Ketone Body Rescued Seizure Behavior Of LRP1 Deficiency By Modulating Glutamate Transport

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Research Article

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Abstract

LRP1, the low-density lipoprotein receptor 1, would be a novel candidate epilepsy gene according to our bioinformatic results and the animal study. In this study, we explored the role of LRP1 in Epilepsy and whether Beta-hydroxybutyrate, the principal ketone body of the ketogenic diet can treat epilepsy caused by LRP1 deficiency. UAS/GAL4 system was used to establish different genotype models. Flies were given Standard, High-sucrose, and ketone body food randomly. The bang-sensitive test was performed on flies and seizure-like behavior was assessed. Morphologic alteration of LRP1 defect in the brain was detected under GPF expression flies. We established global, astrocytic, and neuronal LRP1 knockdown flies. Whole body and glia LRP1 defect flies had a higher seizure rate compared to the control group in the behavior test. Ketone body decreased the seizure rate in behavior test in all LRP1 defect flies, compared to Standard and High sucrose diet. In morphologic experiments, we found that LRP1 deficiency caused partial loss of the ellipsoidal body and partial destruction of the fan-shaped body. Overexpression of glutamate transporter gene Eaat1 could mimic the ketone body effect on LRP1 deficiency flies. This study demonstrated that LRP1 defect globally or in astrocytes or neurons could induce epilepsy. The ketone body efficaciously rescued epilepsy caused by LRP1 knockdown. The results support screening for LRP1 mutations as discriminating conduct for individuals who require clinical attention and further clarify the mechanism of the ketogenic diet in Epilepsy, which could help Epilepsy patients making a precise treatment case by case.

1. Introduction

Epilepsy is a common neurological disorder affecting all age groups and is related to seizures, which are abnormal discharge of brain neurons. It is one of the transient dysfunctions of the brain that can occur in sudden and can be recurring [1]. Recent studies have shown that abnormal brain energy metabolism is closely related to the onset of epilepsy [2]. Myoclonic epilepsy with ragged-red fibers (MERRF), affecting complex I of the electron transport chain, is one of the examples concerning mitochondrial DNA (mtDNA) mutations that result in epileptic phenotypes [3]. Glucose is the predominant energy source in the brain [4]. The imbalance of glucose can lead to seizures [2, 5-7]. However, the mechanisms underlying glucose regulation and altered neuronal excitability remain poorly understood [8].

LRP1, the low-density lipoprotein receptor 1, has more than 40 different ligands and participates in many physiological processes. In the central nervous system (CNS), LRP1 is ubiquitously expressed and serves as a critical transport receptor as well as a modulator of several distinct signaling pathways in neurons [9], astrocytes[10], and microglia. It has been demonstrated that LRP1 is involved in nerve excitability. In neurons, the deletion of LRP1 caused hyperactivity, dystonia [9]. In astrocytes, early astrocyte dysfunction caused by LRP1 deletion is an important factor leading to abnormal excitability and morphologic changes of epileptic seizures during brain development in mice [11]. In embryonic cortical radial glia stem cells located in the telencephalon, the deletion of LRP1 caused a severe epileptic phenotype [9]. However, the mechanism of LRP1 induced epilepsy remains unknown.

The ketogenic diet (KD) is a high-fat, calorie-restricted diet used to treat childhood epilepsies that do not respond to available drugs. Despite its clinical use for nearly 100 years, how the KD controls seizures remains unknown [12]. KD was used to regulate energy metabolism in order to manage epilepsy [13], beta-hydroxybutyrate is the major bioactive metabolite [14, 15]. In this study, we are aimed to explore the role of LRP1 in Epilepsy and whether KD can treat epilepsy caused by LRP1 knockdown by using beta-hydroxybutyrate. Our results highlighted that the knockdown of LRP1 can cause epilepsy and abnormal brain structure, and we also found that LRP1 induced epilepsy is associated with glutamate imbalance and KD can rescue LRP1 induced epilepsy.

2. Methods And Materials

2.1 Data sources

To explore novel epilepsy-related genes by bio-information program., we downloaded the gene expression profiles acquired by high-throughput sequencing of GSE139914 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139914) and GSE134697 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134697) from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) after searching for keywords related to epilepsy. Then the gene expression matrix data of the epilepsy group and the control group were imported into GraphPad Prism (version 7.0.0) for t-tests. $P < 0.05$ was used to indicate a significant difference.

2.2 Fly stocks

Drosophila melanogaster (Fruit fly) is a classical animal model for epilepsy studies [16]. It had been used to investigate the pathogenic genes for epilepsy and other neuro-development disorders [17, 18]. Recently, we also used *Drosophila* as a model to discover the novel epilepsy gene-UNC13B [19]. Thus, we used *Drosophila* as a model here to study the role of LRP1 in epilepsy.

