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The function of Drosophila IP3K2 in behavior and metabolism

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Abstract

In previous studies Type B inositol 1, 4,5-trisphosphate 3-kinase (ITPKB) has been connected to several human diseases, especially the sporadic form of Alzheimer's disease (sAD), where patients had an overexpression of ITPKB in comparison to control individuals [1]. sAD is especially related to memory loss; however these patients also display other symptoms, such as eating disability. Interestingly, dementia has also been associated with anorexia [2]. This paper demonstrated the altered eating behavior in Inositol 1,4,5-triphosphate kinase 2 (IP3K2) knocked down (KD) transgenic flies (*Drosophila melanogaster*). To investigate behaviors related to food consumption numerous assays were conducted, including CAFE assay (capillary feeder), starvation assay and lipid extraction. CAFE assay measures the total food intake and no difference was detected in food intake or the frequency of eating. Starvation assay and lipid extraction were measured both separately and in combination. These assays presented a longer lifetime for KD in comparison to the two control groups of flies where KD had lower levels of lipids. However, combining these results indicated that KD group would survive longer against starvation resistance. Besides, the experimental group presents lower levels of lipid in their body. Additional assays, including CAFE assay, starvation assay and lipid extraction, were conducted in order to perform food consumption behavioral in flies. The CAFE assay also enabled us to measure the total food intake by each fly. A combination of starvation assay and lipid extraction indicate that the KD group survive longer under starvation resistance and at the same time contain lower levels of lipid in their body. Additionally, there were no significant differences in amount of food intake and the frequency of eating between experimental group and the controls in our investigation.

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Aim

The aim of this project was to investigate the impact of eating behavior in IP3K2, KD transgenic flies (*Drosophila* melanogaster) against controls.

Introduction

Drosophila melanogaster as a model organism

Drosophila melanogaster or so-called fruit fly, as a model organism, has simplified biological explorations in the context of human diseases. One of the aspects of the simplicity of *Drosophila,* as a model organism, is the size of its genome. Contrary to the human genome which contains an estimated 20 000 – 25 000 protein-coding genes Drosophila has 13 600 protein-coding genes. The expedient features of this organism include small size, short life duration (almost 12 days in 25℃), the ease with which it can be genetically manipulated, sex dimorphism and maintenance with low-cost equipment [3-4]. *Drosophila* has a lot of genes conserved with the humans which is the reason why *Drosophila* has been used to explain molecular pathways known to be associated to different disorders, including neurodegenerative diseases [9].

Drosophila as a model system for studies of neurodegenerative disease

Due to the efforts of Thomas Hunt Morgan, a prominent Evolutionary biologist, *Drosophila* can be used as a model organism for human diseases to probe mutations, and genetic manipulations. The similarities between human and *Drosophila's* genes enable researchers to employ this tiny creature as a model organism for molecular and cellular surveys regarding neurodegenerative diseases [5]. Using *Drosophila* as a model organism for brain disorders it is possible to investigate pathological pathways [14-15].
Alzheimer's disease is the most common example of dementia among older individuals. According to a survey conducted in 2012 one out of eight of the US's elderly population suffers from the sporadic form of Alzheimer's disease [6].

ITPKB Pathway

Increased expression of ITPKB is known to accumulate a higher level of intracellular calcium, with consequences on the mechanisms involved in neural differentiation, neuritis extension, maturation as well as cell migration [12]. Seen in (Fig. 1) the

activation of the G-protein-coupled receptor (GPCRs) up-regulates the expression of D-myo-inositol-1, 4,5-trisphosphate (IP3) and in turn ITPKB, both increasing the amount of calcium. It has been shown that this elevated level of calcium effects learning and memory [13].

