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·Original Article·

Automated rapid iterative negative geotaxis assay and its use in a genetic screen for modifiers of $A\beta_{42}$-induced locomotor decline in *Drosophila*

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ABSTRACT

The negative-geotaxis climbing assay is used to efficiently study aging and neurodegeneration in *Drosophila*. To make it suitable for large-scale study, a method called the rapid iterative negative geotaxis (RING) assay has been established by simultaneously photographing the climbing of multiple groups of flies when they are manually tapped down in test tubes. Here, we automated the assay by using a well-controlled electric motor to drive the tapping, and a homemade program to analyze the climbing height of flies. Using the automated RING (aRING) assay, we found that the climbing ability of a strain of wild-type flies, males in particular, declined rapidly before day 21 after eclosion, but slowly from day 21 to 35. We also found that the expression of arctic mutant $A\beta_{42}$ accelerated the age-dependent decline in the climbing ability of flies. Moreover, using aRING, we examined the effect of third chromosome deficiencies on the accelerated locomotor decline in $A\beta_{42}$-expressing flies, and isolated 7 suppressors and 15 enhancers.

Keywords: RING; negative geotaxis; giant fiber; age-dependent; locomotor decline; *Drosophila*; beta amyloid

INTRODUCTION

Drosophila has been widely used to model human neural diseases, test chemical compounds for disease modulation, and study the genetics of aging[1-4]. In the modeling of Alzheimer's, Parkinson's, and other neurodegenerative diseases with *Drosophila*, disease-causing proteins or peptides are expressed in neurons, resulting in neuropathological changes and behavioral alterations[5]. The negative-geotaxis climbing assay is frequently used to analyze the behavioral outcome of neuronal dysfunction and the effect of potential disease-modulating factors or chemical compounds in these disease models. The traditional climbing assay involves one transparent plastic tube containing a single or a certain number of flies. During the assay, the tube is vertically placed on a table, and all flies are manually tapped down to the bottom of the vial. Due to an innate escape response, flies ascend the wall of the tube. The climbing behavior is either visually examined

or recorded by a digital video or photography. The climbing ability is usually scored as the percentage of flies reaching a predefined height within a prescribed period of time.

However, the traditional climbing assay is tedious and time-consuming, unsuitable for large high-throughput studies, and has potential variations of the manual force used to tap down the flies and other limitations. To overcome these limitations, Grotewiel and colleagues developed a new method for the climbing assay, named the rapid iterative negative geotaxis (RING) assay[6], in which the climbing of multiple animal groups is examined and recorded simultaneously with a digital camera, and the vertical positions of all individual flies on the photographs are assessed either manually or extracted by commercial software, then the climbing heights of individual flies in each group are averaged. Later, Podratz *et al.* developed a system that can automatically tap flies down[7]. However, similar to the traditional climbing assay, only one group of flies can be examined during a single test, and only the percentage of flies having climbed above a predefined height is calculated.

Compared to the traditional climbing assay, the RING assay is much more powerful and can quantify the performance of all flies in many different groups simultaneously. However, in the RING assay, flies are still manually tapped down to the bottom of test tubes, and the average climbing height of each group is acquired using multiple programs.

To make the RING assay more efficient, in this study we automated it by using a small electric motor to drive the RING apparatus for tapping down flies, and a digital video recorder and a homemade program for measuring the climbing height of each individual fly and calculating the average height of all flies in the same test tube. Then the automated RING (aRING) assay was used to test the climbing ability in a recently developed *Drosophila* model of Alzheimer's disease[8] and to genetically screen for potential modifiers of the climbing ability phenotype.

MATERIALS AND METHODS

The Automated RING Apparatus and Associated Equipment

The modified RING apparatus consists of a rectangular metal frame (32 cm × 21 cm × 6 cm) that can hold 10 transparent plastic tubes (2.1 cm in diameter, 19.0 cm in height), each secured on the frame by a screw (Fig. 1A). The associated equipment includes a horizontal metal base with two metal rods and a foam bar attached to it, a small electric motor, a 2PH micro-step driver (type: 2M2260, Doall Industrial Parts and Equipment, Wuxi, China) and an electronic digital display controller (type: MTPG2-5E2N, Xin'er Electronic, Yueqing, China) (Fig. 1A). The controller controls the step driver, which drives the small electric motor to run 2 rounds in 3 s so that the lever attached to the motor can consecutively rise (5.8 cm) and tap the RING apparatus 4 times (Supplementary Video 1). After 4 consecutive taps, all the flies in each tube drop down to the bottom of the tubes. A digital video recorder mounted on a tripod 50 cm in front of the metal frame, is used to record the climbing behavior of flies.

