for SPG, 90% (36 of 40) for CG, 60% (3 of 5) for EG, 78.95 (75 of 95) for ALG and 96.39 (454 of 471) for Neurons (Fig. 2C).

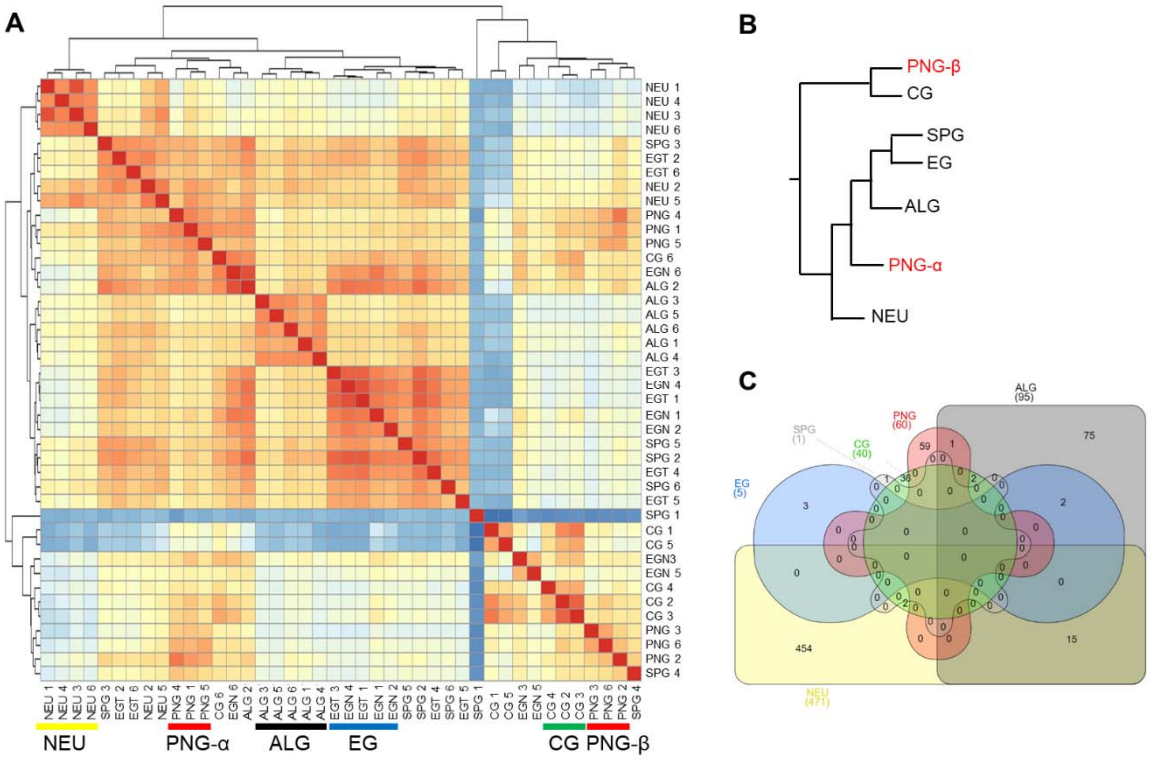


Figure 2. Hierarchical clustering of different neural cell types of *D. melanogaster* based on their transcriptome. A) Hierarchical cluster analysis of gene expression for 42 selected cells from *Drosophila* optic lobe based on the expression level of 17,272 genes for each single cell. Except SPG, all the studied cell types were clustered together suggesting their similarities in expression profile. PNG cells were strongly clustered in two different clusters, suggesting presence of two distinct PNG subtypes in *Drosophila* (PNG-α and PNG-β) B) Dendrogram shows the similarity degree of the clustered cells based on whole transcriptome. C) Number of unique and shared DEGs for each cell type of *D. melanogaster* nervous system. Total number of DEGs for each cell type

are presented in parenthesis. PNG= perineural glia, SPG= subperineural glia, CG= cortex glia, ALG= astrocyte-like glia, EG= ensheathing glia, and NEU= neurons.

Identification of *deathstar* as a gene with specific enrichment in astrocyte-like glia of *D. melanogaster*

After cell type identification, we asked whether specific genes can also characterize the identified cell types. To identify the genes capable of distinguishing different cell types, we performed differential expression analysis in order to compare the transcriptome of the cluster of interest with that of all other cell types. The differentially expressed genes (DEGs) for each cell type are presented in Supplemental file S1.