The flies were fed standard cornmeal and maintained in the incubator at 25℃ and 60–70% humidity on a 12:12-h light/dark cycle. *UAS-LRP1-RNAi* (THU2483/FBst0029548), *UAS-Scn1a-RNAi* (para-RNAi, positive control), and *UAS-Eaat1-RNAi* flies were donated by Tsing Hua Fly Center (Tsinghua University, Beijing, China). The double balancer line was purchased from Bloomington Fly Stock Center (Bloomington, IN, USA). The Gal4 driver line *tub-Gal4*, *elav-Gal4*, *repo-Gal4* were a gift from Prof. LIU Ji-Yong (Guangzhou Medical University, Guangzhou, China), and the *UAS-mCD8::GFP* line was a gift from Prof. KE Ya (The Chinese University of Hong Kong, Hong Kong). Canton-S was used as the WT line in this study. The *tub-Gal4*, *elav-Gal4*, *repo-Gal4* line was crossed with *LRP1-RNAi* to establish global *LRP1* knockdown flies (*tub-Gal4>LRP1-RNAi*), glial LRP1 knockdown flies (*repo-Gal4>LRP1-RNAi*), and neuronal LRP1 knockdown flies (*elva-Gal4>LRP1-RNAi*) [20]. Then, behavioral assessment was conducted.

2.3 Seizure behavior test

Stress sensitivity or bang sensitivity (BS) experiments to assess stress-induced seizures were conducted as originally described by Ganetzky and Wu [21] with some modifications that have been described in

detail previously [22]. The bang-sensitive (BS) test was conducted on flies 3–5 days after eclosion and seizure-like behavior was assessed [16, 23]. Flies were anesthetized with CO_2 and were transferred to new clean food vials one day before testing. About two to five flies were collected in empty plastic fly vials (3-6 flies/vial). Flies were allowed to recover for at least 30 min and mechanically stimulated with a vortex mixer (VWR, Radnor, PA, USA) at maximum speed for 10 second. The time for each fly to recover, which defined as standing, was scored, and a mean value from the scoring was taken for each vial. One vial was calculated as one N number in each group.

2.4 Three types of Diet

Standard medium: 7.12g Cornmeal, 1.68g of yeast powder, 0.56g AGAR, 8.5g sucrose, and 100ml D-D water, was blended and heated to the appropriate temperature (about 100 ℃). After that, 0.47ml propionic acid was added.

High-sucrose medium: 7.12g cornmeal, 1.68g of yeast powder, 0.56g AGAR, 30g sucrose, and 100ml D-D water, was blended and heated and 0.47ml propionic acid was added.

Ketone Body medium: 3mM 0.04g beta-HB was added to the standard medium.

2.5 Morphology

Morphologic alteration of LRP1 defect in the brain was detected using GFP expression flies. The LRP1 knockdown and control flies labeled with membrane GFP, *tub-Gal4>UAS-mCD8::GFP/UAS-Unc13b-RNAi*, and *tub-Gal4>UAS-mCD8::GFP*, were generated by UAS-mCD8::GFP and double balancer flies. The fly brain was dissected, fixed, and permeabilized following the previous description [19]. Samples were observed using a confocal microscope (SP8; Zeiss, Jena, Germany) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6 Statistical analysis

All quantitative data are presented as mean±S.D. The Student's t-test was used to compare 2 independent or paired samples. One-way ANOVA was used to compare multiple samples, and Tukey's posthoc test was used to evaluate differences between two groups. Statistical analyses were performed with GraphPad Prism 7.00 and SPSS 20. The cutoff value for statistical significance is $P<0.05$.

3. Results

3.1 LRP1 Gene was Low Expression in Epilepsy Patients

GSE139914 contained 6 epilepsy samples and 41 controls from the human Brodmann Area 38. Samples of GSE134697 were from Neocortex and consisted of 17 epilepsy samples and 2 controls. With $P <0.05$ as the threshold, we found that LRP1 expression was statistically significantly higher in the Epilepsy group than that in the control group. (Figure 1).

3.2 Global Knockdown of LRP1 Induced Seizure Behavior

To further confirm the role of LRP1 in seizure, we examined BS seizure-like behavior in *tub-Gal4>LRP1-RNAi LRP1* flies. The *tub-Gal4>LRP1-RNAi* flies exhibited the typical seizure-like behavior [24]. About 30.72% of *tub-Gal4>LRP1-RNAi* flies showed obvious seizure-like behavior, which was higher than the rate in *LRP1-RNAi* control flies (30.72%±13.28% [n=10] vs 4.16%±7.20% [n=7]; **P=0.003). The *tub-Gal4>Scn1a-RNAi* positive control flies had a higher rate of seizures than *tub-Gal4>LRP1-RNAi* flies (48.92%±18.85% [n=5] vs 30.72%±13.28% [n=10]; *P=0.05) (Figure. 2B).