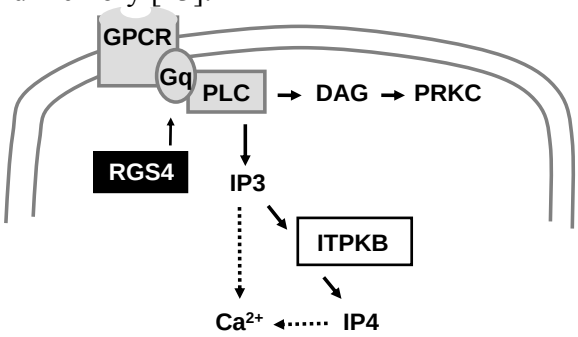

Fig. 1

The above picture presents the hypothesis stated by Emilsson, Saetre and Jazins (2006) that declares that the activation of the GPCR domain will up-regulate the expression of IP3 mRNA consequently increasing the amount of calcium. RGS4 acts as an inhibitor against the GPCR domain. The figure represents a synaptic terminal and includes molecules involved in intracellular signaling related to calcium. The Figure adapted from ref [10]

Targeted Gene Expression

The Gal4/UAS system consists of two main parts; the GAL4 gene that encodes for a transcriptional activator protein and the UAS (Upstream Activation Sequence) that specifically binds to the GAL4 region upon activation. By inserting GAL4 as a transcriptional factor in the genome, different GAL4 lines can be produced. Hence, these lines aid us to express desired gene in different cell types. The gene of interest (GOI) will then be inserted in the downstream section of the UAS. For activating the gene of interest, a crossing of two transgenic lines must be carried out, with a subsequent investigation of the next generation progeny. The GAL4/ UAS system is used to genetically modify animals so they express the gene that you would like to investigate further. The temperature dependent GAL4 system in flies ranges between 16°C to 29°C [11]. In this assay flies were transferred to a 29°C incubator to provide maximal activation of the GAL4 system without any effects on their viability. The system can be applied for either expression or silencing of the desired gene (Fig. 2).

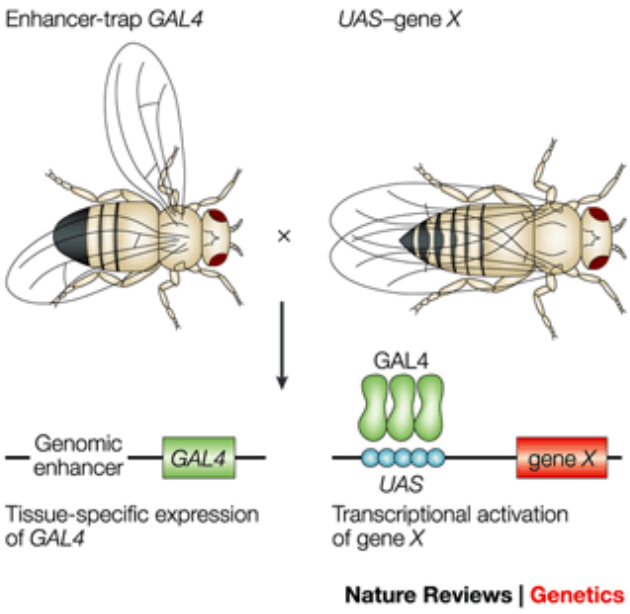

Fig. 2
The two parts system indicates the expression of the GOI (X). To activate the transcription in flies carrying the Upstream Activator System (UAS), gene (X) should be crossed with the flies having GAL 4 gene, which will be expressed in a desire tissue. Then the offspring can either over express or silence gene (X)[7]. The figure is adapted from ref [16]

Silencing of the GOI is obtained by RNA interference (RNAi) and this is a specific and powerful method for loss-of-function studies [22]; RNAi can be used to hinder the translation processes of the mRNA (messenger RNA). In this procedure, insertion of a double-stranded RNA (dsRNA), creating a hairpin-loop structure, will result in either mRNA degradation or translational repression.

Material and method

Fly stocks

Genotypes included in this study are:
Elav-GAL4:GAL 80:
P{w[+mW.hs]=GawB}elav[C155] w[*];
P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=tubP-GAL80}LL2
W1118: w1118
UAS-IP3K2: P{KK112399}VIE-260B
Control 1(c1): w^{1118} *x UASIP3K2,* Control 2*(c2): Elav-GAL4.GAL80 x* w^{1118},
Experimental (exp): *Elav-GAL4.GAL80 x UAS IP3K2*
All Flies were kept on a standard fly food diet (Jazz-Mix, Fisher Scientific). Dry yeast was added to increase the yeast content to 10%. Per 1050 ml requested food, 1200 ml of water was mixed with 226.8 g of Jazz Mix and 8.7 % extra yeast.
Flies were kept at 25℃ on a 12:12 light:dark cycle using standard system.