Automated RING Assay

The aRING assay was conducted in a way similar to that described by Gargano *et al.*[6]. Flies were collected under brief CO_2 anesthesia (1–2 min) and allowed to recover overnight at 25°C under 70% relative humidity prior to assay. aRING was performed in the same environment. To assess aRING, 10 flies were transferred into each negative geotaxis tube, and 10 tubes with flies were loaded into the modified RING apparatus and secured with screws. After 1-min rest, the step controller started to run to control the step driver and the small electric motor so that the RING apparatus was automatically tapped 4 times to tap down all flies, then flies began to ascend the wall. Flies were assessed in 3–5 consecutive trials separated by 60-s intervals, and the whole process was recorded with the digital video recorder (Supplementary Video 1).

Data Analyses and Statistical Tests

Digital videos of aRING were imported into a PC, displayed with Windows Media Player (Microsoft Corp., Redmond, WA), and a snapshot of the video of each trial was taken at the time point when the flies had climbed for a prescribed period. The snapshots were opened with our homemade software "RflyDetection". The scale of a snapshot was defined by the ratio of the test tubes' height on the snapshot to their real height (19 cm) (Fig. 1B). After determination of the vertical scale, a selecting rectangle was drawn to include all flies in a tube, each fly in the tube was

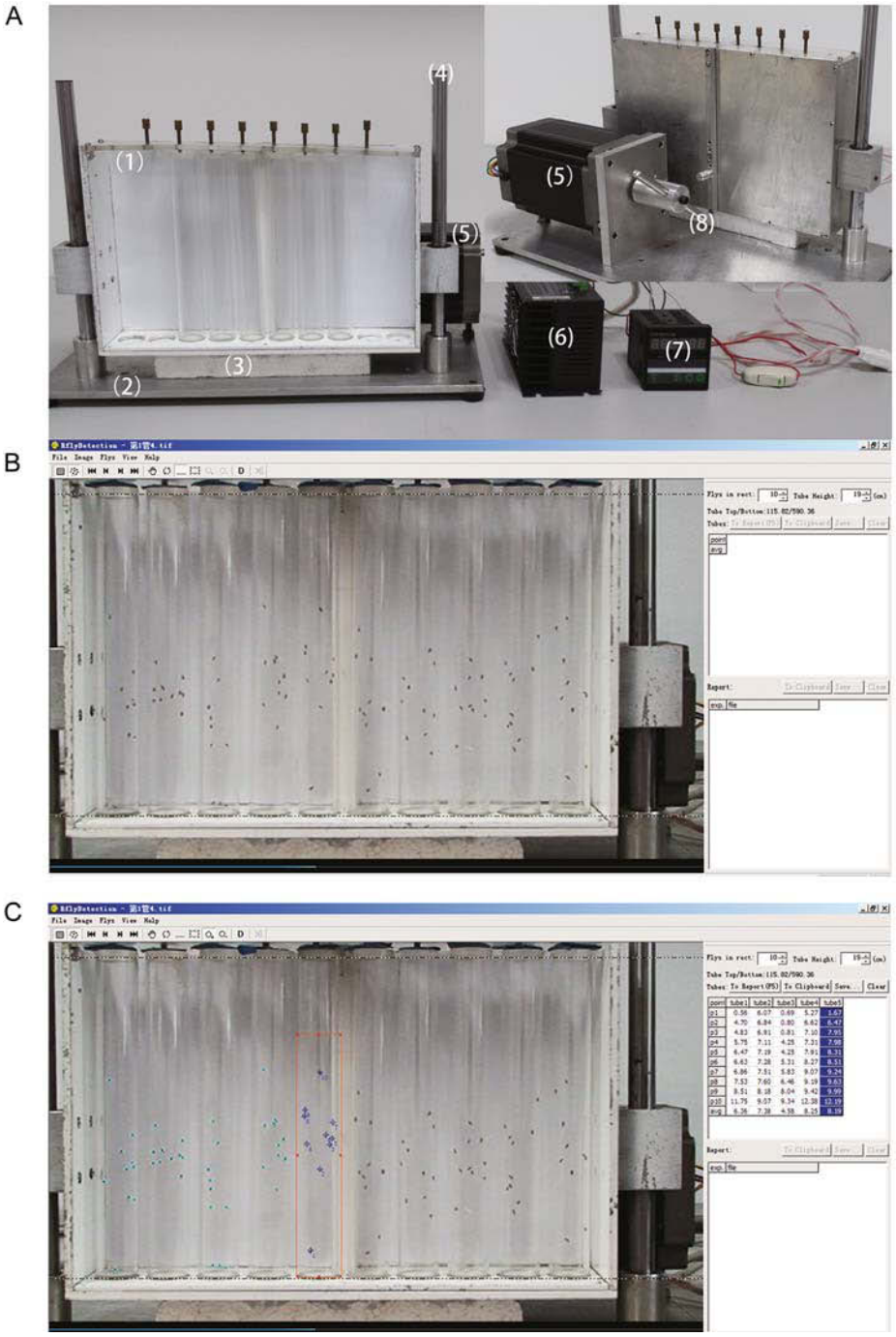

Fig. 1. The aRING assay. A, The equipment and set-up for the aRING. (1) aRING apparatus with test tubes, (2) metal base, (3) foam bar, (4) vertical steel rod, (5) small electric motor, (6) micro-step driver, (7) electronic controller, (8) lever attached to the small electric motor. The insert in the right upper corner is a rear view of the system. A digital video recorder (not shown for clarity) was placed 50 cm in front of the aRING apparatus; B, A snapshot of the video opened with our homemade "RflyDetection" software. Please note the two horizontal dashed lines placed at the top and bottom of the test tubes, which were used for scaling. The number of flies in each test tube and the tube height