東海大學生命科學系

碩士論文

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單磷酸腺苷活化蛋白質激酶(AMPK)在果蠅支鏈胺基酸(BCAA)代謝異常之角色

Role of neuronal AMP-activated protein kinase in *Drosophila* BCAA-catabolizing enzyme mutant

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中華民國 111 年 8 月

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經本委員會審定通過，特此證明。

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目錄

圖目錄

I. 中文摘要

楓糖尿症(maple syrup urine disease, MSUD)是一種罕見的體染色體隱性遺傳疾病，由於支鏈 α - 酮酸脫氫酶(branched-chain α-ketoacid dehydrogenase, BCKDH)複合物及支鏈氨基酸轉氨酶(branched-chain amino acid aminotransferase, BCAT)功能的缺失，使支鏈胺基酸(branched chain amino acids, BCAAs)無法正常代謝進而累積在體內，導致胎兒神經發育損傷，造成發育遲緩及提早神經退化等問題。然而，楓糖尿症迄今並無有效之藥物治療方法，可能與其致病機轉複雜及不明有關。本研究中，藉由先前實驗室已建立 BCKDH (E2)基因剔除之 BCAA 代謝異常果蠅，作為研究楓糖尿症病理機轉之模式生物。我的研究發現 BCKDH 突變果蠅株的大腦中粒線體型態異常，並觀察到其腦內的 ATP 減少，進一步了解能量平衡與 BCAA 代謝異常引發神經性病症的關係，發現生物體內的重要能量傳感器單磷酸腺苷活化蛋白質激酶(AMP-activated protein kinase, AMPK)，其活性在 BCKDH 突變果蠅株的大腦組織中較野生型低，而藉由餵食二甲雙胍(metformin)可活化腦中 AMPK，而在過去的研究中指出餵食 metformin 可改善突變株果蠅發育及行為缺陷之症狀。許多文獻指出自噬作用(autophagy)扮演維持系統性能量平衡的角色且受 AMPK 活性之調控，而相較野生型果蠅，BCKDH 突變果蠅腦內自噬作用受到抑制，經餵食 metformin 能活化其腦中自噬作用，並且餵食突變果蠅株自噬作用活化劑雷帕黴素(rapamycin)後，也可改善其爬行及發育缺陷之症狀。除了討論 AMPK 的下游機制造成 BCKDH 突變果蠅的缺陷，本論文也進一步探討 AMPK 的調控機制，發現 AMPK 的活性有可能因活性氧化物(Reactive oxygen species, ROS)增加而被抑制，並且 ROS 可能是透過活化雷帕黴素靶蛋白(Target of rapamycin, TOR)來抑制 AMPK。在未來的研究中，將進一步

證實果蠅 BCKDH 突變株中 AMPK 與 TOR 之間的調控機制，並探討粒線體的功能異常如何參與 BCAA 代謝異常果蠅之病理機轉。

II. ABSTRACT

Maple syrup urine disease (MSUD) is a rare autosomal recessive inherited disease. The patient's sweat and urine have the smell of maple syrup, and patients may suffer from developmental delays, abnormal muscle tone, and neurological impairments. This disease is caused by a severe deficiency of the branched-chain α-ketoacid dehydrogenase (BCKDH) complex and branched-chain amino acid aminotransferase (BCAT), which disrupt the normal metabolism of branched-chain amino acids (BCAAs). However, there is no effective medication for MSUD potentially due to the complicated and unknown pathogenesis. Through a genetically BCKDH(E2)-deleted *Drosophila* with aberrant BCAA metabolism, this bio-model was manipulated to study pathogenesis for MSUD in this study. Our study found abnormal mitochondrial morphology in the brains of mutant flies and a decrease in adenosine triphosphate (ATP). To study the relationship between energy homeostasis and neurological problems caused by BCAA dysregulation, we found that the activity of AMP-activated protein kinase (AMPK), an energy sensor of the organism, was inhibited as compared to wild type and it could be activated in the mutant brain by orally administered with metformin, and in previous studies, the treatment of metformin can also improve the developmental and behavioral defects in BCKDH mutant flies. As energy homeostasis crucially is maintained by autophagy which was modulated by the AMPK activity. We found that the autophagy activity declined in the brains of mutant flies and could be partially restored after the treatment with metformin. Additionally, the oral feeding of rapamycin, an activator for autophagy, can also improve the development and crawling defects in mutant flies. In addition to investigating the downstream mechanism of AMPK in mutant flies, this study also explored the regulation of AMPK and found that the activity of AMPK was inhibited by the target of rapamycin (TOR) which is activated by reactive oxygen species (ROS). In the future, we proposed studying the regulation between AMPK

and TOR in BCKDH mutant flies and demonstrating that mitochondrial dysfunction involves pathogenesis among dysregulated BCAA metabolism.

III. 論文正文

1. Introduction

1.1 Gene mutations in BCAA catabolism enzymes lead to maple syrup urine disease

Maple syrup urine disease (MSUD) is a rare autosomal recessive disorder that affects approximately 1 in 200,000 babies in the United States (Therrell et al., 2014). Patients with MSUD got neurological dysfunction that leads to developmental delay, encephalopathy, and neurodegeneration.

Previous studies indicated that the neurological dysfunction exhibited by maple syrup urine disease is associated with the elevation of branched-chain amino acids (BCAAs) in the body (Frazier et al., 2014; Strauss et al., 2020; Strauss et al., 2010). BCAAs are essential amino acids, including leucine, isoleucine, and valine, and will be catalyzed by BCAA-catabolic enzymes, such as branched-chain amino acid aminotransferase (BCAT) and branched-chain α-ketoacid dehydrogenase (BCKDH) complex. MSUD is usually caused by the mutations in the genes of the BCAT and BCKDH complex. Loss of BCAA-catabolic enzyme activity leads to abnormal accumulation of BCAAs in the blood of patients, which can cause toxicity and result in neurological dysfunction (Blackburn et al., 2017).

BCAAs in organisms are firstly converted into their relevant α-ketoacids such as α-ketoisocaproate (KIC, ketoleucine), α-keto-β-methyl valerate (KMV, ketoisoleucine), and α-ketoisovalerate (KIV, ketovaline) by BCAT, The BCKDH complex initiated oxidative decarboxylation and converted α-ketoacids into acetoacetate, acetyl-CoA, and succinyl-CoA (Holecek, 2018). The BCKDH complex contains 4 subunits, E1α (branched-chain keto acid decarboxylase alpha), E1β (branched-chain keto acid decarboxylase beta), E2 (dihydrolipoyl transacylase), and E3 (dihydrolipoamide dehydrogenase), which are encoded by

the genes *BCKDHA*, *BCKDHB*, *DBT*, and *DLD*.

MSUD is classified into five distinct clinical phenotypes. The classic form, intermediate type, intermittent type, thiamin-responsive type, and E3-deficiency. The classic form only remains 0–2% of BCKDH activity and is the most common type of MSUD. The intermediate and intermittent types have milder symptoms than the classic type and are associated with higher BCKDH activity (Chuang et al., 2006).

1.2 The main treatments for MSUD and the studies in animal models

Currently, a protein diet and hemodialysis are commonly used to control the course of MSUD. However, the dietary restriction of protein is difficult to execute, and may also lead to the lack of other essential amino acids. Liver transplantation is the most effective treatment of MSUD to date, which substantially corrects whole body BCAA metabolism in MSUD (Khanna et al., 2006), but the high risk and matching problem make the surgery hard to implement. Therefore, it is necessary to find more effective treatments. Although the mechanism of MSUD has been studied for many years, no drug has been developed so far, and the difficulty may be related to its complex and unclear mechanism.

Animal models currently used to study abnormal metabolism of BCAA include mouse (Homanics et al., 2006)and zebrafish (Roberts, 2012), both of which establish the model by knocking out the E2 (*DBT*) gene in the BCKDH complex. The E2 mutant mouse model shows a high BCAA level in blood and the lack of E2 subunit gene cause neonatal lethality in the mouse. On the other hand, E2 mutant zebrafish reveals a defect in its behavior, which also shows in MSUD patients.

The research in animal models makes the study of MSUD more easily. However, the pathogenic mechanisms of MSUD are complicated, and a single type of animal model cannot explain the disease well. Therefore, to further study

the mechanism, establishing more animal models is necessary.

Drosophila is genetically similar to humans and is often used to study human diseases, is a common animal model with many advantages. Previously, our lab has published a BCAA catabolizing enzyme mutant established by CRISPR/Cas9 system to knock out the *Drosophila DBT* gene and confirmed that this mutant can be used as a model to study mechanisms in MSUD (Tsai et al., 2020). In this study, we used the *dDBT* mutant flies to do the experiments. This mutant strain has previously been observed with abnormal accumulation of BCAAs and has several symptoms including decreased mobility and development defect reveals in human MSUD.

1.3 Energy homeostasis is associated with BCAA catabolism

Several studies indicate that obesity and diabetes are associated with abnormal metabolism of BCAA and the concentration of BCAA can be used to predict the level of insulin resistance (Wurtz et al., 2013), but the relationship between energy homeostasis regulation and maple syrup urine disease remains unknown.

In the previous study, it was found that the treatment of metformin can significantly improve the mobility and developmental defect of *dDBT* mutant flies (Tsai et al., 2020). Metformin is medicine for type 2 diabetes and was reported to activate AMPK (AMP-activated protein kinase), the main energy sensor in the organism (Hardie and Hawley, 2001; Hawley et al., 2002; Winder and Hardie, 1999), from this point of view, the association between energy homeostasis and MSUD seems worth investigating.

