ENDOCRINE MODULATORS OF NEUROLOGICAL PROCESSES: POTENTIAL TREATMENT TARGETS OF PEDIATRIC NEUROLOGICAL DISEASES

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ENDOCRINE MODULATORS OF NEUROLOGICAL PROCESSES: POTENTIAL TREATMENT TARGETS OF PEDIATRIC NEUROLOGICAL DISEASES

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Table of Contents

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Editorial: Endocrine Modulators of Neurological Processes: Potential Treatment Targets of Pediatric Neurological Diseases

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Editorial on the Research Topic

Endocrine Modulators of Neurological Processes: Potential Treatment Targets of Pediatric Neurological Diseases

INTRODUCTION

Over the last decade, significant progress has been made in understanding the effects of neuroendocrine regulators, represented by brain gut peptides, leptin, melatonin, as well as ketogenic diet (KD), on brain development, brain injury repair and early warning. This Research Topic contains 10 articles, contributed by 40 authors, from 17 countries (plus 28 experts participating in the review and editing), focusing on most recent understanding of the effects of neuroendocrine regulators on pediatric neurological diseases, including the predictive value of ghrelin, leptin, and other adipokines in the early diagnosis and intervention on pediatric neuropathy, the progress of basic research and the potential translational medicine value, etc.

Gastrointestinal peptides play important roles by regulating feeding and energy homeostasis. Ghrelin is a multifaceted gut hormone that is famously known as a "hunger hormone," just the opposite of cholecystokinin (CCK), which is referred to as a "satiety factor." Both hormones act *via* the peripheral vagal afferent and interact to modulate feeding regulation (1). The anticonvulsant effects of ghrelin and CCK have been reported (2, 3). Notably, ghrelin is expected to be an effective therapy for lean patients with cachexia caused by chronic heart failure, anorexia nervosa, and

functional dyspepsia (4). In this Research Topic, Marchiò et al. evaluated ghrelin and growth during 1 year of ketogenic diet (KD). They examined a small cohort of six children (two males and four females, age range 3–10.4 years) affected by refractory epilepsy, who received the KD as add-on treatment. The results showed that ghrelin plasma levels are consistently reduced in children with refractory epilepsy and maintained on the KD. This change was associated with low growth indexes in the majority of patients.

Although the mechanism of KD's anti-epileptogenic and neuroprotective effects has been studied in recent years (5, 6), there is still a lack of spectral molecular expression research. Here a proteomics study by Zheng et al. analyzed the effects of KD against lithium chloride/pilocarpine-induced status epilepticus (SE) in rats. Seventy-nine proteins in hippocampus showing a significant change in abundance between SE and control (Ctr) groups were reciprocally regulated in the SE + KD group compared to the SE group (i.e., the seizure-induced change was reversed by KD). Of these, five (dystrobrevin, centromere protein V, oxysterol-binding protein, tetraspanin-2, and progesterone receptor membrane component 2) were verified by parallel reaction monitoring. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that proteins of the synaptic vesicle cycle pathway were enriched both among proteins differing in abundance between SE and Ctr groups as well as between SE + KD and SE groups.

Chen et al. investigated the effects of different zinc concentrations in the diet on long-term neurobehavioral and seizure thresholds following lithium chloride/pilocarpine-induced developmental seizures. They found that zinc supplementation for 4 weeks significantly improved injury-related changes, and rescued abnormalities in GPR39, ZnT-3, and MBP expression in the hippocampus, which suggests that the role of zinc ions in developmental convulsive brain injury may not be purely excitotoxic as previously thought (7–9), but may play a protective role in the brain as mentioned earlier (10). In a perspective article by Doenyas, a more comprehensive approach termed the "gut-immune-endocrine-brain" axis, is taken, based on which a personalized treatment plan for autism spectrum disorder (ASD) is presented.

Another clinical Cross-Sectional Study by Chen et al. in the present Research Topic investigated the association among plasma adipokines, mainly leptin, visfatin, adiponectin, or IL-6, and the prognosis of febrile seizures (FS). The main findings were that serum adiponectin and IL-6 levels were significantly higher in the FS group than in the FC and HC groups, while there was no statistical difference between the FC and HC groups, indicating that higher plasma levels of IL-6 and adiponectin could serve as an additional biomarker in the early treatment or follow-up of FS children.

Melatonin is an indoleamine secreted by the pineal gland. It can be used for the treatment of children with developmental disorders, such as ASD and attention deficit/hyperactivity disorder to improve their sleep disturbance (11, 12). Animal experiments and limited human data have confirmed the neuroprotective effect of melatonin on hypoxic-ischemic (HI) or developmental seizure-induced excitotoxic brain damage (13–15). Here, two studies have explored the molecular signaling mechanism of the neuroprotective effect of melatonin through the *in vitro* glutamate excitotoxic injury model and the *in vivo* cerebral palsy model. Wang et al. investigated the impact of melatonin on the parameters of glutamate cytotoxicity in mouse HT22 hippocampal neurons and tested the hypothesis that melatonin confers neuroprotective effects *via* mitochondrial oxidative stress/autophagy signaling. The findings indicate that melatonin exerts neuroprotective effects against glutamate-induced excitotoxicity by reducing mitophagy-related oxidative stress and maintaining mitochondrial function. Sun et al. used plppr5 knockout (plppr5-/-) mice and their wild-type littermates to establish a model of HI injury to further explore the effects of melatonin on brain injury and the role of plppr5 in this treatment in an HI model, which mainly focuses on cognition, exercise, learning and memory. They found that plppr5 knockout aggravated HI damage and partially weakened the neuroprotective effect of melatonin in some aspects (such as novel object recognition tests and partial nerve reflexes).

Interactions between the brain and distinct adipose depots have a key role in maintaining energy balance, thereby promoting brain development, among them, leptin play a key role (16). Recently, there has been renewed interest in the role of leptin in repairing developmental brain damage (17, 18). Here, a review article by Fujita and Yamashita summarize novel functions of leptin in animal models of neurodegenerative diseases. Specifically, they focus on the emerging evidence for the role of leptin in non-neuronal cells in the central nervous system, including astrocytes, microglia, and oligodendrocytes, which provides helpful information to establish therapeutic strategies to address the neurological diseases.

In addition, the study by Zhao et al. explored the significance of the α2 isoform of Na+/K+-ATPase in the regulation of the electrophysiological properties of skeletal muscle cells by β-Catenin. The review article by Wu et al. discussed the emerging roles of long non-coding RNAs in chronic neuropathic pain.

In conclusion, the present clinical and basic studies allow us a better understanding of the effects of endocrine modulators on pediatric neurological diseases. We hope that the information gathered from this Research Topic will help promote clinical translational medical research to better prevent and treat these injuries in the near future.

AUTHOR CONTRIBUTIONS

HN wrote the draft. GB, DU, and AC reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Date Y, Toshinai K, Koda S, Miyazato M, Shimbara T, Tsuruta T, et al. Peripheral interaction of ghrelin with cholecystokinin on feeding regulation. *Endocrinology* (2005) 146:3518–25. doi: 10.1210/en.2004-1240
2. Lee SY, Soltesz I. Cholecystokinin: a multi-functional molecular switch of neuronal circuits. *Dev Neurobiol* (2011) 71:83–91. doi: 10.1002/dneu.20815
3. Coppens J, Aourz N, Walrave L, Fehrentz JA, Martinez J, De Bundel D, et al. Anticonvulsant effect of a ghrelin receptor agonist in 6Hz corneally kindled mice. *Epilepsia* (2016) 57:e195–9. doi: 10.1111/epi.13463
4. Ueno H, Shiiya T, Nakazato M. Translational research of ghrelin. *Ann N Y Acad Sci* (2010) 1200:120–7. doi: 10.1111/j.1749-6632.2010.05509.x
5. Tian T, Li LL, Zhang SQ, Ni H. Long-Term Effects of Ketogenic Diet on Subsequent Seizure-Induced Brain Injury During Early Adulthood: Relationship of Seizure Thresholds to Zinc Transporter-Related Gene Expressions. *Biol Trace Elem Res* (2016) 174:369–76. doi: 10.1007/s12011-016-0730-3
6. Ni H, Jiang YW, Tao LY, Jin MF, Wu XR. ZnT-1, ZnT-3, CaMK II, PRG-1 expressions in .hippocampus following neonatal seizure-induced cognitive deficit in rats. *Toxicol Lett* (2009) 184:145–50. doi: 10.1016/j.toxlet.2008.11.003
7. Ni H, Jiang YW, Tao LY, Cen JN, Wu XR. Effects of penicillin-induced developmental epilepticus on hippocampal regenerative sprouting, related gene expression and cognitive deficits in rats. *Toxicol Lett* (2009) 188:166–6. doi: 10.1016/j.toxlet.2009.04.002
8. Ni H, Feng X, Gong Y, Tao LY, Wu XR. Acute phase expression pattern of ZnTs, LC3, and beclin-1 in rat Hippocampus and its regulation by 3-methyladenine following recurrent neonatal seizures. *Biol Trace Elem Res* (2011) 143:320–31. doi: 10.1007/s12011-010-8836-5
9. Ni H, Li C, Feng X, Cen JN. Effects of forced running exercise on cognitive function and its relation to zinc homeostasis-related gene expression in rat hippocampus. *Biol Trace Elem Res* (2011) 142:704–12. doi: 10.1007/s12011-010-8793-z
10. Bruni O, Alonso-Alconada D, Besag F, Biran V, Braam W, Cortese S, et al. Current role of melatonin in pediatric neurology: clinical recommendations. *Eur J Paediatr Neurol* (2015) 19:122–33. doi: 10.1016/j.ejpn.2014.12.007
11. Maras A, Schroder CM, Malow BA, Findling RL, Breddy J, Nir T, et al. Long-Term Efficacy and Safety of Pediatric Prolonged-Release Melatonin for Insomnia in Children with Autism Spectrum Disorder. *J Child Adolesc Psychopharmacol* (2018) 28:699–710. doi: 10.1089/cap.2018.0020
12. Paprocka J, Kijonka M, Rzepka B, Sokół M. Melatonin in Hypoxic-Ischemic Brain Injury in Term and Preterm Babies. *Int J Endocrinol* (2019) 2019:9626715. doi: 10.1155/2019/9626715
13. Barghout MS, Al-Shahawy AK, El Amrousy DM, Darwish AH. Comparison Between Efficacy of Melatonin and Diazepam for Prevention of Recurrent Simple Febrile Seizures: A Randomized Clinical Trial. *Pediatr Neurol* (2019) 101:33–8. doi: 10.1016/j.pediatrneurol.2019.01.010
14. Ni H, Sun Q, Tian T, Feng X, Sun BL. Prophylactic treatment with melatonin before recurrent neonatal seizures: effects on long-term neurobehavioral changes and the underlying expression of metabolism-related genes in rat hippocampus and cerebral cortex. *Pharmacol Biochem Behav* (2015) 133:25–30. doi: 10.1016/j.pbb.2015.03.012
15. Caron A, Lee S, Elmquist JK, Gautron L. Leptin and brain-adipose crosstalks. *Nat Rev Neurosci* (2018) 19:153–65. doi: 10.1038/nrn.2018.7
16. Jin MF, Ni H, Li LL. Leptin maintained zinc homeostasis against glutamate-induced excitotoxicity by preventing mitophagy-mediated mitochondrial activation in HT22 hippocampal neuronal cells. *Front Neurol* (2018) 9:322:322. doi: 10.3389/fneur.2018.00322
17. Ni H, Chen SH, Li LL, Jin MF. Leptin treatment prevents long-term abnormalities in cognition, seizure threshold, hippocampal mossy fiber sprouting and ZnT3/CB-D28k expression in a rat developmental "twist" seizure model. *Epilepsy Res* (2018) 139C:164–70. doi: 10.1016/j.eplepsyres.2017.12.009
18. Ni H, Zhao DJ, Tian T. Ketogenic diet change cPLA2/clusterin and autophagy related gene expression and correlate with cognitive deficits and hippocampal MFs sprouting following neonatal seizures. *Epilepsy Res* (2016) 120:13–8. doi: 10.1016/j.eplepsyres.2015.11.021

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BRIEF RESEARCH REPORT
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Ghrelin Plasma Levels After 1 Year of Ketogenic Diet in Children With Refractory Epilepsy

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The ketogenic diet (KD) is a high-fat, low carbohydrate nutritional treatment adopted in several countries for refractory epilepsy. However, the use of KD is limited by adverse events including growth retardation. In a previous investigation, we demonstrated that ghrelin is reduced in children maintained on KD for 3 months. As ghrelin regulates growth hormone (GH) secretion, it can be hypothesized that growth retardation depends on the reduced ghrelin availability. To assess this hypothesis, in this study we evaluate ghrelin and growth during 1 year of KD. We examined a small cohort of 6 children (2 males and 4 females, age range 3–10.4 years) affected by refractory epilepsy, who received the KD as add-on treatment. All patients were on drug polytherapy. Endpoints of the study were: (i) ghrelin plasma levels at 0, 15, 30, 90, and 365 days from KD onset, (ii) growth, and (iii) seizure control by ketogenesis. Ghrelin levels were −53 and −47% of basal levels, respectively, at 90 and 365 days ($P < 0.05$ for both). Mean height index *z* scores were reduced, but not significantly, by comparing basal values with those at the end of observation. Instead, body mass index *z* scores slightly increased. Ketosis induced by the KD was within 2–5 mmol/L and satisfactorily reduced the seizure frequency (>50%) in all patients. We show that ghrelin plasma levels are consistently reduced in children with refractory epilepsy and maintained on the KD. This change was associated with low growth indexes in the majority of patients.

Keywords: children, epilepsy, ghrelin, growth, ketogenic diet

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INTRODUCTION

A high-fat, low-carbohydrate and normal protein diet has been developed, and named "ketogenic diet (KD)," to control antiseizure drug (ASD)-refractory epilepsy. This diet was designed to induce ketone body production, so to reproduce the physiological changes associated with prolonged fasting (1). In children with refractory seizures, the KD may be effective in up to 87% of cases (2). In spite of its demonstrated usefulness (3), various adverse events among which the most consistent is growth retardation (4–9) may limit the diffusion of KD as a medical nutritional treatment. Growth impairment in patients on the KD has been related to dysfunctional production of insulin-like growth factor-1 (IGF-1) (8), probably resulting from the alteration of mechanisms

regulating growth hormone (GH) production. To this regard, the growth hormone secretagogue (GHS) ghrelin, which is a major regulator of food intake, metabolism, and GH secretion (10), could be responsible for the growth impairment and maybe for the reduction of IGF-1 observed in children on the KD.

Ghrelin is a peptide hormone prevalently released by gastric P/D1 cells and has also been involved in memory (11), anxiety (12), epilepsy (13), and neuroprotection (14). Interestingly, ghrelin is markedly dysregulated in pediatric patients with epilepsy and treated with ASDs (15). In addition, ghrelin has been associated with body weight gain in children with epilepsy and receiving valproate, thus suggesting an association between ghrelin and dysregulation of metabolism in epilepsy (16). In view of this evidence, we recently observed a time-dependent reduction in ghrelin plasma levels in children with refractory epilepsy and maintained on the KD (2). However, in these children we noticed a stable growth during the short 90 days' period of observation. For this reason, we decided to extend our previous study to establish if ghrelin levels could be stably reduced after 1 year of KD in children with refractory epilepsy, and the possible impact of the hypothesized ghrelin reduction on growth in the same patients.

MATERIALS AND METHODS

Experimental Design

Patients

We considered a small cohort of 6 subjects composed by 2 males and 4 female children with diagnosis of refractory epilepsy, admitted to the pediatric neurology hospitalist service between October 2013 and June 2014. The inclusion criteria were: confirmed diagnosis of epilepsy, refractoriness to ASDs, age ranging from 0 to 14 years, and informed consent signed by parents. Whereas, exclusion criteria were acute or chronic metabolic diseases unrelated to epilepsy and the lack of adherence to the nutritional protocol. The KD was administered by starting with a 2:1 ratio of lipids and proteins + carbohydrates and, after few days, shifting to a 3.5/4:1 ratio as detailed previously (2, 17). KD was monitored and supplemented as previously described (2, 17). Height, weight and body mass index (BMI) were measured before the KD onset and until the end of observation period. Data about demographic features, clinical characteristics, diagnostic findings, therapeutic interventions, and clinical outcomes are reported in **Table 1**. The Ethics Committee of Modena (4206/C.E.) approved the research protocol according to local regulations and informed written consent was obtained from relatives of participants.

Quantitative Analysis of Ghrelin

Reagents and Materials

To block conversion of ghrelin to des-acyl ghrelin (18), we used the protease inhibitor cocktail P2714 from Sigma Aldrich (Milan, Italy). The enzyme-linked immunosorbent assay (ELISA) kit for human plasma acyl ghrelin (EZGRA-88K) was obtained from Merck Millipore (Milan, Italy).

Sample Processing

Fasting blood samples (8:00–9:00 a.m.) were obtained (3 mL) and coded to assure a blind processing for immunoassays. Blood collected in tubes with dipotassium ethylenediaminetetraacetate dihydrate and 10% (v/v) P2714 was gently shaken and quickly placed on ice, then 15 min centrifuged (1,800 g at 4°C) to store plasma into sterile microtubes (200 μL) at −80°C. 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride, contained in P2714, was previously shown to block ghrelin des-acylation effectively even when samples were kept frozen for months (18).

Immunoassays

Immunoassays were performed according to instructions. Samples were processed all together. Standards, controls, and coded samples were added to plates coated with the primary antibody and incubated at room temperature (RT) for 2 h. Then, plates were washed (300 μL to each well) five times and the enzyme added (100 μL) and incubated at RT for 2 h. After rewashing, substrate solution (200 μL) was dispensed to wells. Then, plates were gently shaken in the dark at RT for at least 30 min. Each well was mixed, and absorbance measured at 414 nm using a microplate reader (DTX 880 multimode detector, Beckman Coulter, USA). A cubic polynomial fitting was used to determine concentrations from the calibration curves. The intra-assay coefficient of variation (%) for ghrelin was 3.8, instead the inter-assay coefficient of variation was 7.5.

Statistics

Data from immunoassays, height and body mass index values were all analyzed using one-way analysis of variance with repeated measures followed by Duncan's test for comparisons. All statistical analyses were performed using Sigmaplot 13 (Systat Software, San Jose, CA). Data are presented as mean ± standard error of the mean and regarded significantly different at $P < 0.05$.

RESULTS

We examined a small cohort of patients affected by refractory epilepsy, composed by 2 males and 4 female children. The mean age was 5.9 ± 1.1 year, ranging from 3.0 to 10.4 years. Demographic and clinical features of these children are illustrated in **Table 1**. In order to monitor the response to KD, patients' ketosis was daily assessed and ranged from 2 to 5 mmol/L. The reduction in seizure frequency obtained by administering the KD was satisfactory in all patients (>50%): 2 patients displayed more than 75% reduction, while the other 4 patients rarely presented seizures as recurrence was −90% of previous frequency. However, KD adjustments were required to obtain an effective control of seizures in the majority of children (**Table 2**). Nobody stopped to take ASDs and all were on polytherapy.

Concerning growth, we calculated *z* scores immediately before the KD onset (−0.670 ± 0.739, $n = 5$), 90 days later (0.002 ± 0.295, $n = 5$), and at the end of our study (365 days; −1.198 ± 0.763, $n = 6$). Although we did not find significant differences, changes in the *z* score values were differently influenced by the

TABLE 1 | Demographic and clinical features of patients treated with the ketogenic diet.

Sex	Age	Age at epilepsy onset	Seizure type	Etiology	Concomitant ASDs	Type of KD	Reduction in seizure frequency (%)	β-hydroxybutyric acid (mmol/L, mean ± SEM)	Side effects
F (1)	7.2	2.6	Spasm	Metabolic	CBZ, LEV, NZP	Classic 2:1	>75	3.9 ± 0.2	Hypercholesterolemia; Hyperoxaluria
F (2)	10.4	10.2	Spasm, Drop Attack, TC	Unknown	CBZ, LEV	Classic 4:1	>75	3.7 ± 0.1	Constipation; Vomiting
F (3)	4.2	4.2	Drop Attack, Myocl, Ab	Structural	LEV, TPM	Classic 2:1	>90	4.1 ± 0.1	Hypercholesterolemia
M (1)	5.2	2.3	TC, Drop Attack	Unknown	LEV, TPM	Classic 3:1	>90	3.3 ± 0.2	–
F (4)	5.2	5.2	TC, Drop Attack	Genetic	NZP, RFN, VPA	MCT 3.5:1	>90	3.6 ± 0.1	Nausea; Vomiting
M (2)	3	2.3	TC, Drop Attack	Unknown	TPX, VGB, VPA	Classic 4:1	>90	4.5 ± 0.2	–

Ketosis levels were calculated by averaging all values obtained during each week. Ab, absence; ASDs, antiseizure drugs; CBZ, carbamazepine; KD, ketogenic diet; LEV, levetiracetam; Myocl, myoclonus; NZP, nitrazepam; RFN, rufinamide; SEM, standard error of the mean; TC, tonic-clonic; TPM, topiramate; VPA, valproate; VGB, vigabatrin.

TABLE 2 | Ketogenic diet (KD) was adapted to satisfy every specific requirement, as well as to obtain an adequate seizure control.

Sex	Energy intake (kcal/day)	Lipids (%)	Proteins (%)	Carbohydrates (%)	Number of meals/day	Required adjustments
F (1)	800	82	11	7	4	3
F (2)	1,600	89	8	3	4	2
F (3)	1,300	82	8	10	3	2
M (1)	1,250	87	5	8	4	4
F (4)	1,400	89	6	5	3	0
M (2)	1,350	90	8	2	3	3

When seizure control was not satisfactory, the KD was adjusted. Only final KDs are reported in table.

KD timing (**Figure 1**). Specifically, height *z* scores were reduced in 3 out of 5 patients at the end of the study, and in 2 of them were even lower than those measured at beginning. Surprisingly, this change occurred after an initial increase of *z* scores observed at 90 days in 3 out of 5 children (**Figure 1A**). From 90 to 365 days, height *z* scores declined in 4 out of 5 children and only in one child this trend was the opposite. Concerning BMI *z* scores, a reduction was observed in 3 patients at the end of our study, whereas the other 2 presented a steady increase, at all considered time intervals (**Figure 1B**). Average values were: 0.0560 ± 0.913 prior to KD; -0.712 ± 0.624 at 90 days; 0.155 ± 0.597 at 365 days. All the observed changes did not seem to be related to differences in KDs, as illustrated in **Table 2**.

