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Gr33a Modulates *Drosophila* Male Courtship Preference

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In any gamogenetic species, attraction between individuals of the opposite sex promotes reproductive success that guarantees their thriving. Consequently, mate determination between two sexes is effortless for an animal. However, choosing a spouse from numerous attractive partners of the opposite sex needs deliberation. In *Drosophila melanogaster*, both younger virgin females and older ones are equally liked options to males; nevertheless, when given options, males prefer younger females to older ones. Non-volatile cuticular hydrocarbons, considered as major pheromones in *Drosophila*, constitute females' sexual attraction that act through males' gustatory receptors (Grs) to elicit male courtship. To date, only a few putative Grs are known to play roles in male courtship. Here we report that loss of Gr33a function or abrogating the activity of Gr33a neurons does not disrupt male-female courtship, but eliminates males' preference for younger mates. Furthermore, ectopic expression of human amyloid precursor protein (APP) in Gr33a neurons abolishes males' preference behavior. Such function of APP is mediated by the transcription factor forkhead box O (dFoxO). These results not only provide mechanistic insights into *Drosophila* male courtship preference, but also establish a novel *Drosophila* model for Alzheimer's disease (AD).

To avoid futile reproductive efforts, an animal must distinguish conspecifics from other species and differentiate the sex of conspecific partners. It must also determine the most suitable mates from large amounts of available partners in order to maximize reproductive efficiency. Evolution endows *Drosophila melanogaster* males with the instinct to discriminate conspecifics from other *Drosophila* species[1] and to discern females from males[2]. It also bestows on them the ability to select the most favorable mates among masses of desirable virgin females[3].

In *Drosophila*, non-volatile cuticular hydrocarbons (CHCs) have been recognized as a type of major sex pheromone[4,5], which convey information of an individual such as species and sex. Female-specific CHCs are detected by male gustatory receptors (Grs), a chemosensory receptor family mainly responsible for detecting non-volatile chemicals[6], during tapping and licking steps in stereotypical male courtship behavior[6–9]. So far, only a few Grs, including Gr32a, Gr33a and Gr39a are reported to be engaged in *Drosophila* male courtship behavior[10]. While Gr32a acts to assist males to discriminate conspecifics from other species[11], Gr39a is required for males to distinguish females from males[12]. On the other hand, Gr33a functions to inhibit male-male courtship[13]. Despite these findings, roles of the Grs in males' choices for the most favorable mates have remained largely unknown.

Our previous study sets a paradigm of choice model in which both options (younger virgin females and older ones) are proved to be attractive to *Drosophila* males, but males still intensely prefer younger mates to older ones[3]. Using this model, we explored the mechanisms by which males bias their potential mates. Gene loss-of-function, gain-of-function, and cell-inactivation experiments demonstrated that *Gr33a* and Gr33a neurons are essential for males' preference for younger mates. Since our previous data indicated that pan-neuronal expression of human amyloid precursor protein (APP) ablates males' preference for younger mates[3], we sought to investigate whether APP expression in Gr33a neurons would affect this behavior. Indeed, we found that Gr33a neurons-specific expression of APP abolished males' preference for younger mates, and this function of APP is mediated by the transcription factor forkhead box O (dFoxO).

Methods

Drosophila Strains. *Oregon R*, w^{1118}, w^{1}, $Gr39a^{k05106}$, $Gr33a^{Gal4}$, $Gr33a^{1}$, *UAS*-Gr33a, *UAS*-APPΔCT, *UAS*-GFP and *UAS*-APP were obtained from Bloomington *Drosophila* Stock Center. $Gr32a^{MI04430}$ was a gift from Dr. Zuoren Wang. Δ*ppk23* and Δ*ppk29* were gifts from Dr. Kristin

Scott. *UAS*-TNT and *UAS*-TNT^{in} were gifts from Dr. Aike Guo. *dfoxO*$^{\Delta 94}$ was generously provided by Dr. Linda Partridge. Wild type (WT) controls for the mutants (*Gr32a*, *Gr33a*, *Gr39a*, *ppk23*, *ppk29*) were from the corresponding background strains.

Fly Rearing. *Drosophila* stocks were maintained on a standard corn flour, yeast and agar medium under a 12 h light and 12 h dark cycle at 25°C. Naive male and virgin female flies were collected at eclosion. Males were kept individually for three days before test. Females were housed in groups of 10 per vial and were transferred every three days to new vials containing fresh medium.

Courtship Behavior Assays. Courtship choice assays were performed as described before[3]. All courtship choice assays were carried out at approximately the same time each day (within 1 hour at the beginning of the 12 h illumination half of the cycle)[14] by pairing a naive male together with two younger wild type virgin females and two older ones. Before tests, males were checked and unhealthy ones were excluded from experiments. All tests were recorded for 10 min with an HDR-CX270 digital video camera (Sony). After recording, videos were analyzed by a researcher who was blinded to the genotypes of males or age markers of females, using Noldus EthoVision® XT software (Noldus Information Technology). Given that mating success terminated male courtship behavior and that a successful mating was consensual, thus, either males' or females' choices could bias the mating results[15]. Tests in which male mated successfully within 10 min were excluded from analysis. The courtship index (CI) was calculated as the percentage of time that a male displayed any of the courtship steps towards the females during the 10 min observation period. In courtship choice assays, CI_y or CI_o represents CI towards younger virgin females or older ones, respectively; the total courtship index (CI_t) represents a male's total CIs towards both younger virgin females and older ones in a choice assay. We also defined a preference index (PI): the relative difference between males' courtship percentage towards younger females and that towards older ones in a choice assay.

CHC Extraction. Cuticular hydrocarbon (CHC) extracts were obtained from 3-day (younger) and 30-day (older) old virgin females. Five replicate CHC samples were prepared for each age. For each sample, extract was obtained from five individuals, selected from a vial containing 10 flies. Flies were anesthetized with CO_2, and were placed into an individual glass vial with 30 μl of hexane (Merck KGaA) containing 100 ng/μl of octadecane (C18) and 100 ng/μl of hexacosane (nC26) as injection standards. To achieve efficient extraction, the glass vial was gently agitated for 5 min[16]. The extract was removed and placed in a clean glass vial and stored at −20°C prior to analysis. Extracts were examined by gas chromatography and mass spectrometry (See Supplemental Experimental Procedures).