As the main energy sensor in the organism, AMPK is sensitive to the ratio of AMP: ATP and will be activated by the consumption of ATP, thereby promoting the production of ATP to maintain energy homeostasis, and the activation of AMPK also affects lipid and glucose metabolism, protein synthesis, and can

induce autophagy through either directly or indirectly pathway (Hardie, 2007). The regulation of autophagy has links with various human diseases, including neurodegeneration and cancer (Rubinsztein et al., 2007). However, the relationship between MSUD and autophagy remains unclear.

In this paper, I have continued some of the results from the lab and found that the activation of AMPK in *Drosophila* with abnormal BCAA metabolism may improve neurological defects by promoting autophagy. Using drugs to induce autophagy can also significantly rescue the development and behavior defect of *dDBT* mutants, indicating the importance of energy homeostasis and autophagy in the abnormal BCAA metabolism *Drosophila* model.

2. Materials and methods

2.1 Fly stocks

The *dDBT* mutant (*dDBT*$^{\triangle}$) was established by our lab and published in a previous study (Tsai *et al.*, 2020). *elav-GAL4*, *UAS-SOD2*, *UAS-Atg1-RNAi*, and *W*^{1118} wild type were obtained from the Bloomington Drosophila Stock Center.

2.2 Immunostaining and imaging

Dissect the 3rd instar larval brains in Phosphate buffered saline (PBS), and fixed the samples in 4% paraformaldehyde for 20 min, then washed three times with PBS with 0.5% Triton X-100 (0.5% PBST) for 5 min. The sample was then blocked in 2% bovine serum albumin (BSA) with 0.5% PBST for 30 min at RT, after the blocking, primary antibodies were added to the brain samples and incubate at 4℃ over 8 hours. The following antibodies were used: Anti-ELAV (1:50; 9F8A9, Developmental Studies Hybridoma Bank), Anti-ATP5A (1:400; ab14748, Abcam), Anti-4 HNE (1:400; ab46545, Abcam). The samples were then washed three times with 0.5% PBST for 5 min, and incubated with the secondary antibodies at 4℃ over 8 hours. The secondary antibodies (1:400) are as follows: goat anti-mouse (Abcam) conjugated with Alexa Fluor 488 (A-11001) or goat anti-rabbit conjugated with Alexa Fluor 647 (A-21244). After being washed three times with 0.5% PBST, the brain samples were mounted with VECTASHIELD® mounting medium (H-1500, Vector Laboratories) and imaged by Leica SP5 confocal microscopy.

2.3 Larval crawling assay

Following previous descriptions (Brooks et al., 2016), 6 third instar larvae were selected and placed on a 200 mm×115 mm×30 mm agar plate, which contained Brilliant Blue dye to provide a dark background. The camera was

placed on a tripod and kept still until the end of the experiment, and the selected larvae will allow moving for 60 seconds in each video. The video was converted to .avi and analyzed with ImageJ Plugin wrMTrck (http://www.phage.dk/plugins/wrmtrck.html), to calculate the crawling speed/length and convert the crawling path to a figure, the experiments were performed at least three biological repeats. Related parameters were set as in Brooks et al (Brooks *et al.*, 2016).

2.4 Transmission electron microscopy

The dissection of larval brains was in 0.1 M cacodylate buffer and fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer at 4 °C overnight, then washed with 0.1 M cacodylate buffer. After treating with 1% OsO4 at 4 °C for 1 hour and washing with ddH_2O, 30%, 50%, 70%, 95%, and 100% ethanol solutions were then used to dehydrate the samples at room temperature. Using series epoxy resin to infiltrate the samples, and incubated them in pure resin, then polymerize at 60 °C for 2 days.

2.5 ATP Bioluminescence Assay

The detection of ATP levels is followed by the protocol of ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Cat. No. 11699695001). Dissect 20 brains of the 3rd instar larvae and carefully milled with pestle rods in 100 mM Tris buffer with 4 mM EDTA (pH 7.75). After incubating at 95℃ for 2 min, then centrifuge for 1 min at 4℃ at a rotation rate of 3500rpm and take the supernatant. After adding luciferase, measure its luminescence and compare it with the ATP standard curve. The quantity of protein in each sample is detected by adding sample loading dye and measuring the absorbance at 595 nm.

2.6 Western blotting

After the dissection of *Drosophila* larval brain tissue, every 20 brains were lysed with a mixture of RIPA buffer and sample loading dye, then boiled for 10 min. Samples were loaded in a 15% or 10% SDS-PAGE gel and subjected to electrophoresis, then the separated bands were transferred to PVDF membranes. The membranes were blocked with 5% skim milk in Tris Buffered Saline with Tween-20 (TBST) for 30 minutes at room temperature. After washing with TBST, the membranes were incubated overnight with primary antibodies at 4 °C, the primary antibodies are as follows: Anti-Phospho-AMPK (1:100; 2531S, Cell signaling), Anti-AMPK (1:1000; MABS1232, Merck Millipore), Anti-PS6K (1:1000; 9209, Cell signaling), Anti-ATG8 (1:10000; ABC974, Merck Millipore), Anti-β-Actin (GTX109639, GeneTex). Again, after washing, the membranes were incubated with HRP-conjugated secondary antibody (1:10000) in 1% skim milk for 1 hour and washed with TBST. The target proteins were visualized with chemiluminescent HRP substrate (Millipore, Burlington, MA, USA).

The *ex vivo* experiments in this study is to dissect 20 of the 3rd instar larval brain and incubate them in metformin, rapamycin, or H_2O_2 with Schneider's Insect Medium (S0146, Sigma-Aldrich) for 8hrs, 30 mins, or 1min. The sample will collect for the western blotting.

2.7 Developmental analysis

Picked 30 samples of the first-instar larvae in a group and reared them in density-controlled vials. After a few days, count the number of pupae and the number of adults, and calculate the pupation rate and eclosion rate as the ratio to the original number of larvae. The experiments were performed at least three biological repeats, and the charts were made with prism.
(https://www.graphpad.com/scientific-software/prism/)

3. Result

In this study, we used the *dDBT* mutant, which genetically knock out *BCKDH(E2)* gene, the mutant line with aberrant BCAA metabolism showed developmental and behavioral defects. Previous research has shown abnormal accumulation of cellular ROS (reactive oxygen species) in *dDBT* mutant larval brain (Tsai et al., 2020).

3.1 Treatment with leucine causes oxidative stress in the brain of BCAA-catabolizing enzyme mutant

However, it remains unclear whether the increase in ROS is caused by abnormal accumulation of BCAA, to observe the ROS stress in the *Drosophila* larval brain, we used the immunostaining experiment and measure the 4HNE(4-Hydroxy-2-nonenal) signaling. 4HNE is a product of lipid peroxidation and has been used as a marker of oxidative stress. In the experiment, the 4HNE signaling (green fluorescence) in the brain of *dDBT* mutant larvae was significantly increased compared with wild type, and the 4HNE signaling in the brain of leucine (one of BCAA)-feeding *dDBT* mutant was higher than unfed *dDBT* mutant (Fig.1). These Suggest that leucine uptake may increased oxidative stress in the brain of flies with abnormal BCAA metabolism.

3.2 Mitochondrial ROS in the brain causes behavior defects in BCAA-catabolizing enzyme mutant

Mitochondria are an important source of ROS (Murphy, 2009), and to confirm that the ROS were produced by mitochondria, I designed a locomotion experiment with third instar larvae.

Previous studies have shown that the crawling behavior of *dDBT* mutant larvae is poorer than that of wild-type (Tsai et al., 2020). To examine whether the oxidated stress in *dDBT* mutant's brain was caused by mitochondrial ROS and could affect locomotion, we used the GAL4-UAS system to overexpress SOD2 (Superoxide dismutase 2) in the brain of *dDBT* mutant, which is an enzyme used to reduce mitochondrial ROS level (Fridovich, 1995; Sarsour et al., 2014). The results showed neuron specific driver *elav-gal4* to express *SOD2* could significantly increase the crawling distance and speed of mutant larvae (Fig.2). This result indicates that the abnormal accumulation of ROS in the mutant brain may lead to its behavior defects.

3.3 Defective mitochondrial morphology and reduced mitochondrial signal in the brain of *dDBT* mutant

The increase of mitochondrial ROS often associated with mitochondrial dysfunction (Murphy, 2013). To initially confirm whether abnormal mitochondrial physiology in the *dDBT* mutant brains also change their morphology, we use TEM (transmission electron microscope) technology and observe the mitochondrial morphology in the wild-type (w^{1118}) (Fig. 3A, B) and *dDBT* mutant brain tissue (Fig. 3C, D, E, F). The TEM experiment reveals mitochondria and the loss of mitochondrial inner membrane structure in the *dDBT* mutant brain (Fig. 3C, D, E, F). This result shows that loss of BCAA metabolism leads to abnormal mitochondrial morphology in brain tissues.

In addition to observe the morphology of mitochondria, we also use immunostaining to analyze the range areas of ATP5α (ATP synthase subunit α) protein in larval brains. ATP5α is a catalytic subunit of the mitochondrial ATP synthase complex responsible for the synthesis of ATP (Goldberg et al., 2018) and is mostly used as a mitochondrial marker. The ATP5α signal (green fluorescence) in the *dDBT* mutant brain was lower than that of the wild type

(Fig.4A) and the statistic shows a significantly decreased (Fig.4B), which may represent a decrease in the number of mitochondria and thus may affect ATP production in the *dDBT* mutant brains.

