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Neither insufficiency nor overexpression of *sac1* affects the accumulation of $A\beta_{42}$ in *Drosophila* expressing $A\beta_{42}$

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Abstract. – OBJECTIVE: We investigated the effects of genetic down- and up-regulation of sac1 expression on $A\beta_{42}$ accumulation and the associated neural deficits in flies with direct expression of arctic mutant $A\beta_{42}$ ($A\beta$arc) in the neurons of GF pathway.

MATERIALS AND METHODS: We genetically down-regulated and up-regulated the level of sac1, encoding a major phosphoinositide phosphatases in a disease model, in which arctic mutant $A\beta_{42}$ is directly expressed in the neurons of a neural pathway of adult fruit flies.

RESULTS: We conducted a time-course analysis of $A\beta_{42}$ level in the model and found an age-dependent elevation of $A\beta_{42}$ accumulation, closely correlated to the age-dependent decline of climbing ability in the model flies. Neither sac1 insufficiency nor sac1 over-expression significantly changed the three phenotypes.

CONCLUSIONS: We found that the alterations of sac1 expression did not change $A\beta_{42}$ accumulation and neural deficits in the model.

Key Words:
Alzheimer's disease, Intraneuronal beta amyloid, Phosphoinositides phosphatase, sac1, Drosophila, Giant fiber pathway.

Introduction

Alzheimer's disease (AD) is currently the most common cause of dementia in the elderly people. Sufficient evidence exists to support that accumulation of beta amyloid (Aβ) in the brain plays an important role in AD. Aβ, particularly $A\beta_{42}$, accumulates not only in the extracellular space but also in intracellular organelles and the plasma membrane in AD brains. Neuronal Aβ accumulation is involved in synaptic deficits, formation of amyloid plaque and cell death in AD[1-5].

In *Drosophila*, pan-neuronal expression of $A\beta_{42}$ produces predominant intraneuronal Aβ accumulation and neural deficits[6,7]. We also reported previously that expression of wildtype or arctic mutant $A\beta_{42}$ in the *Drosophila* giant fiber (GF) pathway causes predominant intraneuronal Aβ accumulation, age-dependent synaptic deficits, and premature death[8-10]. Given the powerful genetic tools of *Drosophila*, flies expressing-$A\beta_{42}$ can serve as a convenient platform to study the mechanisms for intraneuronal Aβ accumulation and resultant synaptic deficits *in vivo*.

Phosphatidylinositol (PI) and its metabolites, phosphoinositides (PtdIns) may play important roles in the pathogenesis of Alzheimer's disease[11]. PI, phosphatidylinositol 4,5 phosphate ($PI_{4,5}P$) and other acidic phospholipids may directly interact with Aβ on lipid membranes, and facilitate the conversion of random coil into β-structure in Aβ, leading to Aβ aggregation. $PI_{4,5}P$ on the plasma membrane may suppress $A\beta_{42}$ secretion from cultured cells[12] possibly by inhibiting the activity of γ-secretase[13], and mediate the cellular toxicity of oligomeric $A\beta_{42}$ possibly by up-regulating calcium influx into the cell[14]. Phosphatidylinositol-3-phosphate (PI_3P) regulates the sorting and processing of amyloid precursor protein (APP)[15].

Consistently, some metabolizing enzymes of PtdIns have also been implicated in AD[11]. Phos-

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phatidylinositol-3-kinase (PI_3K) phosphorylates $PI_{4,5}P$ into $PI_{3,4,5}P_3$, while phosphatase and tensin homolog (PTEN) antagonizes the effect of PI3K by converting $PI_{3,4,5}P$ to $PI_{4,5}P$. Aberrant activation of PI_3K/kinase AKT signaling may disrupt the proteolysis of misfolding Aβ and tau in one hand, and mediate the transmission of pathophysiological signals from Aβ to tau in the other hand[16,17]. Moreover, Aβ expression may cause hyper-activation of PI3K and enhancement of long-term depression of synaptic transmission; down-regulation of PI3K and up-regulation of PTEN rescue synaptic defects in Aβ-expressing flies[18]. Synaptojanin 1 dephosphorylates the D-5 position phosphate from $PI_{4,5}P$ and $PI_{3,4,5}P$. Genetic reduction of synaptojanin 1 suppresses $A\beta_{42}$ secretion from cultured cells[12,19], but this effect was ascribed to an increase in both endocytosis and intracellular degradation of Aβ[19]. Down-regulation of Class III PI 3-kinase, which phosphorylates PI into PI_3P[20], enhances amyloidogenic processing of APP in endosomes[15].