3.3 LRP1 defect induced partial loss of ellipsoidal body

We examined possible morphologic changes in LRP1 knockdown flies based on GFP expression. The confocal images showed that LRP1 knockdown did not affect the main brain structures including the ellipsoidal body and fan sharp body (Figure. 3A–C), except for the partial loss of the ellipsoidal body (Figure. 3D) and partial destruction of the fan-shaped body (Figure. 3E, 3F).

3.4 Ketone body can Reduce Seizure-Like Behavior in LRP1 Defect Flies

To further explore the effects of the ketogenic diet and high glucose diet on the incidence of epilepsy in *tub-Gal4>LRP1-RNAi LRP1* knockdown flies and WT flies (*Canton-S*), we performed a bang-sensitive (BS) test to different diet application groups. About 30.72% of *tub-Gal4>LRP1-RNAi* flies feeding Standard food showed obvious seizure-like behavior, which was higher than the rate in *tub-Gal4>LRP1-RNAi* flies feeding Ketone body (beta-hydroxybutyrate, BHB) food (30.72%±13.28% [n=10] vs 2.08%±5.89% [n=8]; P<0.001) (Figure. 2C). The *Canton-s* flies feeding high sucrose food also had a higher rate of seizures than those feeding Standard food (24.44%±10.99% [n=6] vs 3.07%±5.63% [n=7]; P=0.02). However, there is no difference between the two groups of *tub-Gal4>LRP1-RNAi* flies feeding the Standard food and high sucrose food (30.72%±13.28% [n=10] vs 23.94%±16.90% [n=11]; P=0.75) (Figure. 2C).

3.5 Glial LRP1 Defect is Sufficient to Induce Seizure Behavior and sensitive to Ketone body

It is well known that glial LRP1 performs in regulating energy homeostasis, so we speculate that the expression of LRP1 in glia is closely related to epilepsy. Therefore, we established *repo-Gal4>LRP1-RNAi LRP1* flies and elav-*Gal4>LRP1-RNAi* flies. In the same way, we analyzed the BS percentage of the three models. We found that about 17.94% of *repo-Gal4>LRP1-RNAi* flies showed obvious seizure-like behavior, which was higher than the rate in elav-*Gal4>LRP1-RNAi LRP1* knockdown flies (17.94%±13.19% [n=22] vs 4.60%±8.46% [n=21]; ***P=0.001) (Figure. 4A). At the same time, the *tub-Gal4>LRP1-RNAi* flies had a higher rate of seizures than *repo-Gal4>LRP1-RNAi* flies (30.72%±13.28% [n=10] vs 17.94%±13.19% [n=22]; P=0.02). These results indicate that the incidence of epileptic behavior in *Drosophila* by *repo-Gal4>LRP1-RNAi* LRP1 knockdown can be further increased, which further suggests that not only is epilepsy caused by astrocytes closely related to LRP1 but also there are other ways causing epilepsy induced by LRP1

knockdown, suggesting a new research direction for us. To our surprise, contrasting with *tub-Gal4>LRP1-RNAi* flies feeding high sucrose diet previously mentioned, the *elav-Gal4>LRP1-RNAi* flies feeding high sucrose food had a higher rate of seizures than that feeding standard food (30.75%±15.93% [n=5] vs 4.60%±8.46% [n=21]; ****P<0.001), which is worth further discussion. (Figure. 4B). The same as *tub-Gal4>LRP1-RNAi* flies feeding KD, the *repo-Gal4>LRP1-RNAi* flies feeding standard food also had a higher rate of seizures than that feeding BHB (17.94%±13.19% [n=22] vs 3.57%±7.10% [n=14]; **P=0.004). (Figure. 4C).

3.6 Ketone body Rescued LRP1 Defect by Modulating Glutamate transporter

A previous study had shown that LRP1 defect may cause seizure by affecting glutamate transmission. Glutamate transporter is also important to synaptic glutamate transmission. Thus, we hypothesized that loss of function of LRP1 can cause seizure via modulating the glutamate transporters. To investigate this hypothesis, we knock down glutamate transporter, EAAT1, in various tissues. Global (*tub>Eaat1-RNAi*) or glial (*repo>Eaat1-RNAi*) knockdown of EAAT1 could induce seizure behavior (Figure. 5A). Glial of EAAT1 could induce a serious seizure and this seizure could not be inhibited by ketone body (BHB) (39.25% ±18.9%, n=4 v.s. 27.13%±13.16%, n=4, p=0.33). Interestingly, overexpression of EAAT1 in LRP1 knockdown flies could partially rescue (29.69% ± 9.35%, n=9 v.s. 9.06% ± 5.44%, n=7, ***p=0.0001) (Figure. 5B). It suggested that downregulated Eaat1 was the mechanism of LRP1 defect causing seizure and the ketogenic diet could partially rescue it by up-regulating EAAT1.