are shown on the top in a built-in table on the right; C, Tube-by-tube labeling and measurement of flies' vertical positions using "RflyDetection". Please note that the climbing heights of flies and the average values are shown in the built-in table. Please refer to Supplementary Video 2 for a demonstration of using "RflyDetection".

automatically labeled, the climbing height was recorded for each fly and averaged from the 10 flies and displayed in a table (Fig. 1C, Supplementary Video 2), which was saved and opened with Excel (Microsoft Corp.) for further analysis. Sometimes the positions of two flies overlapped, making them hard to distinguish with the homemade software or by visual inspection. In this case, the video was played again around the time point of the snapshot to determine the position of the overlap and label this position twice. In this way, the climbing heights of individual flies in a tube and the average value were obtained. The average values of a tube in 3–5 consecutive trials were further averaged to generate a single data point. As described by Gargano *et al*.[6], negative geotaxis scores obtained from aRING displayed a Gaussian distribution (data not shown), and standard parametric tests in Prism (GraphPad software) were used to assess statistical significance. P <0.05 was considered to be statistically significant.

Fly Stocks and Genetics

Drosophila stocks were cultured on standard medium and entrained into a 12 h/12 h light/dark cycle at 25°C. The recipe of the medium was: ddH_2O 0.65 L/L, baker's yeast 16 g/L, corn flour 80 g/L, agar 6.5 g/L, brown sugar 137.5 g/L, and beer yeast 7.5 g/L; and the recipe of preservative was: methylparaben 2 g/L, alcohol 20 ml/L, ddH_2O 100 mL/L, and propionic acid 6.25 mL/L.

The genetic background of [UAS]Aβ_{arc} and [Gal4]A307 transgenic flies[8] was purified by crossing to an isogenic wild-type line *w* ^{1118} (Bloomington stock number 5905) for 10 generations. The resulting homozygous [UAS]Aβ_{arc} and [Gal4]A307 flies were used to generate Aβ_{arc} flies expressing the arctic mutant Aβ_{42} in the giant fiber system. Aβ_{arc} flies at different ages were subjected to aRING to study the age-dependent locomotor decline.