Starvation assay

Virgin females of *Elav-GAL4* and w^{1118} were crossed with males of the different RNAi lines. The crosses were kept in separate bottles in a 25℃ incubator. Male progenies were transferred in to a 29℃ incubator between five to seven days. In this survey using females were ignored since treatment of female creates problems such as deleterious effects of mutagens on oocyte itself and the tendency of it to keep mutagens, whereas mature sperm are rather subtle to mutagens. 20 males from each group (two controls and one KD) were put in one agarose vial deprived of any food. At least 6 replicates were performed for each strain. Finally, the agarose vials were shifted to the 25℃ incubators and checked for number of dead flies every 12 h.

CAFE assay

Male flies of three strains were transferred in to different vials. Each vial contains 16ml of 1% agarose with 5 % sucrose, 5 % yeast extract and 0.5 % green food in water. Then, a 5 µl capillary was immersed in oil and transmitted rapidly to the food vial until it got stuffed. Afterward, a total of five male flies of each strain were separately transferred to the vials and enclosed the top by parafilm. A marker was used to indicating the boundary of the food and oil. 10 equipped vials were positioned in front of a HD camera for 24 h for each strain. After 24 h the capillaries were removed and the food intake was measured by the checking of new food-oil interface.

Lipid Extraction assay

Anesthetized male flies were kept in groups of 30 and aged 5-7 days. At least 11 replicates were performed for each strain. Immediately after, the vials were relocated to the 60 °C incubator for 1h. Dry weights were calculated with a precision of 0.0001 g scale. 10 ml of ethyl ether was poured in each glass vial to extract all the lipids, which were covered with cotton and left for 24 h in room temperature. After one day the ethyl ether was filtered and flies were transferred again in to the 60 °C incubator for 1h to eliminate remaining ethyl ether. Lipid free weights were calculated as earlier mentioned. To obtain the average lipid concentration per fly the lipid-free weight was subtracted from the dry weight and then the number was divided by the total number of flies in each vial.

Statistics

The log rank test is a nonparametric test that compares the survival distributions of two samples. For the starvation assay, log rank test was used via the given website: http://bioinf.wehi.edu.au/software/russell/logrank/
Log rank test used for the starvation assay computations. Minitab 14 used for the statistical analysis. For calculating the means equality values of flies' groups, ANOVA was used. Moreover, One-Way ANOVA has been used to see differences for more than two groups however for two group's difference the student t-test was used.

Results

Starvation assay

Starvation assay were performed to distinguish the possible role of IP3K2 in increasing or decreasing survival. By performing this assay we could detect that the KD group would survive for longer time in comparison to the control flies (Fig. 3) Flies were kept in groups of 20 and aged for 5-7 days. For this assay at least 5 replicates were performed for each strain.