3.4 The amount of ATP was reduced in the *dDBT* mutant brain and showed a low AMPK activity

To determine if there are changes in ATP production in the dDBT mutant brain and whether the accumulation of BCAA leads to the changes in ATP amount, in order to test this point, we use the ATP Bioluminescence assay kit to measure and compare ATP level from wild-type, *dDBT* mutant, and the leucine-treated *dDBT* mutant brains. The results showed that ATP amount in *dDBT* mutant flies decreases to about 60% compared with wild type, and the treatment of leucine made an exacerbated reduction of ATP amount in mutant flies (Fig.5).

The ATP amount reduced in BCAA catabolizing enzyme mutants, and ATP synthesis was regulated by AMPK, an energy sensor of the organism, which will be activated by the increased AMP: ATP ratio, and further maintain the energy homeostasis(Hardie, 2007). Several studies have revealed that AMPK is a therapeutic target for type 2 diabetes (T2D), and the activity is diminished in humans with T2D or obesity (Coughlan et al., 2014). AMPK is a heterotrimeric complex consisting of α-subunit (catalytic), β-subunit (scaffolding), and γ-subunit (regulatory), and is activated by phosphorylation of threonine 172 (T^{172}) on the α-subunit (Carling, 2017; Hawley et al., 1996).

We use western blotting to measure the p-AMPK (phosphorylated AMPK, active AMPK) in the brains of *dDBT* mutants by Anti-Phospho-AMPK (Cell signaling) and found that p-AMPK level in the mutant brains was significantly lower than wild type. The signaling of reduced ATP activates AMPK and promotes ATP synthesis in a normal situation. However, decreased the AMPK activity was found in the brains of *dDBT* mutants (Fig.6). This finding led us to

suggest that the regulation of energy homeostasis by AMPK may play a key role in abnormal BCAA metabolism.

3.6 The treatment with metformin activates AMPK and induces autophagy in the *dDBT* mutant brain

Several studies have shown a higher level of BCAA in patients with T2D, and the increase in BCAA is also associated with insulin resistance or obesity. The dietary BCAA restriction caused healthy changes in obese rats and also reduced postprandial insulin secretion in T2D patients (Karusheva et al., 2019; McGarrah et al., 2020). In addition, the development of diabetes is related to the disruption of energy homeostasis, and AMPK is the main energy sensor, also a therapeutic target of T2D used in clinical treatment. Currently, the widely used first-line drug metformin improves T2D symptoms by activating AMPK (Hardie and Hawley, 2001; Hawley *et al.*, 2002; Villegas et al., 2009; Winder and Hardie, 1999). However, even know that BCAA metabolism is associated with the progression of obesity and diabetes, the role between energy homeostasis and BCAA metabolism is still unknown.

The previous study showed that the treatment of metformin in *dDBT* mutants can significantly improve their development and behavior (Tsai et al., 2020), but the mechanism remains unclear. To further study the mechanism under the abnormal BCAA metabolism *dDBT* mutant for metformin treatment on AMPK activity, I compared the wild type, *dDBT* mutant, and the metformin-treated *dDBT* mutant by western blot for p-AMPK level. In these brain tissues of *dDBT* mutant flies, p-AMPK increased with metformin treatment in the *ex vivo* experiment and it is dependent on the concentration of metformin (Fig. 7).

Therefore, I suggest that metformin may improve development and behavior defects by activating AMPK in the brains of *dDBT* mutants. I then investigated the downstream mechanisms of the regulation of AMPK activity, and what kind

of downstream mechanisms cause the development and behavior defect under abnormal BCAA metabolism.

Previous studies indicate that autophagy is an important mechanism for regulating the energy homeostasis in organisms, and several pieces of evidence revealed that the activated AMPK can induce autophagy (Hardie, 2007). Therefore, I wondered whether the low activity of AMPK reduced autophagy in the brain of *dDBT* mutant and whether metformin can induce autophagy by activating AMPK.

I use western blot to measure Atg8a-II (lipidated Atg8a, the mammalian LC3-II isoform), and observed autophagy in the *dDBT* mutant brain significantly reduced compared to wild-type (Fig. 8), and the *ex vivo* experiment treated *dDBT* mutant brain with metformin showed an increase in Atg8a-II (Fig. 8A), while the oral feeding experiment also showed similar results (Fig. 8B).

3.7 The induction of autophagy in brain tissue improves *dDBT* mutant defects

Therefore, I speculate that AMPK activity is reduced with abnormal BCAA metabolism, resulting in a decrease in autophagy, and the treatment of metformin may induce autophagy by activating AMPK. Previous research has demonstrated that autophagy is related to neural development and function (Fleming and Rubinsztein, 2020), so I consider whether activating autophagy in the *dDBT* mutant could also rescue the defects caused by abnormal BCAA metabolism.

Rapamycin is an immunosuppressive drug that activates autophagy by inhibits mTOR activity (Sarkar et al., 2009). To study the role of autophagy in *dDBT* mutant, I treated mutant larvae with rapamycin and the data shown that the autophagy marker (Atg8a-II) increased in the brain of the *dDBT* mutant with the treatment of rapamycin (Fig. 9A), confirming that rapamycin can induce autophagy in *dDBT* mutant brains. To confirm whether the activated autophagy

in mutant flies could rescue the developmental defects, I divided thirty of the second instar larvae of the *dDBT* mutant into groups and calculated their pupation rate and eclosion rate after the oral feeding experiment with 50μM rapamycin. Compared with wild-type, the pupation rate of *dDBT* mutant flies was lower than wild-type about 18%, and the eclosion rate has declined by about 95%. With the feeding of rapamycin, there was a significant increase in the *dDBT* mutant's pupation/ eclosion rate, the pupation rate compared to un-fed mutant increased by about 10%, and the eclosion rate increased by about 9% (Fig. 9B, C). This result showed that rapamycin treatment can rescue developmental defects.

A previous study pointed out that abnormal BCAA metabolism not only cause developmental defects but also lead to poor mobility in *Drosophila* (Tsai et al., 2020). To see whether feeding rapamycin also improved *dDBT* mutant behavior defects, I performed the locomotion assay and found that the crawling path of the *dDBT* mutant compared to wild-type showed shorter and twisting lines, but the rapamycin-treated mutant showed a more similar path to wild-type (Fig. 10A). Further analyzing their crawling length and speed, the results showed that the crawling length and speed of *dDBT* mutant larvae were significantly lower than wild-type, but after feeding rapamycin, the poor mobility of mutant has been significantly improved (Fig. 10B, C). This result showed that the treatment with rapamycin not only rescues the developmental defects but also improved mobility in the abnormal BCAA metabolism flies.

As the study above, I suggest that rapamycin can induce autophagy in the brain of the *dDBT* mutant, and it can also be observed that after feeding rapamycin, the development and mobility of mutant larvae are significantly improved, indicating that autophagy may play a role in those defects causing by abnormal BCAA metabolism.

To further demonstrate the role of autophagy, I used the GAL4-UAS system to reduce the expression of Atg1 (the mammalian ULK1 isoform, an upstream kinase of autophagy) in the brains of *dDBT* mutants. Blocking autophagy and

treating *dDBT* mutant with rapamycin at the same time, it was found that mutant larvae could not improve their crawling length and speed (Fig. 11B, C), and their crawling path also remains as untreated mutants (Fig. 11A). Indicating that the activation of autophagy is the main reason for rapamycin to rescue the behavior defects. This evidence suggests that the reduction of autophagy in *dDBT* mutant is responsible for its neuronal defects.

3.8 The regulation of AMPK in BCAA-catabolizing enzyme mutant

It is determined that AMPK-regulated autophagy affects the development and mobility of *dDBT* mutant flies, but the reason is still unknown why the AMPK activity is inhibited under abnormal BCAA metabolism. To confirm that the AMPK activity is regulated by high concentrations of BCAA, I measured the AMPK activity in wild-type and *dDBT* mutant brain tissue by *ex vivo* treating with 10mM Leucine and found that p-AMPK in mutant brains is inhibited with leucine treatment (Fig. 12A). This result is not observed in the wild type (Fig. 12B), indicating that in the model with abnormal BCAA metabolism, the accumulation of leucine causing the loss of AMPK activity, while in a normal situation that could metabolize BCAA, leucine treatment does not cause the loss of AMPK activity.

To further explore the mechanism of how Leucine inhibits AMPK, and as discussed above, leucine also induces the ROS stress in *dDBT* mutant's brain (Fig.1), thus I first discuss whether ROS accumulation triggered by abnormal BCAA metabolism could regulate AMPK activity. Brain tissues of wild-type and *dDBT* mutant were *ex vivo* treated in 10 μM H_2O_2 to simulate an oxidative stress environment. It is found that H_2O_2 treatment significantly inhibited the AMPK activity in the brain of *dDBT* mutant, while the AMPK activity in the wild-type brain tissue was also inhibited by H_2O_2 treatment under the same conditions (Fig. 13).

To find out the reason why AMPK in *dDBT* mutants is inhibited by ROS, I screened the upstream factors of AMPK. The regulation of AMPK is by Ca2+- and Calmodulin-Dependent Protein Kinases, Insulin-Activated Kinase Akt or liver kinase B1 (LKB1) in addition to the AMP/ATP ratio (Hardie and Ashford, 2014), but neither of them plays a main role in *dDBT* mutant to regulate AMPK (data not shown). Apart from the regulators shown above, another research reveals that TOR also regulates AMPK. In previous studies, it was found that AMPK inhibits TOR signaling, and TOR also inhibits the activity of AMPK(Jo et al., 2019).