Among all the clustered cells, ALG could be sufficiently discriminated by a single uncharacterized gene named *CG11000* (here assigned as *deathstar*). Expression level analysis of this gene using the RNA-seq profile of single cells in *Drosophila* optic lobe [12] demonstrated its significant enrichment in 12 cells of all 84 cells, which corresponds to ALG (Fig. 3A). In the same RNA-seq expression data (accession number: GSE103771), a significant positive correlation (R^2=0.6637; *p*-value = 0.0001) was observed between the expression level of the candidate gene (*deathstar*) and that of *Eaat1*, which previously was reported as ALG-specific marker [5] (Fig. 3B). In addition, the expression pattern of *deathstar* and *Eaat1* across developmental time of *D. melanogaster*, derived from a different RNA-seq data (accession number: GSE107049) showed a correlation between these two genes (Fig. 3C and 3D), supporting the observed expression specificity of *deathstar* gene in ALG of *D. melanogaster*.

To corroborate the assigned specificity of *deathstar*, we tested whether the promoter-GAL4 lines of this gene could label ALG in CNS of *D. melanogaster*. Consistently, data showed that *deathstar* (Red signals) is mostly co-labelled with the ALG marker *Eaat-1* (Green signals), in specific bilateral parts of *Drosophila* optic lobe and VNC. Both male and female flies showed the same pattern of *deathstar* expression in *Drosophila* CNS (Fig. 4A and 4B). This experiment was performed twice for males and females, and the same results were obtained (Supplemental file S2).