Figure 2 illustrates ghrelin levels measured before and after the KD onset. Consistently, we observed stably reduced ghrelin plasma levels at 30 (−53%), 90 (−56%), and 365 (−47%) days after the KD onset. All reductions were statistically significant when compared with basal levels ($P < 0.05$ for all the above-mentioned time intervals, Duncan's test). It is worth mentioning that the reduction in ghrelin plasma levels was already detectable after 15 days (−40%), but it did not reach statistical significance because two samples were missing for technical reasons.

DISCUSSION

Growth impairment is an adverse event in children with refractory epilepsy receiving the KD. Although a study initially reported that growth was normal even after 6 months of KD (4), all subsequent studies demonstrated that weight and height were both compromised in the long-term (5–9). Interestingly, lack of changes at early time intervals was also confirmed by others who observed that growth impairment occurs only after 6 months of KD (7). In line with this report, we observed a non-significant change in growth after 3 months of KD (2), which is further confirmed by the present results. However, at the end of the study height *z* scores were more in line with the consistent findings reported by others (5–9).

The impact of KD on growth was initially related to the altered composition of the meal, especially for the reduction in micronutrients such as oligominerals and vitamins. However, authors excluded such an interpretation by comparing the effects on growth of the different KDs currently available to induce the ketosis (7). Also, the imbalance in protein-to-energy ratio has been suggested as responsible for the growth impairment (19). Therefore, we made any effort to carefully maintain a

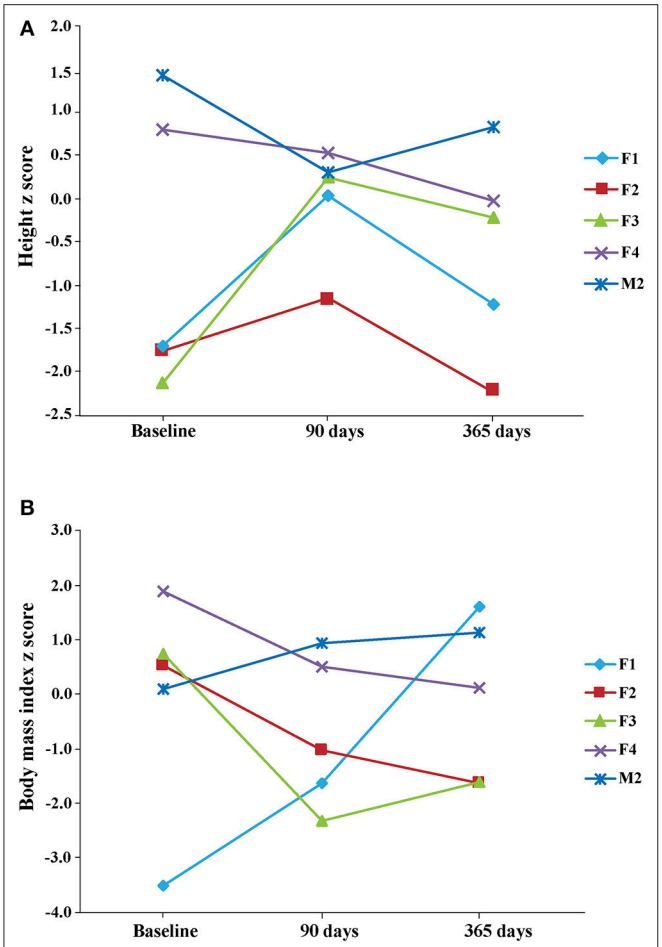

FIGURE 1 | Height **(A)** and body mass index (BMI) **(B)** *z* scores were measured at the beginning of KD (baseline), after 3 months (90 days) and at the end of the observation period (365 days). Note that *z* scores were decreased in 3 patients at the end of the study, with respect to baseline scores. Height mean values and the respective standard deviation were: −0.670 ± 0.739 ($n = 5$) at baseline, 0.002 ± 0.295 ($n = 5$) 90 days later, and −1.198 ± 0.763 ($n = 6$) at the end of study. BMI mean values and the respective standard deviation were: 0.0560 ± 0.913 ($n = 5$) at baseline, −0.712 ± 0.624 ($n = 5$) 90 days later, and 0.155 ± 0.597 ($n = 6$) at the end of study. Note that one patient's data are not illustrated in the figure because height and BMI values were available only for the last time interval considered in our study.

FIGURE 2 | Changes in ghrelin plasma levels in children ($n = 6$) affected by refractory epilepsy and maintained on the ketogenic diet up to 1 year. *$P <$ 0.05, one-way analysis of variance with repeated measures followed by Duncan's test.

more than adequate caloric and protein intake, as well as in giving supplement vitamins and oligoelements to children, as previously described (2). For this reason, we are prone to believe that the malfunctioning of GH axis in children receiving the KD could explain the growth impairment. In line with this interpretation, we found that ghrelin is rapidly reduced and kept to approximately half basal values by the KD. After the KD onset, IGF-I is also immediately reduced and stabilized on low levels up to 1 year (8). As ghrelin is able to stimulate GH release, we suggest that the reduction in ghrelin levels may explain the reported reduction in IGF-I levels. To this regard, caution is needed because lack of any consequence for growth has been paradoxically found in animals in which ghrelin or its receptor was deleted (20). However, a missense mutation of GHS receptor segregating with short stature was reported for two unrelated families and, at least in one family, IGF-1 levels were markedly reduced (21). Consistently, growth velocity was increased in children of these families who received administration of GH.

The reason for this phenomenon could be the reduced gastric ghrelin expression and secretion observed in high-fat fed mice (22), suggesting that the same phenomenon may occur in patients receiving the KD. However, it has to be mentioned that also low carbohydrate high fat diets used for treatment of obesity have been found to modify ghrelin levels (23, 24). In these diets, the lipids and proteins + carbohydrates ratio is much lower than that recommended for refractory seizures. Moreover, restriction in energy intake is always present and adults are the main population involved in the studies, whereas KDs for epilepsy are usually proposed to children for whom no energy restriction is provided. So, the reported changes in ghrelin levels in response to reduction in carbohydrate intake may depend on factors different from those affecting ghrelin in children with epilepsy and on the KD. Additionally, results on changes in ghrelin level associated with reduction in carbohydrates intake were also controversial, since in one study ghrelin was reduced by 18% (23), whereas in another one an increase of 7% was observed (24). The magnitude of these changes was anyway not comparable to that found in our children, suggesting that the reduction in carbohydrates in KD does not completely explain our findings.

The reduction in ghrelin availability could result in other, less evident consequences apart from the growth impairment. Ghrelin has been identified as an anticonvulsant peptide (13). We recently found that ghrelin plasma levels are higher in children responding to ASDs, but not in those with refractory epilepsy (15). For this reason, the observed reduction in ghrelin availability due to KD may be unfavorable for the optimal control of seizures. Contrary to this hypothesis, in our children we observed a more than satisfactory reduction in seizure frequency. However, we suspect that the reduction in ghrelin levels may be involved in other phenomena. For

instance, we described a paradoxical response in patients who developed increased seizure frequency and severity when starting the KD (17). To tentatively investigate this paradoxical response, in a seizure model we observed a prolongation of electrographic discharge induced by 6-Hz corneal stimulation in mice receiving the KD for a week. However, it remains to be established if ghrelin can play a role in these phenomena.

Ghrelin has also been demonstrated to possess protective properties in models of neuronal and vascular lesion [reviewed in Lucchi et al. (14)]. Indeed, ghrelin was found to attenuate kainic acid-induced neuronal cell loss in the mouse hippocampus (25). The ghrelin analog JMV-1843 rescued neurons and astrocytes in the pilocarpine model of *status epilepticus* (26). Beneficial effects were also reported for ghrelin in models of cerebral ischemia, although they have still to be clearly defined (27, 28). Interestingly, low ghrelin levels have been related to enhanced risk of liver damage in children undergoing surgery (29). This suggests that the reduction in ghrelin levels we observed in children with refractory epilepsy and receiving the KD may potentially expose to various consequences and should be addressed in order to prevent any of the possibly related adverse effects.

In conclusion, our study identified a long-lasting reduction in ghrelin levels in children with refractory epilepsy addressed by KD and receiving an adequate energy intake. The reduction in ghrelin availability could be related to the slower growth observed in children on KDs. However, a study based on a larger cohort is required to definitely demonstrate the association between the changes in ghrelin production and growth impairment due to the KD.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

The Ethics Committee of Modena (4206/C.E.) approved the research protocol according to local regulations and informed written consent was obtained from relatives of participants.

AUTHOR CONTRIBUTIONS

GB: concept and design of the study. AG, CL, LR, MB, MM, and TT: data acquisition and analysis. AC, CL, GB, and LI: drafting the manuscript and figures. All authors read and approved the final version of the manuscript.

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REFERENCES

1. Giordano C, Marchiò M, Timofeeva E, Biagini G. Neuroactive peptides as putative mediators of antiepileptic ketogenic diets. *Front Neurol*. (2014) 5:63. doi: 10.3389/fneur.2014.00063
2. Marchiò M, Roli L, Giordano C, Trenti T, Guerra A, Biagini G. Decreased ghrelin and des-acyl ghrelin plasma levels in patients affected by pharmacoresistant epilepsy and maintained on the ketogenic diet. *Clin Nutr*. (2019) 38:954–7. doi: 10.1016/j.clnu.2018.03.009
3. Neal EG, Chaffe H, Schwartz RH, Lawson MS, Edwards N, Fitzsimmons G, et al. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. *Lancet Neurol*. (2008) 7:500–6. doi: 10.1016/S1474-4422(08)70092-9
4. Couch SC, Schwarzman F, Carroll J, Koenigsberger D, Nordli DR, Deckelbaum RJ, et al. Growth and nutritional outcomes of children treated with the ketogenic diet. *J Am Diet Assoc*. (1999) 99:1573–5. doi: 10.1016/S0002-8223(99)00387-9
5. Vining EP, Pyzik P, McGrogan J, Hladky H, Anand A, Kriegler S, et al. Growth of children on the ketogenic diet. *Dev Med Child Neurol*. (2002) 44:796–802. doi: 10.1111/j.1469-8749.2002.tb00769.x
6. Liu YM, Williams S, Basualdo-Hammond C, Stephens D, Curtis R. A prospective study: growth and nutritional status of children treated with the ketogenic diet. *J Am Diet Assoc*. (2003) 103:707–12. doi: 10.1053/jada.2003.50136
7. Neal EG, Chaffe HM, Edwards N, Lawson MS, Schwartz RH, Cross JH. Growth of children on classical and medium-chain triglyceride ketogenic diets. *Pediatrics*. (2008) 122:334–40. doi: 10.1542/peds.2007-2410
8. Spulber G, Spulber S, Hagenäs L, Amark P, Dahlin M. Growth dependence on insulin-like growth factor-1 during the ketogenic diet. *Epilepsia*. (2009) 50:297–303. doi: 10.1111/j.1528-1167.2008.01769.x
9. Kim JT, Kang HC, Song JE, Lee MJ, Lee YJ, Lee EJ, et al. Catch-up growth after long-term implementation and weaning from ketogenic diet in pediatric epileptic patients. *Clin Nutr*. (2013) 32:98–103. doi: 10.1016/j.clnu.2012.05.019
10. Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, et al. Ghrelin. *Mol Metabol*. (2015) 4:437–60. doi: 10.1016/j.molmet.2015.03.005
11. Diano S, Farr SA, Benoit SC, McNay EC, da Silva I, Horvath B, et al. Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci*. (2006) 9:381–8. doi: 10.1038/nn1656
12. Stark R, Santos VV, Geenen B, Cabral A, Dinan T, Bayliss JA. Des-acyl ghrelin and ghrelin O-acyltransferase regulate hypothalamic-pituitary-adrenal axis activation and anxiety in response to acute stress. *Endocrinology*. (2016) 157:3946–57. doi: 10.1210/en.2016-1306
13. Portelli J, Michotte Y, Smolders I. Ghrelin: an emerging new anticonvulsant neuropeptide. *Epilepsia*. (2012) 53:585–95. doi: 10.1111/j.1528-1167.2012.03423.x
14. Lucchi C, Vinet J, Meletti S, Biagini G. Ischemic-hypoxic mechanisms leading to hippocampal dysfunction as a consequence of status epilepticus. *Epilepsy Behav*. (2015) 49:47–54. doi: 10.1016/j.yebeh.2015.04.003
15. Marchiò M, Roli L, Giordano C, Caramaschi E, Guerra A, Trenti T, et al. High plasma levels of ghrelin and des-acyl ghrelin in responders to antiepileptic drugs. *Neurology*. (2018) 91:62–6. doi: 10.1212/WNL.0000000000005741
16. Greco R, Latini G, Chiarelli F, Iannetti P, Verrotti A. Leptin, ghrelin, and adiponectin in epileptic patients treated with valproic acid. *Neurology*. (2005) 65:1808–9. doi: 10.1212/01.wnl.0000187074.27586.d1
17. Lucchi C, Marchiò M, Caramaschi E, Giordano C, Giordano R, Guerra A, et al. Electrographic changes accompanying recurrent seizures under ketogenic diet treatment. *Pharmaceuticals*. (2017) 10:82. doi: 10.3390/ph10040082

18. Delhanty PJ, Huisman M, Julien M, Mouchain K, Brune P, Themmen AP, et al. The acylated (AG) to unacylated (UAG) ghrelin ratio in esterase inhibitor-treated blood is higher than previously described. *Clin Endocrinol*. (2015) 82:142–6. doi: 10.1111/cen.12489
19. Nation J, Humphrey M, MacKay M, Boneh A. Linear growth of children on a ketogenic diet: does the protein-to-energy ratio matter? *J Child Neurol*. (2014) 29:1496–501. doi: 10.1177/0883073813508222
20. Albarran-Zeckler RG, Sun Y, Smith RG. Physiological roles revealed by ghrelin and ghrelin receptor deficient mice. *Peptides*. (2011) 32:2229–35. doi: 10.1016/j.peptides.2011.07.003
21. Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, et al. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest*. (2006) 116:760–8. doi: 10.1172/JCI25303
22. Moesgaard SG, Ahrén B, Carr RD, Gram DX, Brand CL, Sundler F. Effects of high-fat feeding and fasting on ghrelin expression in the mouse stomach. *Regul Pept*. (2004) 120:261–7. doi: 10.1016/j.regpep.2004.03.018
23. Ebbeling CB, Feldman HA, Klein GL, Wong JMW, Bielak L, Steltz SK, et al. Effects of a low carbohydrate diet on energy expenditure during weight loss maintenance: randomized trial. *BMJ*. (2018) 363:k4583. doi: 10.1136/bmj.k4583
24. Hall KD, Bemis T, Brychta R, Chen KY, Courville A, Crayner EJ, et al. Calorie for calorie, dietary fat restriction results in more body fat loss than carbohydrate restriction in people with obesity. *Cell Metab*. (2015) 22:427–36. doi: 10.1016/j.cmet.2015.07.021
25. Lee J, Lim E, Kim Y, Li E, Park S. Ghrelin attenuates kainic acid-induced neuronal cell death in the mouse hippocampus. *J Endocrinol*. (2010) 205:263–70. doi: 10.1677/JOE-10-0040
26. Lucchi C, Curia G, Vinet J, Gualtieri F, Bresciani E, Locatelli V, et al. Protective but not anticonvulsant effects of ghrelin and JMV-1843 in the pilocarpine model of *status epilepticus*. *PLoS ONE*. (2013) 8:e72716. doi: 10.1371/journal.pone.0072716
27. Miao Y, Xia Q, Hou Z, Zheng Y, Pan H, Zhu S. Ghrelin protects cortical neuron against focal ischemia/reperfusion in rats. *Biochem Biophys Res Commun*. (2007) 359:795–800. doi: 10.1016/j.bbrc.2007.05.192
28. Kenny R, Cai G, Bayliss JA, Clarke M, Choo YL, Miller AA, et al. Endogenous ghrelin's role in hippocampal neuroprotection after global cerebral ischemia: does endogenous ghrelin protect against global stroke? *Am J Physiol Regul Integr Comp Physiol*. (2013) 304: R980–90. doi: 10.1152/ajpregu.00594.2012
29. Zenitani M, Hosoda H, Kodama T, Saka R, Takama Y, Ueno T, et al. Postoperative decrease in plasma acyl ghrelin levels after pediatric living donor liver transplantation in association with hepatic damage due to ischemia and reperfusion injury. *Pediatr Surg Int*. (2019) 35:709–14. doi: 10.1007/s00383-019-04463-8

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β-Catenin Controls the Electrophysiologic Properties of Skeletal Muscle Cells by Regulating the α2 Isoform of Na^+/K^+-ATPase

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β-Catenin is a key component of the canonical Wnt signaling pathway. It has been shown to have an important role in formation of the neuromuscular junction. Our previous studies showed that in the absence of β-catenin, the resting membrane potential (RMP) is depolarized in muscle cells and expression of the α2 subunit of sodium/potassium adenosine triphosphatase (α2NKA) is reduced. To understand the underlying mechanisms, we investigated the electrophysiologic properties of a primary cell line derived from mouse myoblasts (C2C12 cells) that were transfected with small-interfering RNAs and over-expressed plasmids targeting β-catenin. We found that the RMP was depolarized in β-catenin knocked-down C2C12 cells and was unchanged in β-catenin over-expressed muscle cells. An action potential (AP) was not released by knockdown or over-expression of β-catenin. α2NKA expression was reduced by β-catenin knockdown, and increased by β-catenin over-expression. We showed that β-catenin could interact physically with α2NKA (but not with α1NKA) in muscle cells. NKA activity and α2NKA content in the cell membranes of skeletal muscle cells were modulated positively by β-catenin. These results suggested that β-catenin (at least in part) regulates the RMP and AP in muscle cells, and does so by regulating α2NKA.

Keywords: electrophysiologic properties, neuromuscular junction, skeletal muscle, Na^+/K^+-ATPase, β-catenin

INTRODUCTION

β-Catenin is a crucial downstream component of the Wnt signaling pathway. β-Catenin translocates to the nucleus and subsequently regulates target–gene transcription (Molenaar et al., 1996; Hecht et al., 2000). β-Catenin also binds to the cytoplasmic domain of α-catenin and cadherins, where it participates in cell adherence and organization of the actin cytoskeleton (Nelson and Nusse, 2004). β-Catenin not only promotes cell proliferation and tissue expansion, it also affects the fate and final differentiation of cells after cell division. β-Catenin is associated with disorders

caused by abnormal development and some tumors (Chilosi et al., 2003; Varallo et al., 2003; Jamieson et al., 2004; Batlle et al., 2005). The function of β-catenin is extensive, but it is associated with several diseases. However, up until now, the underlying mechanisms have not been clear.

In the central nervous system (CNS), β-catenin participates in synaptic assembly: neural development, axonal growth, orientation, and synaptogenesis (Salinas, 2003; Yoshikawa et al., 2003; Zou, 2004). Studies have suggested that Wnt/β-catenin signaling is involved in Alzheimer's disease (Caricasole et al., 2003; Zhao et al., 2005), schizophrenia (Singh et al., 2010), and emotional disorders (Lovestone et al., 2007).

In the peripheral nervous system, the neuromuscular junction (NMJ), which is the synapse between a motor neuron and a muscle fiber, plays an important part in muscle contraction. Appropriate interactions between motor neurons and muscle cells are required for information transmission at the NMJ (Sanes and Lichtman, 2001; Wu et al., 2010).

Agrin is released by motor neurons and binds to low-density lipoprotein receptor-related protein (LRP)-4 and activates muscle-specific kinase (MuSK). Agrin, LRP4, and MuSK are needed for NMJ formation (DeChiara et al., 1996; Gautam et al., 1996; Zong et al., 2012). In contrast, muscle cells produce retrograde signals for presynaptic differentiation (Lyuksyutova et al., 2003). It has been suggested that β-catenin in muscle might play a key part in the formation and development of the NMJ (Zhang et al., 2007; Li et al., 2008; Wang et al., 2008; Wu et al., 2015).

Research has revealed that most mutant mice deficient in β-catenin (and specifically deficient in β-catenin in skeletal muscles) die a few hours after birth (Li et al., 2008; Zhao et al., 2014). Previously, we showed that β-catenin is crucial for maintenance of the resting membrane potential (RMP) of skeletal muscle cells, and that its lack of expression can induce marked reductions in expression of the α2 subunit of sodium/potassium adenosine triphosphatase (α2NKA) (Zhao et al., 2014).

In the present study, we showed that β-catenin plays a part in the RMP and action potential (AP) of skeletal muscle cells at the NMJ. We recorded depolarization following knockdown of the β-catenin gene, but variance was not seen if β-catenin was over-expressed in skeletal muscle cells. The AP was inhibited by up-regulation and down-regulation of functional expression of β-catenin. Also, α2NKA expression was reduced by knocking down β-catenin and was increased by over-expressing β-catenin. We found that α2NKA and β-catenin displayed a "physical" interaction in muscle cells. Finally, NKA activity and α2NKA content in the cell membrane were found to be modulated positively by β-catenin.

We were able to suggest a mechanism underlying the electrophysiologic changes caused by β-catenin expression in muscle cells. We also provided additional evidence that supports a key role for β-catenin at the NMJ. Our data could be used to advance research on the intricate role of β-catenin in neurologic disorders.

MATERIALS AND METHODS

Reagents and Antibodies

Antibodies targeted against β-catenin and α2NKA were obtained from Abcam (AB32572; used at 1:6000 dilution for immunoblotting and 1:200 for staining; AB2871, used at 1:500 for immunoblotting and 1:50 for staining; Cambridge, United Kingdom). Antibodies against K^+-voltage-gated channel subfamily C member 4 (Kv3.4; DF10312; 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AF7021; 1:1000) were purchased from Affinity (Cincinnati, OH, United States). Antibodies against α1NKA (GTX22867; 1:600) were obtained from GeneTex (Irvine, CA, United States). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G (BA1051; 1:50,000), HRP-conjugated goat anti-rabbit IgG (BA1054; 1:50,000), Cy3-conjugaed goat anti-rabbit IgG (BA1032; 1:2000), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BA1101; 1:2000) were purchased from Wuhan Boster (Wuhan City, China). A Na^+/K^+-ATP Enzyme Test kit (BC0060) was obtained from Solarbio Life Sciences (Beijing, China). A Cell Surface Protein Isolation kit (89881) was purchased from Pierce (Rockford, IL, United States).