Statistical Analysis. Since CIs were generally not normally distributed, nonparametric tests were employed in statistical analysis. Comparisons of intragroup CIs (CI_y and CI_o) in courtship choice assays used the Related-samples Wilcoxon Signed Rank test. CI_ts and PIs in choice assays were compared using the Kruskal-Wallis test followed by the post-hoc Dunn's test. Besides, CI_ts and PIs in choice assays to test the roles of Grs in males' interest on females and in cell-inactivation experiments were compared using the Mann-Whitney *U* test. In analysis of GC and MS results, Mann-Whitney *U* test was also applied due to the small volume of samples.

Results

The roles of Grs in males' courtship preference behavior. To investigate the possible function of Gr32a, Gr33a and Gr39a in males' preference for younger mates, we employed their mutant alleles: *Gr32a*^{MI04430}, *Gr39a*^{k05106} and *Gr33a*^{Gal4} (a portion of the *Gr33a* coding region is replaced by *Gal4* using homologous recombination[13]), and introduced these males into courtship choice assays. In courtship choice behavior, a prerequisite of surveying a male's preference for younger or older mates is that the male does not lose the sexual interest in females. The courtship intensity of a male is a good indicator of its sexual interest in females[17]. Accordingly, choices of males displaying drastically impaired courtship might be unrepresentative. To assess a male's sexual interest in females, we defined a total courtship index (CI_t), which represents a male's courtship intensity towards both mates (See methods). CI_t of *Gr32a*^{MI04430} males shows no significant difference from that of wild type controls (Figure 1A), indicating that loss of *Gr32a* does not affect male-female courtship. Likewise, CI_t of *Gr33a*^{Gal4} males is similar to that of wild type controls (Figure 1B), inferring that malfunction of Gr33a does not disrupt male-female courtship. In contrast, mutation in *Gr39a* (*Gr39a*^{k05106}) results in significantly reduced courtship intensity (Figure 1C), consistent with its reported role in male-female courtship[12]. In addition to Grs, chemosensory receptors such as degenerin/epithelial sodium channel/pickpocket (Ppk) family, also modulate male courtship behavior[18]. Therefore, we checked Δ*ppk23* and Δ*ppk29* males, which are loss-of-function mutants of *ppk23* and *ppk29* genes that are known to play critical roles in male courtship behavior[19,20]. We found that CI_t of Δ*ppk23* males decreases remarkably (Figure S1A), consistent with its role in male-female courtship[20]. Intriguingly, Δ*ppk29* males displayed similar CI_t as that of wild type control (Figure S1B), implying that dysfunction of Ppk29 does not significantly reduce male-female courtship intensity.

Since loss of Gr32a, Gr33a or Ppk29 does not affect males' sexual interest in females, we next examined their roles in males' preference behavior. In choice assays, *Gr32a*^{MI04430} males and Δ*ppk29* males still prefer younger virgin females, while *Gr33a*^{Gal4} males court both younger and older virgin females at the same intensity (Figure 1D, E and Figure S2A). To accurately quantify the extent of a male's preference for younger or older mates, we defined a preference index (PI), the relative difference between the courtship percentage towards younger females and that towards older ones in a choice assay: $PI = [CI_y - CI_o]/[CI_y + CI_o]$. PIs of *Gr32a*^{MI04430} and Δ*ppk29* males are not substantially different from those of corresponding wild type controls, whereas PI of *Gr33a*^{Gal4} males is completely abolished (Figure 1F, G and Figure S2B). These data indicate that malfunction of Gr33a eliminates males' preference for younger mates.

***Gr33a* is required for males' preference for younger mates.** To further confirm the role of *Gr33a* in males' preference for younger mates, we examined another *Gr33a* mutant allele, *Gr33a*^{1}, which deletes residues 1 to 199 of Gr33a[13]. *Gr33a*^{1} homozygous and trans-heterozygous (*Gr33a*^{1}/*Gr33a*^{Gal4}) males present no preference behavior, yet the phenotype are fully rescued by the introduction of a *UAS*-Gr33a transgene[13] in *Gr33a*^{1}/*Gr33a*^{Gal4} males (*Gr33a*^{rescue}) (Figure 2A and B). On the other hand, there is no difference among CI_ts of *Gr33a* mutant, *Gr33a*^{rescue} and wild type controls (Figure 2C), suggesting that *Gr33a* is not required for male-female courtship, but is necessary for males' preference for younger mates.

Activity of Gr33a neurons is essential for males' preference for younger mates. Gr33a is expressed widely in gustatory receptor neurons (GRNs) that are responsible for aversive chemicals[13,21]. To test whether the activity of Gr33a neurons is required for males' preference for younger mates, we inhibited synaptic transmissions in these neurons by expressing tetanus toxin light chain (TNT)[22] under the control of *Gr33a*^{Gal4} driver (*Gr33a*^{Gal4}/*UAS*-TNT). These TNT-expressing males display no preference for younger mates, whereas control males expressing an inactive version of the *TNT* transgene (*TNT*^{in})[22] (*Gr33a*^{Gal4}/*UAS*-TNT^{in}) exhibit a strong preference for younger mates (Figure 3A and B). On the other hand, males expressing TNT or TNT^{in} display similar CI_t (Figure 3C). These data demonstrate that the activity of Gr33a neurons is essential for males' preference for younger mates.