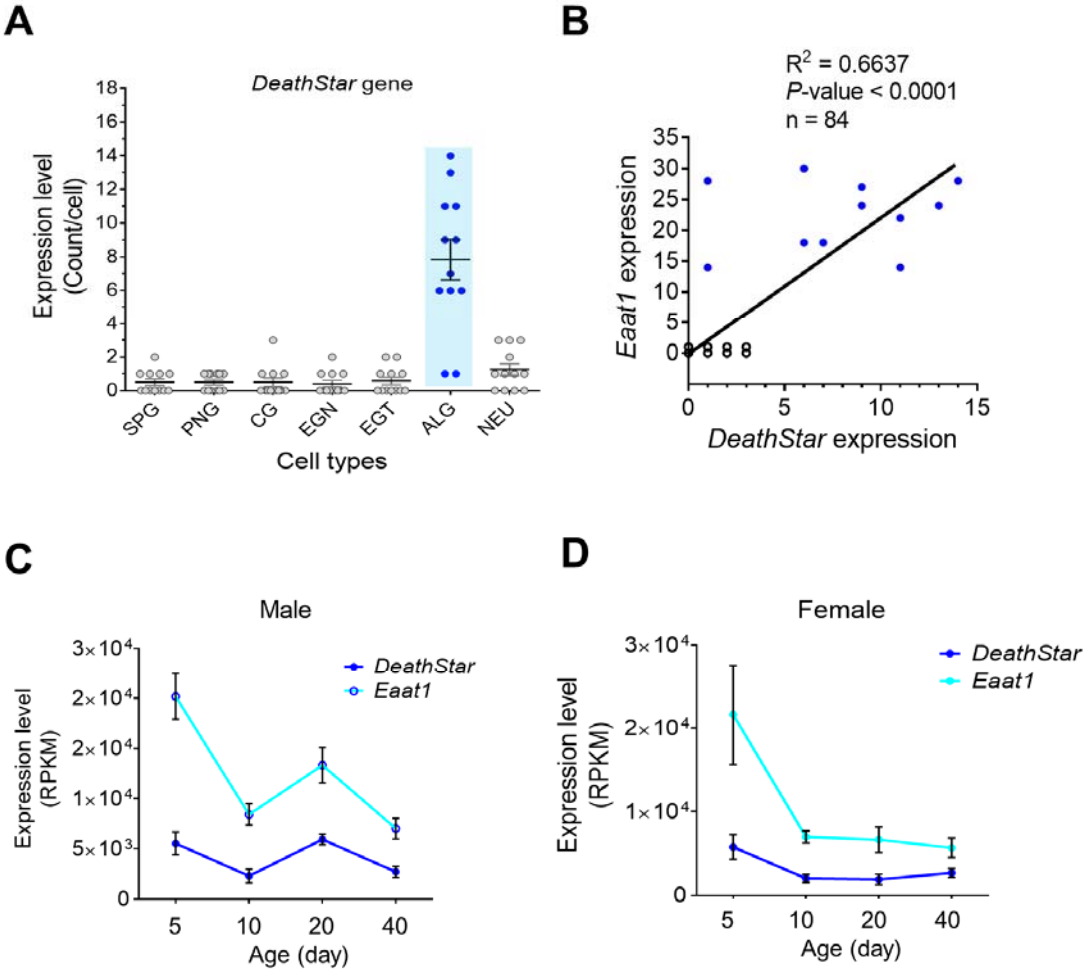


Figure 3. Expression specificity of *deathstar* gene in ALG cells of *D. melanogaster*. A) While the ALG cells exhibited high level of *deathstar* mRNA (Blue points), other glia cells as well as neurons showed low or no expression of this gene (gray points). B) Positive correlation between expression level of known ALG marker (*Eaat1* gene) and that of *deathstar* gene. Blue and gray points respectively denote expression of the genes in ALG and non-ALG single cells. C) Similar expression pattern of *deathstar* (dark blue) and *Eaat1* (bright blue) genes during development of male *D. melanogaster*. D) Similar expression pattern of *deathstar* (dark blue) and *Eaat1* (bright blue) genes during development of female *D. melanogaster*. SPG= subperineural glia, PNG= perineural glia, CG= cortex glia, ALG= astrocyte-like glia, EGN= neuropil ensheathing glia, EGT= tract ensheathing glia, and NEU= neurons.