To confirm that AMPK activity in the *dDBT* mutant is regulated by TOR, I used an *ex vivo* experiment and soaked the *dDBT* mutant larval brains in 50μM rapamycin solution for 20 hours. And the western blot experiment showed that AMPK activity in the brains of *dDBT* mutants was activated by the reduction of phospho-S6K (TOR signaling), which led us to speculate that the loss of AMPK activity in the brain of BCAA catabolizing enzyme mutants was caused by excessive activation of TOR (Fig. 14).

4. Discussion

In this paper, the *Drosophila* with abnormal metabolism of BCAA ($dDBT^{\Delta}$) established by our lab had been used for the experiments, and we found that metformin may induce autophagy by activating AMPK, thereby rescuing the defects caused by abnormal metabolism of BCAA. Inducing autophagy by feeding rapamycin can also increase the pupation rate and eclosion rate of the *dDBT* mutant, moreover, promoting the crawling length and speed, and making its crawling path smoother. The treatment of rapamycin significantly improved defects in *Drosophila* with abnormal BCAA metabolism. According to the result above, I also confirmed that rapamycin improved the mobility of *dDBT* mutant larvae by promoting autophagy rather than through other pathways. In addition to the downstream mechanism of AMPK regulation, I also found that the abnormal accumulation of Leucine is the reason for the loss of AMPK activity, and through experiments, I speculated that the ROS stress caused by the accumulation of BCAA activates the TOR protein, thereby inhibiting the activity of AMPK.

In summary, the accumulation of BCAA in the brain of *Drosophila* with abnormal BCAA metabolism induced mitochondrial ROS. The ROS stress activates TOR, and further inhibits the AMPK activity, while metformin can activate AMPK, induce downstream autophagy, and improve the neuronal defects caused by abnormal BCAA metabolism. Induce autophagy by drugs has a similar effect on improving neuronal defects. (Fig. 15).

4.1 TOR signaling may play a critical role in BCAA-catabolizing enzyme mutant

However, there is still some unknown mechanism in this study, such as how leucines induce mitochondrial ROS, and the largest source of mitochondrial ROS production is generated by respiration, which may be a breakthrough for this

question. There are several interesting findings in the previous researches. It is proposed that leucine increases mitochondrial respiration by activating mTORC1 in Swiss mice (Brunetta et al., 2019). As was mentioned in the previous description, the role of TOR in abnormal BCAA metabolism flies may not only regulate by AMPK or ROS stress and perhaps other mechanisms between leucine and TOR.

It was also found in previous research that the increase of leucine can activate mTORC1 through Leucyl-tRNA Synthetase (LRS), and LRS is a key mediator for amino acid signaling (Han et al., 2012). This research improves the proposed model in this study and also suggests the importance of TOR in abnormal BCAA metabolism. Integrate the studies above, the accumulation of BCAA (Leucine) may activate TOR through LRS and cause a high respiratory rate, thereby increasing mitochondrial ROS, leading to the loss of AMPK activity and the reduction of autophagy.

4.2 Amino acid transporters on the blood-brain barrier may be a new aspect of MSUD research

Nevertheless, although increased AMPK activity in the mutant brain was found to improve defects in development and behavior, the activation of AMPK, inhibition of TOR activity, or reduction of mitochondrial ROS did not rescue the mutant's pupa-lethal, these showed that the mechanism caused by abnormal metabolism of BCAA is complicated, so looking for other mechanisms is necessary to help us understand the reason for this mutant's death.

To explore the pathogenesis of maple syrup in depth, it is necessary to discuss the disease from different aspects. In past studies, the apoptosis in the brains of *dDBT* mutant flies increased (Tsai *et al.*, 2020), but the mechanism remains unclear, and investigating whether BCAAs will induce apoptosis in BCAA-catabolizing enzyme mutants may be an interesting question. There is evidence

that BCAA supplementation induces apoptosis in hepatic tumor cells (Hagiwara et al., 2012), which also increases the possibility of BCAA-induced apoptosis. Therefore, lead to further consideration of a large amount of BCAAs in body fluids may pass through the blood brain barrier (BBB) via transporters into the brain. The L-Type Amino Acid Transporter 1 (LAT1) or the Na^+-dependent transporter system $B^{0,+}$ ($ATB^{0,+}$) on the BBB are transporters of amino acids such as BCAAs (Singh and Ecker, 2018; Van Winkle et al., 2006; Zaragoza, 2020). In the future studies, I will try to observe whether the regulation of the LAT1 and $ATB^{0,+}$ transporters affects the apoptosis in mutant brain tissues in fly, and maybe the study can become a new target for the treatment of abnormal BCAA metabolism.

4.3 The contribution of this research

This study explored the relationship between AMPK and abnormal catabolism of BCAA, and abnormal catabolism of BCAA can cause maple syrup urine disease in humans. The animal model for MSUD include mouse and zebrafish, etc., and it is known that the treatment of metformin could also reduce the metabolite in MSUD mouse (D et al., 2016), but it still few studies on this rare disease and its pathogenesis is mostly unknown. Even though only a few people will get this disease, there is still someone plagued by it, therefore, it is necessary to further research and keep the focus on it. In addition, there is currently no medicine for maple syrup urine disease. The animal model used in this experiment is *Drosophila*, which has the advantages of a short lifespan, rapid generation time, low cost, and nearly 75% of human disease genes have a homolog in it. *Drosophila* is a very suitable drug screening tool, and as was mentioned in the previous chapter, the treatment with metformin improved the *dDBT* mutant's development/ behavior defects. Suggesting that metformin could be further used

in clinical research, and has the greater potential to relieve symptoms in maple syrup urine patients.

In addition to AMPK, the role of autophagy and TOR signaling is also discussed in this paper, the results may become another direction for the development of novel drugs for maple syrup urine disease, and maybe these mechanisms can also explain other diseases related to abnormal BCAA metabolism. Therefore, I suppose that the research on the physiological mechanism of the abnormal BCAA metabolism model will bring progress to the basic research of several diseases.

IV. References

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V. Figure

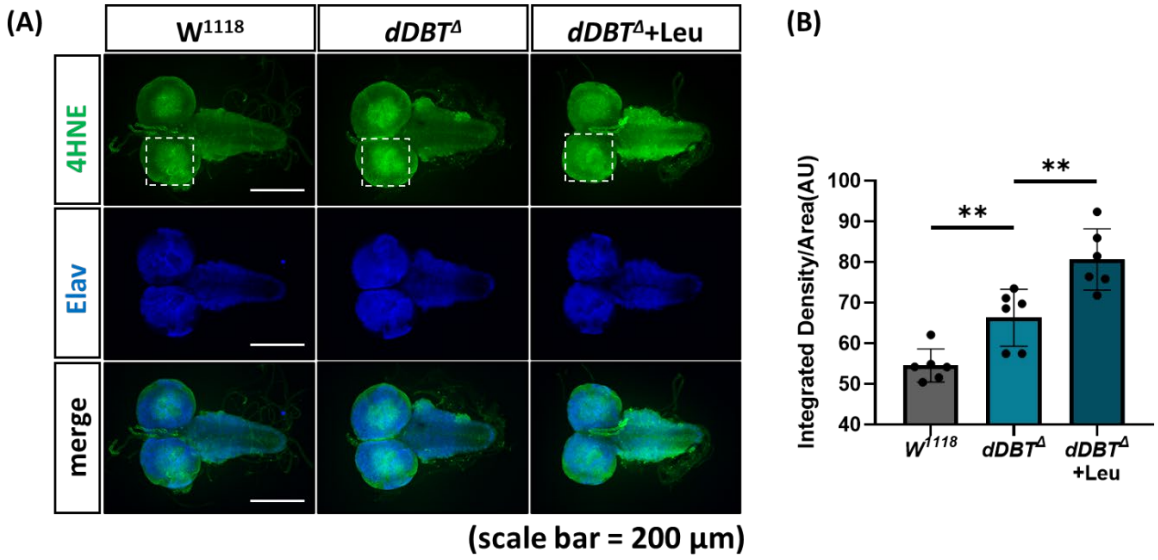

Figure 1. High level of leucine causes oxidative stress in *dDBT* mutants.

(A) The immunostaining experiment shows the 4HNE (green fluorescence) signaling in 3rd instar larval brain, with anti-Elav antibody (blue fluorescence).

(B) Wild type (w^{1118}), *dDBT* mutant and mutant feeding with a leucine-rich diet (50mM). The bar chart on the right side shows the statistics. n=6. **P<0.01.