Constructs

Chemically modified small interfering (si)RNA oligonucleotides were obtained from Shanghai GenePharma (Shanghai, China). The 5′-termini of all siRNAs were labeled with a fluorescent conjugated dye (carboxyfluorescein). The sequences of β-catenin siRNAs (sense and antisense, respectively) were: 5′-CACCU CCCAA GUCCU UUAUT T-3′ and 5′-AUAAA GGACU UGGGA GGUGT T-3′ for siRNA-459; 5′-CCAGG UGGUA GUUAA UAAAT T-3′ and 5′-UUUAU UAACU ACCAC CUGGT T-3′ for siRNA-777; 5′-GGGUU CCGAU GAUAU AAAUT T-3′ and 5′-AUUUA UAUCA UCGGA ACCCT T-3′ for siRNA-1512; 5′-UUCUC CGAAC GUGUC ACGUT T-3′ and 5′-ACGUG ACACG UUCGG AGAAT T-3′ for the negative control (carboxyfluorescein).

The cDNA of β-catenin was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using the primer sequences (forward and reverse, respectively) 5′-GGACT CAGAT CTCGA GATGG CTACT CAAGC TGACC T-3′ and 5′-GTCGA CTGCA GAATT CTTAC AGGTC AGTAT CAAAC CAG-3′. This was followed by sub-cloning into the pIRES2-ZsGreen1 plasmid (**Supplementary Figure 1**). The overexpressed plasmid we constructed was called pIRES2-ZsGreen1-β-catenin.

Culture and Transfection of Cells

C2C12 myoblasts were obtained from American Type Culture Collection (Manassas, VA, United States). They were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; 11965118; Invitrogen, Carlsbad, CA, United States) supplemented with 100 U/mL penicillin–streptomycin, 10% fetal bovine serum (16000-044; Invitrogen, Carlsbad, CA, United States), and 1 mM GlutaMAX (Thermo Fisher, Boston, MA, United States) in 60-mm dishes. Fusion of myoblasts

into myotubes was induced by culture in differentiation medium (high-glucose DMEM supplemented with 2% horse serum). Myoblasts at 70–80% confluence were transfected with Lipofectamine 2000 (11668019; Invitrogen, Carlsbad, CA, United States) according to manufacturer instructions, and then switched to differentiation medium 24-h later.

Electrophysiology Experiment

Fully differentiated myotubes transfected with siRNAs and pIRES2-ZsGreen1-β-catenin were visualized with an inverted microscope (AE31E; Motic, Beijing, China). They were recorded using whole-cell recording techniques employing an amplifier (EPC10), a Patchmaster v2 × 73 analog-to-digital converter, and IGOR 6.0.1.0 (all from HEKA Elektronik, Berlin, Germany). Recording pipettes were pulled to a tip resistance of 3–5 MΩ when filled with an internal solution containing (in mM): 140 potassium gluconate, 5 NaCl, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, 1 $MgCl_2$, and 2 Mg-ATP (pH 7.2 with KOH, and 295 mOsm). Cells were perfused throughout recordings with an external solution containing (in mM): 140 NaCl, 3.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, 10 HEPES, and 1.25 NaH_2PO_4 (pH 7.4 with NaOH and 300 mOsm). Only cells with elongated (rather than flat) morphology were selected. Slightly positive pressure was applied while the pipette was advanced into the bath with the microelectrode manipulator. Once the glass pipette made contact with the cell surface, positive pressure was removed. Then, negative pressure was used to form a pipette-cell seal at >1 GΩ, and gentle suction was applied to rupture the patch membrane. The slow capacitance was compensated, and the membrane capacitance and series resistance were recorded. Seal formation and whole-cell recording were carried out in "current clamp" mode. The RMP was recorded at zero current immediately after breaking the seal between the membrane and the pipette. Cells with a series resistance <20 MΩ and changes <20% throughout recording were used for analyses. Current signals were Bessel-filtered at 5 kHz and sampled at 10 kHz.

Independent APs were evoked by intracellular injection of depolarizing current in a series of 600–1100 pA (at increments of 10–100 pA) rectangular pulses with 10 mS at 20 Hz (Li et al., 2015). All experiments were conducted at room temperature.

Immunoblotting

C2C12 cells were collected and lysed with lysate buffer supplemented with a "protease inhibitor cocktail" [50 mM Tris–Cl (pH 7.4), 1 mM EDTA (pH 8.0), 250 mM NaCl, and 1% Triton-X] (Roche, Basel, Switzerland). The concentration of protein lysates was measured using the Bradford method (5000006; Bio-Rad Laboratories, Hercules, CA, United States). Subsequently, 10 μg of cell lysate with 5 μg of sample buffer [15 g of sodium dodecyl sulfate (SDS), 15.6 mL of 2 M Tris (pH 6.8), 57.5 g of glycerol, 16.6 mL of β-mercaptoethanol] was added to a 10% polyacrylamide gel. Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (162-0177; Bio-Rad Laboratories, Hercules, CA, United States). After sealing with 4% milk containing 0.1% Tween, the PVDF membranes were incubated overnight with primary antibodies against β-catenin, α2NKA, KV3.4, α1NKA, and GAPDH, respectively, at 4°C. They were then washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween. Next, 4% skimmed milk containing 0.1% Tween and HRP-conjugated antibody were added and incubated for 2 h at room temperature. After an electrochemiluminescence developer (170-5060; Bio-Rad Laboratories, Hercules, CA, United States) had been added to PVDF membranes, photographs were obtained using the Gel Doc™ imaging system (Bio-Rad Laboratories, Hercules, CA, United States).

Quantitative RT-PCR (qRT-PCR)

mRNA samples from C2C12 cells were prepared using an RNeasy mini-kit (74106; Qiagen, Stanford, VA, United States) and digested using an RNase-Free DNase kit (79254; Qiagen, Stanford, VA, United States). mRNA samples were reverse-transcribed into cDNA using a high-capacity cDNA Reverse Transcription kit (4368813) according to manufacturer (Applied Biosystems, Foster City, CA, United States) instructions. qRT-PCR was done with a SYBR® Premix Ex Taq™ II kit (DRR041A; TaKaRa Biotechnology, Shiga, Japan) in a real-time PCR instrument (QuantStudio 6; Applied Biosystems, Foster City, CA, United States) with pre-denaturation at 50°C for 10 min, denaturation at 95°C for 30 s, and annealing at 60°C for 30 s for a total of 40 cycles. The primer sequences (forward and reverse, respectively) were: 5′-CTTAC GGCTA CAGAG AGGGG-3′ and 5′-GCAGA GGGAA GCCGT AGTAT-3′ for α1NKA; 5′-ATCAA TGCAG AGGAG GTGGT-3′ and 5′-TGAAC TCAGG AGAAC GGGTC-3′ for α2NKA; 5′-AGACA GCTCG TTGTA CTGCT-3′ and 5′-GTGTC GTGAT GGCGT AGAAC-3′ for β-catenin; 5′-ATGGG TGTGA ACCAC GAGA-3′ and 5′-CAGGG ATGAT GTTCT GGGCA-3′ for GAPDH.

Immunohistochemistry and Co-immunoprecipitation

C2C12 cells were fixed by immersion in 4% paraformaldehyde in PBS for 30 min at 4°C. After addition of Triton X-100 to a final concentration of 0.1%, sections were mounted on gelatin-coated glass slides. Non-specific binding sites were blocked by preincubation for 1 h at room temperature in bovine serum albumin in PBS (PBS/BSA), followed by incubation with the primary antibody in PBS/BSA overnight at 4°C. Bound antibody was visualized by labeling with a FITC-conjugated anti-rabbit antibody or Cy3-conjugated anti-mouse antibody. Secondary antibodies were used at 1:2000 dilution. Fluorescence images were captured using a confocal laser scanning microscope and software (C2; Nikon, Tokyo, Japan).

C2C12 cells were resuspended in immunoprecipitation buffer supplemented with protease inhibitors and phosphatase inhibitors for 10–15 min on ice, and centrifugated at 10,000 × *g* for 10 min at 4°C. The supernatant was precleared with 30 μL of protein A + G agarose beads for 2 h at 4°C to eliminate non-specific binding and reduce the background reading. Samples were incubated with 3 μg of β-catenin antibody at 4°C overnight. Immune complexes were harvested with another 30 μL of protein A + G agarose beads for 3–6 h at

a 4°C. The beads were resuspended in 30 μL of 2× SDS–PAGE buffer and washed three times with PBS. α2NKA and α1NKA were immunoprecipitated under identical conditions using monoclonal antibodies. After heating for 5 min at 100°C in 2× sample buffer, immunoprecipitated proteins were subjected to SDS–PAGE and immunoblotting as described above.

Assays to Measure Enzyme Activity

Cell suspensions of myoblasts at 80–90% confluence were thawed on ice and homogenized for 10 min at1000 × *g*. Then, the homogenate was centrifuged at 1000 × *g* for 10 min at 4°C to obtain the precipitate, which was then resuspended in 1 mL of homogenizing buffer [0.1 mol/L imidazole-HCl, 0.3 mol/L sucrose, and 1 g/L of sodium deoxycholate (without EDTA)] for 10 min at 37°C. The homogenized sample was centrifuged for 10 min at 4000 × *g* and 4°C. The supernatant obtained interfered with subsequent phosphate analyses, so it was mixed with a phosphorus reagent for 10 min in a 40°C water bath, and then cooled to room temperature. Absorbance was determined at 660 nM using an automatic microplate spectrophotometer (Multiskan MK3, Thermo Scientific).

Assays for Na^+/K^+-ATPase activity were determined as described by Ching et al. (2015) using the following formula:

ATPase activity (U/mg protein) = C standard × (A estimated – A control)/(A standard – A blank) × V total/(protein concentration of the sample × V sample)/T = 7.5 × (A estimated – A control)/(A standard – A blank)/protein concentration of the sample.

A, enzymatic activity (the amount of inorganic phosphorus produced by enzymatic decomposition of ATP per milligram of tissue protein and per hour as a unit of enzymatic activity); C, concentration, V, volume, T, the reaction time.

Biotinylation on the Surface of Muscle Cells

Cells were starved with serum-free DMEM overnight, washed with ice-cold PBS, and incubated with PBS containing 0.5 mg/mL non-permeable EZ-link^{TM} Sulfo-NHS-SS-Biotin (pH 8.0; Thermo Scientific) on ice for 30 min. (EZ-link Sulfo-NHS-SS-Biotin is a membrane-impermeable reagent which forms a stable covalent linkage with an extended spacer arm to reduce

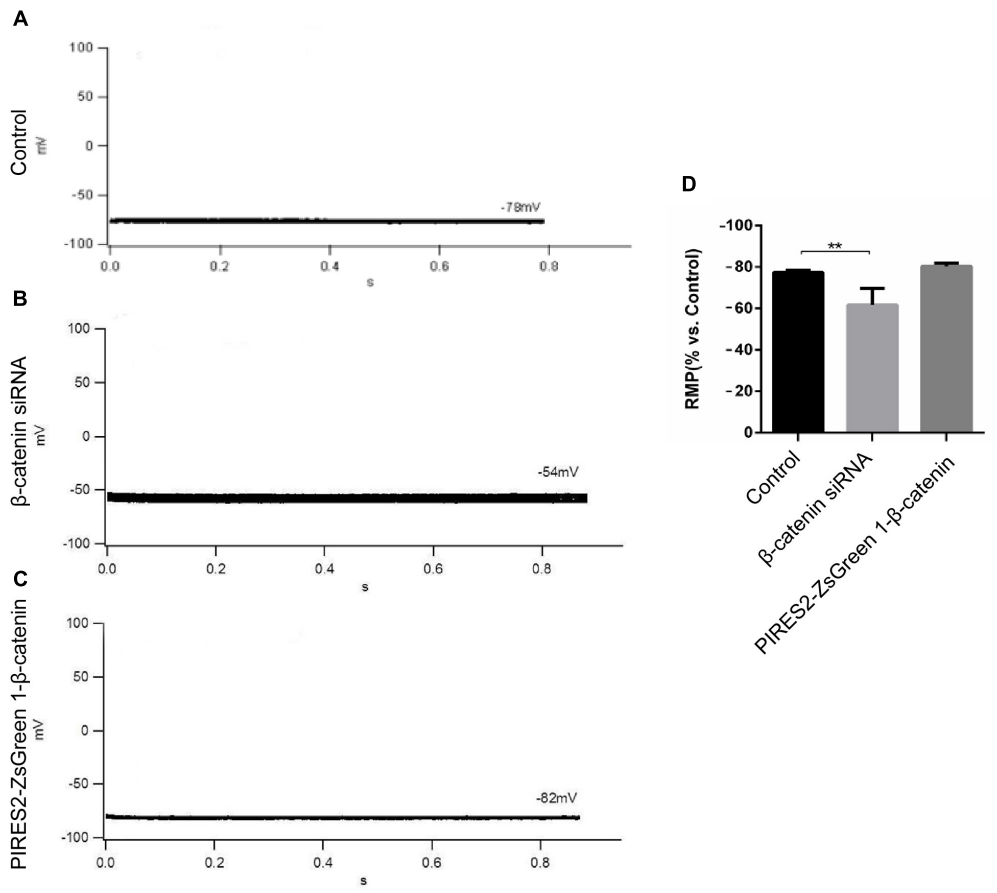

FIGURE 1 | The RMP was depolarized in C2C12 muscle cells by knocking down β-catenin, but was not significantly changed by overexpressing β-catenin. **(A–C)** Whole-cell recordings of the RMP from C2C12 cells of control, β-catenin siRNA, and pIRES2-ZsGreen1-β-catenin, respectively. **(D)** Histograms show the absolute values of the RMP of β-catenin siRNA- and pIRES2-ZsGreen1-β-catenin-transfected C2C12 cells from more than three experiments with values from the control normalized as 1. $n = 3$ cells per group. Values are the mean ± SEM. $^{**}P < 0.01$. Error bars represent SEM.

the steric hindrance associated with avidin binding.) The liquid was suctioned. Cells were incubated with 4 mL of 100 mmol/L glycine at 4°C for 5 min. The step described above was repeated. Cells were washed and harvested by scraping in pre-cooled cell lysate buffer containing 1% Triton-X100 and protease inhibitors (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5). The supernatant was harvested and boiled in 4× buffer solution for 10 min. For pull down of biotinylated proteins, 500 μL of the protein extract was incubated with 30 μL of high-capacity streptavidin agarose beads (Neutravid Agarose; 29202; Pierce, Rockford, IL, United States) overnight at 4°C. Beads were washed, eluted by incubation in Laemmli buffer containing dithiothreitol at 100°C for 10 min, and analyzed by western immunoblotting. The lysate input and elutes were subjected to SDS–PAGE and analyzed by western immunoblotting.

Statistical Analyses

Data are the mean ± SEM. Data were calculated using one-way analysis of variance (ANOVA) with Tukey's honestly significantly different test. Changes were considered significant if alpha values satisfied the limit of $P < 0.05$.

RESULTS

Deletion (but Not Over-Expression of) β-Catenin Depolarized the RMP in Cultured Muscle Cells

Our previous study showed that HSA-β-cat$^{-/-}$ pups died hours after birth having presented with global cyanosis, consistent with previous reports (Li et al., 2008; Zhao et al., 2014). We wished to investigate further the role of β-catenin in muscle cells. Hence, we knocked down and over-expressed β-catenin in C2C12 cells by generating siRNAs targeting β-catenin (labeled with a fluorescent dye to mark successful transfection) and sub-cloning β-catenin cDNA into the pIRES2-ZsGreen1 plasmid, respectively. A siRNA that does not target any known mammalian gene was synthesized as a negative siRNA control. A blank pIRES2-ZsGreen1 plasmid was synthesized as a negative over-expression control.

β-Catenin expression in cells transfected with β-catenin siRNAs and pIRES2-ZsGreen1-β-catenin plasmids was reduced and increased significantly, respectively (**Supplementary Figures 2**, **3**, *t*-test, $P = 0.0027$ for β-catenin siRNA group vs. siRNA NC group, and $P = 0.0014$ for pIRES2-ZsGreen1-β-catenin group vs. pIRES2-ZsGreen1 group). β-Catenin siRNA459 and a number 1 bacterial solution of pIRES2-ZsGreen-β-catenin were selected for subsequent studies. To ascertain if the RMP of β-catenin siRNA- and pIRES2-ZsGreen1-β-catenin plasmid-transfected cells had changed, we undertook whole-cell patch-clamp recording. We found that, in control cells, the RMP was −76 ± 2.83 mV (**Figure 1A**), whereas the RMP of cells transfected with β-catenin siRNA459 and pIRES2-ZsGreen1-β-catenin was −63 ± 7.07 mV (one-way ANOVA, $P = 0.00067$) and −80.75 ± 1.50 mV, respectively (**Figures 1B,C**). These data showed notable depolarization in C2C12 cells in which the β-catenin gene was knocked down, and no significant change in cells in which β-catenin was over-expressed (**Figure 1D**).

AP Was Inhibited by Up- and Down-Regulation of β-Catenin in C2C12 Cells

To evoke an AP, current of duration 10 mS was delivered under a current clamp. To evoke an AP, electrical stimulation (600–1100 pA) was given (increased in increments of 10–100 pA). The AP of muscle cells was evoked in control cells, and the morphology and platform stage were normal (**Figure 2A**). However, in β-catenin knocked-down cells and over-expressed cells, an AP was not evoked in muscle cells (**Figures 2B,C**). Our results showed that the generation of AP required a proper β-catenin expression in the muscle cell membrane of NMJ.

Expression and Transcription of α2NKA Were Associated Positively With β-Catenin in Muscle Cells

Our data showed that β-catenin knockdown depolarized skeletal muscle cells. Furthermore, the AP was not evoked in β-catenin knocked-down or in over-expressed C2C12 cells. Thus, we further explored the possible mechanisms underlying this phenomenon.

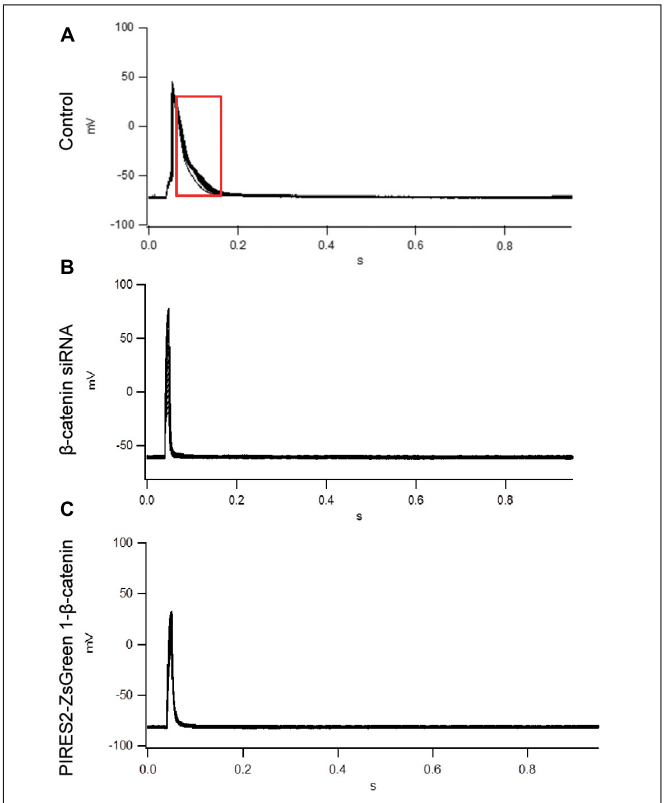

FIGURE 2 | An AP could not be evoked by up- or down-regulation of β-catenin expression in C2C12 cells. **(A)** The AP of muscle cells evoked in control cells. **(B,C)** No AP was seen in β-catenin siRNA or pIRES2-ZsGreen1-β-catenin C2C12 cells. *n* = 3 cells per group.

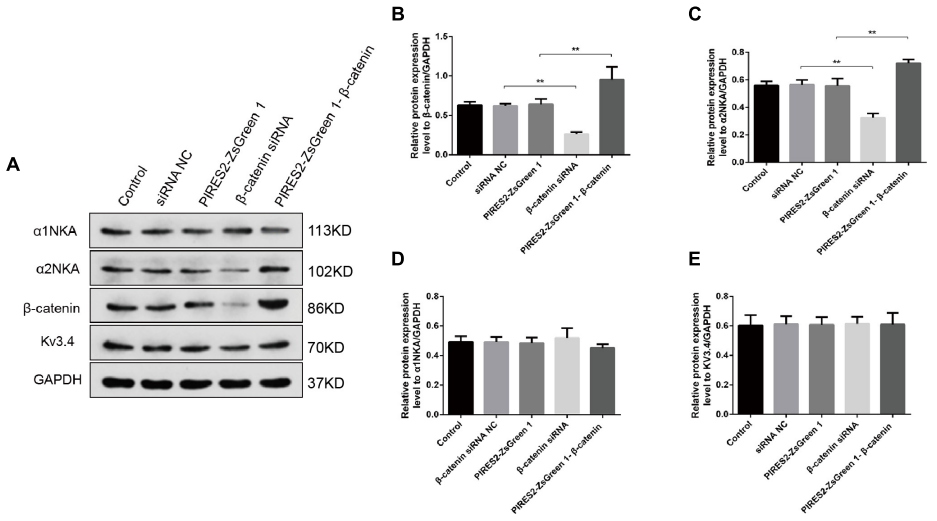

FIGURE 3 | Protein expression of α2NKA, α1NKA, and Kv3.4 in each transfection group. **(A)** Representative expressions of α1NKA, α2NKA, β-catenin, Kv3.4 and GAPDH proteins in five independent groups respectively. Histograms show quantification of β-catenin **(B)**, α2NKA **(C)**, α1NKA **(D)**, and Kv3.4 **(E)** as protein levels relative to GAPDH from five independent experiments. The five groups are the control; siRNA normal control; pIRES2-ZsGreen1; β-catenin siRNA; pIRES2-ZsGreen1-β-catenin. $n = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars represent SEM.