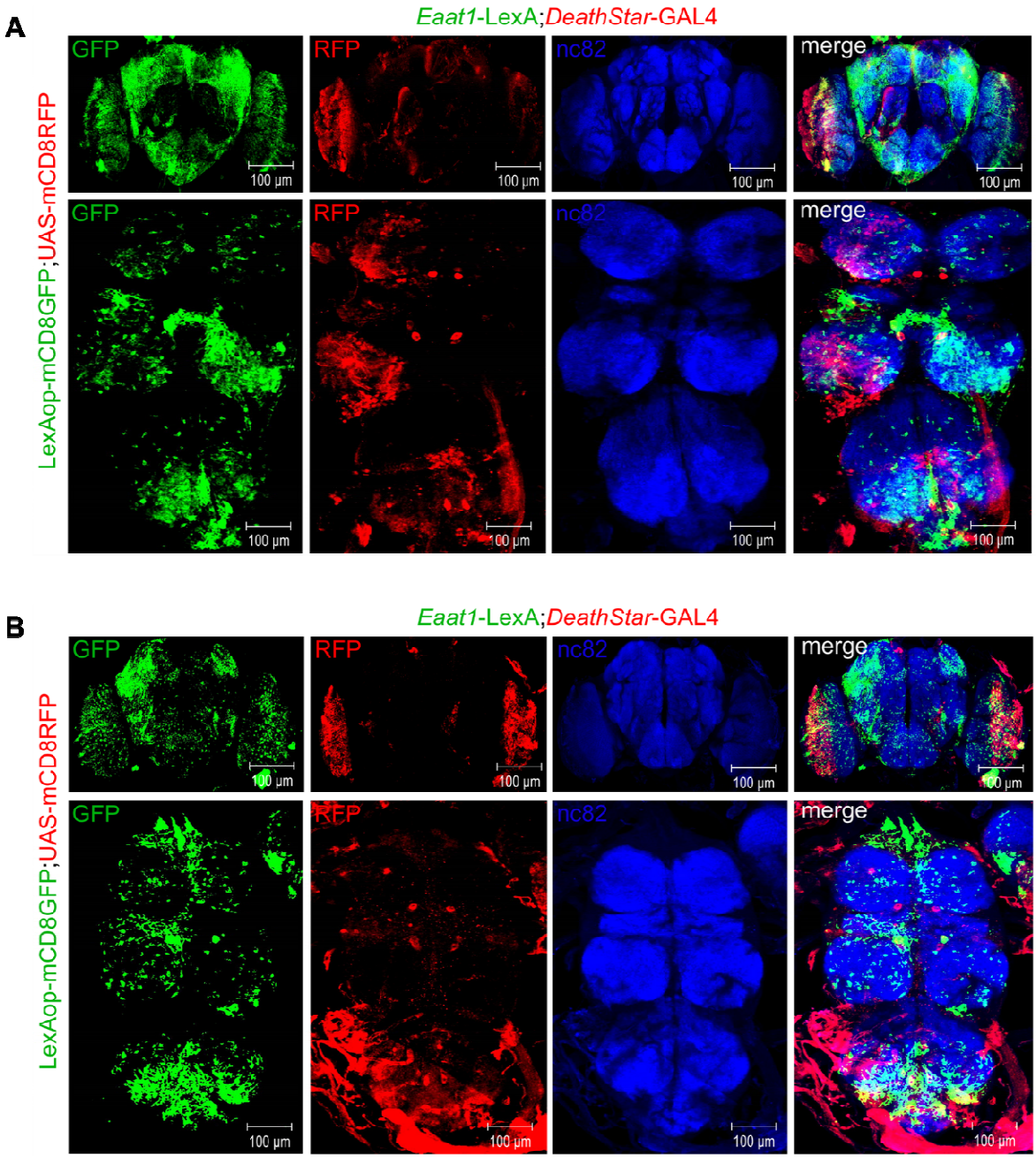


Figure 4. *deathstar* gene is differentially expressed in *Drosophila* ALG cells. Immunostaining result of CNS in male (A) and female (B) flies. In both images, the ALG cells were stained with anti-GFP (green), and the Red signals (anti-RFP) reflect UAS-RFP driven by a GAL4, tagged by the promoter region of *deathstar* gene (*deathstar*-GAL4). Anti-nc82 was used to label neuropil (Blue). Co-labeling of astrocytic cells using *Eaat1*-LexA/LexAop-mCD8GFP (green) together with *deathstar*-GAL4/UAS-RFP (Red) showed ALG-specific expression of *deathstar* gene among other types of *Drosophila* glia cells. Scale bars represent 100 µm in all image parts.

Sex-biased developmental effect of *deathstar* gene in *D. melanogaster*

We crossed GAL4 strains that are expressed in each neural cell types of *D. melanogaster* with the *deathstar*-dsRNA to knockdown deathstar protein level in each neural cell types. Our results showed that most of the males expressing *deathstar*-RNAi in all neural cell types are lethal during development. However, the females with the same genotype developed normally to adult. Both males and females in the control group (harbouring *deathstar*-RNAi without GAL4 expression) showed normal development (Fig. 5A). This result suggests that the *deathstar* gene may have a developmental function exclusively in male flies. However, such developmental effect was not cell type-specific. As the control of this experiment, the *Drosophila CG15765* gene was examined in the same procedure, and a standard ratio of males over females (~1) was obtained for all genotypes (Fig. 5B). The exact number of the progenies of the corresponding crosses are summarized in the supplemental file S3.

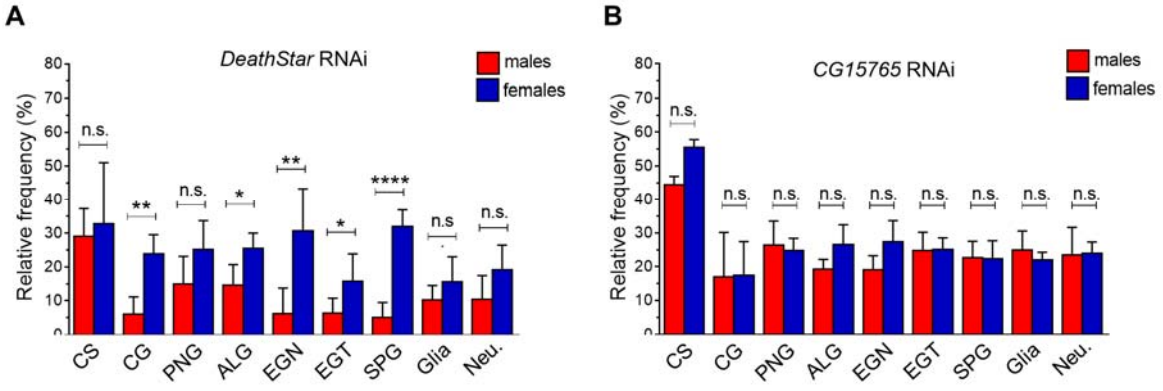


Figure 5. Male-specific developmental effect of *deathstar* gene in *D. melanogaster*. A) While the females with *deathstar* RNAi showed normal development, male flies with the same genotypes could not be matured to adults

and showed overall less frequencies in comparison with the expected ratio of each genotype. B) Using another RNAi as control (*CG15765* RNAi), equal number of males and females were observed which were consistent with the expected ratio of each genotype. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ and $****P < 0.0001$, n.s.= non-significant ($P \geq 0.05$).