Both [UAS]Aβ_{arc} and [Gal4]A307 transgenes were inserted in the 2nd chromosome. To obtain [Gal4]A307-[UAS]Aβ_{arc}/Cyo flies, PCR and DNA sequencing were used to screen the progeny of [UAS]Aβ_{arc}/[Gal4]A307 females. In the genetic screen for modifiers of Aβ_{arc} expression-induced enhancement of age-dependent decline of locomotion, we mated each of the deficient lines of the 3rd chromosome that are included in the Bloomington deficiency kit (Bloomington Stock Center) to [Gal4]A307-[UAS]Aβ_{arc}/Cyo and [Gal4]A307/[Gal4]A307 flies to generate [Gal4]A307-[UAS]Aβ_{arc}/+;df/+ (Aβ_{arc}-df) and [Gal4]A307 /+;df/+ (df) flies. We also mated [Gal4]A307-[UAS]Aβ_{arc}/Cyo to *w*^{1118} flies to generate [Gal4]A307-[UAS]Aβ_{arc}/+;+/+ (Aβ_{arc}) flies.

RESULTS

Using aRING, we first found that the climbing height of wild-type flies at the age of 7 days was linearly correlated with the climbing duration (Fig. 2A), while the position of the test tubes in the RING apparatus did not affect the negative-geotaxis scores (Fig. 2B). The climbing heights of flies of the same gender and age in three consecutive experimental sets were consistent (Fig. 2C). We also examined the climbing ability in another two groups of wild-type flies at the same age using traditional RING, and found no significant difference when compared to those acquired with aRING (Fig. 2D). Similar to traditional RING, aRING did not cause injury to the tested flies as manifested by the survival rate (Fig. 2E). These findings are consistent with the reports of Grotewiel and his colleagues[6, 9]. Please note that the linear correlation between climbing height and duration lasted for the first 10 s rather than 5 s; this was due to the difference in the length of test tubes, and that the climbing speed of our flies was faster than that in their studies, perhaps due to the rougher inner wall of our test tubes.

Then, we conducted the RING assay on isogenic wild-type (*w*^{1118}) flies at the ages of 7, 14, 21, and 35 days after eclosion. We plotted the climbing height against climbing duration, and found an age-dependent decline of climbing ability in both male and female flies (Fig. 3A) as expected. Interestingly, the decline of climbing ability was rapid from days 14 to 21, particularly in males, but relatively slow from days 21 to 35 (Fig. 3A), indicating a rapid locomotor decline during aging from day 14 to 21. The same conclusion was made by comparing the climbing heights at 5 s after the initiation of the negative geotaxis (Fig. 3C).

We have previously reported that wild-type flies exhibit an age-dependent decline in flight ability, and expression of the wild-type (Aβ_{42}) and arctic mutant form (Aβ_{arc}) of beta amyloid 42 in the neurons of the giant fiber system and a subgroup of neurons elsewhere in adult flies accelerates this decline in association with age-dependent alterations of synaptic structure and function[8, 10, 11]. To investigate whether the age-dependent decline in climbing ability could be affected by the expression of Aβ_{arc} in these neurons, we