SAC1 is also a major PtdIns phosphatase that preferentially dephosphorylates the D-4 position phosphate from phosphatidylinositol 4-phosphate (PI_4P)[21], and plays an important role in controlling the cellular level of PtdIns, particularly PI_4P and $PI_{4,5}P$[22-24]. Currently, whether SAC1 plays a role in regulating the assembly and neurotoxicity of Aβ, the trafficking and processing of APP is unknown. Here, we investigated the effects of genetic down- and up-regulation of *sac1* expression on $A\beta_{42}$ accumulation and the associated neural deficits in flies with direct expression of arctic mutant $A\beta_{42}$ ($A\beta_{arc}$) in the neurons of GF pathway.

Materials and Methods

Genetics and Stocks

Drosophila stocks were cultured on standard medium at 23-25°C. After pupation, the adult flies were cultured on standard medium and entrained into 12h/12h light/dark cycle. Transgenic line [UAS] $A\beta_{arc}$, contains the recombinant arctic mutant $A\beta_{42}$ cDNA fused to a secretion signal of the *Drosophila* necrotic gene. The [Gal4] A307[25] was used to drive the expression of Aβ in the GF pathway, and P [Gal4–elav.L][3] was used for pan-neuronal expression. P(GT1)$sac1^{BG02228}$ (Bloomington stock # 12780) and P{EPgy2}$sac1^{EY02269}$ (Bloomington stock # 19651) were used to down-regulate and up-regulate the expression of *sac1* respectively. Before use, all strains of flies were separately backcrossed to an isogenic w^{1118} flies (Bloomington stock # 5905) for at least 5 generations for cleanup of genetic background.

Negative Geotaxis Assays

Gargano et al[26] developed "rapid iterative negative geotaxis (RING) assay" for large scale examination of the climbing ability of flies. We modified the RING assay to allow automatic and highly replicable examination and quantification of the climbing abilit. Briefly, the system includes a rectangular metal frame (32 cm×21 cm×6 cm), which contains 10 vertically-placed transparent plastic vials (2.1 cm in diameter, 19.0 cm in height) fastened onto it. The system also includes an electric motor, which drives the frame to tap in the vertical direction. Controlled by a step driver, the electric motor initiates a group of 4 rapid taps every 1 min. During the assay, 10 male flies are filled in each vial; each group of taps drops the flies to the bottom of the vials; immediately after the taps, the flies were allowed to climb upwards on the walls of the tubes due to negative geotaxis; the climbing process is videotaped and analyzed using a home-made software, which was developed to measure the height of each fly at any given time point. In this study, each experiment includes 5 groups of taps; the heights of all the 10 flies in each vial at the 7^{th} s after each of the 5 groups of taps were averaged as a mean-height; one mean-height was calculated as a single data point.

Longevity Assay

Longevity was examined as previously done. One hundred flies from each genotype were equally separated into five vials containing standard fly food and dry yeast and were cultured at 25°C. The flies were transferred to a new diet after every 3rd day and the number of dead flies was recorded at 3-day intervals until the last one died. Survival rates were analyzed with the SPSS 11 Kaplan-Meier software.

ELISA assay of $A\beta_{42}$ Level in Fly Brain

TO analyze $A\beta_{42}$ level in fly brain, intact brains of 10 flies per strain were dissected out in cold phosphate buffered saline (PBS) and placed immediately into cold ELISA sample buffer supplemented with cocktail protease inhibitor (Calbiochem, San Diego, CA, USA). Brains were homogenized thoroughly, incubated on ice for 3 h

and stored at –20°C. ELISA was performed using $A\beta_{42}$ Human ELISA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RT-PCR of sac1 mRNA

30 fly heads (15 females and 15 males) per strain were homogenized thoroughly with 500 ul Trizol solution (Cat #: 3101-100, Shangai Pufei Biotech Co. Ltd, Shangai, China) and total RNAs were extracted according to the manufacturer's instructions. Oligo dT was used for reverse transcription of mRNA. According to the transcript data of *sac1*, a pair of primers were designed and synthesized by GL Biochem Ltd (Shanghai, China) for real-time PCR quantification of *sac1* mRNA level using the 7500 Real Time PCR system (AB Applied Biosystems, San Francisco, CA, USA), the forward primer is: 5' GCTGCCCAATTTGCGCTATAA 3', and the reverse is: 5' CACGCCCTTGAAAATGCTCTC 3'.