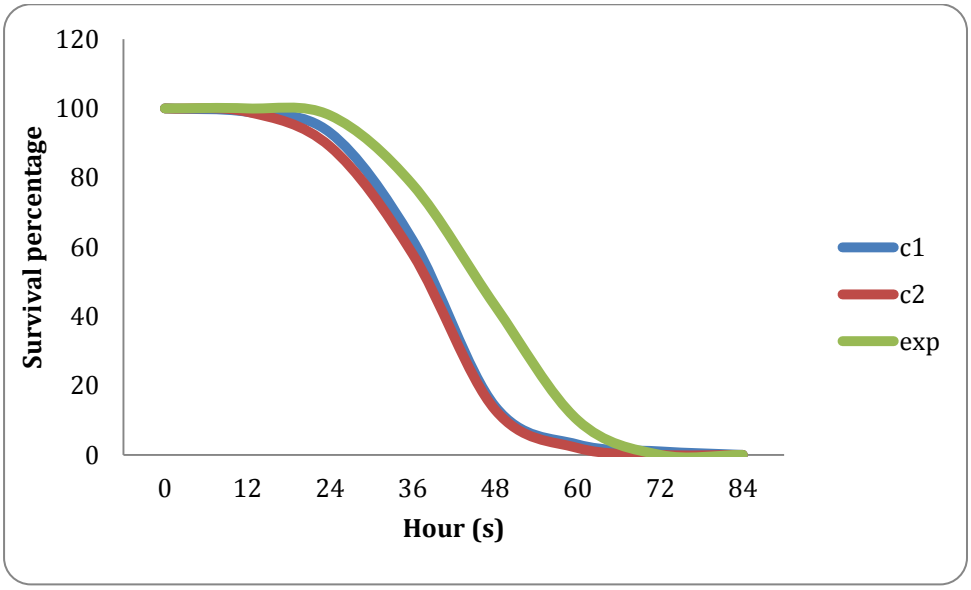

Fig. 3
Starvation assay. Flies were kept in groups of 20 and aged for 5-7 days. KD group would survive longer in comparison to controls. c1: w^{1118} *x UASIP3K2,* n=10*, c2: Elav-GAL4.GAL80 x* w^{1118}*,* n=5, exp: *Elav-GAL4.GAL80 x UAS IP3K2,* n=6 (n=number of replicates) ($p > 0.05$)

CAFE assay (Capillary Feeder)

The CAFE assay measures the food intake as well as the meal volume and the frequency of food intake in individuals or group of flies. Flies were kept in groups of 5 and aged for 5-7 days. At least 10 replicates for each strain were done. The assay was performed with 24 h filming. Average total food intake for the w^{1118} *x UAS IP3K2* was 0.41μl, (SE±0.08). The same criterion was calculated for *Elav-GAL4.GAL 80 x UAS IP3K2* and it indicated 0.44 μl, (SE±0.03). The last group, *Elav-GAL4.GAL80 x* w^{1118} showed 0.62 μl, (SE±0.05). Totally, there were no differences in total food intake between the different strains investigated ($p > 0.05$) (Fig. 4).
Feeding bout was calculated by dividing the frequency by number of the flies in each vial. Average feeding bout for the w^{1118} *x UASIP3K2* was 5.58 μl, (SE±1.24). The other group*, Elav-GAL4.GAL80 x UAS IP3K2* showed 6.88 μl, (SE±0.94) and *Elav-GAL4.GAL80 x* w^{1118} presented 7.86 μl, (SE±0.99) ($p > 0.05$) (Fig. 5).
For computing the average meal size for each strain we divided the average total food intake by average feeding bout which was 0.076 μl for w^{1118} *x UASIP3K2,*

(SE±0.0053). The mentioned criteria for *Elav-GAL4.GAL80 x UAS IP3K2* was 0.070 μl, (SE±0.0055) and for the final group, *Elav-GAL4.GAL80 x w1118* 0.08 μl, (SE±0.007) ($p > 0.05$) (Fig. 6).

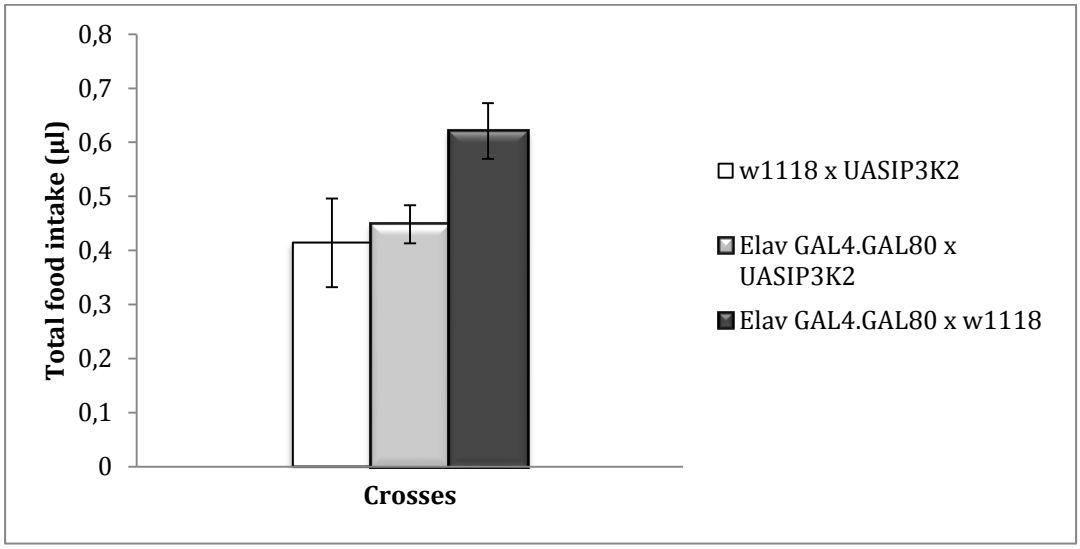

Fig. 4
Average Total food intake of flies with three strains. Calculated by dividing the average total food usage in each capillary by the number of total flies in each vial. 5 flies were kept and aged for 5-7 days. 10 replicates for each strain have been performed. No differences were detected regarding average total food intake between three groups.