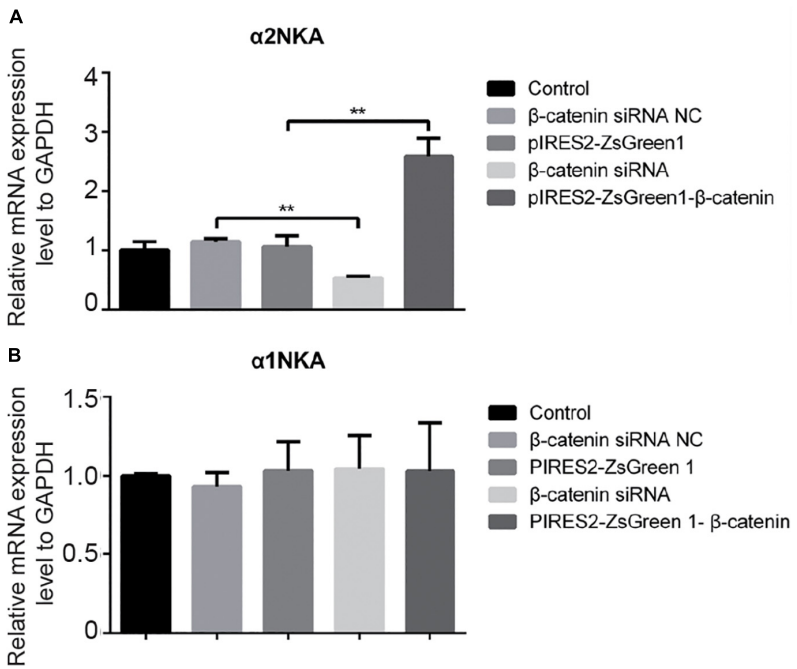

FIGURE 4 | qPCR of α 2NKA mRNA **(A)** and α1NKA **(B)** mRNA in each transfection group. The five groups were: control; siRNA normal control; pIRES2-ZsGreen1; β-catenin siRNA; and pIRES2-ZsGreen1-β-catenin. mRNA expression was relative to that of GAPDH, and by changing the mean $\Delta C(t)$ value of each experimental group to Power $2^{-\Delta\Delta Ct}$. The threshold cycle (Ct) was obtained as the fractional cycle number at which the amount of amplified target reached a fix threshold. Data normalization was performed by subtracting Ct value of the GAPDH from that of the target gene. The $\Delta\Delta Ct$ was calculated as the difference of the normalized Ct value (ΔCt) of the experimental samples and control samples. $\Delta\Delta Ct = \Delta Ct_{\text{experiment}} - \Delta Ct_{\text{control}}$. The control group was nominally considered as 1. $n = 3$ per group. $^{**}P < 0.01$, Error bars represent SEM.

Sodium/potassium adenosine triphosphatase and Kv3.4 channels (Abbott et al., 2001) are crucial for maintaining the RMP in skeletal muscle cells. NKA is important for muscle excitability (Radzyukevich et al., 2013). Skeletal muscles in newborns mainly express α1NKA and α2NKA.

Moreover, β-catenin regulates the expression and transcription of target genes in the Wnt canonical pathway

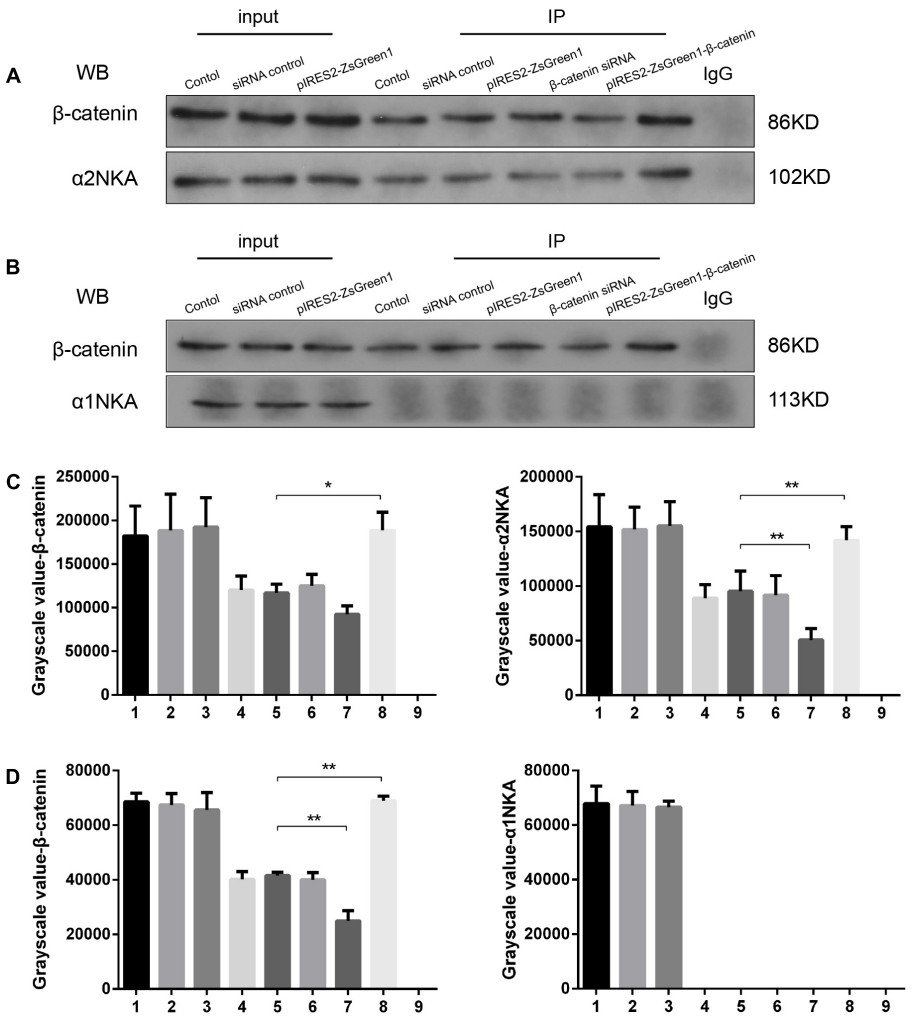

FIGURE 5 | β-Catenin could pull down α2NKA protein **(A)** but not α1NKA protein **(B)** in C2C12 cells. 1. C2C12 input; 2. C2C12 siRNA control input; 3. C2C12 pIRES2-ZsGreen1 input; 4. C2C12 control immunoprecipitation; 5. C2C12 siRNA control immunoprecipitation; 6. C2C12 pIRES2-ZsGreen1 immunoprecipitation; 7. C2C12 β-catenin siRNA immunoprecipitation; 8. C2C12 pIRES2-ZsGreen1-β-catenin immunoprecipitation; 9. IgG control. Histograms show grayscale quantification of β-catenin (**C** left, **D** left), α2NKA (**C** right) and α1NKA (**D** right), from nine independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, Error bars represent SEM.

(Behrens et al., 1996; Clevers, 2006; Salinas and Zou, 2008; Budnik and Salinas, 2011). Thus, we ascertained if β-catenin functions in muscle cells by regulating expression of ion-channel proteins.

We undertook immunoblotting and qRT-PCR in β-catenin knocked-down and over-expressed cells. We measured the protein expression of α1NKA and α2NKA in transfected C2C12 cells. Transfection with β-catenin siRNA caused a significant reduction of α2NKA expression ($P < 0.0001$ for β-catenin siRNA) (**Figures 3A,C**), and transfection with pIRES2-ZsGreen1-β-catenin caused a significant increase in α2NKA expression ($P = 0.0002$ for pIRES2-ZsGreen1-β-catenin) (**Figures 3A,C**). α2NKA expression was reduced to 57.5 ± 3.2% in β-catenin siRNA cells and increased to 129.2 ± 2.8% as compared with controls (**Figure 3C**). No significant difference was found in expression of α1NKA or Kv3.4 in any of the groups (**Figures 3D,E**). The decline in β-catenin expression reduced α2NKA transcription markedly ($P = 0.0014$ for β-catenin siRNA group vs. siRNA NC group, and $P < 0.0001$ for pIRES2-ZsGreen1-β-catenin group vs. pIRES2-ZsGreen1 group). By contrast, enhanced expression of β-catenin increased α2NKA transcription markedly (**Figure 4**).

These data suggested that β-catenin regulates RMP maintenance and partially triggers the AP by altering the transcription and expression of α2NKA in skeletal muscle cells.

α2NKA and β-Catenin Interact Physically in Muscle Cells

We found that β-catenin transfection regulated α2NKA (but not α1NKA) expression positively, so we investigated if there was an interaction between α2NKA and β-catenin. Co-immunoprecipitation studies showed that β-catenin could pull down α2NKA protein but not α1NKA protein in C2C12

cells (**Figure 5**). Furthermore, the abundance of α2NKA protein changed according to β-catenin abundance (**Figure 5A**). When compared with the control group (**Figure 5A**, fourth group), the interaction between α2NKA and β-catenin did not induce obvious changes in β-catenin siRNA normal control cells (**Figure 5A**, fifth group) or in pIRES2-ZsGreen1 cells (**Figure 5A**, sixth group). Compared with siRNA normal control cells (**Figure 5A**, fifth group), the interaction between α2NKA and β-catenin was weakened in β-catenin siRNA cells (**Figure 5A**, seventh group). Compared with pIRES2-ZsGreen1 cells (**Figure 5A**, sixth group), the interaction between α2NKA and β-catenin was enhanced in pIRES2-ZsGreen1-β-catenin cells (**Figure 5A**, eighth group). In C2C12 control cells (**Figure 5B**, fourth group), β-catenin could not pull down α1NKA protein.

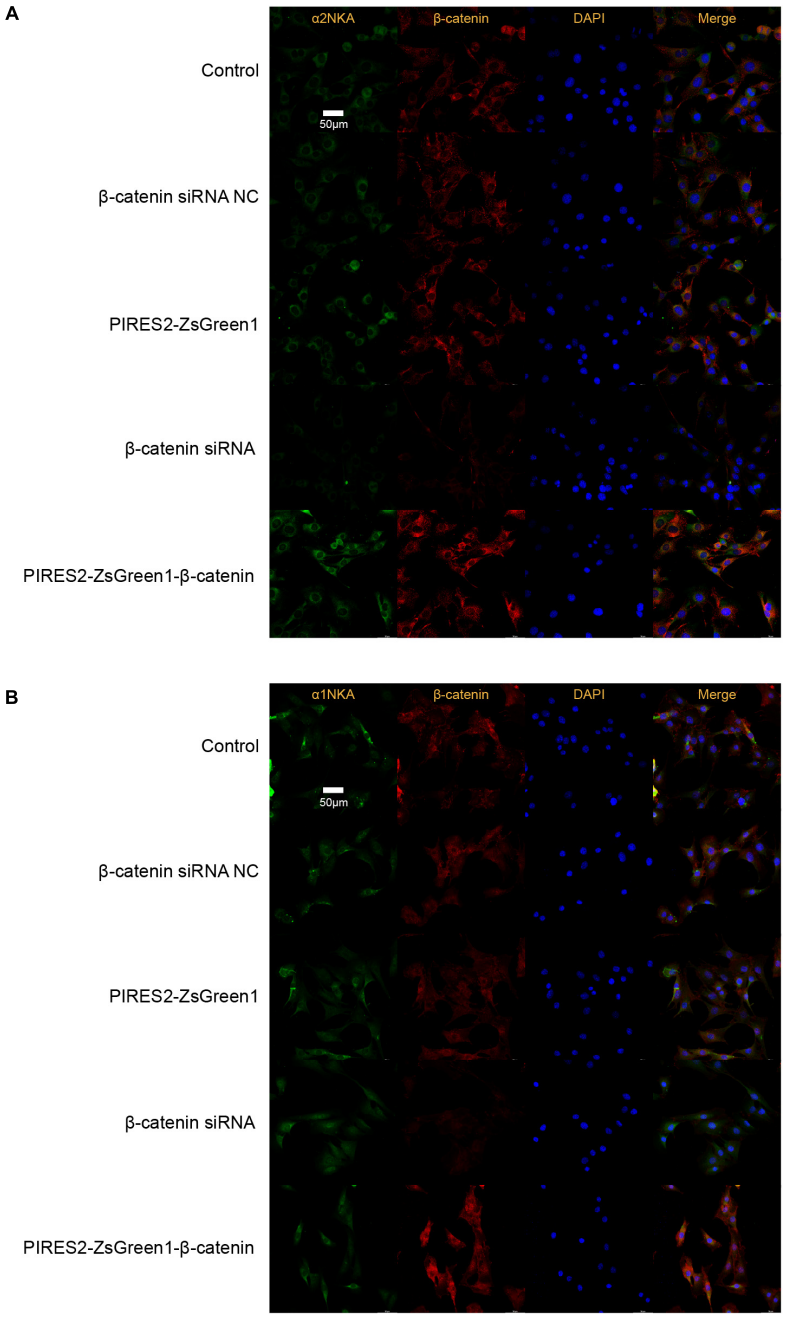

FIGURE 6 | Co-localization of β-catenin and NKA in C2C12 cells, and expression of β-catenin, α2NKA, and α1NKA in C2C12 cells. **(A)** The immunofluorescence intensity of β-catenin and α2NKA in muscle cells from five groups is as described in **Figure 3**. **(B)** The immunofluorescence intensity of β-catenin and α1NKA in muscle cells from five groups is as described in **Figure 3**. Scale bar, 50 μm. *n* = 3 per group.

Thus, in C2C12 siRNA control cells, C2C12 β-catenin over-expressed control cells, C2C12 β-catenin siRNA cells, and C2C12 β-catenin over-expressed cells, α1NKA protein could not be detected, and whether expression of β-catenin protein was decreased or increased was not known (**Figure 5B**). As expected, in cultured skeletal muscle cells, β-catenin co-localized with α2NKA, but it failed to do so with α1NKA. Thus, β-catenin could physically influence the abundance and activity of proteins (**Figures 5C,D**).

Confocal microscopy revealed that β-catenin protein co-localized with the proteins of α2NKA and α1NKA in C2C12 cells (**Figure 6**). In addition, α2NKA fluorescence was reduced in β-catenin knocked-down cells, and was increased in β-catenin over-expressed cells compared with control cells (**Figure 6B**). Otherwise, there was no change in the fluorescence of α1NKA protein compared with each C2C12 group (**Figure 6A**). These findings suggested that β-catenin could interact physically with α2NKA in muscle cells.

NKA Activity Was Changed Throughout β-Catenin Expression in Muscle Cells

The results mentioned above showed that β-catenin regulates the electrophysiologic properties of muscle cells and α2NKA expression. In addition, NKA activity has an important role in maintenance of the RMP in skeletal muscles. We determined whether deletion and increases in β-catenin expression affected NKA activity.

The percentage activity of NKA was not significantly different among the siRNA control group, PIRES2-ZsGreen1 group, or control group. When compared with siRNA control cells, the percentage activity of NKA was reduced significantly in the β-catenin knockdown group (**Figure 7**, $P = 0.0046$). Compared with the PIRES2-ZsGreen1 group, the percentage activity of NKA was increased significantly in the β-catenin over-expressed group (**Figure 7**, $P = 0.0002$).

Our results showed that interference with β-catenin inhibited NKA activity, whereas β-catenin over-expression promoted NKA activity.

α2NKA Content in the Cell Membrane Was Regulated Positively by β-Catenin in Muscle Cells

Sodium/potassium adenosine triphosphatase is an integral protein in cell membranes. We measured the content of α2NKA and α1NKA in cell membranes by cell-surface biotinylation.

Compared with the siRNA control group, the content of total α2NKA, α2NKA in the cell membrane, and α2NKA/total α2NKA protein in the cell membrane was reduced significantly in cells treated with β-catenin siRNA (**Figure 8A**, $P < 0.0001$). However, the content of total α2NKA, α2NKA in the cell membrane, and α2NKA/total α2NKA protein in the cell membrane was not significantly different between the two groups (**Figure 8B**). In addition, when compared with the pIRES2-ZsGreen 1 control plasmid-transfected group, the content of total α2NKA, α2NKA in the cell membrane, and α2NKA/total α2NKA protein in the cell membrane was increased significantly in β-catenin over-expressed cells (**Figure 8A**, $P = 0.0032$). The content of total α2NKA, α2NKA in the cell membrane, and α2NKA/total α2NKA protein in the cell membrane was not significantly different when comparing the two groups (**Figure 8B**).

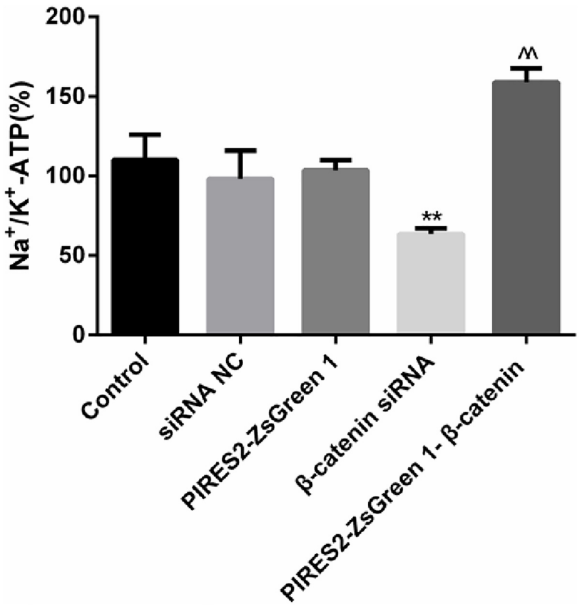

FIGURE 7 | NKA activity changed throughout β-catenin expression in C2C12 cells. The experiment was designed according to the five groups described above. Relative activity from five independent experiments. $^{**}P < 0.01$ vs. siRNA normal control group; $^{\wedge}P < 0.01$ vs. PIRES2-ZsGreen 1 group. Error bars represent SEM.

These data further demonstrated an interaction between β-catenin and α2NKA, but not with α1NKA. Over-expression of β-catenin could improve the expression and content of α2NKA in cell membranes. Furthermore, interference by β-catenin could reduce the expression and content of membrane-expressed α2NKA.

DISCUSSION

The present study demonstrated that β-catenin is involved in regulation of the RMP in skeletal muscle cells (Zhao et al., 2014). We revealed a novel mechanism underlying the early postnatal death of mice deficient in β-catenin in skeletal muscle. We also found that evoking the AP in skeletal muscle cells was affected by the deletion and over-expression of β-catenin.

We demonstrated that the protein expression and mRNA transcription of α2NKA were reduced if β-catenin in muscle cells was knocked down, and were increased if β-catenin in muscle cells was over-expressed. Furthermore, our results suggested that β-catenin and α2NKA interacted physically with muscle cells. Finally, we found that the activity of NKA and α2NKA content in the cell membrane was modulated positively by β-catenin.

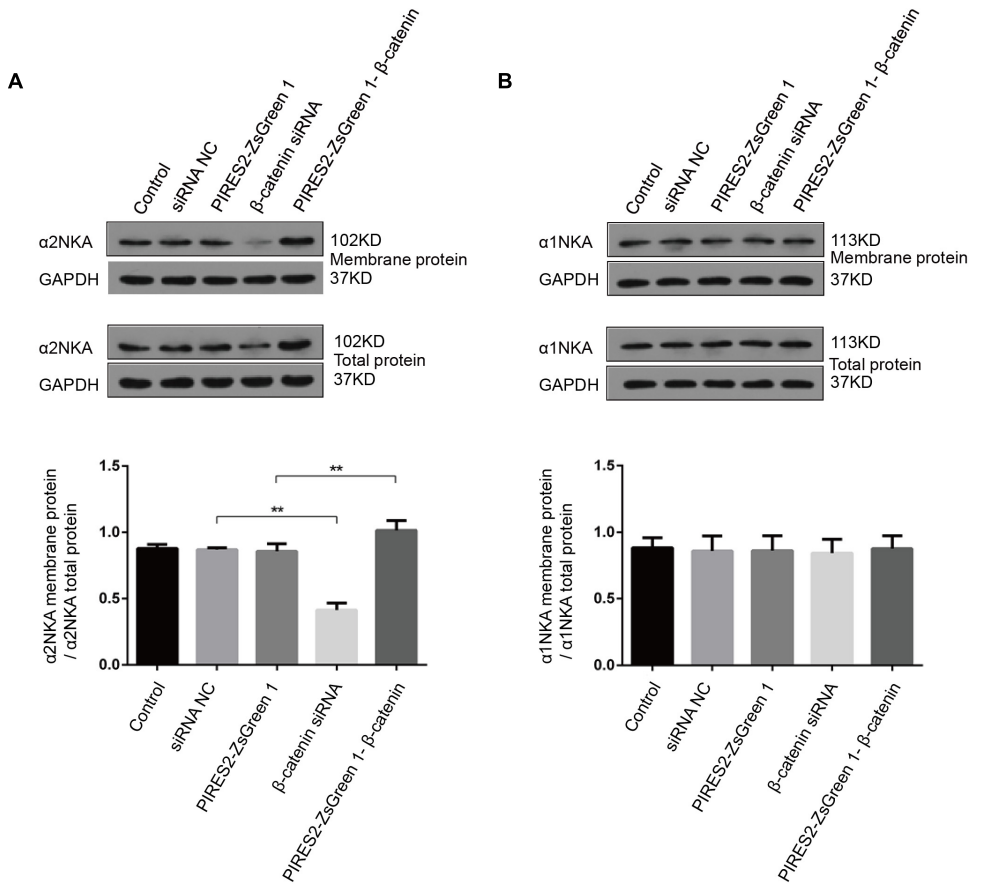

FIGURE 8 | α2NKA content in the cell membrane was regulated by β-catenin expression in C2C12 cells. **(A)** α2NKA content in the cell membrane and total α2NKA protein in cells from five groups as described above (upper two lines). Histograms show quantification of α2NKA/total α2NKA protein in cell membranes from five independent experiments (bottom line). **(B)** Content of α1NKA in the cell membrane and total α1NKA protein in cells from five groups as described above (upper two lines). Histograms show quantification of membrane α1NKA/total α1NKA protein in the cell membrane from five independent experiments (bottom line). $n = 3$ per group. $^{**}P < 0.01$ error bars represent SEM.