Statical Analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The data were presented as mean ± SEM. The criterion for significant difference is $p < 0.05$.

Results

Previously, we reported that expression of wildtype and arctic mutant $A\beta_{42}$ in the *Drosophila* GF pathway and a small group of neurons elsewhere in the adult nervous system causes intraneuronal Aβ accumulation, a variety of age-dependent synaptic changes, accelerated decline of flight ability and premature death[8-10]. To examine whether change of *sac1* expression level affects the Aβ accumulation and associated neural deficits, we genetically manipulated the expression level of *sac1* expression in flies by using two strains of flies, $sac1^{BG02228}$ and $sac1^{EY02269}$. $sac1^{BG02228}$ is a mutation of *Drosophila sac1* gene caused by a transposon insertion. Homozygous $sac1^{BG02228}$ mutation causes embryonic lethality[27]. Our real-time PCR (RT-PCR) revealed that heterozygous mutant flies of $sac1^{BG02228}$ displayed about 50% reduction in the level of *sac1* mRNA (Figure 1a,c). $sac1^{EY02269}$ is a transgene that is inserted into a site upstream of *Drosophila sac1* gene and contains upstream activation elements, which can binds to transcription factor Gal4. RT-PCR revealed that in the presence of Gal4, one copy of $sac1^{EY02269}$ increased the mRNA level of *sac1* by about 250% (Figure 1b&d).

Six groups of flies were generated for next experiments, they are: "control" containing one copy of P[Gal4]A307 transgene; "$sac1^{BG02228/+}$"containing one copy of P[Gal4]A307 and $sac1^{BG02228}$; "$sac1^{EY02269/+}$" containing one copy of P[Gal4]A307 and $sac1^{EY02269}$; "$A\beta_{arc}$" containing one copy of P[Gal4]A307 and [UAS]$A\beta_{arc}$; "$A\beta_{arc}$-$sac1^{BG02228/+}$" containing one copy of P[Gal4]A307, [UAS]$A\beta_{arc}$ and $sac1^{BG02228}$; "$A\beta_{arc}$-$sac1^{EY02269/+}$" containing one

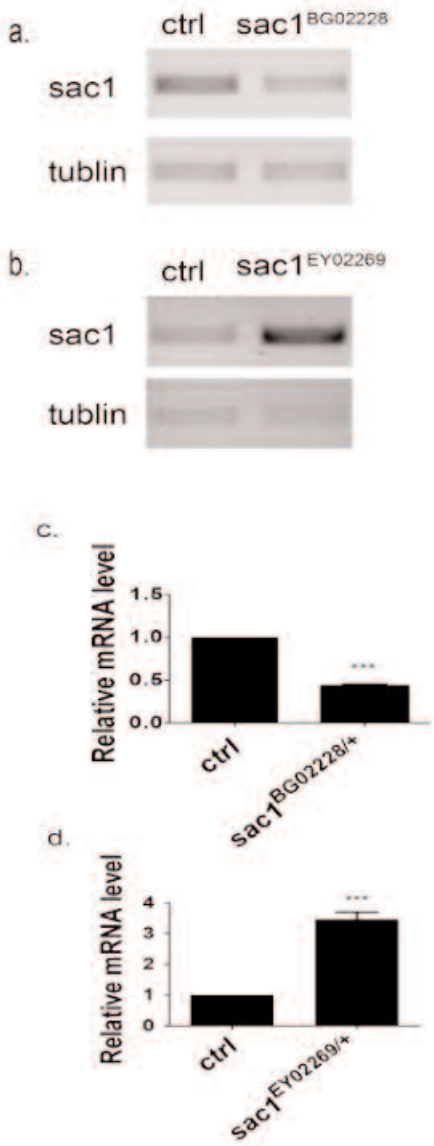

Figure 1. efficacy of genetic manipulation of sac1 expression level. Representative images *(A,B)* and quantification *(C,D)* of sac1 mRNA level in the fly brains with sac1 insufficiency ($sac1^{BG02228/+}$) and sac1 overexpression ($sac1^{EY02269/+}$) respectively, n = 3-5. *T*-test, "***" indicates $p < 0.001$."

copy of P[Gal4]A307, [UAS]$A\beta_{arc}$ and *sac1*^{EY02269}. $A\beta_{arc}$ was expressed in the last three groups, but not in the first three groups.