Expression of APP in Gr33a neurons results in dFoxO-dependent choice defect. Our previous findings suggest that pan-neuronal expression of APP, a potential causative protein of Alzheimer's disease (AD)[23–25], abolishes males' preference for younger mates[3]. We asked whether expression of APP in Gr33a neurons (*Gr33a*^{Gal4}/*UAS*-APP) could interfere with their neuronal activity and affect males' preference behavior. We found that *Gr33a*^{Gal4}/*UAS*-APP males court both younger and older females at similar intensity (Figure 4A). The PI of *Gr33a*^{Gal4}/*UAS*-APP males is remarkably lower than that of control males (*Gr33a*^{Gal4}/+ and +/*UAS*-APP) (Figure 4B), whereas CI_ts among them remain unchanged (Figure 4C). Thus, expression of APP in Gr33a neurons is sufficient to block males' courtship preference for younger females. This perturbation is likely caused by a selective loss of Gr33a neurons, since ectopic expression of APP in *Drosophila* results in

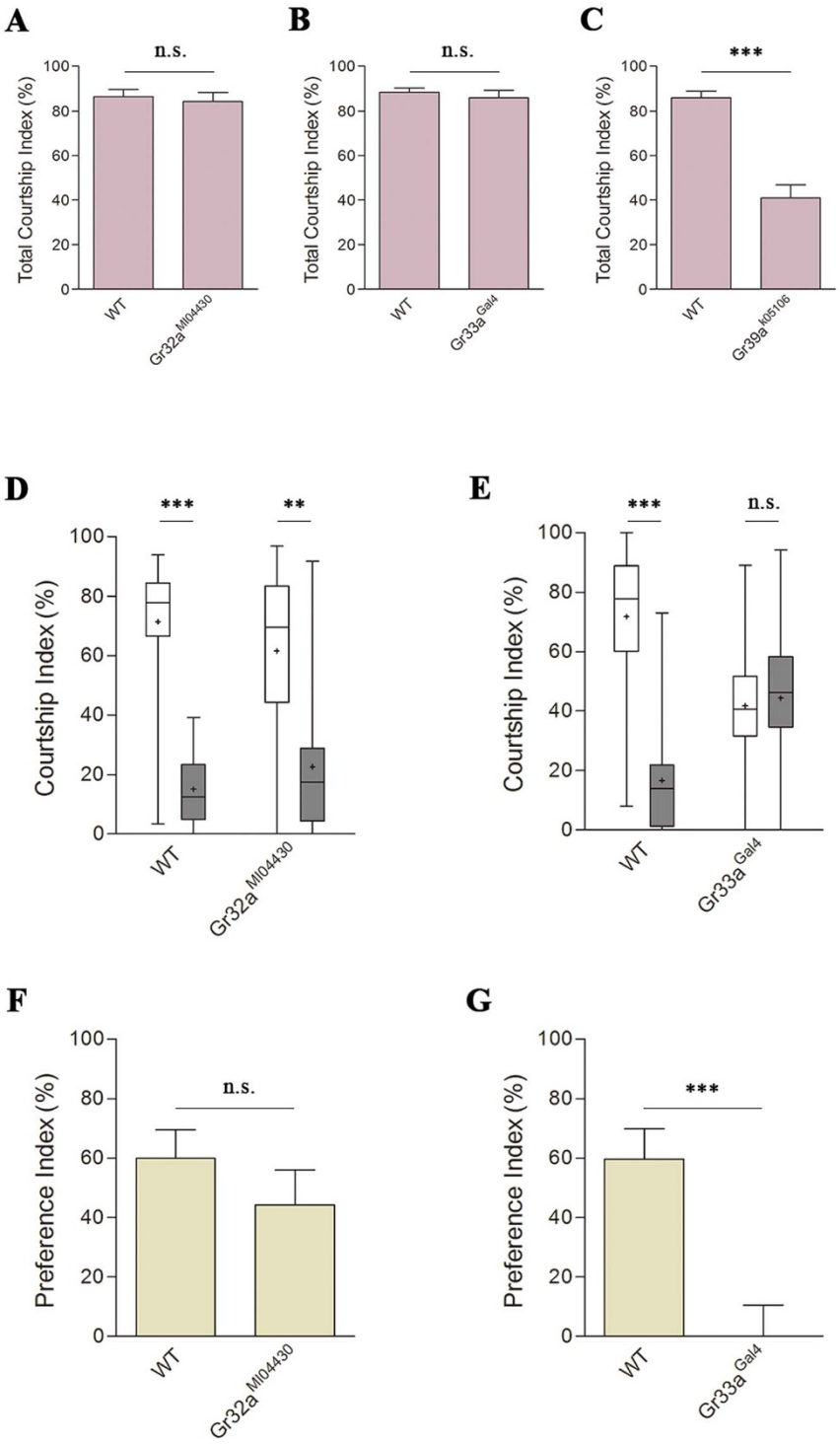

Figure 1 | The roles of Grs in males' courtship preference behavior. (A) Total courtship indices of wild type males and *Gr32a* mutant males in courtship choice assays. Mean ± standard error of the mean (SEM), n = 21 for wild type males and 24 for *Gr32a* mutant males. n.s., $p > 0.5$, Mann-Whitney *U* test. (B) Total courtship indices of wild type males and *Gr33a* mutant males in courtship choice assays. Mean ± standard error of the mean (SEM), n = 21 for wild type males and 22 for *Gr33a* mutant males. n.s., $p > 0.5$, Mann-Whitney *U* test. (C) Total courtship indices of wild type males and *Gr39a* mutant males in courtship choice assays. Mean ± standard error of the mean (SEM), n = 20 for wild type males and 22 for *Gr39a* mutant males. Three asterisks, $p < 0.001$, Mann-Whitney *U* test. (D) Courtship indices of wild type males and *Gr32a* mutant males in courtship choice assays towards younger mates (bar colored white) and older ones (bar colored grey), respectively. Box-and-whisker plots for CIs show 1–99 percentiles and mean (+). Two asterisks, $p < 0.01$, three asterisks, $p < 0.001$, Related-samples Wilcoxon Signed Rank test. (E) Courtship indices of wild type males and *Gr33a* mutant males in courtship choice assays towards younger mates (white) and older ones (grey), respectively. Box-and-whisker plots for CIs show 1–99 percentiles and mean (+). n.s., $p > 0.5$, three asterisks, $p < 0.001$, Related-samples Wilcoxon Signed Rank test. (F) Preference indices of wild type males and *Gr32a* mutant males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.5$, Mann-Whitney *U* test. (G) Preference indices of wild type males and *Gr33a* mutant males in courtship choice assays. Mean ± standard error of the mean (SEM). Three asterisks, $p < 0.001$, Mann-Whitney *U* test.