4. Discussion

Epilepsy is a chronic brain disease that affects approximately 65 million people worldwide [25]. Therefore, it is important to understand the underlying mechanisms for epilepsy, which can improve our management to this disease. In our present study, the bioinformation results indicated that LRP1 would be a potential epilepsy gene. Animal experiments confirmed that LRP1 defects could induce seizures and abnormal brain structure. Additionally, we found that KD is a specific treatment with excellent outcomes, through the modulation of glutamate transporters, for epilepsy induced by LRP1 deficiency.

LRP1 is plays a role in neural excitability. Here we used bioinformatic and fly models to further confirm its important role in Epilepsy. Our flies' data indicated that LRP1 defect could induce seizure behavior and abnormal brain structure, which is consistent with the previous mouse study [26].

It has been reported that LRP1 is closely related to glucose homeostasis. Neuronal LRP1 deficiency impairs insulin signal transduction and downregulates GLUT3 expression in neurons, leading to reduced glucose uptake. [27]. In astrocytes, LRP1 mediates the molecular interaction between insulin-like growth factor 1 receptor (IGF1-R) and GLUT1 [28]. Glucose imbalance leads to seizures, and LRP1 plays an important role in maintaining glucose homeostasis. Thus, we use high sucrose and KD to feed the flies. Our data demonstrated that high sucrose diet can induce epilepsy in wild type flies, consistent with previous studies that high glucose concentrations provoked seizure in the adult rat model [8]. However, to

our surprise, LRP1 deficiency in global cells did not show a higher seizure rate in high sucrose feeding. This suggested the presence of another mechanism that LRP1 deficiency could results in further glucose accumulation, which meant that great seizure susceptibility could not be further aggravated by high sucrose. Further study required to demonstrate this hypothesis.

In this study, we found that LRP1 deficiency can rescue epilepsy in a ketone body-specific manner. The ketone body triggers a systemic shift from glucose metabolism toward the metabolism of fatty acids yielding ketone bodies, such as acetoacetate and BHB as substrates for energy [29]. Ketones, unlike glucose, are not likely deliver an immediate and large amount of energy necessary to initiate or sustain seizure activity [30]. But in our case of LRP1 deficiency, the BHB should not only affected by the glucose metabolism although high sucrose feeding did not significantly affect LRP1 knockdown flies (figure 2C). Instead, BHB can rescue seizure in LRP1 deficiency by modulating synaptic glutamate cycling. Our behavior data showed that overexpression of glutamate transport gene EAAT1 could reduce the seizure rate of LRP1 deficiency flies, and BHB could not decrease seizure rate in EAAT1 knockdown flies. This set of data suggested that glutamate transporter would be one of the targets of KD for epilepsy. Additionally, in a mouse study, LRP1 deficiency result in NMDA receptor decrease [26]. These evidences suggested that KD may rescue epilepsy in LRP1 deficiency by modulating synaptic glutamate cycling.

In summary, we found that LRP1 can be a potential novel epilepsy gene. Screening for LRP1 mutations can identify individuals who require clinical attention for possibly frequent daily seizures. And we firstly found that BHB showed a rescue effect in the LRP1 deficiency model by modulating glutamate transport, which may help to understand the KD mechanism and establish precise treatment toward epilepsy case by case.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Jin-Ming Zhang and Jing-Da Qiao designed this study, Ya-Ping Li performed the bioinformatic experiment, all the authors performed the animal experiments and wrote the manuscript.

Data availability

Raw data were generated at Institute of Neuroscience, The Second Affiliated Hospital of Guangzhou Medical University. Derived data supporting the findings of this study are available from the corresponding author on request.

Ethics approval

No ethical approval is required in this study.