We examined the lifespans of the 6 groups of flies. In consistence with previous reports[6,8], expression of $A\beta_{arc}$ significantly reduced the lifespans of $A\beta_{arc}$-*sac1*$^{BG02228/+}$ and $A\beta_{arc}$-*sac1*$^{EY02269/+}$ flies (Figure 2a). However, the lifespans of $A\beta_{arc}$-*sac1*$^{BG02228/+}$ and $A\beta_{arc}$-*sac1*$^{EY02269/+}$ flies were indistinguishable from that of $A\beta_{arc}$ flies, suggesting that the alterations in the expression level of *sac1* did not change the effect of $A\beta_{arc}$ expression-induced neural deficit on lifespan. Moreover, the lifespans of the three groups without $A\beta_{arc}$ expression were also indistinguishable from each other (Figure 2a), demonstrating that neither decrease nor increase in the expression level of *sac1* changed the lifespan of *Drosophila*. Please note the culture temperature in the present study is 23-25°C, lower than that in previous studies[8]. Thus, the lifespans of both flies with and without $A\beta_{arc}$ and expression in the present study were longer than those in previous studies.

To test the effect of the *sac1* alterations on $A\beta_{arc}$ expression-induced acceleration of age-dependent motor decline, we examined the climbing ability of the 6 groups of flies at the ages of 3, 16 and 26 d after eclosion by using the modified rapid negative geotaxis assay (method). By the age of 16 d, the climbing ability of all 6 groups of animals was not different from each other (Figure 2b). By the age of 26 d, climbing ability of the 3 groups of flies with $A\beta_{arc}$ expression was markedly lower than that of the 3 groups of flies without $A\beta_{arc}$ expression. In consistence with the result of lifespan analysis, the alterations in the expression level of *sac1* did not change the climbing ability in flies with and without $A\beta_{arc}$ expression (Figure 2b).

To examine whether the alterations of *sac1* expression could change the $A\beta_{arc}$ level in $A\beta_{arc}$-flies, we dissected out the brains of $A\beta_{arc}$, $A\beta_{arc}$-*sac1*$^{BG02228/+}$ and $A\beta_{arc}$-*sac1*$^{EY02269/+}$ flies at the ages of 13, 17 and 27 d, and quantified the $A\beta_{42}$ level in the brains bywith ELISA. We found the $A\beta_{42}$ levels in the 3 groups of flies remained un-