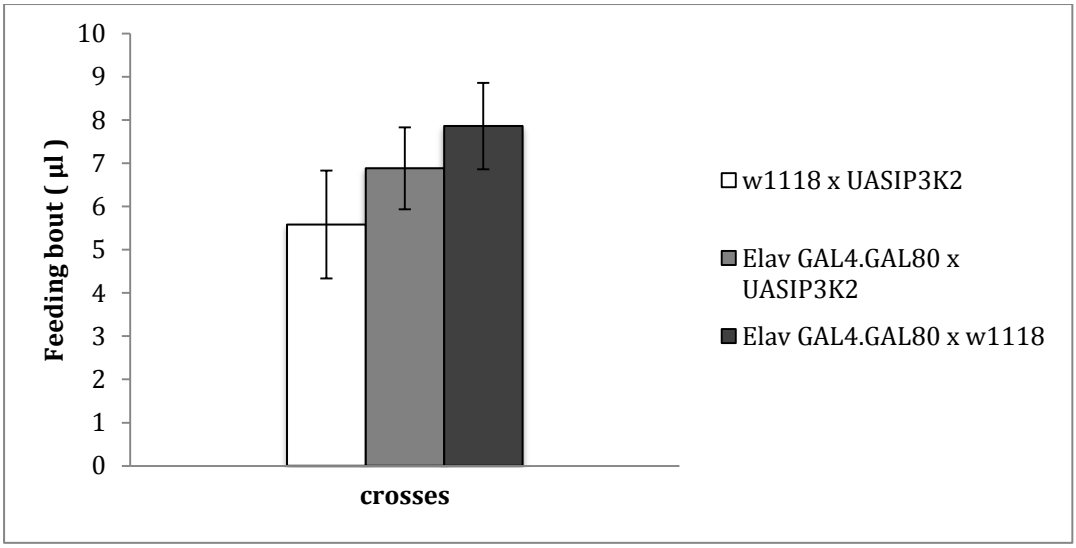

Fig. 5
Average Feeding bout. Calculated form frequency divided by number of flies. 5 flies were kept and aged for 5-7 days. 10 replicates for each strain have been performed. No differences were detected in feeding bout between the KD group and controls.