Consent to participate

Not applicable

Consent to publish

Not applicable

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Figures

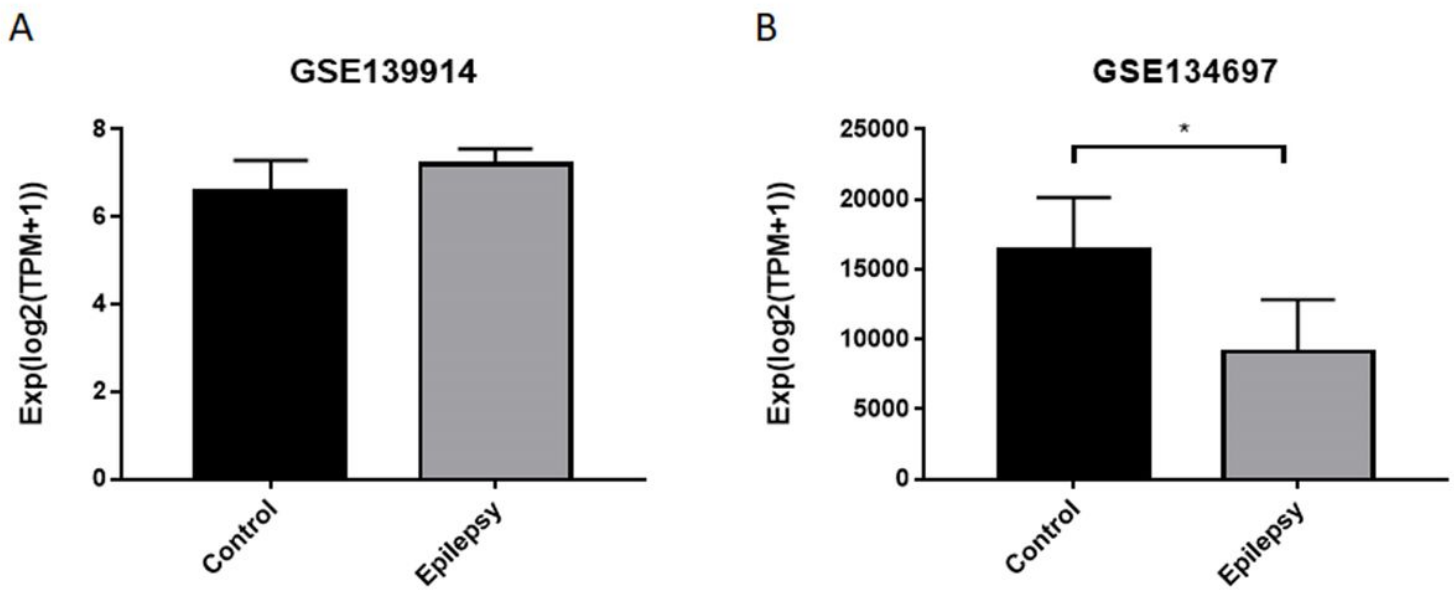

Figure 1

LRP1 expression in Epilepsy patient. (A) There is no different between normal person (Control) and epilepsy patients in GSE139914 database. (B) The LRP1 level of epilepsy patient is lower than that of normal person in GSE14697 database.