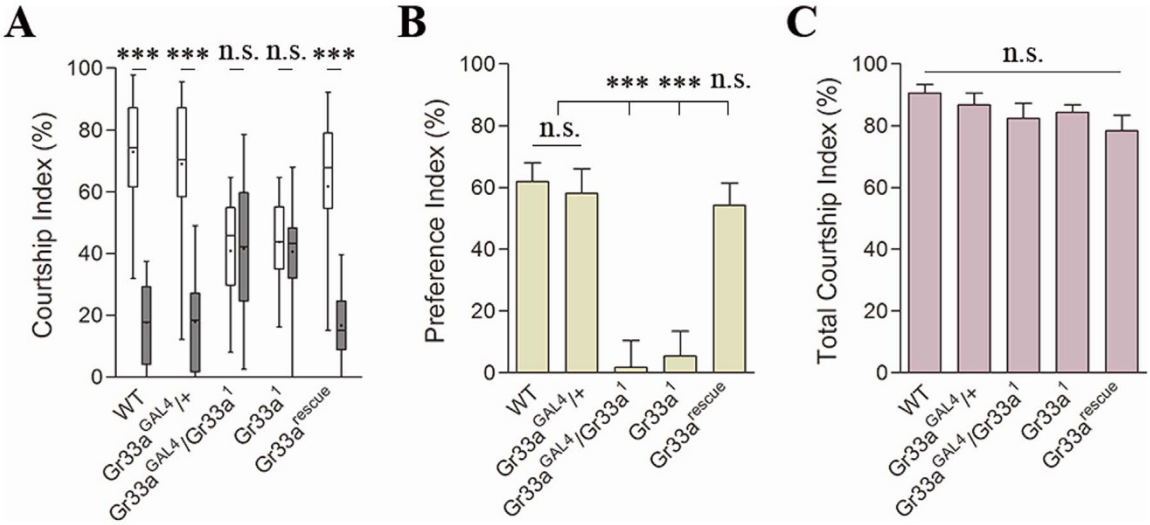

Figure 2 | Gr33a modulates males' preference for younger mates. (A) Courtship indices of wild type males, *Gr33a*^{Gal4}/+ males, *Gr33a*^{Gal4}/*Gr33a*^{1} males, *Gr33a*^{1} males, and *Gr33a*^{rescue} males (*Gr33a*^{Gal4}/*Gr33a*^{1}; *UAS*-Gr33a^{+}/+) in courtship choice assays towards younger mates (white) and older ones (grey), respectively. Box-and-whisker plots for CIs show 1–99 percentiles and mean (+), n = 22 for wild type males, 23 for *Gr33a*^{Gal4}/+ males, 22 for *Gr33a*^{Gal4}/*Gr33a*^{1} males, 22 for *Gr33a*^{1} males, and 23 for *Gr33a*^{rescue} males. n.s., $p > 0.05$, three asterisks, $p < 0.001$, Related-samples Wilcoxon Signed Rank test. (B) Preference indices of wild type males, *Gr33a*^{Gal4}/+ males, *Gr33a*^{Gal4}/*Gr33a*^{1} males, *Gr33a*^{1} males, and *Gr33a*^{rescue} males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.05$, three asterisks, $p < 0.001$, Kruskal-Wallis test, Dunn's post-hoc. (C) Total courtship indices of wild type males, *Gr33a*^{Gal4}/+ males, *Gr33a*^{Gal4}/*Gr33a*^{1} males, *Gr33a*^{1} males, and *Gr33a*^{rescue} males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.05$, Kruskal-Wallis test, Dunn's post-hoc.

neuronal cell death[26]. Alternatively, overexpression of APP interferes with the expression of Gr33a or other critical signaling components, perhaps by causing ER stress. To address the issue, we examined Gr33a neuron in males' labella and foreleg tarsi, two sensory structures expressing Gr33a[13], by the expression of GFP (*Gr33a*^{Gal4}; *UAS*-GFP), and found that overexpression of APP resulted in a significant loss of Gr33a neuron in labella and foreleg tarsi (Figure S3). Consistent with our recent study that the C-terminal APP intracellular domain (AICD) is required for APP to trigger cell death[27], males overexpressing in Gr33a neurons a truncated APP that lacks AICD (APPΔCT) exhibit similar PI and CI to those of control males (Figure 4A, B and C).

The forkhead box O (FoxO) transcription factors have been highly conserved during evolution, and are postulated to modulate various physiological processes, including cell proliferation and differentiation, apoptosis and metabolism[28–31]. Recently, we identified dFoxO as a crucial downstream factor that mediates APP-induced cell death and locomotion defect in *Drosophila*[27]. To examine whether dFoxO is also involved in APP-triggered male courtship preference defect, we introduced a *dfoxO* null mutation (*dfoxO*$^{\Delta 94}$)[32,33] into males