Knockdown of *deathstar* gene affects locomotion activity of *D. melanogaster*

It has been previously reported that ALG cells that cover specific territories of *Drosophila* CNS have significant effects on locomotion behaviours [18]. To investigate the role of *deathstar* gene in locomotion behaviour in *D. melanogaster*, we knocked down the deathstar protein level using RNAi in different cell types of CNS. Then we performed the climbing assay. Results showed that the flies that specifically express *deathstar* RNAi in astrocytes show significant defects in climbing ability (Fig. 6A), suggesting the crucial role of this gene in this cell type. Interestingly, we found that the knockdown of deathstar protein does not affect the climbing activity in female flies (Fig. 6B). When we knocked down the *deathstar* in EGN and SPG, however, female flies showed severe defects in climbing activity (Fig. 6B). These data suggest that the expression of *deathstar* in ALG cells is crucial only in males, not females regarding the climbing activity.

To test whether the behavioural locomotive effect of *deathstar* gene is altered during the ageing of *D. melanogaster*, the climbing assay was performed on the flies with different ages. Results showed that the older flies expressing *deathstar*-RNAi in all neural cell types (three- and four-week-old flies) exhibited significant climbing deficiency (Fig. 6C). However, when we tested the same genotype of flies in the one- and two-week stages, the climbing defects appeared in ALG-specific manner (Fig. 6C). The climbing defects were not observed in the flies with neuron-

specific suppression of *deathstar* gene, suggesting the climbing defects related to *deathstar* is glial-specific in old flies (Fig. 6C). Unlike the male flies, both young and old females did not exhibit any meaningful pattern of climbing defects (Fig. 6D). All these data suggest that the expression of *deathstar* in male ALG cells is crucial for climbing ability in the early adult stage.