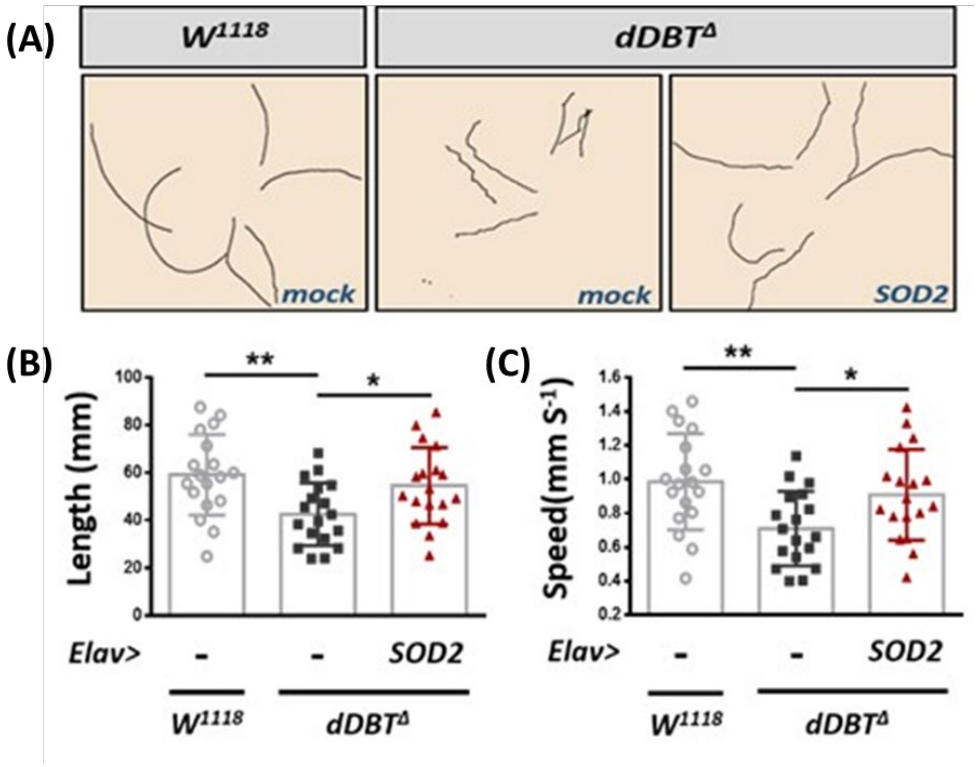

Figure 2. Depletion of mitochondrial ROS by expression of SOD2 in neuron in brain tissue improves larval locomotion defect.

(A) Crawling path of wild-type(w^{1118}), *dDBT* mutant ($dDBT^{\Delta}$), and using the GAL4-UAS system to overexpress *SOD2* in $dDBT^{\Delta}$ brains ($dDBT^{\Delta}$; *elav* > UAS-*SOD2*).

(B) (C) The statistics bar charts show differences in crawling length and speed among w^{1118}, $dDBT^{\Delta}$, and $dDBT^{\Delta}$; *elav* > UAS-*SOD2*. n=6. *P<0.05, **P<0.01.

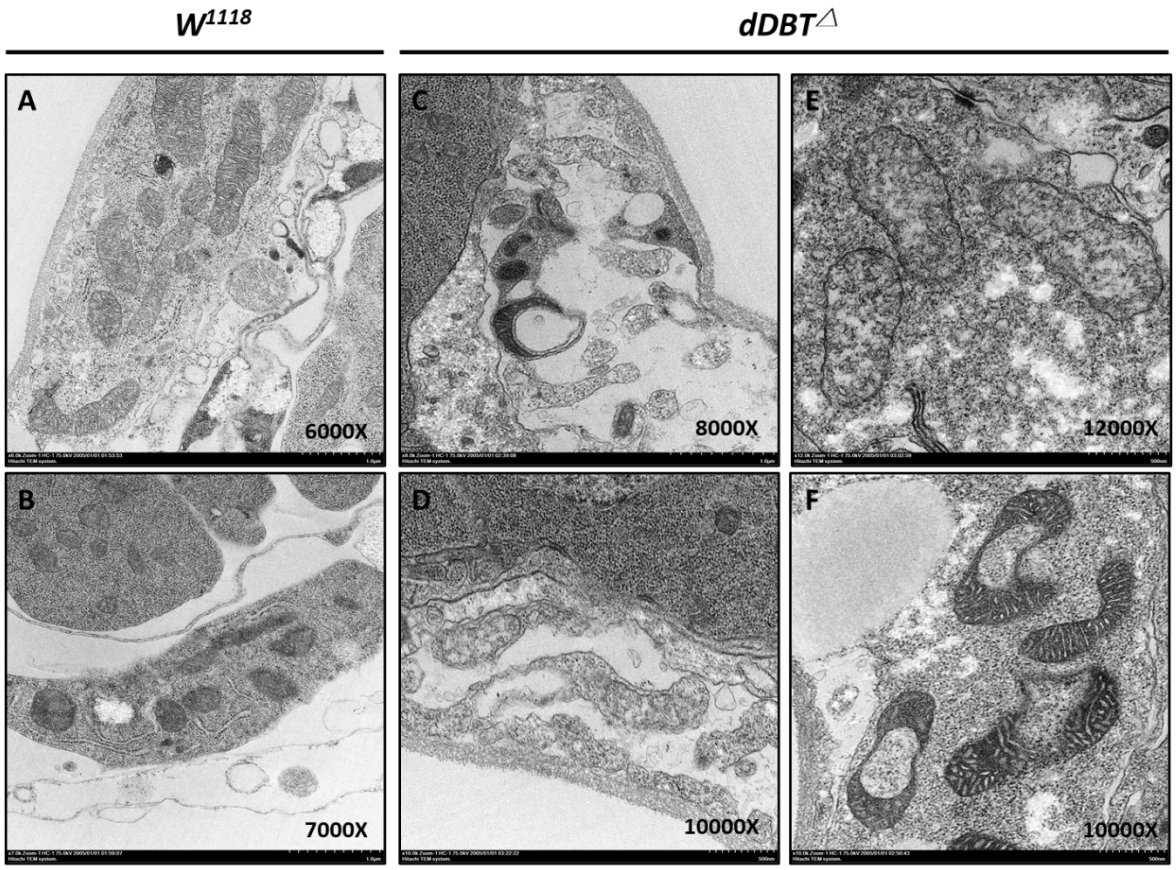

Figure 3. Abnormal mitochondrial morphology in *dDBT* (*dDBT*$^{\Delta}$) mutant brain tissue.

TEM (transmission electron microscope) photos showed abnormal mitochondrial morphology in *dDBT* mutant larval brain tissue compared with wild-type(w^{1118}), the area focuses on the larval central brain at different magnifications.

(A)(B) Mitochondrial in wild-type larval brain shows a healthy morphology.

(C)(D)(E)(F) Mitochondrial morphologies in *dDBT* mutant larval brain are broken and the inner membrane structure had a loss.

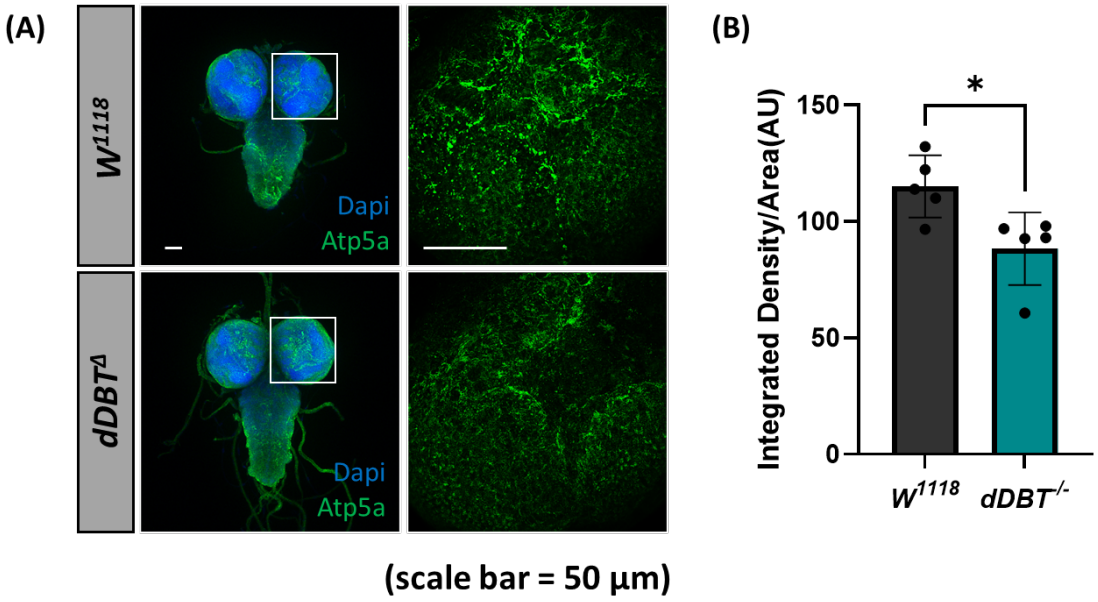

Figure 4. Mitochondria range area decreases in *dDBT* mutant larval brains.

(A) Using immunostaining with anti-ATP5α to observe the mitochondrial range area in wild-type (w^{1118}) and *dDBT* mutant larval brain. The images show Dapi (blue) and ATP5α (green).

(B) Analyze the ATP5α signaling with ImageJ and shows statistic with bar charts for both of them. n=5. *P<0.05.

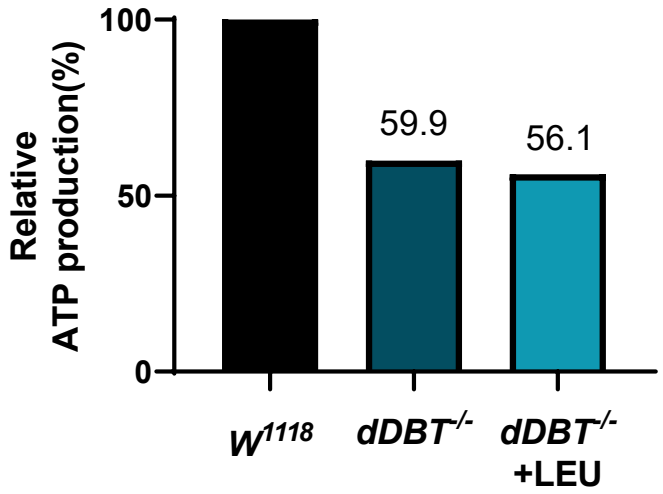

Figure 5. Loss of *dDBT* reduces ATP production in Drosophila larval brain tissue

Using ATP Bioluminescence assay kit (Roche Diagnostics), ATP level for wild-type (w^{1118}), *dDBT* mutant, and the leucine-treated *dDBT* mutant larval brain.