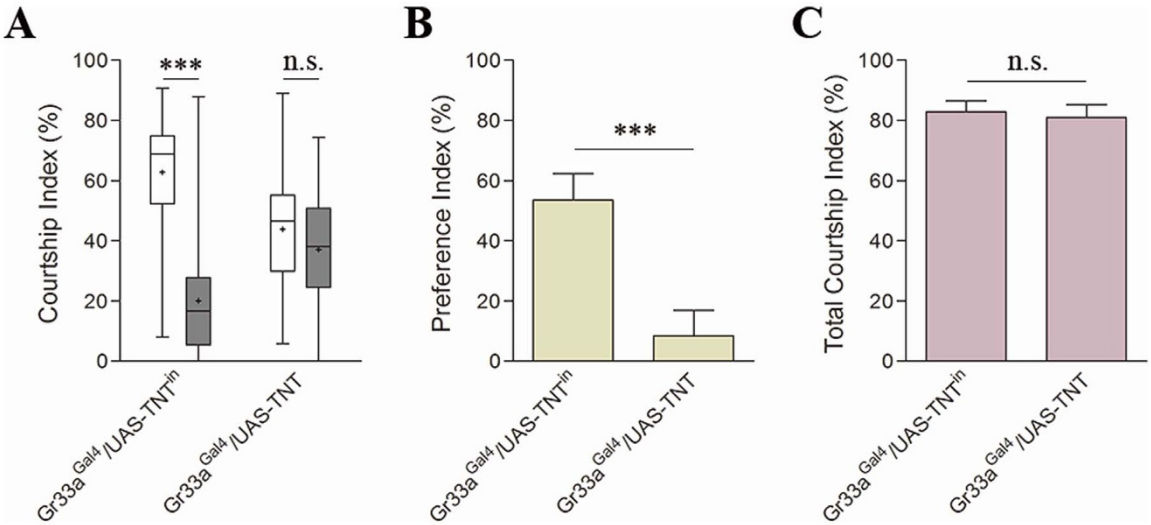

Figure 3 | Activity of Gr33a neurons is essential for males' preference for younger mates. (A) Courtship indices of control males (*Gr33a*^{Gal4}/*UAS*-TNT^{in}) and TNT expressing males (*Gr33a*^{Gal4}/*UAS*-TNT) in courtship choice assays towards younger mates (white) and older ones (grey), respectively. Box-and-whisker plots for CIs show 1-99 percentiles and mean (+), n = 23 for control males and 22 for TNT expressing males. n.s., $p > 0.05$, three asterisks, $p < 0.001$, Related-samples Wilcoxon Signed Rank test. (B) Preference indices of control males and TNT expressing males in courtship choice assays. Mean ± standard error of the mean (SEM). Three asterisks, $p < 0.001$, Mann-Whitney *U* test. (C) Total courtship indices of control males and TNT expressing males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.5$, Mann-Whitney *U* test.

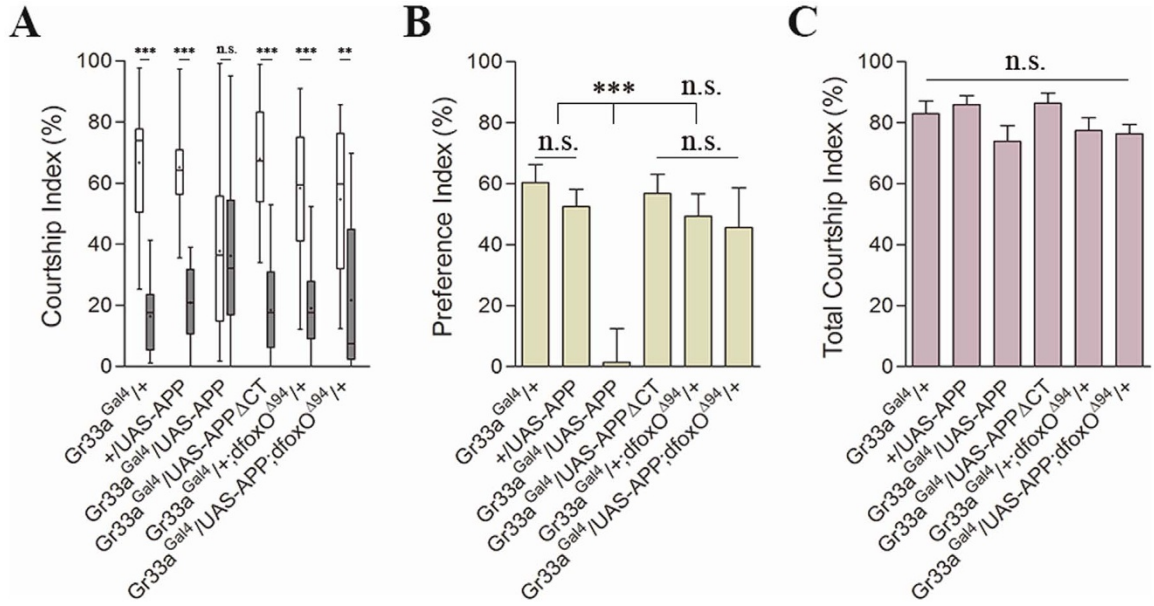

Figure 4 | APP expression in Gr33a neurons abolishes males' preference. (A) Courtship indices of $Gr33a^{Gal4}$/+ and +/*UAS*-APP control males, $Gr33a^{Gal4}$/*UAS*-APP males, $Gr33a^{Gal4}$/*UAS*-APPΔCT males, $Gr33a^{Gal4}$/+; $dfoxO^{\Delta 94}$/+ males and $Gr33a^{Gal4}$/*UAS*-APP; $dfoxO^{\Delta 94}$/+ males in courtship choice assays towards younger mates (white) and older ones (grey), respectively. Box-and-whisker plots for CIs show 1–99 percentiles and mean (+), n = 22 for control males, 21 for +/*UAS*-APP males, 23 for $Gr33a^{Gal4}$/*UAS*-APP males, 25 for $Gr33a^{Gal4}$/*UAS*-APPΔCT males, 21 for $Gr33a^{Gal4}$/+; $dfoxO^{\Delta 94}$/+ males and 21 for $Gr33a^{Gal4}$/*UAS*-APP; $dfoxO^{\Delta 94}$/+ males. n.s., $p > 0.05$, two asterisks, $p < 0.01$, three asterisks, $p < 0.001$, Related-samples Wilcoxon Signed Rank test. (B) Preference indices of $Gr33a^{Gal4}$/+ and +/*UAS*-APP control males, $Gr33a^{Gal4}$/*UAS*-APP males, $Gr33a^{Gal4}$/*UAS*-APPΔCT males, $Gr33a^{Gal4}$/+; $dfoxO^{\Delta 94}$/+ males and $Gr33a^{Gal4}$/*UAS*-APP; $dfoxO^{\Delta 94}$/+ males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.05$, three asterisks, $p < 0.001$, Kruskal-Wallis test, Dunn's post-hoc. (C) Total courtship indices of $Gr33a^{Gal4}$/+ and +/*UAS*-APP control males, $Gr33a^{Gal4}$/*UAS*-APP males, $Gr33a^{Gal4}$/*UAS*-APP*Δ*CT males, $Gr33a^{Gal4}$/+; $dfoxO^{\Delta 94}$/+ males and $Gr33a^{Gal4}$/*UAS*-APP; $dfoxO^{\Delta 94}$/+ males in courtship choice assays. Mean ± standard error of the mean (SEM). n.s., $p > 0.05$, Kruskal-Wallis test, Dunn's post-hoc.

expressing APP in Gr33a neurons. We found that deleting one copy of endogenous *dfoxo* gene significantly rescues the preference defect of $Gr33a^{Gal4}$/*UAS*-APP males (Figure 4A and B), while the CI_ts show no discrepancy among all groups (Figure 4C). These results, taken together, manifest that ectopic expression of APP in Gr33a neurons disrupts the activity of these neurons and acutely dispels males' preference for younger mates. Such function of APP in choice behavior, which is mediated by dFoxO, could be employed as a potential AD model for cognitive study.