These data suggested a possible mechanism underlying the changes in the RMP and AP concordant with changes in β-catenin in muscle cells, and provided additional evidence supporting a role for β-catenin at the NMJ.

The Important Role of β-Catenin in Muscle Cells at NMJs

Increasing evidence suggests that Wnt/β-catenin signaling plays an important part in the development and maturation of the CNS (Wodarz and Nusse, 1998; Ciani and Salinas, 2005). Agrin-induced clustering of acetylcholine (ACh) receptors is inhibited by up- and down-regulation of presynaptic β-catenin at the NMJ (Zhang et al., 2007; Wang et al., 2008). Furthermore, studies have suggested that β-catenin in muscle cells (but not in neurons) is involved in the morphology and development of NMJs (Li et al., 2008). In addition, increasing β-catenin expression in muscle leads to increased clustering of ACh receptors (Wu et al., 2012). These data suggest that development of the NMJ requires an intricate balance of β-catenin activity in muscle tissue.

HSA-β-cat$^{flox(ex3)/+}$ mice (i.e., β-catenin gain of function in skeletal muscle cells) are viable at birth, whereas HSA-β-cat$^{-/-}$ mice (i.e., β-catenin deletion of function in skeletal muscle cells) die soon after birth (Li et al., 2008; Wu et al., 2012; Zhao et al., 2014). Furthermore, we have shown that knockdown and disruption of β-catenin depolarizes the RMP in skeletal muscle cells (Zhao et al., 2014). We showed that the RMP in C2C12 cells was refractory to change when we increased β-catenin expression. However, the AP could not be evoked in β-catenin knocked-down or over-expressed C2C12 cells irrespective of the extent of the applied electrical stimulation. These data suggest that deletion (but not an increase) in β-catenin induced depolarization of the RMP in muscle cells. In addition, evoking the AP requires a proper expression of β-catenin in muscle cells. These changes in the electrical properties of muscle cells might contribute to (or result from) abnormalities seen at the NMJ (Jurdana et al., 2009).

Mechanisms Underlying the Functional Change of Skeletal Muscle Regulated by β-Catenin

Scholars have shown that β-catenin deletion reduces the frequency of miniature endplate potentials (Li et al., 2008; Zhao et al., 2014). We found that the RMP in skeletal muscle cells was regulated by β-catenin expression. In addition, the AP could not be evoked by β-catenin knocked-down or over-expressed C2C12 cells. Changes in β-catenin expression in muscle cells might reduce the excitability of muscle cells (Clausen and Everts, 1991), which might modulate their contractility and fatigability. The mechanism by which β-catenin regulates the electrical properties of skeletal muscle warrants further study.

Critical Factors in the NKA-Mediated Regulation of the RMP and AP in Muscle Cells

Sodium/potassium adenosine triphosphatase is an integral protein in cell membranes because it maintains the RMP and regulates intracellular and extracellular osmotic balance (Bannett et al., 1984; Brodie et al., 1987). NKA is a heteromer that mainly contains an α-subunit and a glycosylated β-subunit. The α-subunit executes the transport and catalytic activity of NKA (Lingrel and Kuntzweiler, 1994). The β-subunit is also in modulating the affinity of Na^+ and K^+ (Eakle et al., 1995). There are four types of α-subunit, and they display tissue-specific expression: the α1 isoform appears in nearly all tissues; the α2 isoform is restricted to smooth muscle, skeletal muscle, and the brain; the α3 subunit is found in neurons and the ovaries; the α4 subunit is found in sperm (Shull et al., 1985, 1986; Sverdlov et al., 1987; Lingrel and Kuntzweiler, 1994).

The skeletal muscles of newborns mainly express the α2 and α1 subunits. Heiny et al. (2010) showed that electrogenic transport by NKA contributes −15 to −20 mV to the RMP of skeletal muscle cells. We found that α2NKA expression decreased if β-catenin expression was deficient, and was increased if β-catenin expression was increased. However, expression of α1NKA and Kv3.4 [both of which can regulate the RMP in skeletal muscle cells (Abbott et al., 2001)] was not altered significantly. We also demonstrated that NKA activity was influenced positively by changes in β-catenin expression in muscle cells.

Sodium/potassium adenosine triphosphatase is central to the excitability, contractility, and fatigability of muscles and nerves (Radzyukevich et al., 2013). In β-catenin knocked-down cells, the AP could not be evoked because cells had been depolarized and were fatigued. Canonical Wnt/β-catenin signaling has been shown to decrease the activity of Na^+ channels in cardiomyocytes (Wang et al., 2016). In β-catenin over-expressing cells, the generation of AP might be affected not only by α2NKA activity, but also by other ion channels. These data showed a likely mechanism by which β-catenin regulates the RMP and AP and, thus, affects the normal function of skeletal muscle cells.

β-Catenin Displays a Physical Interaction With α2NKA in Muscle Cells

β-Catenin has two major functions: (i) it acts as a co-transcriptional activator of T-cell factor/lymphocyte enhancer factor-1 and (ii) it regulates expression of target genes (Molenaar et al., 1996; Hecht et al., 2000). β-Catenin also participates in cell adherence (Nelson and Nusse, 2004; Drees et al., 2005). Our study indicated that the effect of β-catenin does not require interaction with α-catenin but, instead, it requires its transactivation domain, which suggests the involvement of transcriptional regulation, but not a cell-adhesion function (Wu et al., 2015). Our study showed that the mRNA expression of α2NKA changed in accordance with altered functional expression of β-catenin. In addition, studies have shown that endogenous NKA current is enhanced by β-catenin over-expression (Sopjani et al., 2010). Thus, β-catenin is likely to regulate α2NKA by its co-transcriptional function.

Furthermore, our results suggested that β-catenin and α2NKA exerted a physical interaction with C2C12 cells. We also revealed that NKA activity and α2NKA content in the cell membrane were modulated positively by β-catenin.

CONCLUSION

We provided further evidence that β-catenin is crucial for maintaining the RMP and evoking the AP in skeletal muscle cells. Skeletal muscle cells lacking β-catenin expression and with high expression of β-catenin showed decreased expression and increased expression of α2NKA, respectively. Hence, β-catenin appears to regulate the electrical properties and affects the normal functioning of skeletal muscle cells.

We showed that β-catenin could interact physically with α2NKA (but not with α1NKA) in muscle cells. β-Catenin could regulate the enzymatic activity and content of α2NKA in cell membranes. These results suggested that β-catenin (at least in part) regulates the RMP and AP in muscle cells, and does so by regulating α2NKA.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

FG, CZ, KJ, YY, and JS conceptualized and designed the study. CZ, YY, YZ, JS, LJ, GS, LX, and WZ acquired and analyzed the data. CZ, SM, and PJ drafted the text and prepared the figures. All authors approved the final version of the manuscript to be published.

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REFERENCES

Abbott, G. W., Butler, M. H., Bendahhou, S., Dalakas, M. C., Ptacek, L. J., and Goldstein, S. A. (2001). MiRP2 forms potassium channels in skeletal muscle with Kv3.4 and is associated with periodic paralysis. *Cell* 104, 217–231. doi: 10.1016/s0092-8674(01)00207-0

Bannett, R. R., Sampson, S. R., and Shainberg, A. (1984). Influence of thyroid hormone on some electrophysiological properties of developing rat skeletal muscle cells in culture. *Brain Res.* 294, 75–82. doi: 10.1016/0006-8993(84)91311-8

Batlle, E., Bacani, J., Begthel, H., Jonkheer, S., Gregorieff, A., van de Born, M., et al. (2005). EphB receptor activity suppresses colorectal cancer progression. *Nature* 435, 1126–1130. doi: 10.1038/nature03626

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., et al. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382, 638–642. doi: 10.1038/382638a0

Brodie, C., Bak, A., Shainberg, A., and Sampson, S. R. (1987). Role of Na-K ATPase in regulation of resting membrane potential of cultured rat skeletal myotubes. *J. Cell. Physiol.* 130, 191–198. doi: 10.1002/jcp.1041300204

Budnik, V., and Salinas, P. C. (2011). Wnt signaling during synaptic development and plasticity. *Curr. Opin. Neurobiol.* 21, 151–159. doi: 10.1016/j.conb.2010.12.002

Caricasole, A., Copani, A., Caruso, A., Caraci, F., Iacovelli, L., Sortino, M. A., et al. (2003). The Wnt pathway, cell-cycle activation and beta-amyloid: novel therapeutic strategies in Alzheimer's disease? *Trends Pharmacol. Sci.* 24, 233–238. doi: 10.1016/s0165-6147(03)00100-7

Chilosi, M., Poletti, V., Zamo, A., Lestani, M., Montagna, L., Piccoli, P., et al. (2003). Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am. J. Pathol.* 162, 1495–1502. doi: 10.1016/s0002-9440(10)64282-4

Ching, B., Woo, J. M., Hiong, K. C., Boo, M. V., Choo, C. Y., Wong, W. P., et al. (2015). Na+/K+-ATPase alpha-subunit (nkaalpha) isoforms and their mRNA expression levels, overall Nkaalpha protein abundance, and kinetic properties of Nka in the skeletal muscle and three electric organs of the electric eel, Electrophorus electricus. *PLoS One* 10:e0118352. doi: 10.1371/journal.pone.0118352

Ciani, L., and Salinas, P. C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat. Rev. Neurosci.* 6, 351–362. doi: 10.1038/nrn1665

Clausen, T., and Everts, M. E. (1991). K(+)-induced inhibition of contractile force in rat skeletal muscle: role of active Na(+)-K+ transport. *Am. J. Physiol.* 261, C799–C807.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* 127, 469–480. doi: 10.1016/j.cell.2006.10.018

DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85, 501–512. doi: 10.1016/s0092-8674(00)81251-9

Drees, F., Pokutta, S., Yamada, S., Nelson, W. J., and Weis, W. I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 123, 903–915. doi: 10.1016/j.cell.2005.09.021

Eakle, K. A., Lyu, R. M., and Farley, R. A. (1995). The influence of beta subunit structure on the interaction of Na+/K(+)-ATPase complexes with Na+. A chimeric beta subunit reduces the Na+ dependence of phosphoenzyme formation from ATP. *J. Biol. Chem.* 270, 13937–13947. doi: 10.1074/jbc.270.23.13937

Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P., et al. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85, 525–535. doi: 10.1016/s0092-8674(00)81253-2

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* 19, 1839–1850. doi: 10.1093/emboj/19.8.1839

Heiny, J. A., Kravtsova, V. V., Mandel, F., Radzyukevich, T. L., Benziane, B., Prokofiev, A. V., et al. (2010). The nicotinic acetylcholine receptor and the Na,K-ATPase alpha2 isoform interact to regulate membrane electrogenesis in skeletal muscle. *J. Biol. Chem.* 285, 28614–28626. doi: 10.1074/jbc.M110.150961

Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N. Engl. J. Med.* 351, 657–667. doi: 10.1056/nejmoa040258

Jurdana, M., Fumagalli, G., Grubic, Z., Lorenzon, P., Mars, T., and Sciancalepore, M. (2009). Neural agrin changes the electrical properties of developing human skeletal muscle cells. *Cell Mol. Neurobiol.* 29, 123–131. doi: 10.1007/s10571-008-9304-z

Li, J., Xie, D., Huang, J., Lv, F., Shi, D., Liu, Y., et al. (2015). Cold-Inducible RNA-binding protein regulates cardiac repolarization by targeting transient outward potassium channels. *Circ. Res.* 116, 1655–1659. doi: 10.1161/CIRCRESAHA.116.306287

Li, X. M., Dong, X. P., Luo, S. W., Zhang, B., Lee, D. H., Ting, A. K., et al. (2008). Retrograde regulation of motoneuron differentiation by muscle beta-catenin. *Nat. Neurosci.* 11, 262–268. doi: 10.1038/nn2053

Lingrel, J. B., and Kuntzweiler, T. (1994). Na+,K(+)-ATPase. *J. Biol. Chem.* 269, 19659–19662.

Lovestone, S., Killick, R., Di Forti, M., and Murray, R. (2007). Schizophrenia as a GSK-3 dysregulation disorder. *Trends Neurosci.* 30, 142–149. doi: 10.1016/j.tins.2007.02.002

Lyuksyutova, A. I., Lu, C. C., Milanesio, N., King, L. A., Guo, N., Wang, Y., et al. (2003). Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 302, 1984–1988. doi: 10.1126/science.1089610

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., et al. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 86, 391–399. doi: 10.1016/s0092-8674(00)80112-9

Nelson, W. J., and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303, 1483–1487. doi: 10.1126/science.1094291

Radzyukevich, T. L., Neumann, J. C., Rindler, T. N., Oshiro, N., Goldhamer, D. J., Lingrel, J. B., et al. (2013). Tissue-specific role of the Na,K-ATPase alpha2 isozyme in skeletal muscle. *J. Biol. Chem.* 288, 1226–1237. doi: 10.1074/jbc.M112.424663

Salinas, P. C. (2003). Synaptogenesis: Wnt and TGF-beta take centre stage. *Curr. Biol.* 13, R60–R62.

Salinas, P. C., and Zou, Y. (2008). Wnt signaling in neural circuit assembly. *Annu. Rev. Neurosci.* 31, 339–358. doi: 10.1146/annurev.neuro.31.060407.125649

Sanes, J. R., and Lichtman, J. W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* 2, 791–805. doi: 10.1038/35097557

Shull, G. E., Greeb, J., and Lingrel, J. B. (1986). Molecular cloning of three distinct forms of the Na+, K+-ATPase alpha-subunit from rat brain. *Biochemistry* 25, 8125–8132. doi: 10.1021/bi00373a001

Shull, G. E., Schwartz, A., and Lingrel, J. B. (1985). Amino-acid sequence of the catalytic subunit of the (Na+ + K+)ATPase deduced from a complementary DNA. *Nature* 316, 691–695. doi: 10.1038/316691a0

Singh, K. K., Ge, X., Mao, Y., Drane, L., Meletis, K., Samuels, B. A., et al. (2010). Dixdc1 is a critical regulator of DISC1 and embryonic cortical development. *Neuron* 67, 33–48. doi: 10.1016/j.neuron.2010.06.002

Sopjani, M., Alesutan, I., Wilmes, J., Dermaku-Sopjani, M., Lam, R. S., Koutsouki, E., et al. (2010). Stimulation of Na+/K+ ATPase activity and Na+ coupled glucose transport by beta-catenin. *Biochem. Biophys. Res. Commun.* 402, 467–470. doi: 10.1016/j.bbrc.2010.10.049

Sverdlov, E. D., Monastyrskaya, G. S., Broude, N. E., Ushkaryov Yu, A., Allikmets, R. L., Melkov, A. M., et al. (1987). The family of human Na+,K+-ATPase genes. No less than five genes and/or pseudogenes related to the alpha-subunit. *FEBS Lett.* 217, 275–278. doi: 10.1016/0014-5793(87)80677-4

Varallo, V. M., Gan, B. S., Seney, S., Ross, D. C., Roth, J. H., Richards, R. S., et al. (2003). Beta-catenin expression in Dupuytren's disease: potential role for cell-matrix interactions in modulating beta-catenin levels in vivo and in vitro. *Oncogene* 22, 3680–3684. doi: 10.1038/sj.onc.1206415

Wang, J., Ruan, N. J., Qian, L., Lei, W. L., Chen, F., and Luo, Z. G. (2008). Wnt/beta-catenin signaling suppresses Rapsyn expression and inhibits acetylcholine receptor clustering at the neuromuscular junction. *J. Biol. Chem.* 283, 21668–21675. doi: 10.1074/jbc.M709939200

Wang, N., Huo, R., Cai, B., Lu, Y., Ye, B., Li, X., et al. (2016). Activation of Wnt/beta-catenin signaling by hydrogen peroxide transcriptionally inhibits NaV1.5 expression. *Free Radic. Biol. Med.* 96, 34–44. doi: 10.1016/j.freeradbiomed.2016.04.003

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* 14, 59–88.

Wu, H., Barik, A., Lu, Y., Shen, C., Bowman, A., Li, L., et al. (2015). Slit2 as a beta-catenin/Ctnnb1-dependent retrograde signal for presynaptic differentiation. *eLife* 4:e07266. doi: 10.7554/eLife.07266

Wu, H., Lu, Y., Barik, A., Joseph, A., Taketo, M. M., Xiong, W. C., et al. (2012). beta-Catenin gain of function in muscles impairs neuromuscular junction formation. *Development* 139, 2392–2404. doi: 10.1242/dev.080705

Wu, H., Xiong, W. C., and Mei, L. (2010). To build a synapse: signaling pathways in neuromuscular junction assembly. *Development* 137, 1017–1033. doi: 10.1242/dev.038711

Yoshikawa, S., McKinnon, R. D., Kokel, M., and Thomas, J. B. (2003). Wnt-mediated axon guidance via the Drosophila Derailed receptor. *Nature* 422, 583–588. doi: 10.1038/nature01522

Zhang, B., Luo, S., Dong, X. P., Zhang, X., Liu, C., Luo, Z., et al. (2007). Beta-catenin regulates acetylcholine receptor clustering in muscle cells through interaction with rapsyn. *J. Neurosci.* 27, 3968–3973. doi: 10.1523/jneurosci.4691-06.2007

Zhao, C., Aviles, C., Abel, R. A., Almli, C. R., McQuillen, P., and Pleasure, S. J. (2005). Hippocampal and visuospatial learning defects in mice with a deletion of frizzled 9, a gene in the Williams syndrome deletion interval. *Development* 132, 2917–2927. doi: 10.1242/dev.01871

Zhao, C., Zhang, J., Li, K., Yang, J., Yu, H., Duan, S., et al. (2014). beta-Catenin regulates membrane potential in muscle cells by regulating the alpha2 subunit of Na,K-ATPase. *Eur. J. Neurosci.* 40, 2216–2224. doi: 10.1111/ejn.12564

Zong, Y., Zhang, B., Gu, S., Lee, K., Zhou, J., Yao, G., et al. (2012). Structural basis of agrin-LRP4-MuSK signaling. *Genes Dev.* 26, 247–258. doi: 10.1101/gad.180885.111

Zou, Y. (2004). Wnt signaling in axon guidance. *Trends Neurosci.* 27, 528–532. doi: 10.1016/j.tins.2004.06.015

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MINI REVIEW
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The Effects of Leptin on Glial Cells in Neurological Diseases

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It is known that various endocrine modulators, including leptin and ghrelin, have neuroprotective roles in neurological diseases. Leptin is a hormone produced by adipocytes and was originally identified as a gene related to obesity in mice. The leptin receptors in the hypothalamus are the main target for the homeostatic regulation of body weight. Recent studies have demonstrated that leptin receptors are also expressed in other regions of the central nervous system (CNS), such as the hippocampus, cerebral cortex, and spinal cord. Accordingly, these studies identified the involvement of leptin in the regulation of neuronal survival and neural development. Furthermore, leptin has been shown to have neuroprotective functions in animal models of neurological diseases and demyelination. These observations also suggest that dysregulation of leptin signaling may be involved in the association between neurodegeneration and obesity. In this review, we summarize novel functions of leptin in animal models of neurodegenerative diseases. Specifically, we focus on the emerging evidence for the role of leptin in non-neuronal cells in the CNS, including astrocytes, microglia, and oligodendrocytes. Understanding leptin-mediated neuroprotective signals and molecular mechanisms underlying remyelination will be helpful to establish therapeutic strategies against neurological diseases.

Keywords: leptin, neuron, astrocyte, microglia, oligodendrocyte

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INTRODUCTION

The endocrine hormone leptin was originally identified in 1994 and is composed of 167 amino acids (aa). Leptin is encoded by the obese (*ob*) gene (Halaas et al., 1995). Leptin is predominantly produced by adipocytes and exerts its function both peripherally and centrally. This adipokine regulates food intake, metabolism, and energy homeostasis by activating receptors in the central nervous system (CNS). Peripheral leptin binds to leptin receptors in the choroid plexus and is transported across the blood–brain barrier (BBB) into the CNS. Circulating leptin levels are correlated with the body fat mass. Leptin deficiency phenotypes were first reported in mice carrying the obese (*ob*/*ob*) mutation in 1950 (Ingalls et al., 1950), which was later identified as a recessive mutation of leptin (Zhang et al., 1994). Administration of leptin completely recovers the obesity phenotype of *ob*/*ob* mice and induces anorexia and body weight loss in normal mice, suggesting that leptin negatively regulates feeding behaviors and inhibits obesity (Halaas et al., 1995; Pelleymounter et al., 1995; Rentsch et al., 1995; Weigle et al., 1995). The association of leptin and leptin receptor

mutations with obesity has also been reported in humans (Montague et al., 1997; Clement et al., 1998; Strobel et al., 1998).

Leptin receptors (ObRs) are encoded by diabetes (*db*) genes and belong to the class I cytokine receptor superfamily. Six isoforms, ObRa-f, have been identified with identical N-terminal domains. Mice and rats have five ObR isoforms (ObRa-e in mice; ObRa-c, e, and f in rats), whereas humans have four (ObRa, b, c, and e) (Cioffi et al., 1996; Ahima and Flier, 2000; Wauman and Tavernier, 2011). All ObR isoforms except the ObRe isoform are expressed at the plasma membrane. The extracellular domain of all ObRs consists of two cytokine receptor homology (CRH) domains separated by an immunoglobulin-like (Ig-like) domain, followed by two membrane-proximal fibronectin type III (FN III) domains (**Figure 1**). The membrane-proximal CRH2 domain is essential for leptin binding (Fong et al., 1998). The two FN III domains are required for receptor activation and transduction of leptin signaling (Prokop et al., 2012). The length of the intracellular domain differs among isoforms. The long isoform of the mouse ObRb has a 302-aa intracellular domain (Uniprot[1]) and fully transduces leptin receptor signaling. The short isoforms (ObRa, c, d, and f) have intracellular domains of 30–40 aa and transduce only some of the ObRb-induced signals. Because ObRe has no transmembrane region, it is considered to serve as an antagonist for leptin signaling and to mediate leptin transport across the BBB (Tu et al., 2008), as well as to support the stability and availability of circulating leptin (Basharat et al., 2014). Leptin binding to ObRb increases Janus tyrosine kinase 2 (JAK2) phosphorylation, leading to the activation of various intracellular signaling molecules, including signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) (Chen et al., 2015; Fazolini et al., 2015).