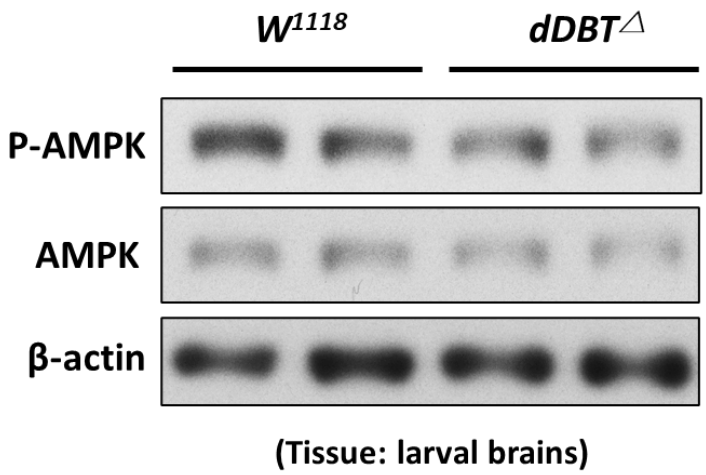

Figure 6. AMPK activity reduced in *dDBT* mutant larval brain

Western blot for the p-AMPK (phosphorylated AMPK, active AMPK) in wild-type (W^{1118}) and *dDBT* mutant, and compare to total AMPK. The total AMPK and β-actin as an internal control. Each sample contains 20 larval brains.

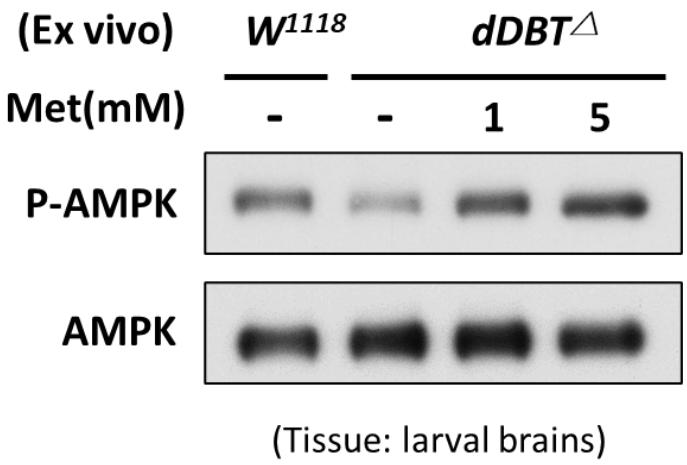

Figure 7. The treatment with metformin improves AMPK activity in the *dDBT* mutant larval brain.

Western blot observes the p-AMPK (phosphorylated AMPK, active AMPK) in wild-type (W^{1118}), *dDBT* mutant, and the metformin-treated *dDBT* mutant with the *ex vivo* experiments. The total AMPK as an internal control. Each sample contains 20 larval brains.

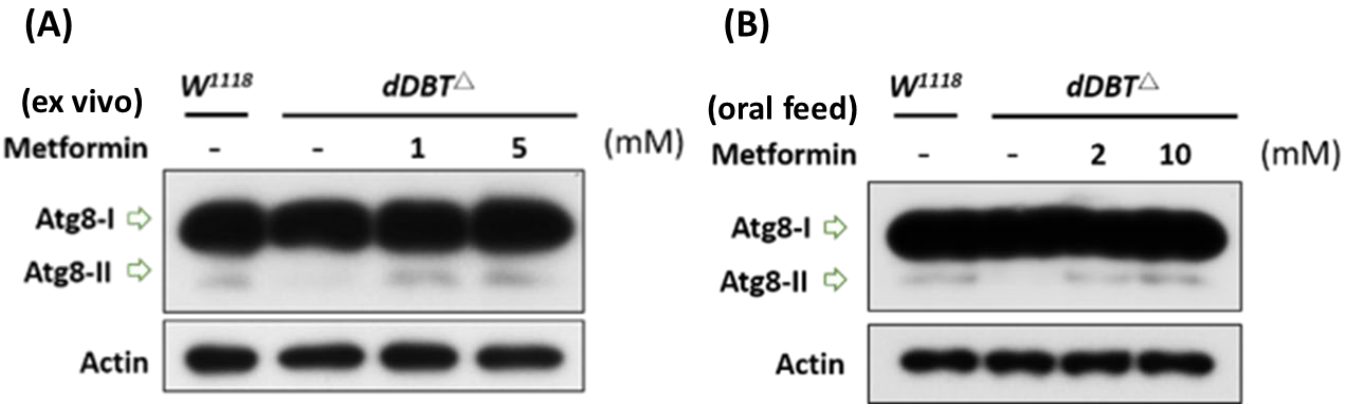

Figure 8. The treatment with metformin improves autophagy in *dDBT* mutant larval brains.

(A) Western blot of Atg8a-II (lipidated Atg8a, the mammalian LC3-II isoform) in wild-type, *dDBT* mutant, and *ex vivo* metformin-treated *dDBT* mutant, and use β-actin as an internal control, each sample contains 20 larval brains.

(B) Western blot of Atg8a-II in wild-type, *dDBT* mutant, and *dDBT* mutant oral feeding with metformin (1, 5mM), and β-actin as an internal control, each sample contains 20 larval brains.

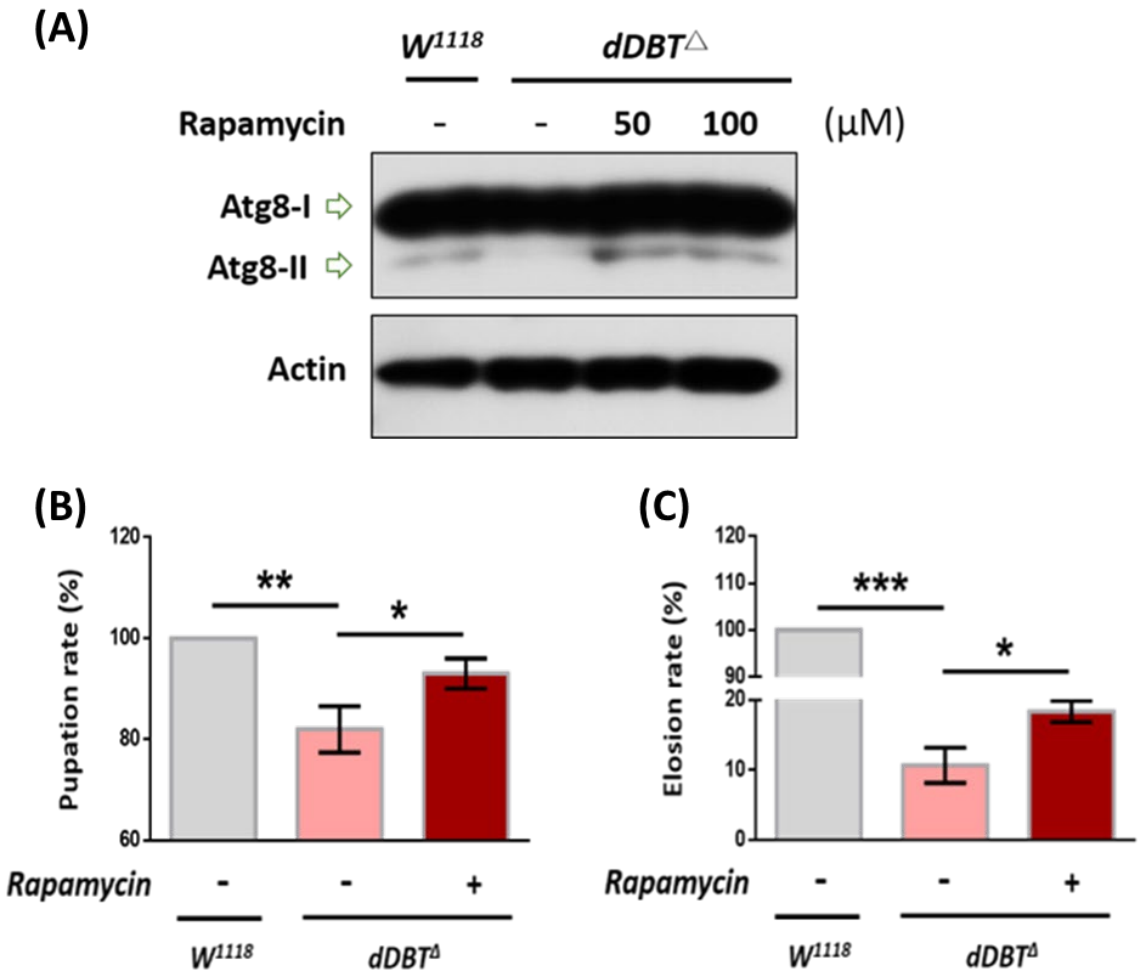

Figure 9. Rapamycin induces autophagy in *dDBT* mutant larval brains and improves its development defect.

(A) Western blot of lipidated Atg8a in wild-type, *dDBT* mutant, and rapamycin-feeding *dDBT* mutant, use β-actin as an internal control, and each sample contains 20 larval brains.