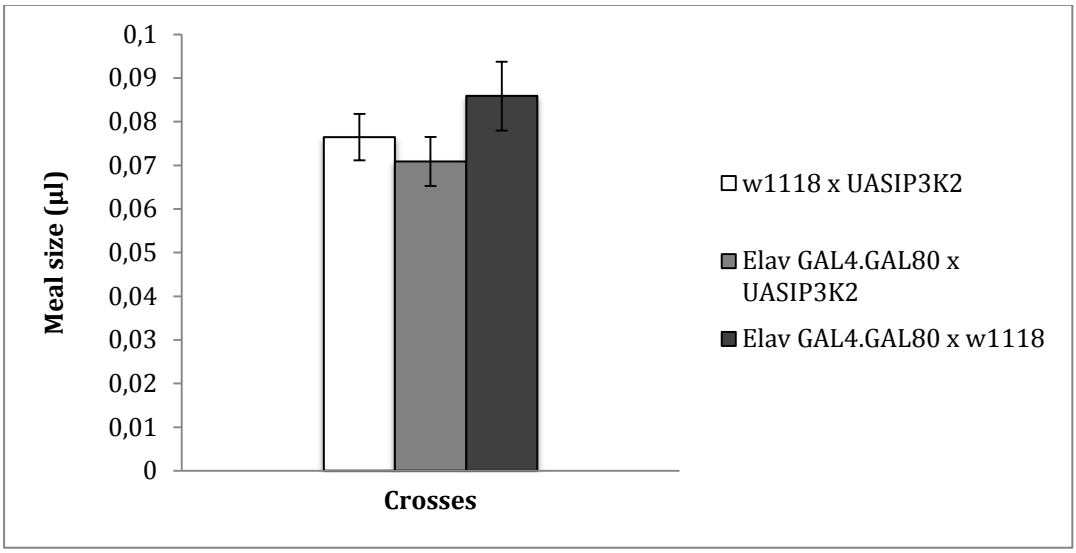

Fig. 6

Average meal size analyzed form dividing the amount of average total food intake by average feeding bout. 5 flies were kept and aged for 5-7 days. 10 replicates for each strain have been performed. No differences were detected in meal size between the KD and controls.

Lipid extraction

Lipid extraction was performed.to investigate the average lipid content in each group. Information about lipid content determines the metabolism differences, which will give info about metabolism processes. There were at least 11 replicates of each group containing 30 males in each glass vial. Flies were aged for 5-7 days. The figure shows that KD group has a smaller amount of lipid in comparison to the controls. Average lipid content of the *Elav-GAL4.GAL80 x UAS IP3K2* was 7.62 µg, (SE±0.14), the next group, *Elav-GAL4.GAL80 x* w^{1118} showed 8.13 µg (SE ± 0.11) , and the last strain, w^{1118} *x UASIP3K2* had 7.97µg,(SE ± 0.09) ($p<0.05$) (Fig. 7).

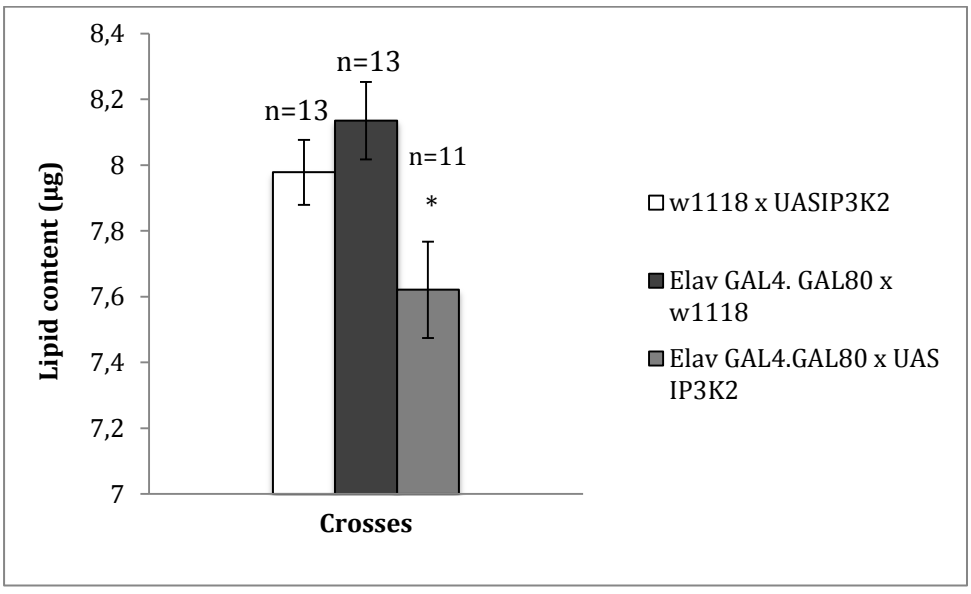

Fig. 7

Lipid content. 30 male flies were kept and aged between 5-7 days. The experimental group has a smaller amount of lipid content comparing to the controls. n=number of replicates
Significant level p> 0.05, * p< 0.05, ** p< 0.01

Combining starvation and lipid extraction

To disentangle the question if flies with less amount of lipids have more resistance to starvation the two previous assays, starvation and lipid content, were combined and investigated at 12 h and 24 h. Starvation and lipid extraction at 12h was done by running at least 10 replicates of each strain. 30 flies were kept in vials between 5-7 days. Average lipid content for the *Elav-GAL4.GAL80 x* w^{1118} was 7.04 µg, (SE±0.12), next group, for *Elav-GAL4.GAL80 x UAS IP3K2* showed 6.94 µg, (SE ± 0.10) and the last group w^{1118} *x UASIP3K2* implied 6.93 µg,(SE ± 0.13) ($p > 0.05$) (Fig. 8).
For the 24 h assay, 30 flies were kept in each vial and aged for 5-7 days. We used at least 12 replicate for each strain. Average lipid content for *Elav-GAL4.GAL80 x* w^{1118} was 6.46 µg, (SE±0.14), next group , for *Elav-GAL4.GAL80 x UAS IP3K2* showed 6.95 µg, (SE±0.1) and final group , w^{1118} *x UASIP3K2* indicated 6.82 µg, (SE±0.11) ($p<0.05$) (Fig. 9).