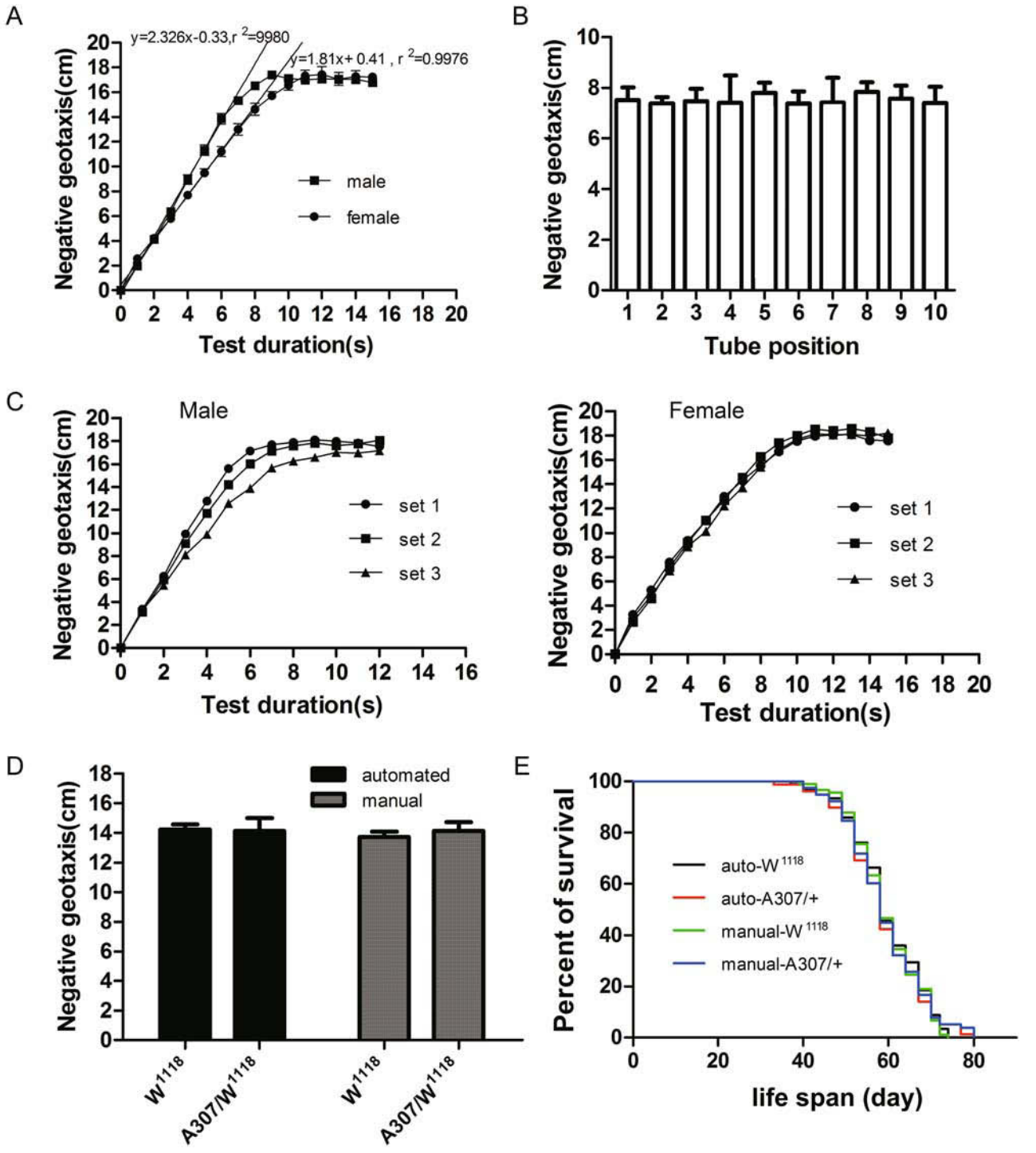

Fig. 2. Validation of the aRING assay. A, Linear correlation of climbing duration and height in both male and female wild-type flies, n = 3 for each line. B, The positions of the test tubes did not change the average climbing height of flies, *n* = 3 for each bar. C, The climbing heights of male (left) and female (right) wild-type flies of the same age in three independent experimental sets were consistent, demonstrating that the aRING assay is highly reproducible; *n* = 3 for each line. D, Comparison of flies' climbing ability examined by traditional RING and aRING. No significant difference, one-way ANOVA. E, The life spans of RING- and aRING-tested flies. No significant difference, one-way ANOVA.