Female CHC profiles change with age. Cuticular hydrocarbons (CHCs) are reported to serve as sex pheromones which act through gustatory receptors to modulate male courtship behavior[7]. Among all the CHCs produced by *Drosophila melanogaster*, 7-tricosene (7-T) (C23) is repulsive to males; 7-pentacosene (7-P) (C25) has complex roles in courtship whereas 7,11-heptacosadiene (7,11-HD) (C27) and 7,11-nonacosadiene (7,11-ND) (C29) are attractive to males[4]. To explore the difference in CHC profiles between younger and older virgin females, we performed gas chromatography and mass spectrometry. We found that the concentrations of most female specific dienes and alkenes were lower on younger virgin females than on older ones (Figure 5 and Table S1). Thus, we conclude that female CHC profiles change with age, with the major female specific CHCs increase by aging.

Discussion

The function of Grs in males' preference behavior. *Drosophila* male courtship choice has been frequently applied for studying decision making in animals[34], yet most of the past studies have focused on male courtship choices between likes and dislikes, such as court towards females vs. males[35], or virgin vs. non-virgin females[36,37]. We have previously characterized a choice behavior between two equally-liked options: mature virgin females, whether younger or older, were similarly attractive to naive males; nevertheless, when given the option, males turn out to be picky and prefer younger virgin females to older ones[3]. Here we found that a gustatory receptor, Gr33a, is necessary for males' preference for younger mates. Gr33a is thought to be necessary to inhibit homosexual behavior[13,21]; its role in heterosexual behavior, however, is rarely pondered. In this study, we reveal the critical role of Gr33a in males' preference for younger mates. Furthermore, ectopic expression of APP in Gr33a neurons eliminates males' preference behavior, and such function is mediated by dFoxO, a recently reported downstream factor of APP[27]. Therefore, our work demonstrates the genetic interaction of APP and dFoxO in Gr33a neurons, which modulates males' preference for younger mates.

APP is identified as a potential causative protein of AD, a common progressive neurodegenerative disorder[25], in which cognitive decline is the prime symptom[23,24,38]. Although *Drosophila* has long been utilized for building AD models to investigate the pathogenesis and possible cure for AD[39,40], accepted *Drosophila* AD models are limited to locomotion model and life span model[41–43], which have little correlation with cognitive ability. Our findings, however, have offered the possibility for establishing a novel *Drosophila* AD model that is related to cognitive ability.

The role of CHCs in males' preference. In all CHCs produced by files, 7, 11-HD and 7, 11-ND have been identified as female specific aphrodisiac pheromones to *Drosophila melanogaster* males[20]. Our GC and MS results suggest that both 7, 11-HD and 7, 11-ND are expressed at lower concentration in younger virgin females than the older ones. Hence, it appears unlikely that 7, 11-HD or 7, 11-ND is the cause that leads males to court younger virgin females more vigorously than the older ones. On the contrary, since Gr33a has been reported as a receptor of aversive odors[13,21], it is more likely that older females produce certain aversive odors that can be