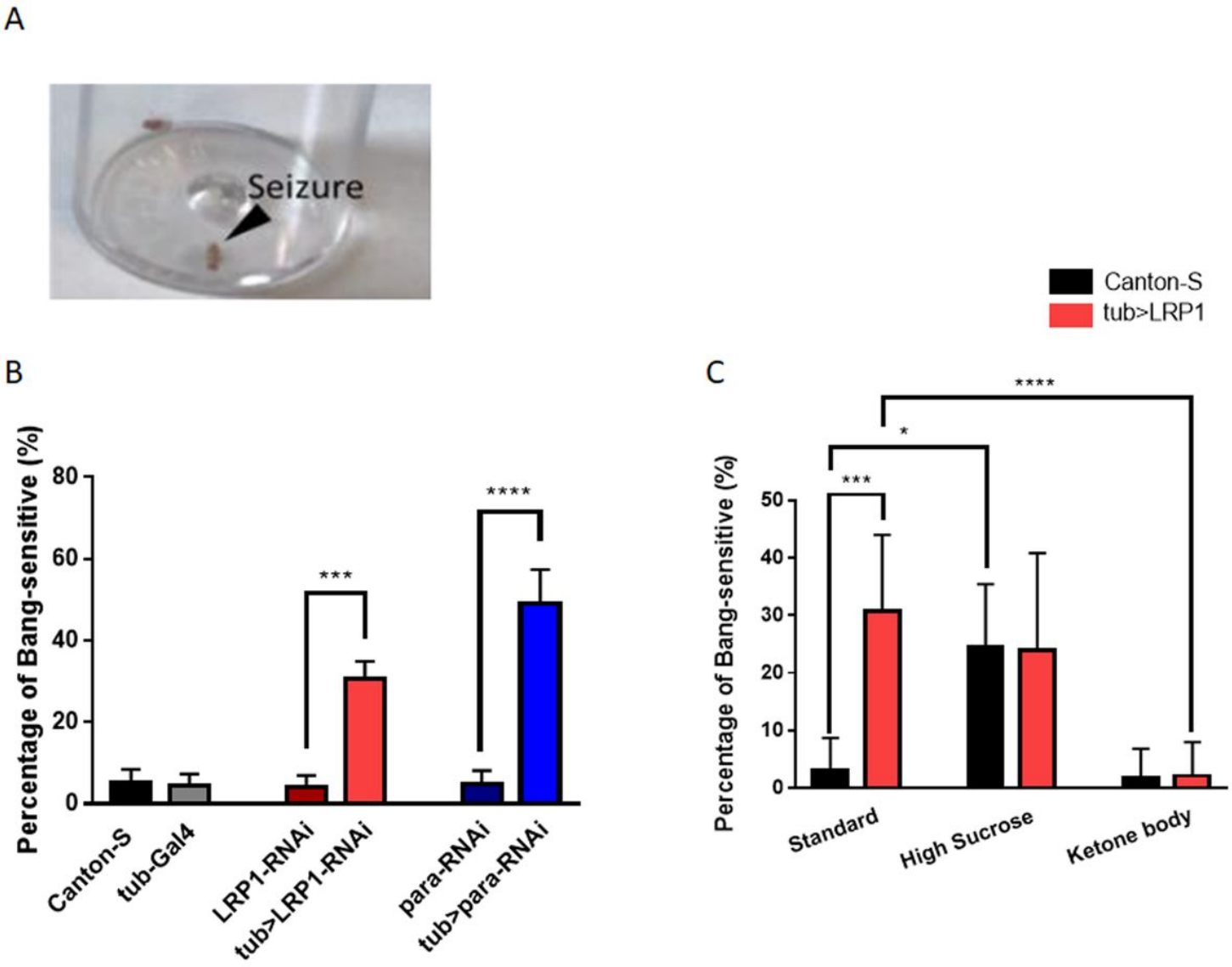

Figure 2

Knockdown of LRP1 induces seizure-like behavior in *Drosophila*. (A) Seizure behavior in flies. (B) Seizures occurred at a higher rate in LRP1 knockdown flies (*tub-Gal4>LRP1-RNAi*) than in WT flies (*Canton-S*) and other control groups. The *tub-Gal4>Scn1a-RNAi* positive controls had a higher rate of seizures than the *tub-Gal4>LRP1-RNAi* group. (C) Ketone body diet largely inhibited the seizure in LRP1 global knockdown flies.