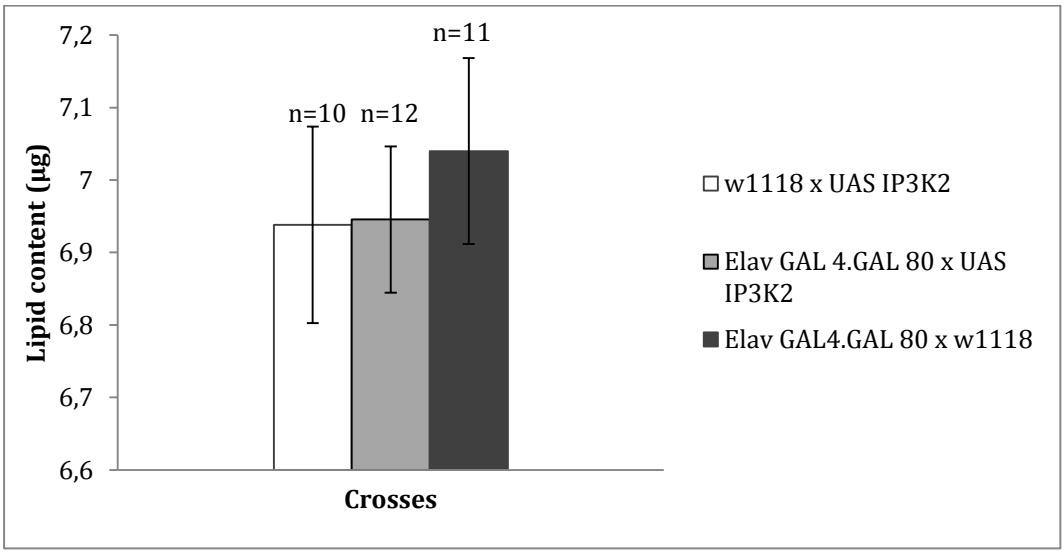

Fig. 8
12 h of starvation and lipid extraction. 30 flies were kept in vials between 5-7 days. There is no significant difference in lipid content after 12 h of starvation between the experimental group and the controls. n=number of replicates.