(B)(C) The pupation and eclosion rate of wild-type, *dDBT* mutant, and rapamycin-feeding *dDBT* mutant in percentage. Each sample contains 30 larvae, and made 3 bio-repeat. Three biological replicates were performed. *P<0.05, **P<0.01.

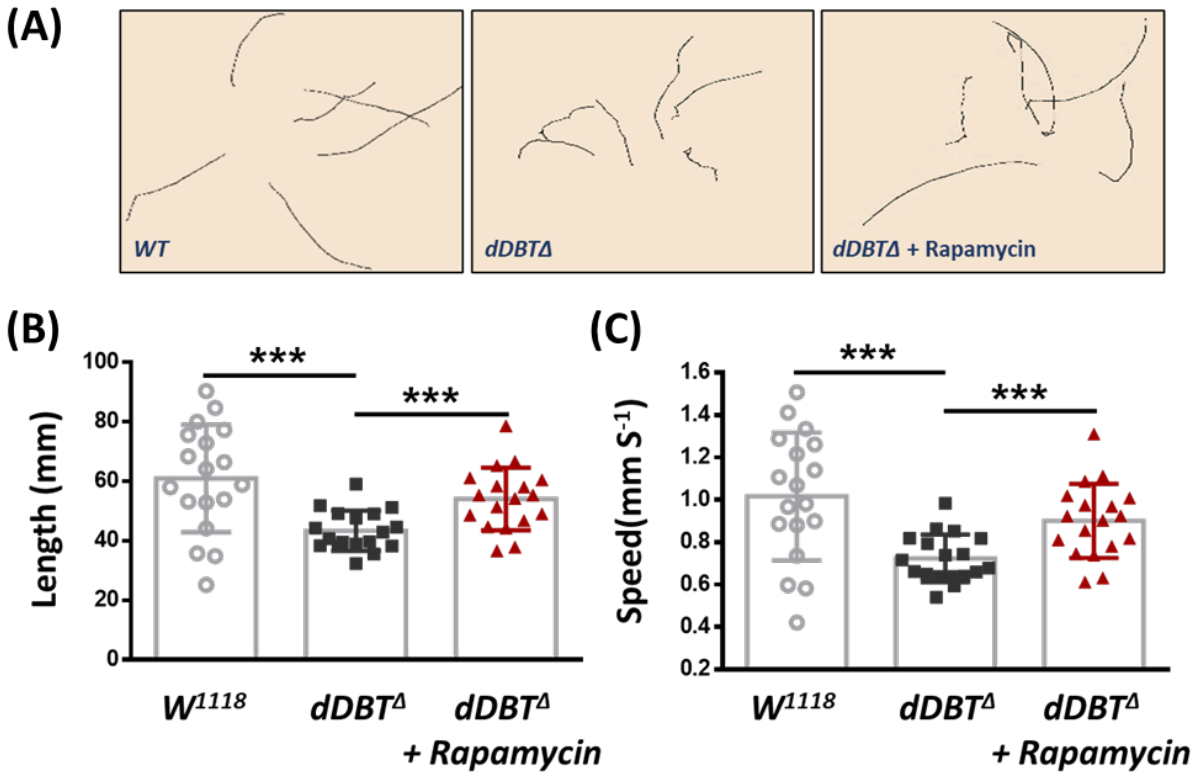

Figure 10. The feeding of rapamycin improves behavior defects in *dDBT* mutant larvae.

(A) The 3rd instar larval crawling path of wild type (W^{1118}) with control food, *dDBT* mutant with control food, and *dDBT* mutant with 50uM rapamycin food, and the experiment was performed after one day of feeding.

(B)(C) The crawling length and speed of 3rd instar larvae analyze with ImageJ and converted to statistics bar charts. ***P<0.001.

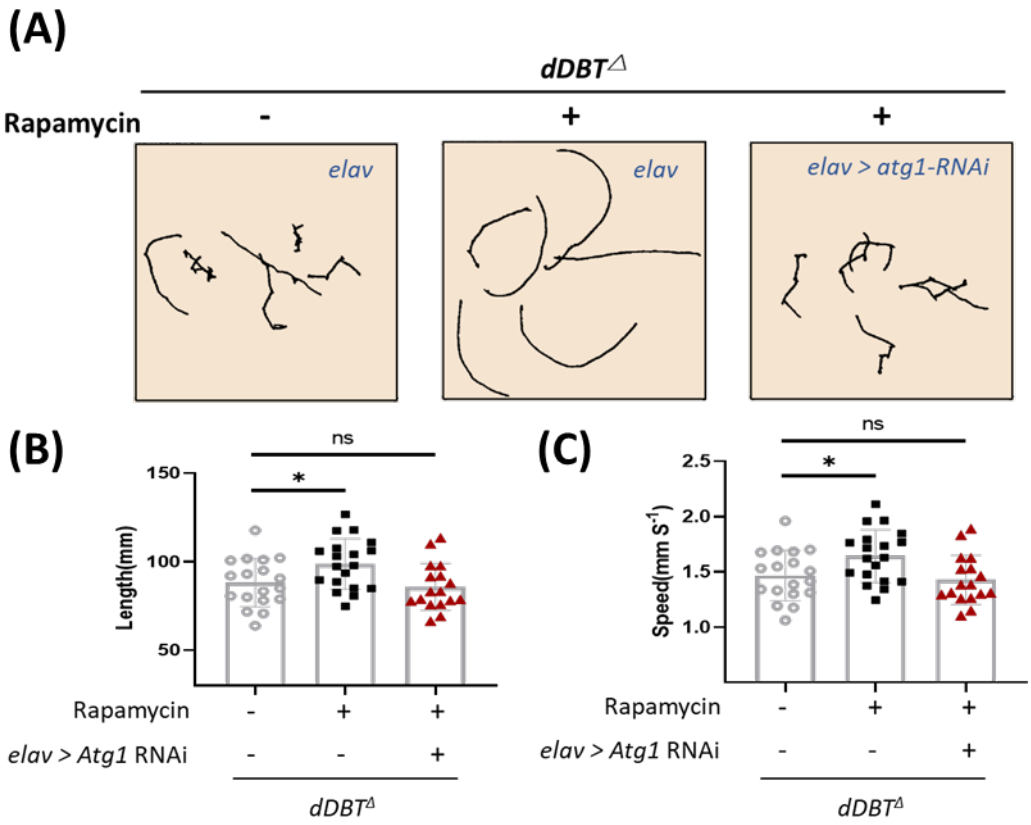

Figure 11. The process of autophagy regulates mobility in *dDBT* mutants.

(A) The 3rd instar larval crawling path of *dDBT* mutant (*dDBT*$^{\Delta}$) with control food, *dDBT*$^{\Delta}$ with rapamycin(50μM) treatment, and using the GAL4-UAS system to knock down Atg1 in *dDBT*$^{\Delta}$ brains and feed larvae with rapamycin (*dDBT*$^{\Delta}$; *elav* > UAS-*Atg1* RNAi).

(B)(C) The crawling length and speed of 3rd instar larvae analyze with ImageJ and converted to statistics bar charts. *P<0.05.

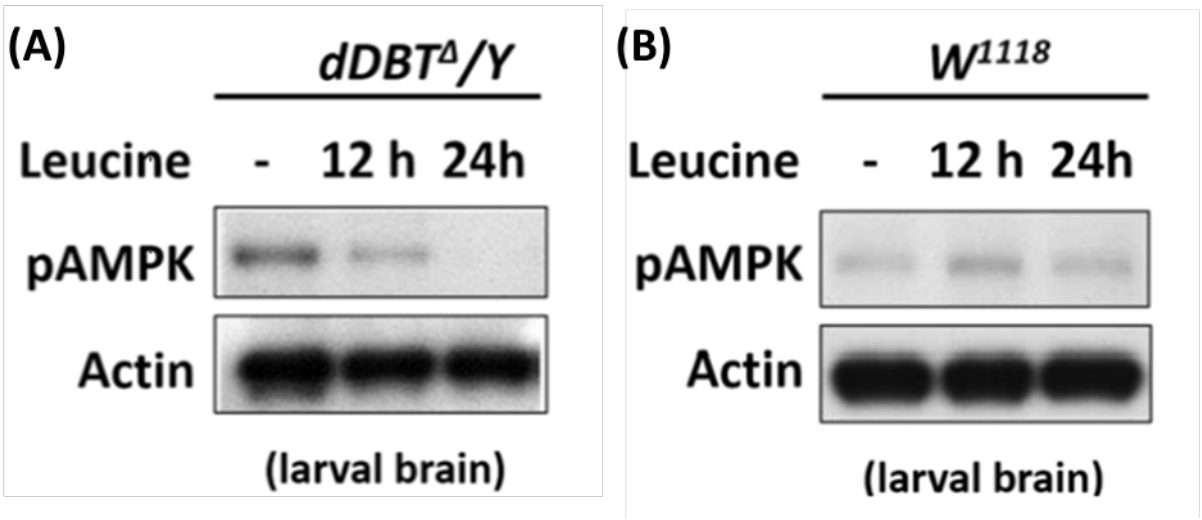

Figure 12. Leucine inhibits AMPK activity in the *dDBT* mutant larval brain.
(A) Western blot observes the p-AMPK in *dDBT* mutant, *ex vivo* treating with 2mM leucine for 0,12,24 hours. The β-actin as an internal control, and each sample contains 20 larval brains.
(B) p-AMPK in wild-type (w^{1118}), *ex vivo* treating with 2mM leucine for 0,12,24 hours. The β-actin as an internal control, and each sample contains 20 larval brains.