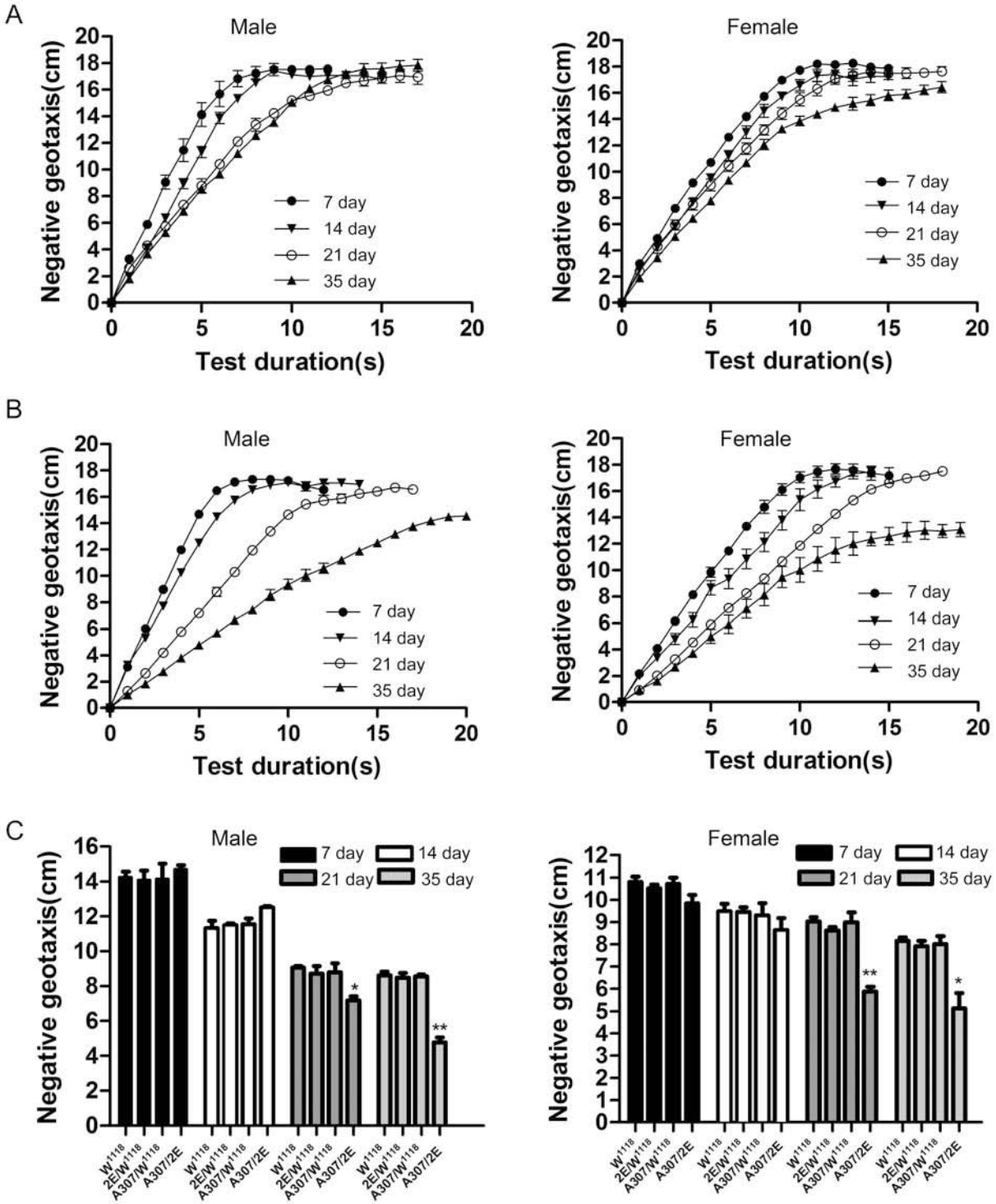

Fig. 3. Distinct age-dependent decline of climbing ability in both wild-type and $A\beta_{arc}$-expressing flies. (A, B) Plots of the climbing height against climbing duration in male and female wild-type (w^{1118}) (A) and $A\beta_{arc}$-expressing flies (B), on days 7, 14, 21, and 35 after eclosion; n = 3 for each line. C, Histograms showing the average climbing height at 5 s after the initiation of negative geotaxis in wild-type (w^{1118}), $A\beta_{arc}$-expressing (A307/2E) flies, and two control lines (2E/w^{1118} and A307/w^{1118} containing [UAS]$A\beta_{arc}$ and [Gal4]A307 transgenes respectively, but without expression of $A\beta_{arc}$). n = 3 for each bar, *P <0.05 and **P <0.01 compared with w^{1118} flies, one-way ANOVA.

conducted the aRING assay on $A\beta_{arc}$-expressing flies, wild-type, and two groups of other control flies (2E/w^{1118} and A307/w^{1118}) at the ages of 7, 14, 21, 35 days. All four groups exhibited an age-dependent decline in climbing ability, but the decline was significantly faster from days 14 to 21 and 21 to 35 in $A\beta_{arc}$-expressing flies than in the other groups (Fig. 3A&B), although the climbing ability of all four groups was not significantly different from each at the age of 7 and 14 days (Tables 1–3). Therefore, expression of $A\beta_{arc}$ in the giant fiber system also accelerated the age-dependent decline of climbing ability. Again, the same conclusion can be made by comparing the climbing heights at 5 s after initiation of the negative geotaxis in $A\beta_{arc}$-expressing flies with those in control flies (Fig. 3C).