Because leptin receptors are expressed not only in the hypothalamus, but also in other areas of the brain, including the cerebral cortex, hippocampus, ventral tegmental area, substantia nigra, medulla, and cerebellum, the role of leptin extends beyond its usual hypothalamic function. Leptin signaling is thought to play a role in processes such as learning, memory, and neuroprotection following neurodegeneration. In this review, we discuss the neuroprotective effects of leptin in CNS diseases. Accumulating studies have reported that leptin functions in non-neuronal cells in the CNS. Here, we also focus on the current understanding of glial leptin functions and signaling mechanisms.

EXPRESSION OF LEPTIN AND LEPTIN RECEPTORS

Leptin is produced mainly in adipose tissue, but is also produced in organs such as the stomach, mammary glands, placenta, skeletal muscle, heart, kidney, and brain (Bado et al., 1998; Smith-Kirwin et al., 1998; Morash et al., 1999; Wilkinson et al., 2000; Lin et al., 2011; Shan and Yeo, 2011; Tessier et al., 2013; Wang et al., 2014; Feijoo-Bandin et al., 2015). These findings suggest that leptin has many diverse functions. Accordingly, disturbance of leptin signaling affects various systems, including the cardiovascular, immune, reproductive, and nervous systems (Donato et al., 2011; Procaccini et al., 2012).

The long isoform of the leptin receptor ObRb is expressed in the hypothalamic nuclei, including the arcuate (ARC) nucleus, the dorsomedial (DMH) nucleus, the paraventricular (PVN) nucleus, the ventromedial hypothalamic (VMH) nucleus, and the lateral hypothalamic (LH) nucleus (Mercer et al., 1996; Schwartz et al., 1996; Friedman and Halaas, 1998; Yi et al., 2013). The expression of leptin in these areas implicates that leptin functions as a metabolic hormone, and many other reviews have already summarized the role of leptin in the hypothalamus (Ahima and Flier, 2000; Margetic et al., 2002; Leinninger, 2011). The expression of other ObR isoforms has also been found in extrahypothalamic nuclei in the cerebral cortex, hippocampus, ventral tegmental area, substantia nigra, medulla, and cerebellum (Elmquist et al., 1998; Figlewicz et al., 2003; Mutze et al., 2006). More specifically, ObRb is expressed in the neurogenic niche in the dentate gyrus of the adult hippocampus (Garza et al., 2008), suggesting that leptin signaling may play a role in the regulation of neurogenesis. The short isoforms ObRa and ObRc, but not ObRb, are abundantly expressed in the cerebral microvessels composing the BBB, suggesting that these receptors may be associated with leptin transport (Tartaglia et al., 1995; Bjorbaek et al., 1997; Golden et al., 1997).

Leptin receptor expression has been observed in various types of neurons, including glutamatergic, GABAergic, and dopaminergic neurons (Figlewicz et al., 2003; Vong et al., 2011; Xu et al., 2013; Yi et al., 2013). Therefore, neurons have been well-studied as the primary leptin targets in the CNS. However, leptin receptor mRNA expression has also been found in glial cells in normal CNS tissue such as the mouse spinal cord and the rat hypothalamus (Hsuchou et al., 2009b; **Figure 2**). The following section "Leptin Signaling in Glial Cells" focuses on the expression and function of leptin and leptin receptors in glial cells.

NEUROPROTECTIVE ROLES OF LEPTIN

Besides the inhibition of food intake, leptin signaling helps regulate neurogenesis, synaptogenesis, and neuronal excitability (Bouret, 2010; Paz-Filho et al., 2010; Arnoldussen et al., 2014). ObRs are highly expressed in synaptic regions of cultured hippocampal neurons (Shanley et al., 2002). Consistent with this synaptic expression pattern, leptin potently regulates hippocampal synaptic function (McGregor and Harvey, 2018). Furthermore, leptin regulates neurogenesis during the development of the CNS. These functions suggest that leptin may have protective functions in neurological diseases and insults. The association of increased leptin levels in obesity and increased risk of neurodegenerative diseases in obese

[1] www.uniprot.org/uniprot/P48356

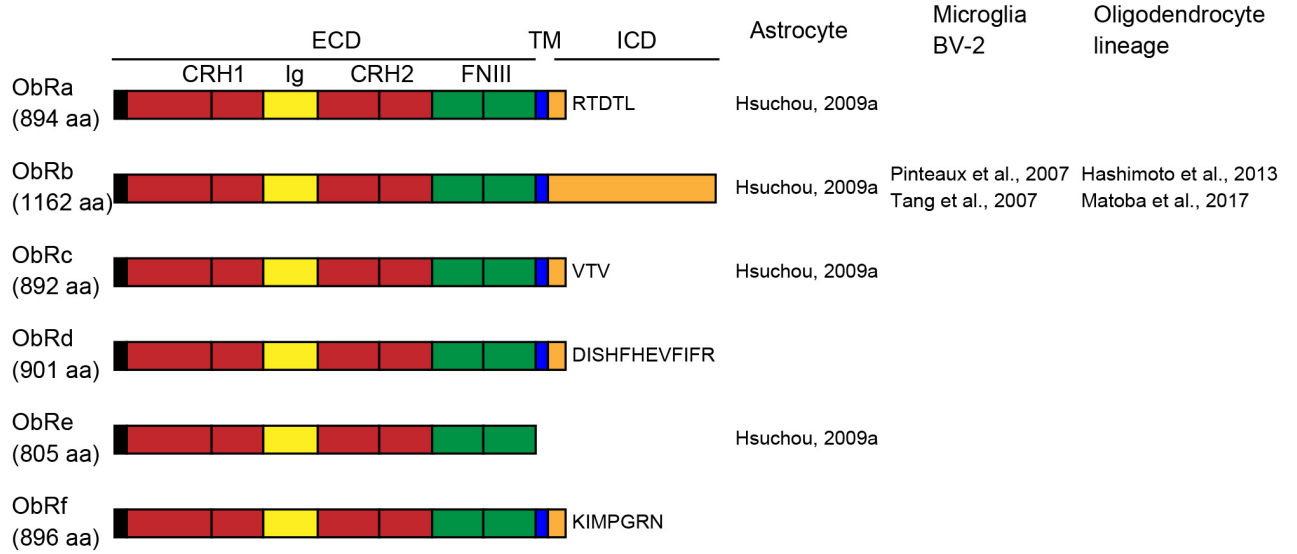

FIGURE 1 | Schematic representation of leptin receptor isoforms and their domains and distribution of leptin receptor (ObR) isoforms in glial cells. The extracellular domain (ECD) of ObRs is composed of two cytokine receptor homology (CRH, red) domains, separated by an immunoglobulin-like (Ig-like, yellow) domain, followed by two membrane-proximal fibronectin type III (FN III, green) domains. ObRe lacks the transmembrane (TM, blue) and intracellular (ICD, orange) domains. The length of the intracellular domains differs among isoforms. aa, amino acids.

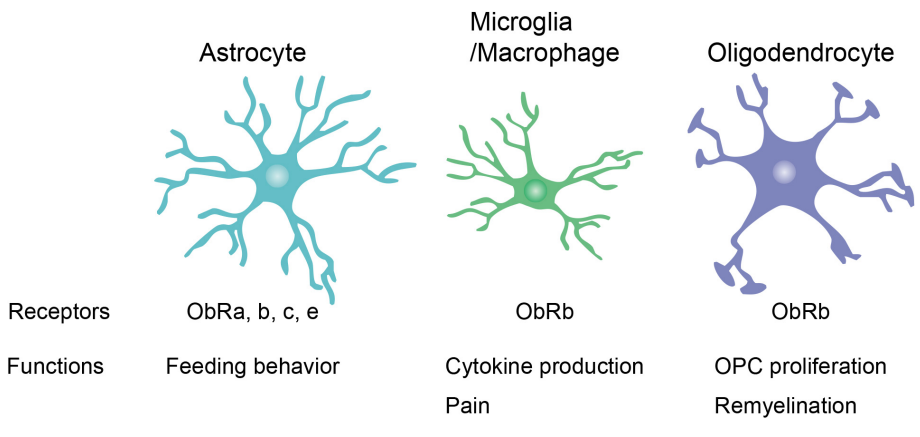

FIGURE 2 | Leptin receptor (ObR) expression and function in glial cells. Reported expression of ObR isoforms and functions of ObRs in glial cells are summarized. OPC, oligodendrocyte precursor cell.

individuals has been widely discussed (Forny-Germano et al., 2018; Lloret et al., 2019). In animal disease models, neurotoxin 1-methyl-4-phenyl-1, 2, 4, 5-tetrahydropyridine (MPTP)- or 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease (PD) model show greater neurodegeneration with obesity compared to littermates fed a normal diet (Choi et al., 2005; Morris et al., 2010). Midlife adiposity is associated with the risk of PD (Abbott et al., 2002; Procaccini et al., 2016). Furthermore, midlife obesity is also linked to a higher risk of developing cognitive disorders, including Alzheimer's disease (Kivipelto et al., 2005; Rosengren et al., 2005; Whitmer et al., 2005). It has been reported that plasma levels of leptin are decreased in PD and Huntington's disease (Pratley et al., 2000; Evidente et al., 2001; Popovic et al., 2004). Leptin also shows the protective effects on pediatric neurological diseases. The human seizures frequently occur early in life. Zn^{2+} is one of the most abundant divalent metal ions in the CNS (Chimienti et al., 2003; Devirgiliis et al., 2007), and zinc transporters show dynamic expression pattern in animal seizure model (Ni et al., 2010, 2011a,b). Zinc transporters ZnT3, ZnT4, and ZIP7 are upregulated in cultured HT22 cells and flurothyl-induced recurrent neonatal seizures, and these altered expressions were reversed by leptin treatment (Jin et al., 2018; Li et al., 2018). Leptin suppresses aberrant mossy fiber sprouting and hippocampal-related cognitive impairment in rat developmental seizure models (Li et al., 2018; Ni et al., 2018). Thus, there is emerging evidence for the neuroprotective roles of leptin in neurological diseases. As there are systematic reviews on the neuroprotective effects of leptin in CNS diseases

(Signore et al., 2008; Tang, 2008; Davis et al., 2014; Li et al., 2016), here we focus on the role of leptin signaling in non-neuronal cells in the CNS, including astrocytes, microglia, and oligodendrocytes.

LEPTIN SIGNALING IN GLIAL CELLS

The Role of Leptin in Astrocytes

Astrocytes are glial cells involved in homeostatic control and neuroprotection (Verkhratsky and Nedergaard, 2018). Astrocytes express several ObR isoforms, including ObRa, ObRb, ObRc, and ObRe (Hsuchou et al., 2009a). Primary cultured astrocytes prepared from the mouse hypothalamus mainly express the isoforms ObRa and ObRb. In addition to these isoforms, ObRc and ObRe are expressed in cells prepared from the mouse hippocampus. ObR mRNA expression has also been detected in astrocytes in the rat hypothalamus via fluorescent *in situ* hybridization and immunohistochemistry (Hsuchou et al., 2009b). ObRs are expressed by glial fibrillary acidic protein (GFAP)-positive cells in the hypothalamus. There have been reports on regional differences of the cell types expressing ObRs. In the ARC, ObR immunofluorescence is predominantly observed in neurons, whereas it is mainly detected in cells with a morphology resembling astrocytes in the DMH hypothalamus (Pan et al., 2008). Additionally, in the ARC, a specialized subpopulation of astrocytes that shows intense immunoreactivity for brain fatty acid-binding protein, which is considered to be involved in the regulation of feeding, is in close proximity to leptin-sensitive neurons (Young, 2002).

It has been reported that upregulation of astrocytic ObRs is involved in adult-onset obesity (Pan and Kastin, 2007; Pan et al., 2008; Hsuchou et al., 2009a). The agouti viable yellow (A^{vy}) mouse has a spontaneous mutation consisting of an insertion of a retrotransposon in the promoter region of the gene encoding the agouti signaling protein, which induces ectopic overexpression of the agouti-related protein (AgRP), leading to antagonism of the melanocortin receptors (MCRs). This produces two prominent phenotypes in the A^{vy} mouse. The first is dysregulation of MCR-1 signaling in the skin, which increases pheomelanin synthesis and results in the agouti coat. The second phenotype results from defects in MCR-4 signaling, which causes hyperphagia and obesity (Dickies, 1962; Wolff, 1965; Wolff et al., 1986; Shimizu et al., 1989; Duhl et al., 1994; Wolff and Whittaker, 2005). The A^{vy} mouse shows increased ObR expression in astrocytes and increased ObRb mRNA in microvessels, which may play crucial roles in the reduced apparent influx of leptin from the blood to the brain (Pan and Kastin, 2007). Furthermore, inhibition of astrocytes in A^{vy} mice via intracerebroventricular treatment with the astrocyte-specific metabolic inhibitor fluorocitrate reduces the uptake of fluorescently labeled leptin into astrocytes and attenuates leptin-induced phosphorylation of STAT3, whereas neuronal uptake in the ARC and DMH hypothalamus is increased (Pan et al., 2011). These observations suggest opposite roles of astrocytic leptin signaling in neurons of the A^{vy} mouse. ObR-positive astrocytes are also increased in diet-induced obesity in adult B6 mice (Hsuchou et al., 2009a).

Leptin has also been reported to affect astrocyte morphology and synaptic protein levels in the hypothalamus, and it can rapidly induce synaptic changes in feeding circuits of *ob*/*ob* mice (Pinto et al., 2004; Horvath et al., 2010; Garcia-Caceres et al., 2011). Chronic intracerebroventricular leptin treatment modulates the astrocyte-specific glucose transporters (GLUT)-2, GLUT-3, and glutamate transporter (GLAST) (Fuente-Martin et al., 2012) in the hypothalamus, suggesting that leptin signaling in astrocytes may influence glutamate levels in the synaptic cleft, as well as energy homeostasis. Conditional deletion of astrocytic leptin receptors using Cre-loxP systems (GFAP-Cre; leptin receptor flox/flox) alters the astrocyte morphology and increases the number of synapses on proopiomelanocortin (POMC) and AgRP neurons, which are involved in the hypothalamic feeding control (Kim et al., 2014). Deletion of ObR in astrocytes diminishes leptin-induced anorexia and enhances fasting- or ghrelin-induced hyperphagia. These observations suggest that astrocytic leptin signaling actively controls feeding behavior.

The Role of Leptin in Microglia and Macrophages

The murine BV-2 cell line and rat microglia express both the long and short isoforms of the leptin receptor (Pinteaux et al., 2007; Tang et al., 2007). Semi-quantitative RT-PCR showed that ObRb mRNA expression is elevated in microglia compared to that in astrocytes and neurons, whereas ObRa mRNA expression levels are higher in astrocytes and microglia compared to that in neurons (Pinteaux et al., 2007).

Leptin has been reported to have an effect on cytokine production in microglia/macrophages (Pinteaux et al., 2007; Tang et al., 2007; Lafrance et al., 2010). Leptin activates ObRb in rat primary microglial cells and induces interleukin (IL)-1β production via STAT3 activation (Pinteaux et al., 2007). In primary hypothalamic microglia, leptin treatment induces IL-1β and tumor necrosis factor-α (TNF-α) but not ionized calcium binding adaptor molecule 1 (Iba1) expression, and disruption of leptin signaling in *ob*/*ob* or *db*/*db* mice alters the expression of genes involved in microglial functions (Gao et al., 2014). Pretreatment of rat brain microglial cells with leptin increases the lipopolysaccharide (LPS)-induced expression of IL-1β, TNF-α, and chemokines such as cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) (Lafrance et al., 2010). Leptin treatment has also been shown to increase IL-6 production in BV-2 cells via the leptin receptor/IRS-1/PI3K/Akt/NF-κB/p300 signaling pathway, as leptin-induced IL-6 production is diminished by inhibitors for these signaling molecules (Tang et al., 2007). Plasma leptin levels are positively correlated with the number of Iba1-positive cells in the ARC of mice fed with a high saturated fat diet, raising the possibility that overnutrition-dependent inflammation is associated with leptin signaling in microglia (Andre et al., 2017). Furthermore, deletion of

the leptin receptor in myeloid cells, including microglia and macrophages, by crossing Cx3cr1-Cre mice with ObR-loxP mice disrupts hypothalamic neuronal circuits, that regulate metabolism, and increases body weight (Gao et al., 2018). In this mouse, the number of POMC neurons in the ARC and alpha-melanocyte-stimulating hormone (α-MSH) projections from the ARC to the PVN are decreased, concurrent with the presence of less ramified microglia with impaired phagocytic capacity in the PVN. These observations suggest that the microglial ObR mediates, at least in part, the action of leptin in regulating inflammation.

Leptin has been shown to be involved in the activation of microglia and macrophages in the context of neuropathic pain. Microglia have been known to play a critical role in mechanisms of neuropathic pain (Coull et al., 2005; Koizumi et al., 2007; Beggs and Salter, 2010; Yasui et al., 2014; Inoue and Tsuda, 2018). Microglial activation in the spinal cord is involved in nerve injury-induced pain hypersensitivity, which characterizes neuropathic pain. Intrathecal administration of a leptin antagonist prevents the development of thermal hyperalgesia and mechanical allodynia in the rat chronic constriction injury (CCI) model (Lim et al., 2009). Leptin-deficiency in mice also abolishes CCI-induced neuropathic pain behaviors. Expression of both leptin and ObRb is increased in the ipsilateral dorsal horn of the spinal cord after injury. In *in vitro* organotypic lumbar spinal cord cultures, leptin treatment increases OX-42 (CD11b/c)-positive cells and upregulates IL-1β, as well as the NR1 subunit of the NMDA receptor, via the JAK/STAT pathway, suggesting that microglia are a possible source of IL-1β. In the partial sciatic nerve ligation (PSL, Seltzer model) model (Seltzer et al., 1990), leptin expression is increased in adipocytes and in the epineurium of the injured sciatic nerve. However, PSL-induced tactile allodynia does not develop in the leptin-deficient *ob/ob* mouse (Maeda et al., 2009). In this model, macrophages are recruited to the perineurium of the sciatic nerve and express the leptin receptor, and leptin stimulates macrophages through the JAK–STAT pathway. Treating the macrophage cell line J774A.1 with leptin induces mRNA expression of matrix metalloproteinase-9 (MMP-9) and inducible nitric oxide synthase (iNOS), which are molecules known to underlie allodynia development via phosphorylated STAT3. These findings suggest that leptin may be associated with the development of neuropathic pain via activation of macrophages.

By contrast, it has been reported that leptin reduces microglial activation and contributes to functional recovery in the rat spinal cord injury (SCI) model (Fernandez-Martos et al., 2012). Both leptin and ObRb mRNA expression levels are increased after SCI. Intraparenchymal injection of leptin enhances functional motor recovery and prevents neuropathic pain after SCI. Leptin administration decreases the expression of inflammatory genes and decreases the area of Iba1-positive microglia/macrophages after SCI. Thus, leptin-controlled inflammatory modulation in microglia shows diverse effects and depends on the animal model. Further investigations using cell-type-specific deletions of leptin and leptin receptors will be helpful in clarifying the role of leptin signaling in microglia.

The Role of Leptin in Oligodendrocytes

Expression of ObRs is also detected in oligodendrocyte lineage cells. ObRb is expressed in NG-2 positive oligodendrocyte precursor cells (OPCs) but not in A2B5-positive oligodendrocyte progenitors in the postnatal day (P)7 mouse cerebrum. By P14, ObRb expression is detected in O4-positive mature oligodendrocytes and OPCs (Hashimoto et al., 2013), indicating that ObRb expression depends on the differentiation status of the cells. Embryonic day (E)18 *ob/ob* mice show increased numbers of OPCs compared to wild-type mice, suggesting that leptin can attenuate oligodendrocyte development in the mouse embryonic cerebral cortex (Udagawa et al., 2006). By contrast, myelin basic protein (MBP) mRNA expression is at P14 and P28 lower in *ob*/*ob* mice than in wild-type mice, and leptin-treated *ob/ob* mice exhibit increased myelination, suggesting that leptin regulates differentiation and/or myelination of oligodendrocytes (Hashimoto et al., 2013). Obesity also seems to be associated with oligodendrocyte maturation and myelination. High fat diet-induced adult-onset obesity may reduce myelin thickness and inhibit oligodendrocyte maturation. OPC differentiation following focal white matter stroke is impaired in obese mice, while their platelet-derived growth factor receptor alpha (PDGFRα)-positive OPC response in the early phase after stroke is exaggerated.

Immunohistochemistry in spinal cord tissue showed that ObRb protein expression is observed in PDGFRα-positive OPCs, GFAP-positive astrocytes, and NeuN-positive neurons (Matoba et al., 2017). Demyelination induced by lysophosphatidylcholine (LPC) injection does not affect ObRb expression in these CNS cells. However, intrathecal administration of leptin-neutralizing antibodies decreases bromodeoxyuridine (BrdU)- and PDGFRα-double-positive proliferating OPCs after LPC injection and induces a reduction in myelin formation, as characterized by a decrease in MBP-positive area, suggesting that leptin signaling supports an increase in OPC proliferation and remyelination in LPC-induced demyelination. In the SCI model, immunohistochemistry showed that ObRb expression is increased in APC-positive oligodendrocytes and, to a lesser extent, in GFAP-positive astrocytes, OX-42-positive microglia/macrophages, and NeuN-positive neurons 24 h after injury (Fernandez-Martos et al., 2012). Leptin administration also increases myelin preservation caudal to the lesion epicenter. Although whether expression of ObRb increases differs from the injury model, leptin appears to be increased in the spinal cord after both demyelination and injury. Thus, these studies suggest that leptin signaling in oligodendrocyte lineage cells may contribute to cell and tissue repair after injury or other pathological conditions in the CNS.