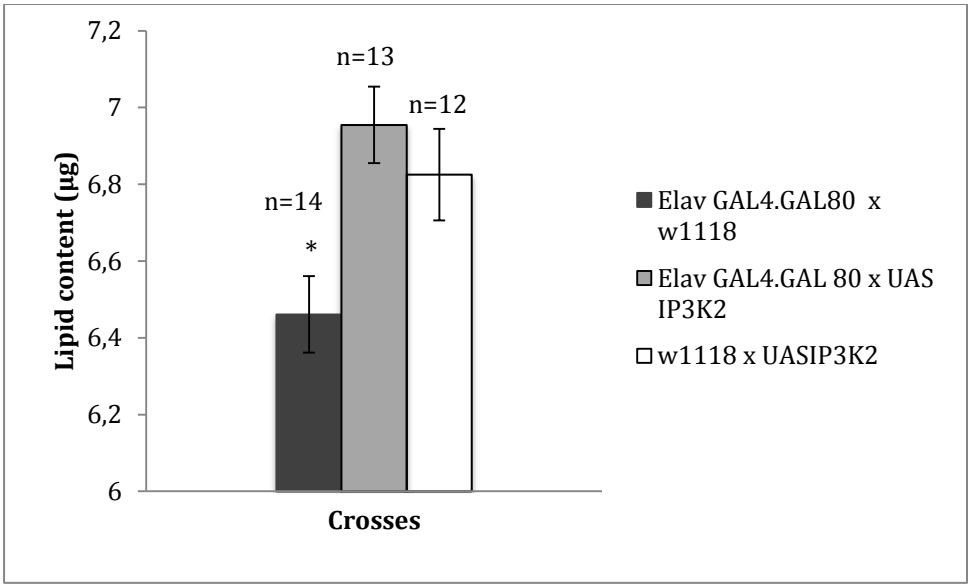

Fig. 9

24 h of starvation and lipid extraction. 30 flies were kept in vials between 5-7 days. A significant difference was detected between the KD group and one of the controls according to lipid content after 24 h of starvation. n=number of replicates. Significant level $p > 0.05$, * $p < 0.05$, ** $p < 0.01$

Discussion

Patients with AD are often faced with feeding disabilities, which could be realized through understanding of feeding behavioral attitudes [19]. According to previous studies, the protein-coding-gene ITPKB is involved in cellular activities and calcium signaling and is highly expressed in AD patients [20]. In this survey ortholog of this gene in *Drosophila* known as, IP3K2 was used to investigate the eating behavior. According to molecular functions of ITPKB, cellular regulation, immune system regulator and neural calcium signaling could be interpreted as lipid metabolism disorders (show as a result from genetic manipulation [18]). Concerning the ITPKB pathway, Inositol triphosphate or inositol 1,4,5-trisphosphate known as InsP3 or IP3, together with diacylglycerol (DAG), are engaged in signal transduction and lipid signaling [17]. Lipid metabolism plays a key role in growth processes of an organism but is also a vital energy resource when faced with starvation [8].

A starvation assay was performed to find the survival ratio between the KD group compared with the control groups. As shown in the results the KD cluster had a higher survival curve compared to the control groups, concluding that the KD cluster was more resistant to starvation, indicating that IP3K2 may be involved in lipid storage.

As showed in the lipid extraction assay the KD group had less lipid content than the control groups, thus we would expect their survivability ratio to be less than the control groups. However, as shown in the starvation assay the KD group survived longer than the control groups consequently we speculate that the rate of lipid metabolism throughout starvation could differ between the KD group and control groups. To investigate this hypothesis, lipid content was measured by extraction of adding ether for each 12 h or 24 h to accumulate lean dry weight. Consequently, the distinction between dry weight and lean dry weight are supposed to be the total quantity of lipid content. Due to practical benefits from using males in this survey,

such as being proficient of doing conjugal duties after treatments and the capability of mating with several females and prorogate the treated chromosome, females were not used in the experiments. Interestingly, according to the results, the KD group had a smaller amount of lipid compared to the control whereas the survival curve showed higher starvation resistance. Regarding inositol's function as a chemical compound of the gene of concern, two families of inositol phosphoglycans, IPG-A and IPG-P types are involved in pathways of glucose and lipid metabolism which would cause higher rate of lipogenesis and lower fat weight [21]. Additionally, 12 h of starvation showed no difference regarding lipid storage; conversely after 24 h of starvation the experimental group had contained a higher lipid amount. These results suggest that KD flies might be more dynamically proficient after 24h, making them less susceptible to starvation. By reviewing the capillary feeder assay we hypothesized that there will be differences in eating appetite between the controls and KD group. Here, the point was that to distinguish eating behavior between overeaters and anorexic flies. The assay was performed in nearly 20% humidity in our laboratory. Flies have a daily feeding behavior and usually eat early in the day and during dawn. The examination enabled us to apprehend the food consumption.
To investigate the relation between metabolism and cognitive abilities in association to Alzheimer's disease, these results should be further evaluated in regards to the role of ITPKB in memory behavioral assays. Moreover, the impact of environmental cues such as different diets, especially high protein or high sugar intake would be interesting to explore in the future.

Conclusion

IP3K2, KD flies survive longer compered to controls when exposed to starvation. The IP3K2, KD group also has a reduced amount of lipid. However, IP3K2 KD flies have higher lipids content after 24 h of starvation. Furthermore no food consumption differences were detected between the investigated fly strains. In conclusion we show that IP3K2 is involved in metabolic processes in flies. To investigate if IP3K2 altered metabolic processes affect cognitive behaviors (related to the pathophysiological processes in sAD), memory and learning should be evaluated in relation to altered IP3K2 levels in *Drosophila melanogaster*.

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