Table 1. Differences in the climbing ability among different groups of flies during aging

Genotype	Age							
	7 days		14 days		21 days		35 days	
	Male	Female	Male	Female	Male	Female	Male	Female
w^{1118} *vs* 2E/w^{1118}	0.997	0.873	0.967	1.000	0.914	0.738	0.965	0.972
w^{1118} *vs* A307/w^{1118}	0.999	0.994	0.940	0.990	0.956	0.999	0.997	0.995
w^{1118} *vs* A307/2E	0.936	0.146	0.064	0.550	0.031*	<0.001*	<0.001*	0.004*
2E/w^{1118} *vs* A307/w^{1118}	1.000	0.958	0.999	0.994	0.999	0.798	0.992	0.997
2E/w^{1118} *vs* A307/2E	0.860	0.388	0.12	0.574	0.076	0.001*	<0.001*	0.006
A307/w^{1118} *vs* A307/2E	0.893	0.207	0.14	0.716	0.062	<0.001*	<0.001*	0.005*

P values acquired from one-way ANOVA, "*" represents significant difference.

Table 2. Statistical analysis of age-dependent decline of climbing ability in each group of flies

Age	Genotype							
	w^{1118}		2E/w^{1118}		A307/w^{1118}		A307/2E	
	Male	Female	Male	Female	Male	Female	Male	Female
7 *vs* 14 days	<0.001*	0.018*	0.007*	0.024*	0.038*	0.164	<0.001*	0.375
7 *vs* 21days	<0.001*	0.003*	<0.001*	0.001*	0.001*	0.078	<0.001*	0.002*
7 *vs* 35days	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.009*	<0.001*	0.001*
14 *vs* 21 days	0.002*	0.558	0.004*	0.070*	0.029*	0.948	<0.001*	0.017*
14 *vs* 35 days	0.001*	0.019*	0.003*	0.003*	0.019*	0.220	<0.001*	0.005*
21 *vs* 35 days	0.727	0.123	0.970	0.129	0.990	0.428	<0.001*	0.705

P values acquired from one-way ANOVA,"*" represents significant difference.

Table 3. Statistical significance of the differences in age and genotype

	Age	Genotype	Age & Genotype
Male	<0.001*	0.02*	<0.001*
Female	<0.001*	<0.001*	<0.001*

P values acquired from two-way ANOVA, "*" represents significant difference.

The modified RING assay was further used in a genetic screen for modifiers of the accelerated age-dependent decline of climbing ability in $A\beta_{arc}$-expressing flies. All the lines (180 in total) of the 3rd chromosome of the deficiency (df) kit from the Bloomington Stock Center were used for the genetic screening. As the acceleration of age-dependent locomotor decline by $A\beta_{arc}$-expression was most prominent in males from day 21 to 35, the

negative geotaxis of $A\beta_{arc}$-df flies was examined at the age of 25 days, and compared with that of $A\beta_{arc}$ flies. Since some dfs may change the normal age-dependent decline of climbing ability in the absence of $A\beta_{arc}$ expression, we also compared the negative geotaxis scores of df flies with that of w^{1118} flies (please refer to the methods for detailed information on the $A\beta_{arc}$-df, $A\beta_{arc}$, df and w^{1118} flies). As shown in Fig. 4, when the climbing heights of $A\beta_{arc}$-df flies at 5 s after the initiation of negative geotaxis were significantly higher or lower than that of $A\beta_{arc}$ flies, while df and w^{1118} were not significantly different, then the df was identified as a suppressor or enhancer. A total of 180 df lines were examined, and 7 suppressors and 15 enhancers were identified (Table 4). Interestingly, two enhancers (stock numbers 23714 and 24137) cover the same gene CG3731, whose protein product is a homolog of the mammalian peptidase (mitochondrial processing) beta (PMPCB). The inhibition of its processing of the pre-sequences of mitochondrial proteins was proposed to be a general mechanism for diverse Aβ-induced mitochondrial dysfunctions[12]. Nevertheless, it should be pointed out that the genetic backgrounds of the deficient lines might differ from each other, and from wild-type and $A\beta_{arc}$ flies, so further experiments are needed to validate these suppressors and enhancers.