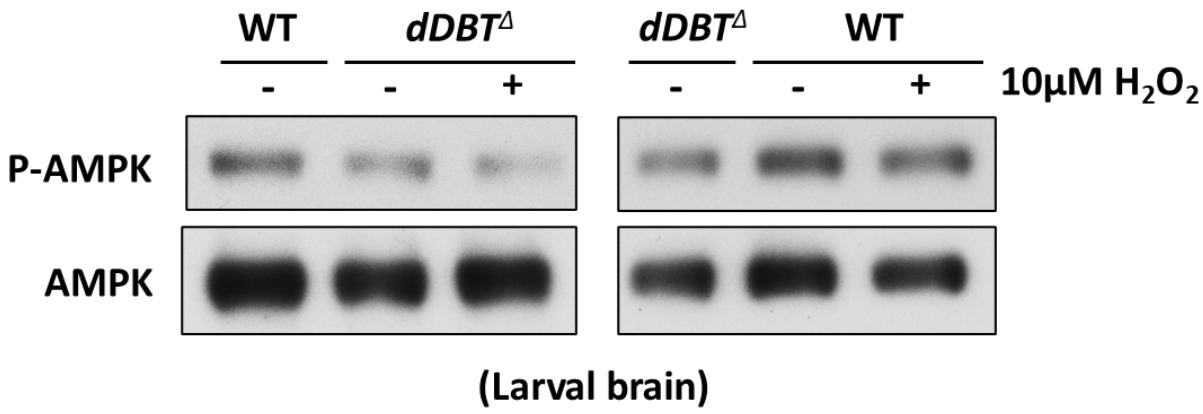

Figure 13. ROS inhibits AMPK activity in the *dDBT* mutant larval brain. Western blot observes the p-AMPK in wild-type (w^{1118}) and *dDBT* mutant and treats them with 10μM H_2O_2 (ex vivo). The total AMPK as an internal control, and each sample contains 20 larval brains.

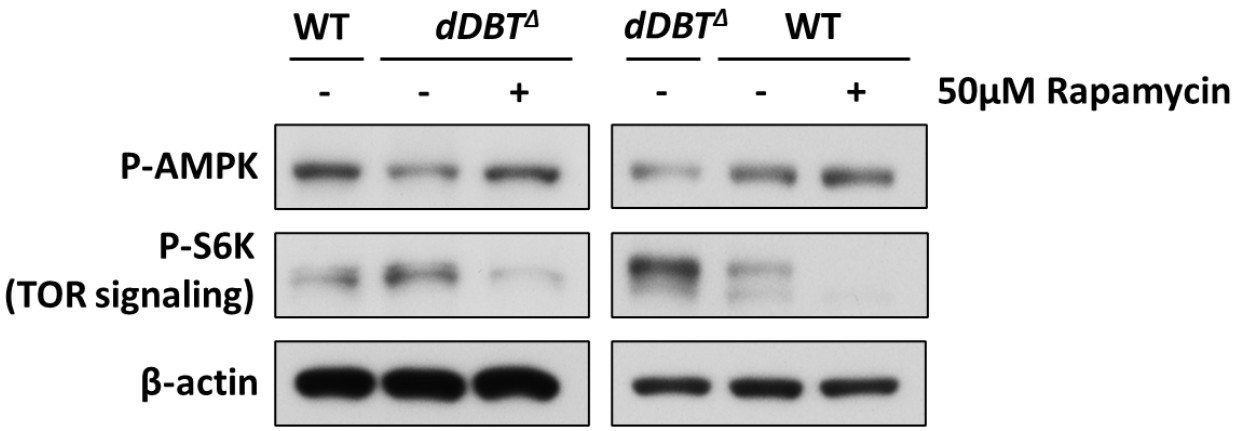

Figure 14. Rapamycin inhibits TOR signaling to activate AMPK in the *dDBT* mutant larval brain.

Western blot observes the p-AMPK and phospho-S6K (P-S6K, TOR signaling) in wild-type (w^{1118}), *dDBT* mutant and treat *dDBT* mutant with 50μM rapamycin *ex vivo* for 20 hours. The β-actin as an internal control, and each sample contains 20 larval brains.

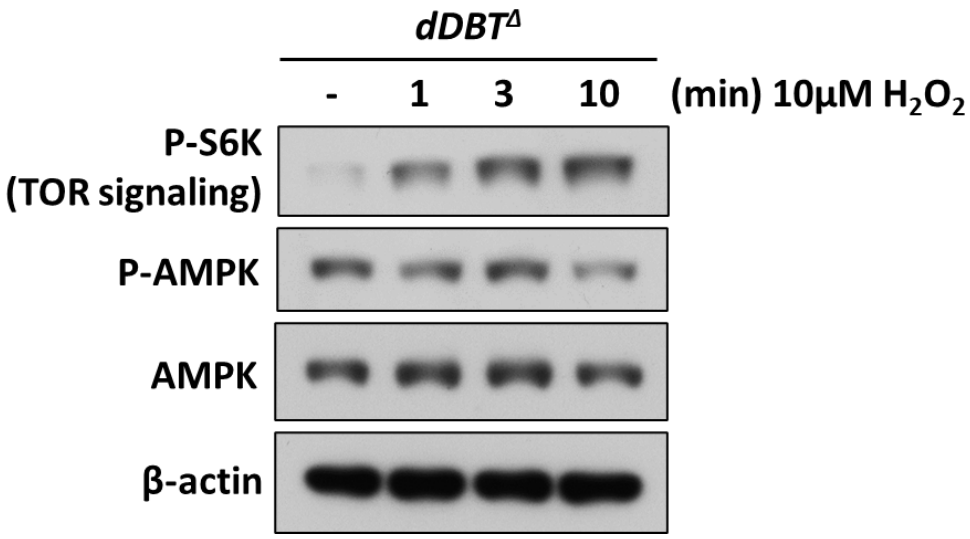

Figure 15. ROS stress(H_2O_2) induces P-S6K (TOR signaling) in *dDBT* mutant brains

Western blot of p-AMPK and phospho-S6K (P-S6K, TOR signaling) in *dDBT* mutant and treat it with 10μM H_2O_2 *ex vivo* for 0, 1, 3, and 10 mins. The total AMPK and β-actin as an internal control, and each sample contains 20 larval brains.

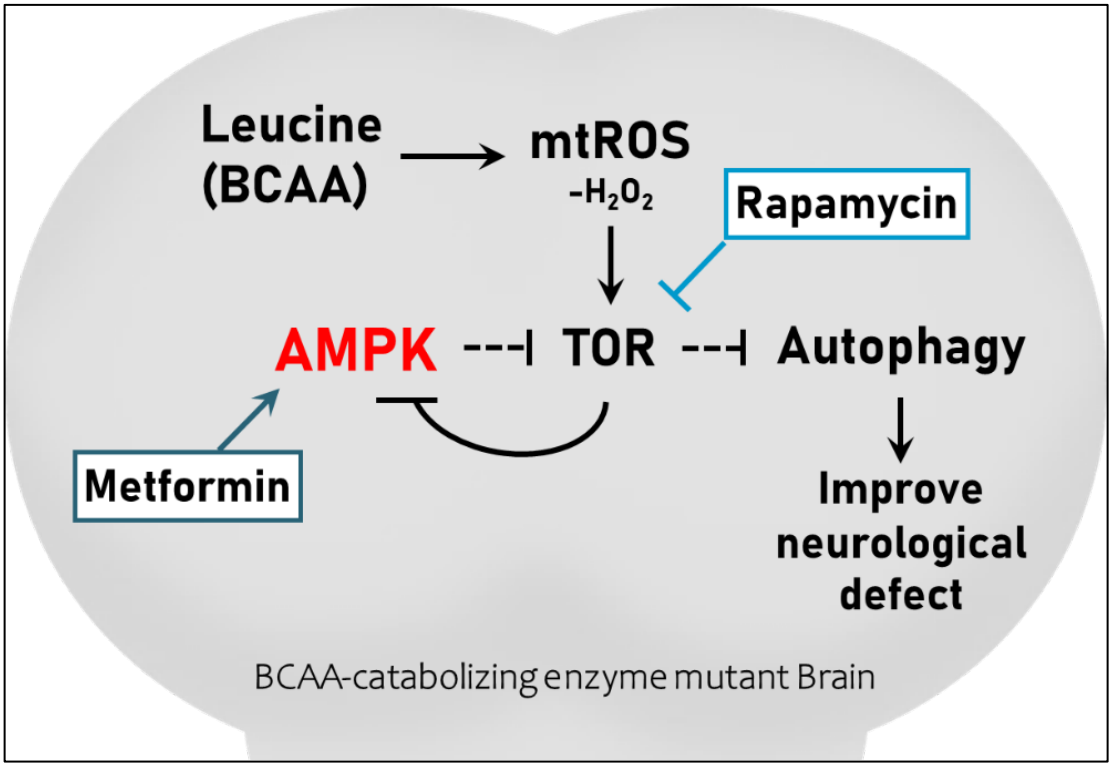

Figure 16. Model of the mechanism in the brain of *Drosophila* with abnormal BCAA metabolism.

The accumulation of BCAAs in the brain induces mitochondrial ROS. The ROS (H_2O_2) stress activates TOR and further inhibits AMPK activity. The low activity of AMPK causes autophagy decreases and leads to neuronal defects in BCAA-catabolizing enzyme mutants.

VI. 個人資料

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