CONCLUSION

Growing evidence from basic research supports that glial functions control the metabolism (Garcia-Caceres et al., 2019). Beyond its roles in food intake and energy homeostasis, leptin has been found to have protective roles in the CNS. Accumulating results from studies on the neuroprotective functions of

leptin signaling in animal models of neurodegeneration and observations in individuals with neurodegenerative disorders support the hypothesis that leptin and leptin receptors are potential therapeutic targets in neurodegenerative diseases. However, there might be some limitations to the therapeutic use of leptin. For example, glial leptin can have both beneficial and detrimental effects depending on the cell type and the context of the neurological disease or insult. To enhance the neuroprotective functions and to prevent the detrimental processes and unwanted effects, it will be important to further our understanding of leptin signaling in glial cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Abbott, R. D., Ross, G. W., White, L. R., Nelson, J. S., Masaki, K. H., Tanner, C. M., et al. (2002). Midlife adiposity and the future risk of Parkinson's disease. *Neurology* 59, 1051–1057. doi: 10.1212/wnl.59.7.1051

Ahima, R. S., and Flier, J. S. (2000). Leptin. *Annu. Rev. Physiol.* 62, 413–437. doi: 10.1146/annurev.physiol.62.1.413

Andre, C., Guzman-Quevedo, O., Rey, C., Remus-Borel, J., Clark, S., Castellanos-Jankiewicz, A., et al. (2017). Inhibiting microglia expansion prevents diet-induced hypothalamic and peripheral inflammation. *Diabetes* 66, 908–919. doi: 10.2337/db16-0586

Arnoldussen, I. A., Kiliaan, A. J., and Gustafson, D. R. (2014). Obesity and dementia: adipokines interact with the brain. *Eur. Neuropsychopharmacol.* 24, 1982–1999. doi: 10.1016/j.euroneuro.2014.03.002

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bortoluzzi, M. N., et al. (1998). The stomach is a source of leptin. *Nature* 394, 790–793. doi: 10.1038/29547

Basharat, S., Parker, J. A., Murphy, K. G., Bloom, S. R., Buckingham, J. C., and John, C. D. (2014). Leptin fails to blunt the lipopolysaccharide-induced activation of the hypothalamic-pituitary-adrenal axis in rats. *J. Endocrinol.* 221, 229–234. doi: 10.1530/JOE-13-0249

Beggs, S., and Salter, M. W. (2010). Microglia-neuronal signalling in neuropathic pain hypersensitivity 2.0. *Curr. Opin. Neurobiol.* 20, 474–480. doi: 10.1016/j.conb.2010.08.005

Bjorbaek, C., Uotani, S., Da Silva, B., and Flier, J. S. (1997). Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J. Biol. Chem.* 272, 32686–32695. doi: 10.1074/jbc.272.51.32686

Bouret, S. G. (2010). Neurodevelopmental actions of leptin. *Brain Res.* 1350, 2–9. doi: 10.1016/j.brainres.2010.04.011

Chen, Y., Wu, R., Chen, H. Z., Xiao, Q., Wang, W. J., He, J. P., et al. (2015). Enhancement of hypothalamic STAT3 acetylation by nuclear receptor Nur77 dictates leptin sensitivity. *Diabetes* 64, 2069–2081. doi: 10.2337/db14-1206

Chimienti, F., Aouffen, M., Favier, A., and Seve, M. (2003). Zinc homeostasis-regulating proteins: new drug targets for triggering cell fate. *Curr. Drug Targets* 4, 323–338. doi: 10.2174/1389450033491082

Choi, J. Y., Jang, E. H., Park, C. S., and Kang, J. H. (2005). Enhanced susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in high-fat diet-induced obesity. *Free Radic. Biol. Med.* 38, 806–816. doi: 10.1016/j.freeradbiomed.2004.12.008

Cioffi, J. A., Shafer, A. W., Zupancic, T. J., Smith-Gbur, J., Mikhail, A., Platika, D., et al. (1996). Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat. Med.* 2, 585–589. doi: 10.1038/nm0596-585

Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., et al. (1998). A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 392, 398–401. doi: 10.1038/32911

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., et al. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438, 1017–1021. doi: 10.1038/nature04223

Davis, C., Mudd, J., and Hawkins, M. (2014). Neuroprotective effects of leptin in the context of obesity and metabolic disorders. *Neurobiol. Dis.* 72(Pt A), 61–71. doi: 10.1016/j.nbd.2014.04.012

Devirgiliis, C., Zalewski, P. D., Perozzi, G., and Murgia, C. (2007). Zinc fluxes and zinc transporter genes in chronic diseases. *Mutat. Res.* 622, 84–93. doi: 10.1016/j.mrfmmm.2007.01.013

Dickies, M. M. (1962). A new viable yellow mutation in the house mouse. *J. Hered.* 53, 84–86. doi: 10.1093/oxfordjournals.jhered.a107129

Donato, J. Jr., Cravo, R. M., Frazao, R., and Elias, C. F. (2011). Hypothalamic sites of leptin action linking metabolism and reproduction. *Neuroendocrinology* 93, 9–18. doi: 10.1159/000322472

Duhl, D. M., Vrieling, H., Miller, K. A., Wolff, G. L., and Barsh, G. S. (1994). Neomorphic agouti mutations in obese yellow mice. *Nat. Genet.* 8, 59–65. doi: 10.1038/ng0994-59

Elmquist, J. K., Bjorbaek, C., Ahima, R. S., Flier, J. S., and Saper, C. B. (1998). Distributions of leptin receptor mRNA isoforms in the rat brain. *J. Comp. Neurol.* 395, 535–547. doi: 10.1002/(sici)1096-9861(19980615)395:4<535::aid-cne9>3.3.co;2-7

Evidente, V. G., Caviness, J. N., Adler, C. H., Gwinn-Hardy, K. A., and Pratley, R. E. (2001). Serum leptin concentrations and satiety in Parkinson's disease patients with and without weight loss. *Mov. Disord.* 16, 924–927. doi: 10.1002/mds.1165

Fazolini, N. P., Cruz, A. L., Werneck, M. B., Viola, J. P., Maya-Monteiro, C. M., and Bozza, P. T. (2015). Leptin activation of mTOR pathway in intestinal epithelial cell triggers lipid droplet formation, cytokine production and increased cell proliferation. *Cell Cycle* 14, 2667–2676. doi: 10.1080/15384101.2015.1041684

Feijoo-Bandin, S., Portoles, M., Rosello-Lleti, E., Rivera, M., Gonzalez-Juanatey, J. R., and Lago, F. (2015). 20 years of leptin: role of leptin in cardiomyocyte physiology and physiopathology. *Life Sci.* 140, 10–18. doi: 10.1016/j.lfs.2015.02.016

Fernandez-Martos, C. M., Gonzalez, P., and Rodriguez, F. J. (2012). Acute leptin treatment enhances functional recovery after spinal cord injury. *PLoS One* 7:e35594. doi: 10.1371/journal.pone.0035594

Figlewicz, D. P., Evans, S. B., Murphy, J., Hoen, M., and Baskin, D. G. (2003). Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. *Brain Res.* 964, 107–115. doi: 10.1016/s0006-8993(02)04087-8

Fong, T. M., Huang, R. R., Tota, M. R., Mao, C., Smith, T., Varnerin, J., et al. (1998). Localization of leptin binding domain in the leptin receptor. *Mol. Pharmacol.* 53, 234–240. doi: 10.1124/mol.53.2.234

Forny-Germano, L., De Felice, F. G., and Vieira, M. (2018). The role of leptin and adiponectin in obesity-associated cognitive decline and Alzheimer's disease. *Front. Neurosci.* 12:1027. doi: 10.3389/fnins.2018.01027

Friedman, J. M., and Halaas, J. L. (1998). Leptin and the regulation of body weight in mammals. *Nature* 395, 763–770. doi: 10.1038/27376

Fuente-Martin, E., Garcia-Caceres, C., Granado, M., De Ceballos, M. L., Sanchez-Garrido, M. A., Sarman, B., et al. (2012). Leptin regulates glutamate and glucose transporters in hypothalamic astrocytes. *J. Clin. Invest.* 122, 3900–3913. doi: 10.1172/JCI64102

Gao, Y., Ottaway, N., Schriever, S. C., Legutko, B., Garcia-Caceres, C., De La Fuente, E., et al. (2014). Hormones and diet, but not body weight, control hypothalamic microglial activity. *Glia* 62, 17–25. doi: 10.1002/glia.22580

Gao, Y., Vidal-Itriago, A., Milanova, I., Korpel, N. L., Kalsbeek, M. J., Tom, R. Z., et al. (2018). Deficiency of leptin receptor in myeloid cells disrupts hypothalamic metabolic circuits and causes body weight increase. *Mol. Metab.* 7, 155–160. doi: 10.1016/j.molmet.2017.11.003

Garcia-Caceres, C., Balland, E., Prevot, V., Luquet, S., Woods, S. C., Koch, M., et al. (2019). Role of astrocytes, microglia, and tanycytes in brain control of systemic metabolism. *Nat. Neurosci.* 22, 7–14. doi: 10.1038/s41593-018-0286-y

Garcia-Caceres, C., Fuente-Martin, E., Burgos-Ramos, E., Granado, M., Frago, L. M., Barrios, V., et al. (2011). Differential acute and chronic effects of leptin on hypothalamic astrocyte morphology and synaptic protein levels. *Endocrinology* 152, 1809–1818. doi: 10.1210/en.2010-1252

Garza, J. C., Guo, M., Zhang, W., and Lu, X. Y. (2008). Leptin increases adult hippocampal neurogenesis in vivo and in vitro. *J. Biol. Chem.* 283, 18238–18247. doi: 10.1074/jbc.M800053200

Golden, P. L., Maccagnan, T. J., and Pardridge, W. M. (1997). Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. *J. Clin. Invest.* 99, 14–18. doi: 10.1172/JCI119125

Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., et al. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269, 543–546. doi: 10.1126/science.7624777

Hashimoto, R., Matsumoto, A., Udagawa, J., Hioki, K., and Otani, H. (2013). Effect of leptin administration on myelination in ob/ob mouse cerebrum after birth. *Neuroreport* 24, 22–29. doi: 10.1097/WNR.0b013e32835ba875

Horvath, T. L., Sarman, B., Garcia-Caceres, C., Enriori, P. J., Sotonyi, P., Shanabrough, M., et al. (2010). Synaptic input organization of the melanocortin system predicts diet-induced hypothalamic reactive gliosis and obesity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14875–14880. doi: 10.1073/pnas.1004282107

Hsuchou, H., He, Y., Kastin, A. J., Tu, H., Markadakis, E. N., Rogers, R. C., et al. (2009a). Obesity induces functional astrocytic leptin receptors in hypothalamus. *Brain* 132, 889–902. doi: 10.1093/brain/awp029

Hsuchou, H., Pan, W., Barnes, M. J., and Kastin, A. J. (2009b). Leptin receptor mRNA in rat brain astrocytes. *Peptides* 30, 2275–2280. doi: 10.1016/j.peptides.2009.08.023

Ingalls, A. M., Dickie, M. M., and Snell, G. D. (1950). Obese, a new mutation in the house mouse. *J. Hered.* 41, 317–318. doi: 10.1093/oxfordjournals.jhered.a106073

Inoue, K., and Tsuda, M. (2018). Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nat. Rev. Neurosci.* 19, 138–152. doi: 10.1038/nrn.2018.2

Jin, M. F., Ni, H., and Li, L. L. (2018). leptin maintained zinc homeostasis against glutamate-induced excitotoxicity by preventing mitophagy-mediated mitochondrial activation in HT22 hippocampal neuronal cells. *Front. Neurol.* 9:322. doi: 10.3389/fneur.2018.00322

Kim, J. G., Suyama, S., Koch, M., Jin, S., Argente-Arizon, P., Argente, J., et al. (2014). Leptin signaling in astrocytes regulates hypothalamic neuronal circuits and feeding. *Nat. Neurosci.* 17, 908–910. doi: 10.1038/nn.3725

Kivipelto, M., Ngandu, T., Fratiglioni, L., Viitanen, M., Kareholt, I., Winblad, B., et al. (2005). Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. *Arch. Neurol.* 62, 1556–1560. doi: 10.1001/archneur.62.10.1556

Koizumi, S., Shigemoto-Mogami, Y., Nasu-Tada, K., Shinozaki, Y., Ohsawa, K., Tsuda, M., et al. (2007). UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature* 446, 1091–1095. doi: 10.1038/nature05704

Lafrance, V., Inoue, W., Kan, B., and Luheshi, G. N. (2010). Leptin modulates cell morphology and cytokine release in microglia. *Brain Behav. Immun.* 24, 358–365. doi: 10.1016/j.bbi.2009.11.003

Leinninger, G. M. (2011). Lateral thinking about leptin: a review of leptin action via the lateral hypothalamus. *Physiol. Behav.* 104, 572–581. doi: 10.1016/j.physbeh.2011.04.060

Li, L. L., Jin, M. F., and Ni, H. (2018). Zinc/CaMK II associated-mitophagy signaling contributed to hippocampal mossy fiber sprouting and cognitive deficits following neonatal seizures and its regulation by chronic leptin treatment. *Front. Neurol.* 9:802. doi: 10.3389/fneur.2018.00802

Li, X. M., Yan, H. J., Guo, Y. S., and Wang, D. (2016). The role of leptin in central nervous system diseases. *Neuroreport* 27, 350–355. doi: 10.1097/WNR.0000000000000546

Lim, G., Wang, S., Zhang, Y., Tian, Y., and Mao, J. (2009). Spinal leptin contributes to the pathogenesis of neuropathic pain in rodents. *J. Clin. Invest.* 119, 295–304. doi: 10.1172/JCI36785

Lin, T. C., Lee, T. C., Hsu, S. L., and Yang, C. S. (2011). The molecular mechanism of leptin secretion and expression induced by aristolochic acid in kidney fibroblast. *PLoS One* 6:e16654. doi: 10.1371/journal.pone.0016654

Lloret, A., Monllor, P., Esteve, D., Cervera-Ferri, A., and Lloret, M. A. (2019). Obesity as a risk factor for Alzheimer's disease: implication of leptin and glutamate. *Front. Neurosci.* 13:508. doi: 10.3389/fnins.2019.00508

Maeda, T., Kiguchi, N., Kobayashi, Y., Ikuta, T., Ozaki, M., and Kishioka, S. (2009). Leptin derived from adipocytes in injured peripheral nerves facilitates development of neuropathic pain via macrophage stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13076–13081. doi: 10.1073/pnas.0903524106

Margetic, S., Gazzola, C., Pegg, G. G., and Hill, R. A. (2002). Leptin: a review of its peripheral actions and interactions. *Int. J. Obes. Relat. Metab. Disord.* 26, 1407–1433. doi: 10.1038/sj.ijo.0802142

Matoba, K., Muramatsu, R., and Yamashita, T. (2017). Leptin sustains spontaneous remyelination in the adult central nervous system. *Sci. Rep.* 7:40397. doi: 10.1038/srep40397

McGregor, G., and Harvey, J. (2018). Regulation of hippocampal synaptic function by the metabolic hormone, leptin: implications for health and neurodegenerative disease. *Front. Cell Neurosci.* 12:340. doi: 10.3389/fncel.2018.00340

Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., and Trayhurn, P. (1996). Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett.* 387, 113–116. doi: 10.1016/0014-5793(96)00473-5

Montague, C. T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., et al. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 387, 903–908. doi: 10.1038/43185

Morash, B., Li, A., Murphy, P. R., Wilkinson, M., and Ur, E. (1999). Leptin gene expression in the brain and pituitary gland. *Endocrinology* 140, 5995–5998. doi: 10.1210/endo.140.12.7288

Morris, J. K., Bomhoff, G. L., Stanford, J. A., and Geiger, P. C. (2010). Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R1082–R1090. doi: 10.1152/ajpregu.00449.2010

Mutze, J., Roth, J., Gerstberger, R., Matsumura, K., and Hubschle, T. (2006). Immunohistochemical evidence of functional leptin receptor expression in neuronal and endothelial cells of the rat brain. *Neurosci. Lett.* 394, 105–110. doi: 10.1016/j.neulet.2005.10.031

Ni, H., Chen, S. H., Li, L. L., and Jin, M. F. (2018). Leptin treatment prevents long-term abnormalities in cognition, seizure threshold, hippocampal mossy fiber sprouting and ZnT3/CB-D28k expression in a rat developmental "twist" seizure model. *Epilepsy Res.* 139, 164–170. doi: 10.1016/j.eplepsyres.2017.12.009

Ni, H., Feng, X., Gong, Y., Tao, L. Y., and Wu, X. R. (2011a). Acute phase expression pattern of ZnTs, LC3, and beclin-1 in rat hippocampus and its regulation by 3-methyladenine following recurrent neonatal seizures. *Biol. Trace Elem. Res.* 143, 320–331. doi: 10.1007/s12011-010-8836-5

Ni, H., Li, C., Feng, X., and Cen, J. N. (2011b). Effects of forced running exercise on cognitive function and its relation to zinc homeostasis-related gene expression in rat hippocampus. *Biol. Trace Elem. Res.* 142, 704–712. doi: 10.1007/s12011-010-8793-z

Ni, H., Jiang, Y. W., Xiao, Z. J., Tao, L. Y., Jin, M. F., and Wu, X. R. (2010). Dynamic pattern of gene expression of ZnT-1, ZnT-3 and PRG-1 in rat brain following flurothyl-induced recurrent neonatal seizures. *Toxicol. Lett.* 194, 86–93. doi: 10.1016/j.toxlet.2010.02.008

Pan, W., Hsuchou, H., He, Y., Sakharkar, A., Cain, C., Yu, C., et al. (2008). Astrocyte leptin receptor (ObR) and leptin transport in adult-onset obese mice. *Endocrinology* 149, 2798–2806. doi: 10.1210/en.2007-1673

Pan, W., Hsuchou, H., Xu, C., Wu, X., Bouret, S. G., and Kastin, A. J. (2011). Astrocytes modulate distribution and neuronal signaling of leptin in the hypothalamus of obese A vy mice. *J. Mol. Neurosci.* 43, 478–484. doi: 10.1007/s12031-010-9470-6

Pan, W., and Kastin, A. J. (2007). Mahogany, blood-brain barrier, and fat mass surge in AVY mice. *Int. J. Obes.* 31, 1030–1032. doi: 10.1038/sj.ijo.0803536

Paz-Filho, G., Wong, M. L., and Licinio, J. (2010). The procognitive effects of leptin in the brain and their clinical implications. *Int. J. Clin. Pract.* 64, 1808–1812. doi: 10.1111/j.1742-1241.2010.02536.x

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., et al. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269, 540–543. doi: 10.1126/science.7624776

Pinteaux, E., Inoue, W., Schmidt, L., Molina-Holgado, F., Rothwell, N. J., and Luheshi, G. N. (2007). Leptin induces interleukin-1beta release from rat microglial cells through a caspase 1 independent mechanism. *J. Neurochem.* 102, 826–833. doi: 10.1111/j.1471-4159.2007.04559.x

Pinto, S., Roseberry, A. G., Liu, H., Diano, S., Shanabrough, M., Cai, X., et al. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 304, 110–115. doi: 10.1126/science.1089459

Popovic, V., Svetel, M., Djurovic, M., Petrovic, S., Doknic, M., Pekic, S., et al. (2004). Circulating and cerebrospinal fluid ghrelin and leptin: potential role in altered body weight in Huntington's disease. *Eur. J. Endocrinol.* 151, 451–455. doi: 10.1530/eje.0.1510451

Pratley, R. E., Salbe, A. D., Ravussin, E., and Caviness, J. N. (2000). Higher sedentary energy expenditure in patients with Huntington's disease. *Ann. Neurol.* 47, 64–70. doi: 10.1002/1531-8249(200001)47:1<64::aid-ana11>3.3.co;2-j

Procaccini, C., Jirillo, E., and Matarese, G. (2012). Leptin as an immunomodulator. *Mol. Aspects Med.* 33, 35–45. doi: 10.1016/j.mam.2011.10.012

Procaccini, C., Santopaolo, M., Faicchia, D., Colamatteo, A., Formisano, L., De Candia, P., et al. (2016). Role of metabolism in neurodegenerative disorders. *Metabolism* 65, 1376–1390. doi: 10.1016/j.metabol.2016.05.018

Prokop, J. W., Duff, R. J., Ball, H. C., Copeland, D. L., and Londraville, R. L. (2012). Leptin and leptin receptor: analysis of a structure to function relationship in interaction and evolution from humans to fish. *Peptides* 38, 326–336. doi: 10.1016/j.peptides.2012.10.002

Rentsch, J., Levens, N., and Chiesi, M. (1995). Recombinant ob-gene product reduces food intake in fasted mice. *Biochem. Biophys. Res. Commun.* 214, 131–136. doi: 10.1006/bbrc.1995.2266

Rosengren, A., Skoog, I., Gustafson, D., and Wilhelmsen, L. (2005). Body mass index, other cardiovascular risk factors, and hospitalization for dementia. *Arch. Intern. Med.* 165, 321–326. doi: 10.1001/archinte.165.3.321

Schwartz, M. W., Seeley, R. J., Campfield, L. A., Burn, P., and Baskin, D. G. (1996). Identification of targets of leptin action in rat hypothalamus. *J. Clin. Invest.* 98, 1101–1106. doi: 10.1172/JCI118891

Seltzer, Z., Dubner, R., and Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43, 205–218. doi: 10.1016/0304-3959(90)91074-s

Shan, X., and Yeo, G. S. (2011). Central leptin and ghrelin signalling: comparing and contrasting their mechanisms of action in the brain. *Rev. Endocr. Metab. Disord.* 12, 197–209. doi: 10.1007/s11154-011-9171-7

Shanley, L. J., O'malley, D., Irving, A. J., Ashford, M. L., and Harvey, J. (2002). Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. *J. Physiol.* 545, 933–944. doi: 10.1113/jphysiol.2002.029488

Shimizu, H., Shargill, N. S., Bray, G. A., Yen, T. T., and Gesellchen, P. D. (1989). Effects of MSH on food intake, body weight and coat color of the yellow obese mouse. *Life Sci.* 45, 543–552. doi: 10.1016/0024-3205(89)90105-7

Signore, A. P., Zhang, F., Weng, Z., Gao, Y., and Chen, J. (2008). Leptin neuroprotection in the CNS: mechanisms and therapeutic potentials. *J. Neurochem.* 106, 1977–1990. doi: 10.1111/j.1471-4159.2008.05457.x

Smith-Kirwin, S. M., O'connor, D. M., De Johnston, J., Lancey, E. D., Hassink, S. G., and Funanage, V. L. (1998). Leptin expression in human mammary epithelial cells and breast milk. *J. Clin. Endocrinol. Metab.* 83, 1810–1813. doi: 10.1210/jcem.83.5.4952

Strobel, A., Issad, T., Camoin, L., Ozata, M., and Strosberg, A. D. (1998). A leptin missense mutation associated with hypogonadism and morbid obesity. *Nat. Genet.* 18, 213–215. doi: 10.1038/ng0398-213

Tang, B. L. (2008). Leptin as a neuroprotective agent. *Biochem. Biophys. Res. Commun.* 368, 181–185. doi: 10.1016/j.bbrc.2008.01.063

Tang, C. H., Lu, D. Y., Yang, R. S., Tsai, H. Y., Kao, M. C., Fu, W. M., et al. (2007). Leptin-induced IL-6 production is mediated by leptin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, akt, NF-kappaB, and p300 pathway in microglia. *J. Immunol.* 179, 1292–1302. doi: 10.4049/jimmunol.179.2.1292

Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., et al. (1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83, 1263–1271.