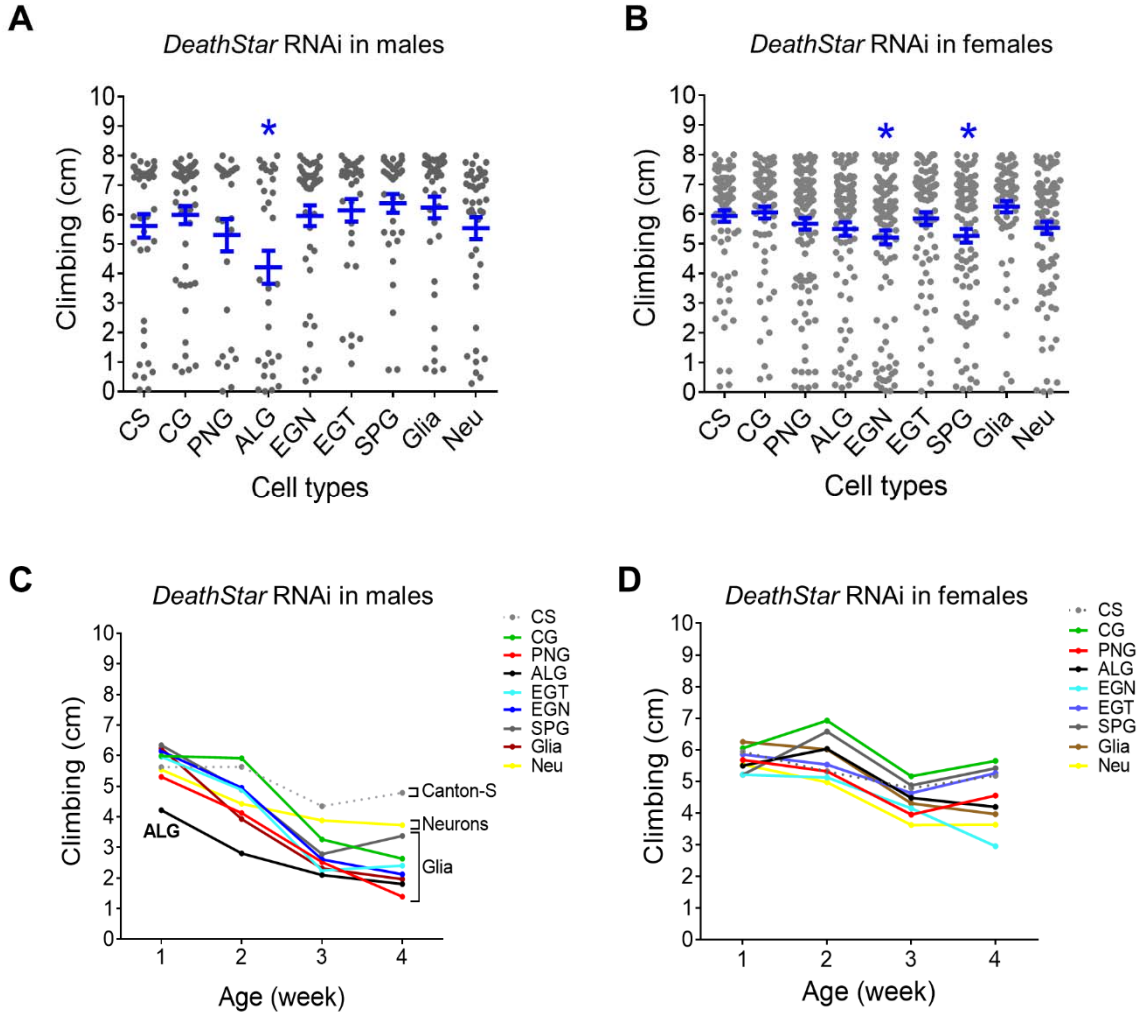


Figure 6. Effects of *deathstar* gene suppression on locomotion behavior of *D. melanogaster*. A) ALG-specific reduction of climbing activity in male flies under suppression of *deathstar* gene. B) Slight EGN- and SPG-specific reduction of climbing behavior in female *D. melanogaster* under suppression of *deathstar* gene. C) Climbing behaviors of male *D. melanogaster* expressing *deathstar* RNAi during different times of development (weeks 1-4) showing ALG-specific effect of the gene. D) Climbing behaviors of female *D. melanogaster* expressing *deathstar* RNAi during different times of development (weeks 1-4) showed no significant effect in ageing flies.

Astrocyte-specific suppression of *deathstar* gene shortens lifespan in *D. melanogaster*

To test the possible function of *deathstar* gene in *D. melanogaster* lifespan, we knocked down *deathstar* in distinct CNS cell types (CG, PNG, ALG, EGN, EGT, SPG, Glia and Neurons) and measured their life span for 40~50 days. We observed a significant reduction in lifespan for the flies that had ALG-specific expression of *deathstar* RNAi, supporting the specific effect of *the deathstar* gene in ALG on the life span of *D. melanogaster* (7A). The ALG-specific effect of *deathstar* RNAi on lifespan was observed in both male and female flies (Fig. 7A and 7B), suggesting that the role of *deathstar* on the lifespan is not sexually dimorphic.