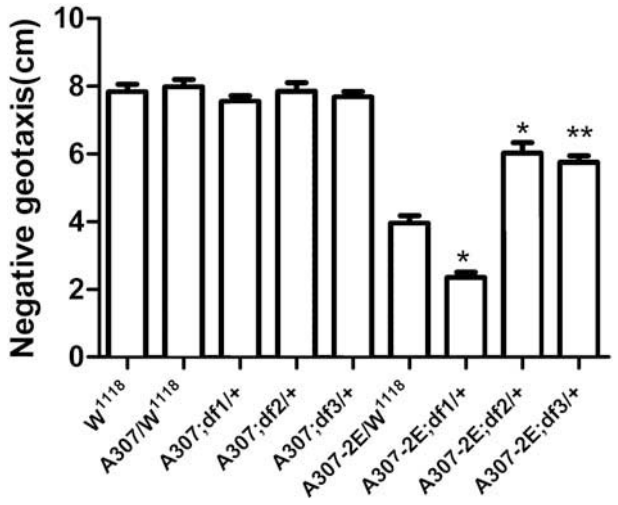

Fig. 4. Representative suppressors and enhancers of the accelerated age-dependent decline of climbing ability induced by expression of $A\beta_{arc}$. The histogram showing the average climbing height at 7 s after the initiation of negative geotaxis in 25-day-old male w^{1118}, A307/w^{1118}, control lines containing the [Gal4]A307 transgene and one 3rd chromosome deficiency, $A\beta_{arc}$-expressing (A307-2E), and A307-2E flies containing one 3rd chromosome deficiency. Df1, df2, and df3 represent the deficiencies in Bloomington stock numbers 23417, 9697 and 7591 respectively; *n* = 4 for each group, **P* <0.05 and ***P* <0.01 *versus* A307-2E flies, one-way ANOVA.

Table 4. Isolated suppressors and enhancers with the corresponding Bloomington stock numbers and deleted segments on the 3rd chromosome

Suppressors		Enhancers	
Stock #	Deleted segment	Stock #	Deleted segment
9693	62A11;62B7	8059	63C1;63F5
7591	66B5;66C8	24392	63F1;64A4
8975	67B11;67C5	8060	63F6;64B9
26828	68F7;69E6	8061	64B9;64C13
9697	75F1;76A1	7588	65C3;65D3
7675	95C12;95D8	8066	66D12;67B3
7681	96D;96D1	8068	68A6;68E1
		8069	68C13;69B4
		8097	70A3;70C10
		8074	70F4;71E1
		27888	71D3;72A1
		8089	79C2;80A4
		25077	83A6;83B6
		23714	88C9;88D8
		24137	88D1;88E3

DISCUSSION

In this study, we automated the RING assay by using a small electric motor to drive the tapping of the RING apparatus, using a digital video recorder, and writing a program to measure the climbing heights of individual flies and the average value. Our aRING assay is superior to the traditional RING assay[6] and the automated climbing apparatus[7] in the following ways: (1) automatically tapping flies down in a highly reliable and reproducible way; (2) simultaneously testing many groups of flies with different genotypes or treatments; (3) suitable for large-scale studies; and (4) efficient and quantitative analysis of the climbing ability of all individual flies.

With the aRING assay, we found that the tested flies

exhibited a distinct age-dependent decline in climbing ability, whether controls or expressing $A\beta_{arc}$, and the rate of decline was not linear when flies, males in particular, were aging from day 7 to 35. The climbing ability declined rapidly from day 14 to 21 but was slower from day 21 to 35, indicating a turning point of the decline rate from day 14 to 21 after eclosion. Also, the decline rate was clearly faster in $A\beta_{arc}$-expressing flies than in controls. Further experiments are required to pinpoint the turning point of the decline rate of climbing ability, profile the gene expression in the nervous system or the muscles just before and after the turning point, and study the functional mechanisms of $A\beta_{arc}$ in the nervous and locomotor systems, which may help understand the aging process.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-014-1526-0.

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