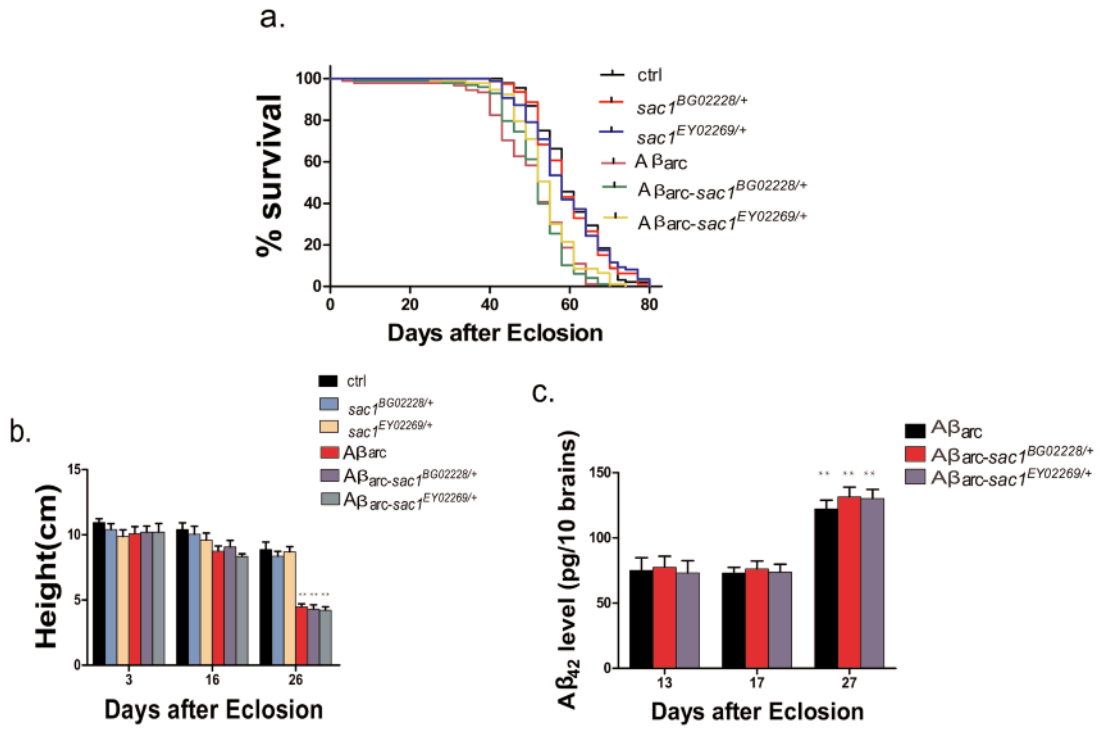

Figure 2. The effects of sac1 alterations on the lifespan, climbing ability and $A\beta_{42}$ level. ***(A)***, survival rate against age, n=100 flies for each genotype, the lifespans of the three groups of flies expressing Aβarc are significantly shorter ($p < 0.001$) than those of the three groups of flies without expression of $A\beta_{arc}$, Log Rank test; ***(B)***, negative geotaxis score against age, n=3-5, one-way Anova; ***(C)*** ELISA quantification of $A\beta_{42}$ in the brains against age, n=3-5, one-way Anova. "*" and "**" indicate $p < 0.5$ and $p < 0.01$ respectively.

changed when flies aged from 13 to 17 d, but markedly increased from 17 to 27 d (Figure 2c), which closely correlated with the age-dependent decline of climbing ability in the 3 groups of animals (Figure 2b). Again, consistent with the results in the examinations of lifespan and climbing ability, the $A\beta_{42}$ levels in the 3 groups of flies were indistinguishable from each other at all stages (Figure 2c).

Discussion

Previously, we generated a *Drosophila* AD model by directly expressing Aβ in a well-characterized neural pathway, the GF pathway, in adult fruit flies with a tissue-specific driver for investigating age-dependent alterations of synaptic function and ultrastructure induced by Aβ accumulation[8]. Herein this paper we refer to this model as the we call this model as neural pathway AD model. In consistence with other studies of fruit flies with direct expression of Aβ in the nervous system[6,7], the neural pathway AD model also exhibits a predominant intraneuronal accumulation of Aβ, and an age-dependent decline of flight ability and premature death[8]. In the present study, we conducted a time-course analysis of $A\beta_{42}$ level in the model and found an age-dependent elevation of $A\beta_{42}$ accumulation, closely correlated to the age-dependent decline of climbing ability in the model flies.

SAC1 is a trans-membrane protein that localizes to the endoplasmic reticulum (ER) and Golgi apparatus, and performs important roles in the metabolism and signaling of phosphoinositide lipids, particularly PI_4P and $PI_{4,5}P$[22-24]. Plasmalemma $PI_{4,5}P$ level was reported to inversely correlate $A\beta_{42}$ secretion from cultured cells[12]. We speculated that the neuronal $A\beta_{42}$ accumulation in the neural pathway AD model might be affected by alterations of the expression level of *sac1*. Thus, we examined the effects of down- and up-regulation of *sac1* on the lifespan, age-dependent Aβ accumulation and associated motor decline in the model. Unexpectedly, neither *sac1* insufficiency nor *sac1* over-expression significantly changed the three phenotypes.

Possible explanations are: (1) SAC1 is mainly localized in ER and Golgi apparatus, and its role is largely restricted to the two compartments; (2) plasmalemma PI_4P and $PI_{4,5}P$ are tightly controlled by other phosphoinositide phosphatases and kinases, neither the insufficiency nor the overexpression of *sac1* could not produce a significant change in the levels of PI_4P and $PI_{4,5}P$.

Conclusions

We investigated the effects of genetic down-regulation and up-regulation of *sac1* expression on $A\beta_{42}$ accumulation and the associated neural deficits in flies with direct expression of arctic mutant $A\beta_{42}$ ($A\beta_{arc}$) in the neurons of GF pathway. We found that the alterations of *sac1* expression did not change $A\beta_{42}$ accumulation and neural deficits in the model and the three phenotypes were not significantly influenced by either *sac1* insufficiency or *sac1* over-expression.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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