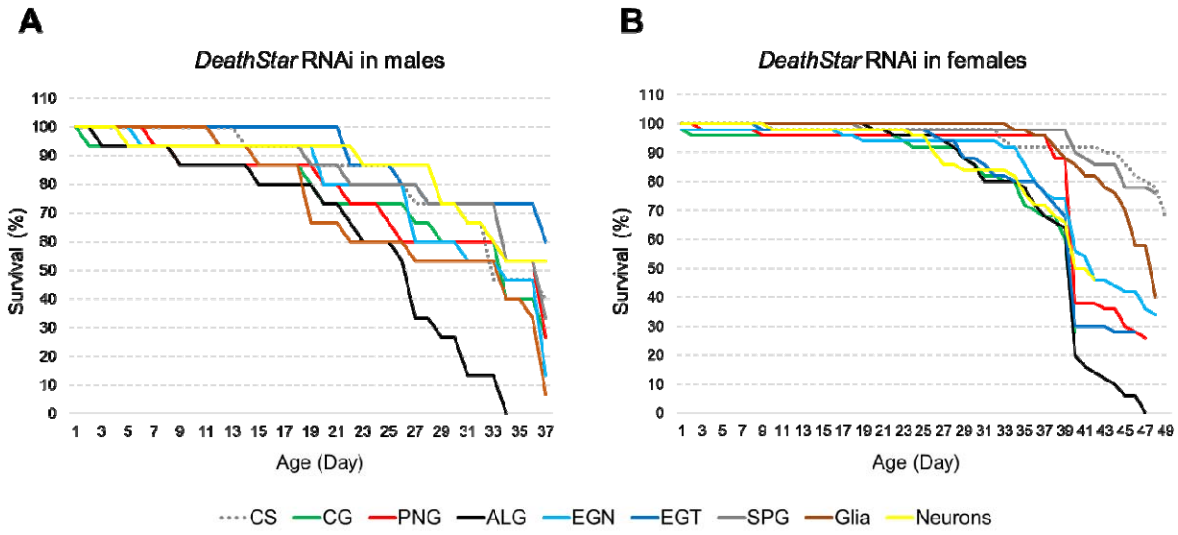


Figure 7. Lifespan effect of *deathstar* gene suppression in *D. melanogaster*. Suppression of *deathstar* gene shortened lifespan of male and female flies in an ALG-specific manner.

DISCUSSION

The brain of *D. melanogaster* is a complex organ with a diverse set of cells serving analogous functions to those of human [7]. Differences between the transcriptome of these cells can determine their fate to be a particular cell type with specific functional characteristics [12]. Previous studies identified and reported the existence of several cell types in nervous system of *D. melanogaster* assigned as; perineural glia (PNG), subperineural glia (SPG), cortex glia (CG), neuropil ensheathing glia (EGN), tract ensheathing glia (EGT), astrocyte-like glia (ALG) and neurons (Neu) [6].

The specific marker genes were for each cell type has been reported. These cell type-specific markers were frequently used for structural and functional annotation of different circuits in the nervous system of *D. melanogaster* [6]. In this study, the transcriptome of 55,000 single cells within the optic lobe of *D. melanogaster* brain was analyzed to identify novel cell-type-specific genes followed by functional analysis of the genes. The optic lobe occupies about two-third of whole *D. melanogaster* brain [19] with diverse set of cells including the above-mentioned cell types [12].

To determine the type of the sequenced single cells, we used previously-reported specific marker genes and the cells with the highest expression level of a particular marker and lack of expression for other markers. Then we classified our bioinformatical analysis with a specific cell type (Fig. 1). In order to test the accuracy of this classification, the selected single cells were compared to each other based on their whole transcriptome (e.i. 17272 *Drosophila* genes).

Results showed successful clustering of most single cells into their corresponding cell type (Fig. 2A). Only the predicted SPG cells could not be classified in a single cluster possibly because of expression heterogeneity of their marker genes (*Mdr65*, *moody* and *Gli* genes) or differences in transcriptome due to their functional specification or physical territory changes within CNS. SPG cells were shown to be equivalent to the vertebrate blood-brain barrier (BBB). Since they may possess the hallmarks of a potent interface between *Drosophila* brain and environment, their transcriptome can be influenced by their positioning and environmental circumstance [20]. Therefore, each single SPG cell may have its profile and could not be categorized in a single cluster based on the transcriptome. Similarly, shreds of evidence showed that ensheathing glia (EG) cells could change their expression profile to be involved in the immune system, thereby their transcriptome will be different from their initial identity [21].

While two specific markers classified PNG cells, *CG4797* and *gem*, (Fig. 1), they were categorized well in two separate clusters based on their transcriptome, here assigned as PNG-α and PNG-β (Fig. 2). This data suggests a new subtype of PNG in *D. melanogaster*. However, further computational and experimental works need to be performed for confirmation of the suggested subtypes.

The best clustering result was found for ALG. Among the six analyzed cells, five cells were clustered together, and the other one was very close to this cluster (Fig. 2). Through comparison of the transcriptome of the cells in the ALG cluster to the other single cells of *Drosophila* optic lobe, the *deathstar* gene was found to be differentially enriched in ALG (Fig. 3A). Therefore, we

hypothesized that this gene could be a novel ALG-specific gene and could mediate specific functions of ALG cells within the central nervous system of *D. melanogaster*.

In order to test this hypothesis, its expression was compared with the previously-known marker for ALG (*Eaat1* gene) using two different expression datasets (GSE103771 and GSE107049). These data showed a significant positive correlation between *deathstar* and *Eaat1* genes which suggest their similar pattern of expression in single cells of the optic lobe (Fig. 3B) and also across different stages of *Drosophila* brain development (Fig. 3C and 3D). Given the ALG-specificity of *deathstar* gene based on the analyzed RNA sequencing data, we decided to determine its expression pattern in the central nervous system of *D. melanogaster* using fluorescent labelling of the *deathstar* gene (by RFP) and the known ALG marker (*Eaat1*) by GFP. Results showed strong bilateral signals corresponding to six single cells for each side of ventral nerve cored and also many single cells within optic lobe (Fig. 4, red signals).