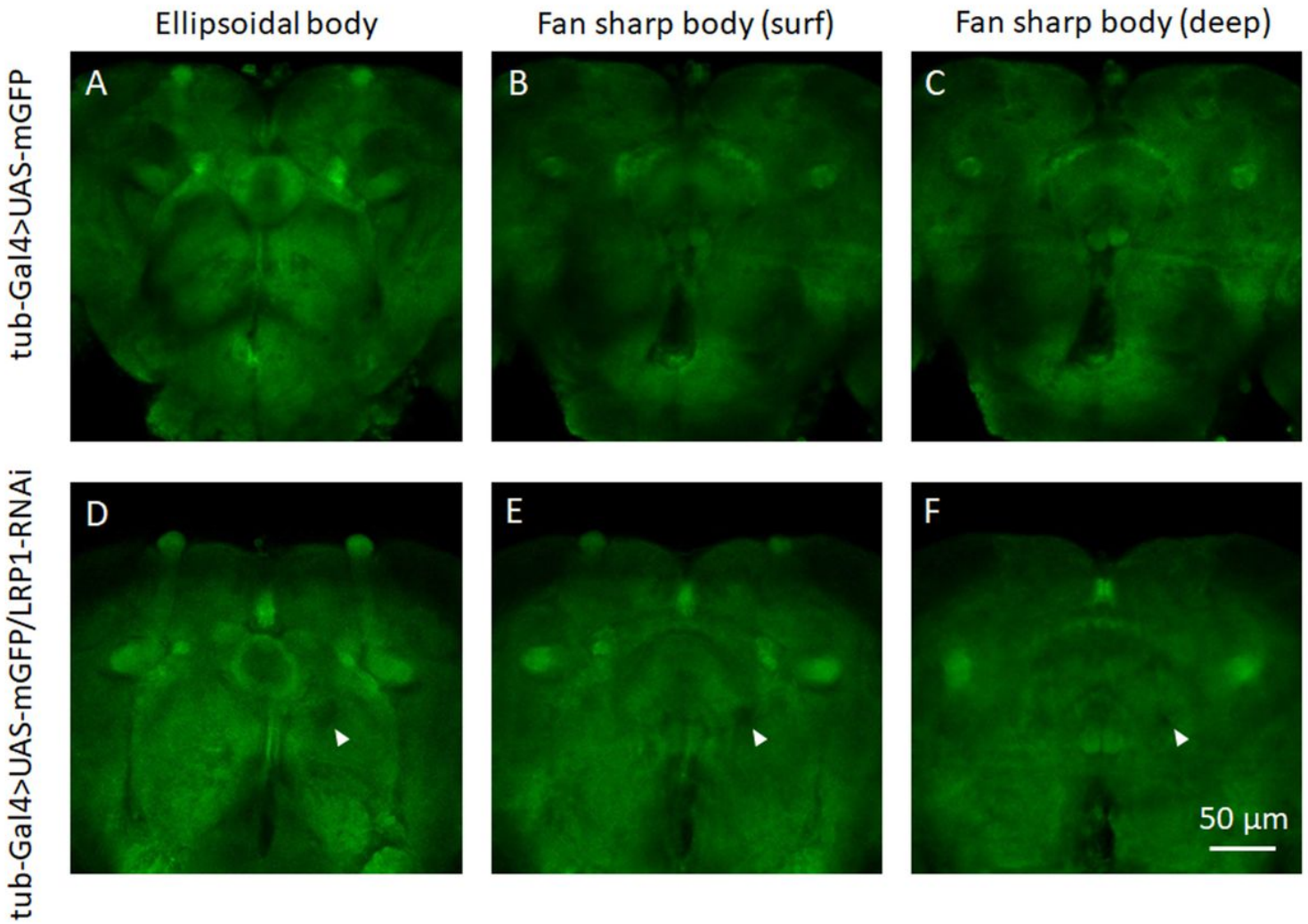

Figure 3

LRP1 defect induced partial loss of ellipsoidal body (A-C) Serial images of the brain of WT flies from anterior to posterior showing the structure of Ellipsoidal body (A), fan shaped body (surf) (B), fan shaped body (deep) (C). (D-F) Serial images of the brain of a LRP1 knockdown fly from anterior to posterior showing the structure of Ellipsoidal body (D), fan shaped body (surf) (E), fan shaped body (deep) (F). Scale bar 50 µm.

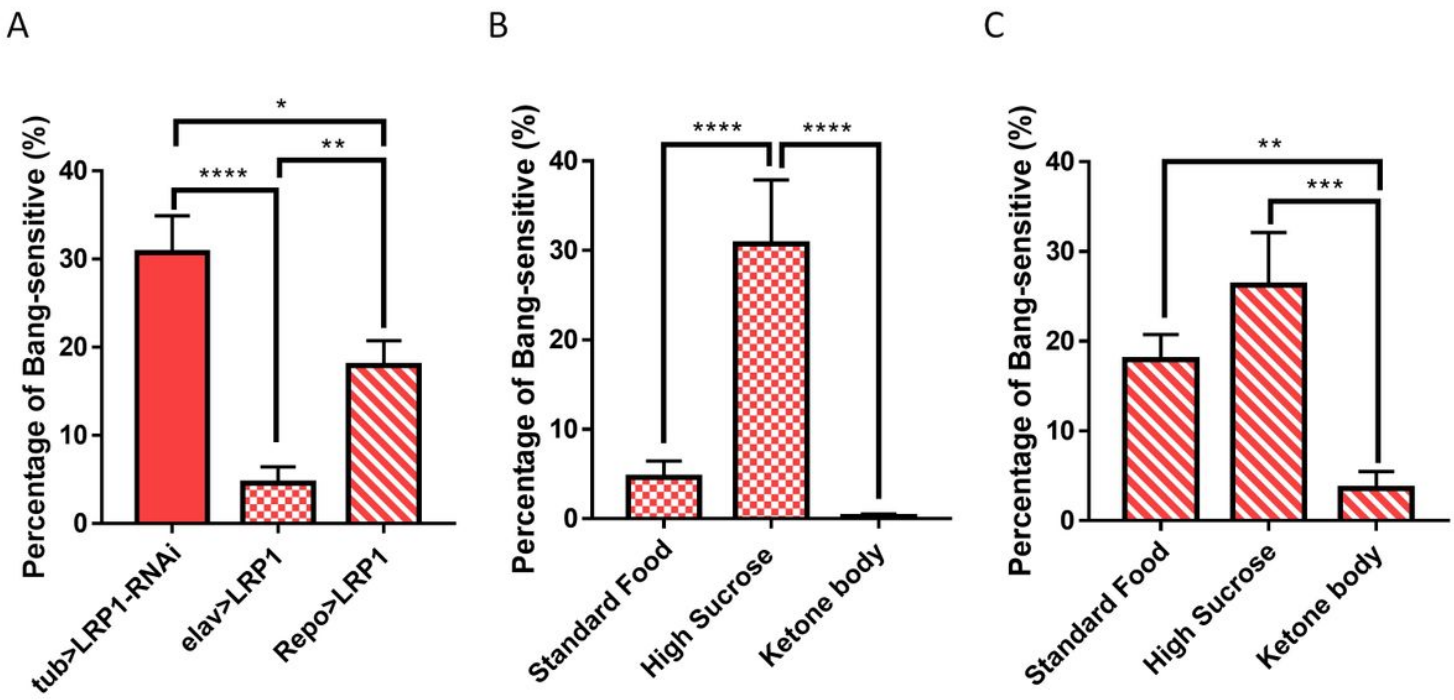

Figure 4

Glial knockdown of LRP1 sufficiently induced Seizure behavior. (A) Seizures occurred at a higher rate in *tub-Gal4>LRP1-RNAi* flies than in *repo-Gal4>LRP1-RNAi* flies. The *repo-Gal4>LRP1-RNAi* flies had a higher rate of seizures than of the *elva-Gal4>LRP1-RNAi* group. (B) The pan-neural LRP1 knockdown (*elav-Gal4>LRP1-RNAi*) flies feeding high sucrose food had a higher rate of seizures than that feeding standard food. Ketone body diet completely inhibited the seizure of those flies. (C) Seizures occurred at a higher rate in glial knockdown (*repo-Gal4>LRP1-RNAi*) flies feeding standard food than that feeding for ketone body diet.