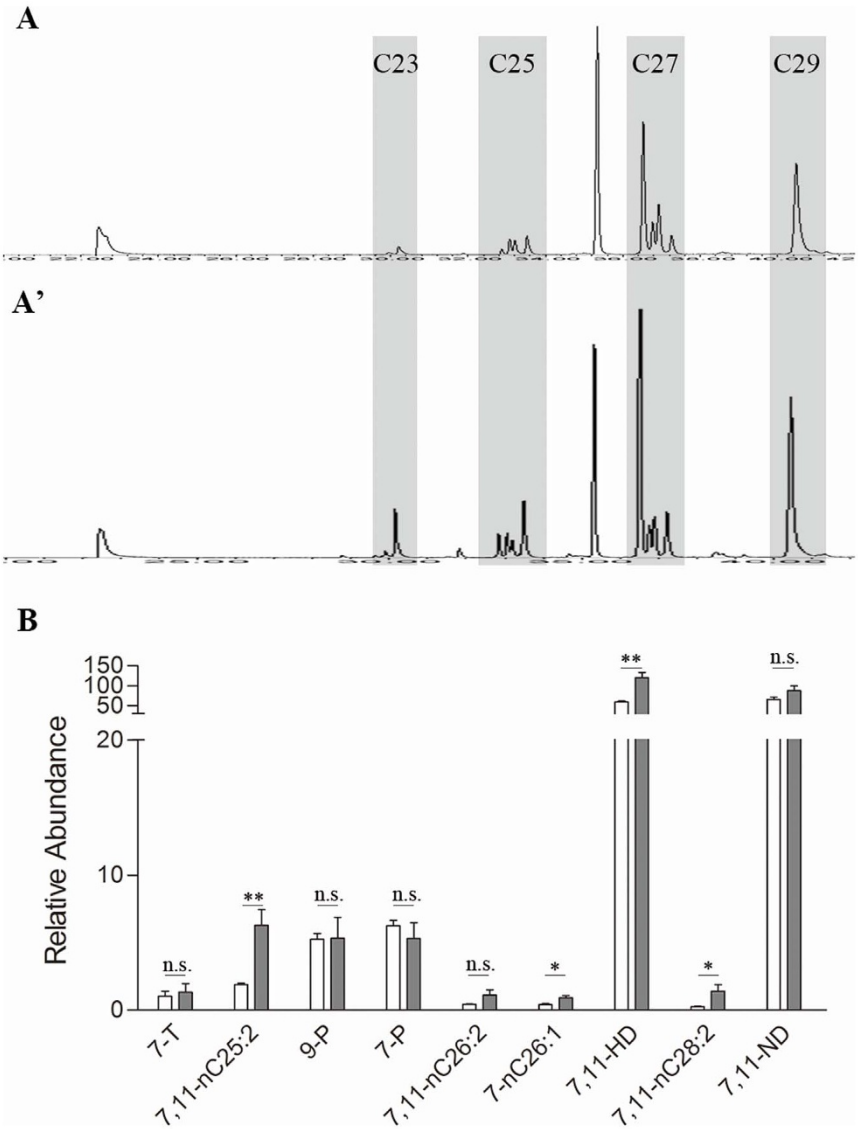

Figure 5 | CHC Profiles of younger and older virgin females. (A) and (A′) The chromatogram plots the area of each peak associated with a compound in units of abundance with column retention time on the x-axis. (A) shows the profile of younger virgin females and (A′) shows that of older ones. (B) Relative abundance (injection standard nC26 is 100) of candidate dienes and alkenes on virgin females. Bar colored white indicates younger virgin females and bar colored grey indicates older ones. n = 4–5 for each compound. n.s., $p > 0.05$, asterisk, $p < 0.05$, two asterisks, $p < 0.01$, Mann-Whitney *U* test.

recognized by males and repel them. Consistent with this explanation, we found that the concentrations of most detected CHCs are significantly higher on the older virgin females than the younger ones. Nevertheless, at this stage we are unable to identify the CHC(s) that serves as the aversive pheromone to males. Besides, we cannot exclude the possibility that younger virgin females produce unknown attractive pheromones other than 7, 11-HD or 7, 11-ND. However, all CHCs we have detected on younger virgin females also present on older ones at a similar or higher lever. Thus, we draw the tentative conclusion that younger virgin females do not produce more attractive pheromones than the older ones. Our results, taken together, unravel the role of bitter sensory Gr33a neurons in males' preference for younger mates and infer that older females might produce certain aversive odors that cause males to turn to younger mates.

1. Dukas, R. Male fruit flies learn to avoid interspecific courtship. *Behav Ecol* **15**, 695–698, doi:DOI 10.1093/beheco/arh068 (2004).
2. Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G. & Levine, J. D. Specialized cells tag sexual and species identity in Drosophila melanogaster. *Nature* **461**, 987–U250, doi:Doi 10.1038/Nature08495 (2009).
3. Hu, Y. J., Han, Y., Wang, X. J. & Xue, L. Aging-related neurodegeneration eliminates male courtship choice in Drosophila. *Neurobiol Aging* **35**, 2174–2178, doi:DOI 10.1016/j.neurobiolaging.2014.02.026 (2014).
4. Ferveur, J. F. Cuticular hydrocarbons: Their evolution and roles in Drosophila pheromonal communication. *Behav Genet* **35**, 279–295, doi:DOI 10.1007/s10519-005-3220-5 (2005).
5. Ferveur, J. F. *et al.* Genetic feminization of pheromones and its behavioral consequences in Drosophila males. *Science* **276**, 1555–1558, doi:DOI 10.1126/science.276.5318.1555 (1997).
6. Ebbs, M. L. & Amrein, H. Taste and pheromone perception in the fruit fly Drosophila melanogaster. *Pflug Arch Eur J Phy* **454**, 735–747, doi:DOI 10.1007/s00424-007-0246-y (2007).
7. Amrein, H. & Thorne, N. Gustatory perception and behavior in Drosophila melanogaster. *Curr Biol* **15**, R673–R684, doi:DOI 10.1016/j.cub.2005.08.021 (2005).
8. Thorne, N., Chromey, C., Bray, S. & Amrein, H. Taste perception and coding in Drosophila. *Curr Biol* **14**, 1065–1079, doi:DOI 10.1016/j.cub.2004.05.019 (2004).
9. Siegel, R. W. & Hall, J. C. Conditioned responses in courtship behavior of normal and mutant Drosophila. *Proc Natl Acad Sci U S A* **76**, 3430–3434 (1979).