Such specific territories of ALG cells in the ventral nerve cord of *D. melanogaster* were also reported by Peco et al., who attributed some locomotion behaviours of *D. melanogaster* to these cells [18]. According to these findings, the locomotion behaviour of *D. melanogaster* was assessed under the suppression of *the deathstar* gene in male and female flies. For this purpose, male flies expressing GAL4 in different neural cell types were crossed with virgin females harbouring the UAS-*deathstar*-RNAi (Table 1). Unexpectedly, we found that the number of newly-eclosed male progenies was drastically lower than females. The collection of male and female flies was repeated for six times, and the same result was obtained (Supplemental file S3).

After counting and quantification of the data, we found that while the progenies of the control group (Canton-S × UAS-*deathstar*-RNAi) shows the same ratio of male and female flies, all other progenies expressing *deathstar* RNAi shows the female-biased ratio of progenies (Fig. 5A). For the control of the experiment, another *Drosophila* gene named *CG15765* was analyzed in the same procedure parallel with *the deathstar* gene. Results of *CG15765* gene showed the same ratio for males and females in all conditions (Fig. 5B). Suppression of *the deathstar* gene in female flies did not show any developmental defect or visible phenotype change (Fig. 5A). These data suggest that *the deathstar* gene is specifically involved in the development of male *D. melanogaster*.

Interestingly, the survived male flies showed a reduced locomotion activity, exclusively when the *deathstar* gene was suppressed in ALG cell type, supporting the specificity of this gene in ALG cells. However, *deathstar* suppression in females did not show any significant changes in climbing activity (Fig. 6). Females showed an EGN- and SPG-specific reduction of climbing activity. However, this effect was not observed throughout their lifespan (Fig. 6B and 6D). These results suggest that while the expression pattern of *the deathstar* gene is similar in both sexes of *D. melanogaster* (Fig. 4), it has differential effects on locomotion behaviours in male and female flies.

There are other pieces of evidence for ALG-enriched gene that have different patterns of expression during early and late stages of *D. melanogaster* development. ALG-enriched genes in larval stages are mainly involved in metabolism, energy production, and protein synthesis, consistent with the known role of astrocytes in metabolic support of neurons [22]. In comparison with the larval astrocytes, ALG cells in adult *D. melanogaster* acquire a new function for regulation of behaviours, additional to their functions in earlier stages [22].

Therefore, it is expected that the suppression of *the deathstar* gene in larval stages is lethal in males. However, this suppression at adulthood stages mediates climbing defects in males (Fig. 5 and 6).

Analysis of lifespan in the flies expressing *deathstar* RNAi in different cell types showed an ALG-specific reduction of lifespan in both sexes which could be another supportive evidence for specificity of *deathstar* gene to ALG cell type (Fig. 7). It is known that *Drosophila* astrocytes (ALG) are significant contributors to modulate neurotransmitter homeostasis within synapses [23] and also regulators of circadian rhythm [24].

Therefore, it is likely that abolishing the functions of ALG or ALG-specific genes could interfere with normal locomotion properties and lifespan of *D. melanogaster*, which are the direct outcome of dysregulations in neurotransmission and circadian rhythm, respectively. Moreover, it was recently reported that mitochondrial dysfunctions in ALG cells contribute to neurodegeneration [23, 25], which could give rise to shortened lifespan of *D. melanogaster* [26].

Taken together, *deathstar* (previously known as *CG11000*) was reported as a protein-coding gene in a fruit fly with 332 amino acids. Its functional role was not known yet in *Drosophila*, and it has no homologue gene in human (according to Flybase reports). In this study, we firstly annotated *deathstar* as an ALG-specific gene which could mediate the developmental process of the male flies additional to its functions in locomotion and lifespan properties of *D. melanogaster*. Further experiments need to be performed in order to elucidate the molecular

mechanisms underlying the observed developmental and behavioural effects of this gene in *D. melanogaster*.

AUTHOR CONTRIBUTIONS

Concep and design of experiments: WJK, HN and KW; performing the experiments: HN, KW and WJK; acquisition of data: HN, KW and WJK; analysis and interpretation of data: HN, KW and WJK; drafting or revising the article: HN and WJK.

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