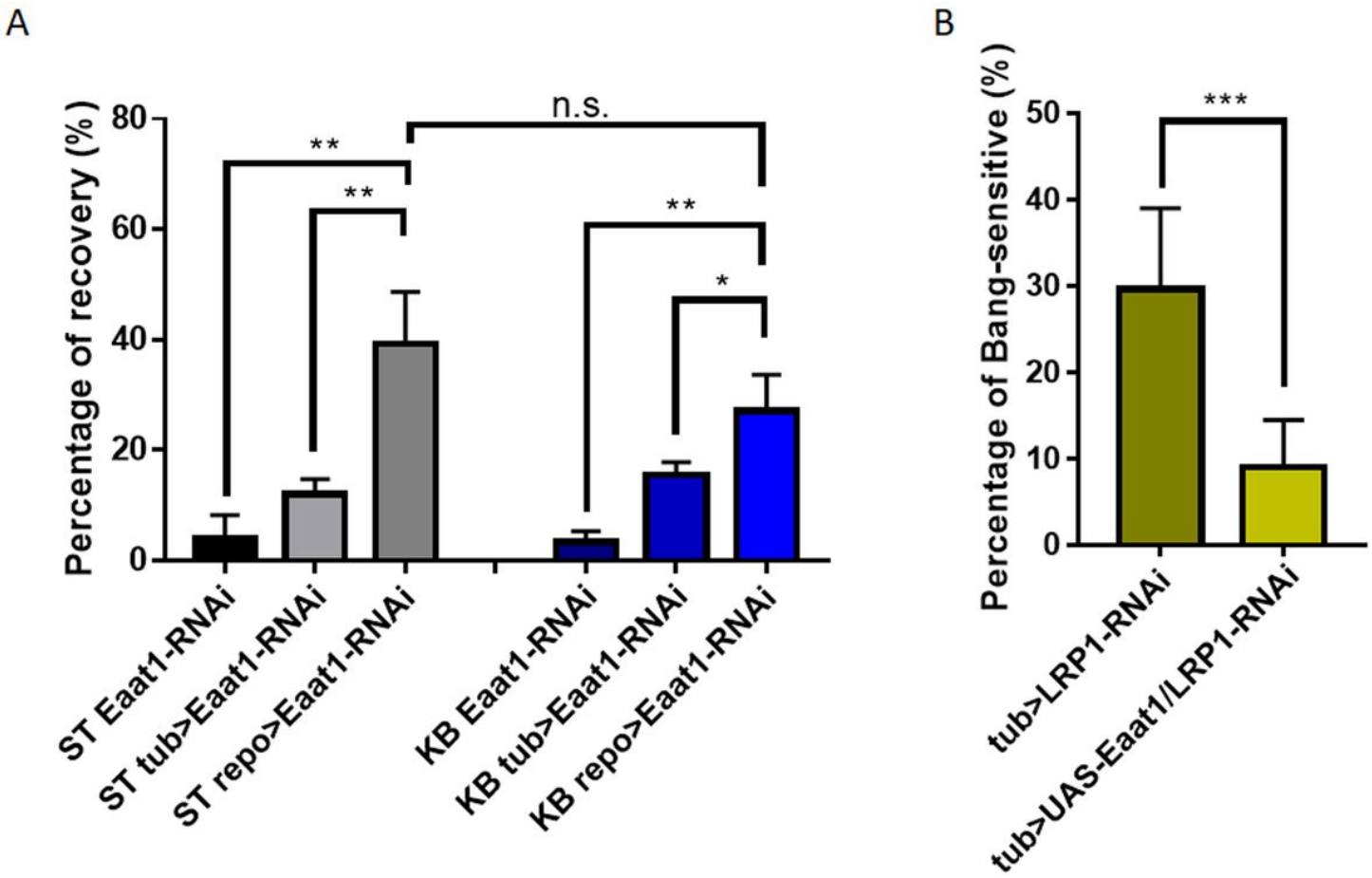

Figure 5

Ketone body diet rescued LRP1 deficiency by downregulating glutamate transmission. (A) Knockdown of glial glutamate transport (*repo>Eaat1-RNAi*) induce serious seizure behavior, and ketone body diet could not inhibit the seizure rate in glial glutamate transporter knockdown flies. (B) Over-expression of Eaat1 could inhibit seizure caused by LRP1 deficiency. ST is short for Standard diet; KB is short for Ketone body diet.