10. Yamamoto, D. & Koganezawa, M. Genes and circuits of courtship behaviour in Drosophila males. *Nat Rev Neurosci* **14**, 681–692, doi:10.1038/nrn3567 (2013).
11. Fan, P. *et al.* Genetic and Neural Mechanisms that Inhibit Drosophila from Mating with Other Species. *Cell* **154**, 89–102, doi:DOI 10.1016/j.cell.2013.06.008 (2013).
12. Watanabe, K., Toba, G., Koganezawa, M. & Yamamoto, D. Gr39a, a Highly Diversified Gustatory Receptor in Drosophila, has a Role in Sexual Behavior. *Behav Genet* **41**, 746–753, doi:DOI 10.1007/s10519-011-9461-6 (2011).
13. Moon, S. J., Lee, Y., Jiao, Y. & Montell, C. A Drosophila Gustatory Receptor Essential for Aversive Taste and Inhibiting Male-to-Male Courtship. *Curr Biol* **19**, 1623–1627, doi:DOI 10.1016/j.cub.2009.07.061 (2009).
14. Grosjean, Y. *et al.* An olfactory receptor for food-derived odours promotes male courtship in Drosophila. *Nature* **478**, 236–U123, doi:Doi 10.1038/Nature10428 (2011).
15. Moehring, A. J. & Mackay, T. F. C. The quantitative genetic basis of male mating behavior in Drosophila melanogaster. *Genetics* **167**, 1249–1263, doi:DOI 10.1534/genetics.103.024372 (2004).
16. Ejima, A., Smith, B. P. C., Lucas, C., Levine, J. D. & Griffith, L. C. Sequential learning of pheromonal cues modulates memory consolidation in trainer-specific associative courtship conditioning. *Curr Biol* **15**, 194–206, doi:DOI 10.1016/j.cub.2005.01.035 (2005).
17. Hall, J. C. The Mating of a Fly. *Science* **264**, 1702–1714, doi:DOI 10.1126/science.8209251 (1994).
18. Liu, T., Starostina, E., Vijayan, V. & Pikielny, C. W. Two Drosophila DEG/ENaC Channel Subunits Have Distinct Functions in Gustatory Neurons That Activate Male Courtship. *J Neurosci* **32**, 11879–11889, doi:Doi 10.1523/Jneurosci.1376-12.2012 (2012).
19. Toda, H., Zhao, X. L. & Dickson, B. J. The Drosophila Female Aphrodisiac Pheromone Activates ppk23(+) Sensory Neurons to Elicit Male Courtship Behavior. *Cell reports* **1**, 599–607, doi:DOI 10.1016/j.celrep.2012.05.007 (2012).
20. Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L. & Scott, K. Contact Chemoreceptors Mediate Male-Male Repulsion and Male-Female Attraction during Drosophila Courtship. *Cell* **149**, 1140–1151, doi:DOI 10.1016/j.cell.2012.03.045 (2012).
21. Lee, Y., Kim, S. H. & Montell, C. Avoiding DEET through Insect Gustatory Receptors. *Neuron* **67**, 555–561, doi:DOI 10.1016/j.neuron.2010.07.006 (2010).
22. Sweeney, S. T., Broadie, K., Keane, J., Niemann, H. & Okane, C. J. Targeted Expression of Tetanus Toxin Light-Chain in Drosophila Specifically Eliminates Synaptic Transmission and Causes Behavioral Defects. *Neuron* **14**, 341–351, doi:Doi 10.1016/0896-6273(95)90290-2 (1995).
23. Salehi, A. *et al.* Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron* **51**, 29–42, doi:DOI 10.1016/j.neuron.2006.05.022 (2006).
24. Greeve, I. *et al.* Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic Drosophila. *J Neurosci* **24**, 3899–3906, doi:Doi 10.1523/Jneurosci.0283-04.2004 (2004).
25. Lovestone, S. The neurobiology and genetics of Alzheimer's disease. *Hum Psychopharm Clin* **10**, S243–S246, doi:DOI 10.1002/hup.470101008 (1995).
26. Gunawardena, S. & Goldstein, L. S. B. Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in Drosophila. *Neuron* **32**, 389–401, doi:Doi 10.1016/S0896-6273(01)00496-2 (2001).
27. Wang, X. *et al.* FoxO mediates APP-induced AICD-dependent cell death. *Cell Death Dis* **5**, doi:Artn E1233 Doi 10.1038/Cddis.2014.196 (2014).
28. Hwangbo, D. S., Gershman, B., Tu, M. P., Palmer, M. & Tatar, M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body (vol 429, pg 562, 2004). *Nature* **434**, 118–118, doi:Doi 10.1038/Nature03446 (2005).
29. Giannakou, M. E. *et al.* Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science* **305**, 361, doi:10.1126/science.1098219 (2004).
30. Puig, O., Marr, M. T., Ruhf, M. L. & Tjian, R. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* **17**, 2006–2020, doi:10.1101/gad.1098703 (2003).
31. Junger, M. A. *et al.* The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *Journal of biology* **2**, 20, doi:10.1186/1475-4924-2-20 (2003).
32. Nechipurenko, I. V. & Broihier, H. T. FoxO limits microtubule stability and is itself negatively regulated by microtubule disruption. *J Cell Biol* **196**, 345–362, doi:DOI 10.1083/jcb.201105154 (2012).
33. Slack, C., Giannakou, M. E., Foley, A., Goss, M. & Partridge, L. dFOXO-independent effects of reduced insulin-like signaling in Drosophila. *Aging cell* **10**, 735–748, doi:DOI 10.1111/j.1474-9726.2011.00707.x (2011).
34. Dickson, B. J. Wired for Sex: The Neurobiology of Drosophila Mating Decisions. *Science* **322**, 904–909, doi:DOI 10.1126/science.1159276 (2008).
35. Everaerts, C., Lacaille, F. & Ferveur, J. F. Is mate choice in Drosophila males guided by olfactory or gustatory pheromones? *Anim Behav* **79**, 1135–1146, doi:DOI 10.1016/j.anbehav.2010.02.013 (2010).
36. Zhou, C. *et al.* Molecular Genetic Analysis of Sexual Rejection: Roles of Octopamine and Its Receptor OAMB in Drosophila Courtship Conditioning. *J Neurosci* **32**, 14281–14287, doi:Doi 10.1523/Jneurosci.0517-12.2012 (2012).
37. Keleman, K. *et al.* Dopamine neurons modulate pheromone responses in Drosophila courtship learning. *Nature* **489**, 145–U210, doi:Doi 10.1038/Nature11345 (2012).
38. Zhao, X. L. *et al.* Expression of beta-Amyloid Induced Age-Dependent Presynaptic and Axonal Changes in Drosophila. *J Neurosci* **30**, 1512–1522, doi:Doi 10.1523/Jneurosci.3699-09.2010 (2010).
39. Crowther, D. C. *et al.* Intraneuronal A beta, non-amyloid aggregates and neurodegeneration in a Drosophila model of Alzheimer's disease. *Neuroscience* **132**, 123–135, doi:DOI 10.1016/j.neuroscience.2004.12.025 (2005).
40. Crowther, D. C., Kinghorn, K. J., Page, R. & Lomas, D. A. Therapeutic targets from a Drosophila model of Alzheimer's disease. *Curr Opin Pharmacol* **4**, 513–516, doi:DOI 10.1016/j.coph.2004.07.001 (2004).
41. Bonner, J. M. & Boulianne, G. L. Drosophila as a model to study age-related neurodegenerative disorders: Alzheimer's disease. *Exp Gerontol* **46**, 335–339, doi:DOI 10.1016/j.exger.2010.08.004 (2011).
42. Rival, T. *et al.* Fenton chemistry and oxidative stress mediate the toxicity of the beta-amyloid peptide in a Drosophila model of Alzheimer's disease. *Eur J Neurosci* **29**, 1335–1347, doi:DOI 10.1111/j.1460-9568.2009.06701.x (2009).
43. Iijima-Ando, K. *et al.* Mitochondrial Mislocalization Underlies A beta 42-Induced Neuronal Dysfunction in a Drosophila Model of Alzheimer's Disease. *Plos One* **4**, doi:Artn E8310 Doi 10.1371/Journal.Pone.0008310 (2009).

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Author contributions

Y.H. and L.X. conceived the project and designed the experiments; Y.H., Y.H. and Y.S. performed the experiments; X.W., Y.M. and E.L. contributed unpublished reagents; Y.H. and Y.H. analyzed the data; Y.H. and L.X. wrote the manuscript.

Additional information

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