Tessier, D. R., Ferraro, Z. M., and Gruslin, A. (2013). Role of leptin in pregnancy: consequences of maternal obesity. *Placenta* 34, 205–211. doi: 10.1016/j.placenta.2012.11.035

Tu, H., Kastin, A. J., Hsuchou, H., and Pan, W. (2008). Soluble receptor inhibits leptin transport. *J. Cell Physiol.* 214, 301–305. doi: 10.1002/jcp.21195

Udagawa, J., Nimura, M., and Otani, H. (2006). Leptin affects oligodendroglial development in the mouse embryonic cerebral cortex. *Neuro Endocrinol. Lett.* 27, 177–182.

Verkhratsky, A., and Nedergaard, M. (2018). Physiology of astroglia. *Physiol. Rev.* 98, 239–389. doi: 10.1152/physrev.00042.2016

Vong, L., Ye, C., Yang, Z., Choi, B., Chua, S. Jr., and Lowell, B. B. (2011). Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. *Neuron* 71, 142–154. doi: 10.1016/j.neuron.2011.05.028

Wang, H., Luo, W., and Eitzman, D. T. (2014). Leptin in thrombosis and atherosclerosis. *Curr. Pharm. Des.* 20, 641–645. doi: 10.2174/13816128113199990015

Wauman, J., and Tavernier, J. (2011). Leptin receptor signaling: pathways to leptin resistance. *Front. Biosci.* 16:2771–2793.

Weigle, D. S., Bukowski, T. R., Foster, D. C., Holderman, S., Kramer, J. M., Lasser, G., et al. (1995). Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. *J. Clin. Invest.* 96, 2065–2070. doi: 10.1172/JCI118254

Whitmer, R. A., Gunderson, E. P., Barrett-Connor, E., Quesenberry, C. P. Jr., and Yaffe, K. (2005). Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study. *BMJ* 330, 1360. doi: 10.1136/bmj.38446.466238.E0

Wilkinson, M., Morash, B., and Ur, E. (2000). The brain is a source of leptin. *Front. Horm. Res.* 26:106–125.

Wolff, G. L. (1965). Body composition and coat color correlation in different phenotypes of "Viable Yellow" mice. *Science* 147, 1145–1147. doi: 10.1126/science.147.3662.1145

Wolff, G. L., Roberts, D. W., and Galbraith, D. B. (1986). Prenatal determination of obesity, tumor susceptibility, and coat color pattern in viable yellow (Avy/a) mice. The yellow mouse syndrome. *J. Hered.* 77, 151–158. doi: 10.1093/oxfordjournals.jhered.a110206

Wolff, G. L., and Whittaker, P. (2005). Dose-response effects of ectopic agouti protein on iron overload and age-associated aspects of the Avy/a obese mouse phenome. *Peptides* 26, 1697–1711. doi: 10.1016/j.peptides.2004.12.033

Xu, Y., Kim, E. R., Zhao, R., Myers, M. G. Jr., Munzberg, H., and Tong, Q. (2013). Glutamate release mediates leptin action on energy expenditure. *Mol. Metab.* 2, 109–115. doi: 10.1016/j.molmet.2013.01.004

Yasui, M., Yoshimura, T., Takeuchi, S., Tokizane, K., Tsuda, M., Inoue, K., et al. (2014). A chronic fatigue syndrome model demonstrates mechanical allodynia and muscular hyperalgesia via spinal microglial activation. *Glia* 62, 1407–1417. doi: 10.1002/glia.22687

Yi, C. X., Meyer, C. W., and Jastroch, M. (2013). Leptin action in the brain: how (and when) it makes fat burn. *Mol. Metab.* 2, 63–64. doi: 10.1016/j.molmet.2013.03.002

Young, J. K. (2002). Anatomical relationship between specialized astrocytes and leptin-sensitive neurones. *J. Anat.* 201, 85–90. doi: 10.1046/j.1469-7580.2002.00068.x

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372, 425–432. doi: 10.1038/372425a0

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Reduction of Mitophagy-Related Oxidative Stress and Preservation of Mitochondria Function Using Melatonin Therapy in an HT22 Hippocampal Neuronal Cell Model of Glutamate-Induced Excitotoxicity

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Recent evidence indicates that autophagy-mediated mitochondrial homeostasis is crucial for oxidative stress-related brain damage and repair. The highest concentration of melatonin is in the mitochondria of cells, and melatonin exhibits well-known antioxidant properties. We investigated the impact and mechanism involved in mitochondrial function and the mitochondrial oxidative stress/autophagy regulator parameters of glutamate cytotoxicity in mouse HT22 hippocampal neurons. We tested the hypothesis that melatonin confers neuroprotective effects via protecting against mitochondrial impairment and mitophagy. Cells were divided into four groups: the control group, melatonin alone group, glutamate injury group, and melatonin pretreatment group. We found that glutamate induced significant changes in mitochondrial function/oxidative stress-related parameters. Leptin administration preserved mitochondrial function, and this effect was associated with increased superoxide dismutase, glutathione (GSH), and mitochondrial membrane potential and decreased GSSG (oxidized glutathione) and mitochondrial reactive oxygen species. Melatonin significantly reduced the fluorescence intensity of mitophagy via the Beclin-1/Bcl-2 pathway, which involves Beclin-1 and Bcl-2 proteins. The mitophagy inhibitor CsA corrected these glutamate-induce changes, as measured by the fluorescence intensity of Mitophagy-Tracker Red CMXROS, mitochondrial ROS, and mitochondrial membrane potential changes. These findings indicate that melatonin exerts neuroprotective effects against glutamate-induced excitotoxicity by reducing mitophagy-related oxidative stress and maintaining mitochondrial function.

Keywords: melatonin, mitochondria, mitophagy, glutamate, HT22

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INTRODUCTION

The developing brain undergoes constant maturation. The developing brain exhibits higher brain excitability, and it is more prone to convulsions than the adult brain. The incidence of neonatal seizures is 1.1–8.6/1,000 live births (1). Status epilepticus (SE) occurs more frequently in children than adults, and 40–50% occur in children under 2 years of age (2). Gluckman et al. (3) reported that ∼75% of neonates with hypoxic ischemic encephalopathy (HIE) developed seizures, and some of these children developed epilepsy in adulthood. Conventional antiepileptic drugs that are currently used in clinical practice have adverse side effects, such as white matter damage, in convulsive children caused by HIE (4). To make things worse, ∼20% of children with epilepsy develop drug-resistant epilepsy (DRE), in which seizures cannot be controlled with further manipulation of antiepileptic drugs (5). Phenobarbital (PB) is a first-line antiepileptic drug (AED) for the treatment of neonatal seizures, but it produces long-term cognitive and intellectual impairments in children with febrile seizures (6). Therefore, there is still a need to better understand the molecular mechanisms underlying the effects of developmental seizures on brain maturation and dysfunction before optimizing age-appropriate treatments.

Unlike traditional antiepileptic drugs, significant progress was made recently in understanding the effects and mechanisms of endocrine modulators, such as melatonin, leptin and ghrelin, on pediatric neurological diseases, particularly epilepsy (7–10). The ketogenic diet (KD) has a history of nearly 100 years of success in the treatment of epilepsy that is refractory to antiepileptic drugs. However, a KD also affects children's normal growth and development, especially brain development (11). The mechanisms of action of KD are poorly defined but certainly involve a change in metabolism. A KD modulates leptin, ghrelin, and melatonin plasma levels and gene expression in the brain, which suggests that these endocrine regulatory molecules have neuroprotective or antiepileptic effects in epilepsy and serve as potential markers of antiepileptic drug responses (12, 13). Clinical and experimental results show that melatonin reduces sleep disorders and circadian rhythm changes alone, and it reduces seizures in combination with AEDs (14). Therefore, it may be used as an adjuvant treatment for epilepsy to lower cost and reduce toxicity. Intensive research by our group and others demonstrated that melatonin was beneficial in experimental models of brain damage caused by developmental seizures (15–17). However, previous research, including our own work, was primarily performed using an *in vivo* animal model, but there are very few studies using cell models. Only three studies on Pubmed used a cultured hippocampal neuron excitotoxic cell model to examine the neuroprotective effects and mechanisms of melatonin. Quiros et al. (18) showed that melatonin reduced glucocorticoid-induced toxicity in hippocampal HT22 cells in the presence of neurotoxins. Herrera et al. (19) demonstrated that melatonin prevented mitochondrial ROS production *in vitro* against glutamate-induced oxytosis in the HT22 mouse hippocampal cell line. Lezoualc'h et al. (20) used cells of the clonal hippocampal cell line HT22 and organotypic hippocampal rat brain slice cultures and found that melatonin protected HT22 cells and organotypic hippocampal slices from glutamate- and H_2O_2-induced cell death. We recently demonstrated that mitophagy-mediated mitochondrial activation contributed to glutamate-induced HT22 neuronal cell damage, and leptin treatment counteracted these adverse effects. However, whether the antioxidant and neuroprotective abilities of melatonin in glutamate-induced *in vitro* neuronal injury is dependent on the same signaling pathway is not known.

The highest concentration of melatonin is in the mitochondria of cells, and it has well-known antioxidant properties (21). Nopparat et al. (22) demonstrated that melatonin protected methamphetamine-induced cell death via by inhibition of Bcl-2/Beclin-1 mediated autophagy. Melatonin inhibited hypoxia-mediated mitophagy in human hepatocarcinoma cells (23). We recently demonstrated that melatonin induced long-term expression changes of energy metabolism-related genes in the hippocampus, including Kcnj11, leptin receptor, dopamine receptor D2, melanocortin 4 receptor, ACAT1, and Cathepsin-E, in a rat model of brain injury induced by neonatal seizures (24, 25). These genes are highly associated with mitochondrial function and autophagy. Therefore, we hypothesized that melatonin provides beneficial effects on mitochondrial function via reducing autophagy and, more specifically, mitophagy.

The present study investigated mitochondrial function, mitochondrial oxidative stress, and mitophagy parameters using an *in vitro* model of glutamate-induced cytotoxicity in mouse HT22 hippocampal neurons. Cell viability, parameters of mitochondrial function and oxidative stress, and biomarkers for mitophagy, including Bcl-2/Beclin-1 protein levels, were measured.

MATERIALS AND METHODS

Cell Lines

The HT22 mouse hippocampal neuronal cell line was purchased from the cell bank of Institute of Cell Biology, Chinese Academy of Sciences.

Reagents and Antibodies

DMEM/high glucose medium and the protein molecular weight marker were purchased from Thermo Fisher Scientific Company (USA). Fetal bovine serum (FBS) was purchased from Serana Company (Germany). L-glutamate, cyclosporine A (CsA), and melatonin were purchased from Sigma-Aldrich Company (USA). The BCA Protein Assay Kit and RIPA lysis buffer were purchased from Beyotime Institute of Biotechnology. An antibody against β-actin was purchased from Sigma-Aldrich. Primary antibodies against Beclin-1 were purchased from Cell Signaling Technology (USA). Antibodies against Bcl-2 and secondary antibodies for immunoblots were HRP-conjugated anti-rabbit and anti-mouse IgGs from Santa Cruz Biotechnology (USA).

Cell Culture Conditions

HT22 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100

mg/ml of streptomycin in humidified air at 37°C with 5% CO_2.

Drug Treatment and Grouping

Cells were seeded into each well of a 6-well or 96-well plate the day before the experiment. The control group (Control), melatonin alone group (Melatonin), glutamate injury group (Glutamate) and melatonin pretreatment group (Glutamate + Melatonin) were established. Melatonin in the melatonin alone group was added to the culture medium to obtain a final concentration at 10^{-7} mol/l. Glutamate in the glutamate injury group was added to the culture medium to obtain a final concentration at 5 mM (26). Cells in the melatonin pretreatment group were pretreated with 10^{-7} mol/l melatonin in culture for 2 h before glutamate was added to the medium to obtain a final concentration at 5 mM. Cells were subjected to various measurements as described below after further incubation for 24 h.

Cell Viability Assay

Cells were seeded at a cell density of 5×10^3 cells/well in 96-well tissue culture plates. After various treatments, we switched the medium in each well with 100 μl DMEM medium containing 10% CCK-8 Cell Proliferation Reagent Cell Counting Kit-8 (CCK8) (5 mg/ml) (Dojindo Molecular Technologies, Kumamoto, Japan) and incubated for an additional 2 h. The absorbance (OD) of the samples was measured at 450 nm using a 96-well plate reader (27).

Lactate Dehydrogenase (LDH) Assay

The LDH release assay was used to determine membrane integrity. The cell density was adjusted to a concentration of 5×10^4, and the cells were cultured in 96-well plates with 100 μl per well. After treatment, 50 μl of supernatant from each well was collected and incubated with the same volume of reaction mixture from the LDH cytotoxicity detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of LDH was calculated from the absorbance at 440 nm in a multi-functional microporous plate reader (28, 29).

Biochemical Analysis of Oxidative Stress Markers

Cells in the logarithmic phase were collected and cultured in 6-well plates at 2×10^5 cells/well. After treatment, the cells were lysed in 200 μL RIPA buffer. Cells were lysed on ice for 30 min, and proteins were crushed using an ultrasonic cell crusher. After centrifugation for 10 min at 12,000 rpm, the supernatant was deproteinated using a buffer solution containing metaphosphoric acid (10% w/v) and triethanolamine (53.1% v/v) to remove proteins and avoid interference from protein-sulfhydryl groups. SOD, GSH, and GSSG levels were measured using SOD, GSH, and GSSG assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions, and the absorbance of each group was measured using a microplate reader (30, 31).

Analysis of Mitochondrial Membrane Potentials Using Mito-Tracker

Mito-Tracker Green FM and Mito-Tracker Red CMXROS were included in the mitochondrial fluorescence probe Mito-Tracker kit (Invitrogen, USA). Mito-Tracker Green FM is a mitochondrial green fluorescent dye that locates stable mitochondria, and it is not affected by mitochondrial membrane potential. The Mito-Tracker Red CMXROS probe is a red fluorescent dye that is passively transported through the cell membrane and directly assembled in active mitochondria. The accumulation of this dye depends on the mitochondrial membrane potential. Cells in logarithmic growth phase were collected and cultured in 6-well plates. Cells were collected after treatment, digested with trypsinase, washed three times with serum-free medium, and re-suspended in a mixed solution of 0.5 ml 200 nM Mito-Tracker Green FM and 25 nM Mito-Tracker Red CMXROS for a 30-min incubation at 37°C in darkness. Cells were washed three times with PBS containing 1% serum, and re-suspended in an appropriate amount of PBS containing 1% serum. A Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) was used to detect the fluorescence ratio of the FL-1 channel (green fluorescence) and FL-2 channel (red fluorescence) and determine the changes of mitochondrial membrane potential. The data were analyzed using FlowJo Analysis Software.

Another group of HT22 cells were seeded in confocal dishes and cultured overnight. Cells were washed three times with serum-free medium after treatment, and a mixed solution of 500 μl prewarmed 200 nM Mito-Tracker Green FM and 25 nM Mito-Tracker Red CMXROS was added. Cells were incubated in an incubator for 30 min, washed three times with serum-free medium and provided with fresh medium. Confocal fluorescence microscopy was used to observe the results of mitochondrial staining, and photographs were taken (32, 33).

Analysis of Mitochondrial Membrane Potentials Using JC-1

Cells were stained after treatment with the mitochondrial membrane potential sensitive probe JC-1 (Beyotime, Shanghai, China). Cells were incubated in an incubator for 30 min at a final concentration of 2 μM in PBS and washed twice with PBS. JC-1 aggregate was measured at the FL-2 channel, and green fluorescence (JC-1 monomer) was measured at the FL-1 channel. The data were analyzed using the FlowJo analysis software, and the results are displayed in a dot plot of J-aggregate red fluorescence (y-axis) against JC-1 green fluorescence (x-axis) (34).

Determination of Mitochondria Reactive Oxygen Species (ROS) (MitoSOX)

MitoSOX™ (Thermo Fisher Scientific, Waltham, MA, USA) Red Reagent penetrates into living cells and produces red fluorescence under oxidative damage of mitochondria. Cells were collected after treatment, washed with HBSS three times and suspended in a mixed solution of 0.5 ml 5 μM MitoSOX and 200 nM Mito-Tracker Green FM. Cells were incubated for 30-min in an

incubator, washed with HBSS three times, and suspended in an appropriate amount of HBSS. The red-green fluorescence ratio was measured using a Gallios flow cytometer.

Another group of cells in logarithmic phase were cultured in 6-well plates at 2×10^5 cells per well. The medium was discarded after treatment, and cells were washed with HBSS three times. One milliliter of a 5-μM MitoSOX solution was added, and cells were incubated at 37°C for 10 min. Cells were washed with HBSS three times, stained with 1 μg/ml Hoechst 33342 for 10 min, washed with HBSS three times and provided with fresh medium. Confocal fluorescence microscopy was used to observe and take photographs for analysis (35–37).

Mitophagy Assay

The Mitophagy Detection Kit (Dojindo Molecular Technologies, Kumamoto, Japan) contained Mitophagy Dye and Lyso Dye, which were used to monitor mitophagy. Mitochondrial autophagosome fuse with lysosomes during mitophagy, and the fluorescence intensity of Mtphagy Dye increases. Cells in logarithmic phase were seeded in confocal dishes and cultured overnight. The cells were washed with Hanks-HEPES buffer 3 times and incubated with 500 μl of 100 nmol/l Mtphagy Dye solution for 30 min. Cells were washed and treated with glutamate and/or melatonin. The medium was removed after a 24-h culture, and cells were incubated with 500 μl of a 1-μmol/l Lyso Dye solution at 37°C in the absence of light. Cells were washed three times, and the co-localization of Mtphagy and Lyso Dyes was observed using confocal fluorescence microscopy (38).

Western Blot Assay

Treated cells were retrieved, and an appropriate amount of lysis buffer was added for lysing on ice for 30 min. An ultrasonic crusher was used to ensure complete crushing of proteins, and the supernatant was recovered after 12,000 r/min centrifugation for 30 min. A BCA protein quantitative kit was used to determine the amount of protein in each sample group. Proteins were boiled for 10 min in the sample buffer, and samples were loaded for electrophoresis. Proteins were transferred to a PVDF membrane, which was incubated in a blocking solution at room temperature for 2 h. Beclin-1 and Bcl-2 antibodies were incubated overnight at 4°C, and the secondary antibody was incubated at room temperature for 1 h. Proteins were imaged using chemiluminescent autography. The strip optical density of the image was formed and analyzed using image analysis software.

Cell Culture With Cyclosporine A and Mitochondrial Function Detection

Cyclosporine A (CsA) (a mitophagy inhibitor) was used to observe the effect of glutamate on mitophagy. Cells were divided into a glutamate group and cyclosporine A intervention group. The cyclosporine A intervention group received 0.5 μM CsA 2 h before glutamate followed by 24 h in culture. The function of mitochondria was observed using JC-1, Mito-Tracker and MitoSOX assay kits, and the level of mitophagy was determined using a mitophagy detection kit and Western blotting (39).

Statistical Analysis

GraphPad Prism version 5.0 was used for data processing. The experimental results are expressed in means ± SEM. One-way ANOVA followed by bonferoni *post-hoc* analysis was used to compare the means of more than two groups. A P-value < 0.05 indicated that the difference was statistically significant.

RESULTS

Effects of Melatonin on Glutamate-Induced Morphological Changes

According to the relevant literature (40, 41) and our preliminary experiments, we used different concentrations of melatonin (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) for efficacy tests. We found that 10^{-7} M melatonin significantly increased cell viability (at 5 mmol/ml glutamate). We further examined the effect of melatonin alone at a concentration of 10^{-7} M on cell survival and found that this concentration produced no cytotoxic effects on the cells. Therefore, 10^{-7} M melatonin was used for the experimental studies.

Light microscopy revealed that cells in the control group adhered to the wall, exhibited a round nucleus, a smooth and intact cell membrane, obvious protrusions and synapses that were interwoven into a network. There was no significant difference between the melatonin alone group and the control group. Cells in the glutamate injury group exhibited weak adherence, significantly inhibited cell proliferation, smaller cell volume, concentrated nuclei, an interrupted synaptic network between cells, and increasingly shorter protrusions. Cells in the melatonin pretreatment group exhibited restored normal morphology and characteristics of obvious proliferation and established synaptic connections between cells. These results demonstrated that melatonin inhibited cell necrosis and apoptosis induced by glutamate neurotoxicity (**Figure 1**).

Evaluation of the Protective Effect of Melatonin on HT22 Cells Using the CCK8 Assay and LDH Release Assay

The well-validated survival assay (CCK-8) demonstrated that exposure of HT22 cells to 5 mM L-glutamine for 24 h elicited maximum cell death. The results show that glutamate induced an obvious decrease in cell viability, and cell viability in the melatonin pretreatment group rebounded significantly (**Figure 2A**).

LDH release was also measured. An increase in LDH release into the culture medium suggests cell injury. The LDH release in melatonin alone group was unchanged in the present study, but it increased in the glutamate injury group. LDH release in the melatonin pretreatment group decreased compared to the glutamate group, which is consistent with the CCK8 assay results (**Figure 2B**). These results suggest that melatonin protects HT22 cells from glutamate-induced damage.

FIGURE 1 | Observation of cell morphology in each group under light microscopy (×10).

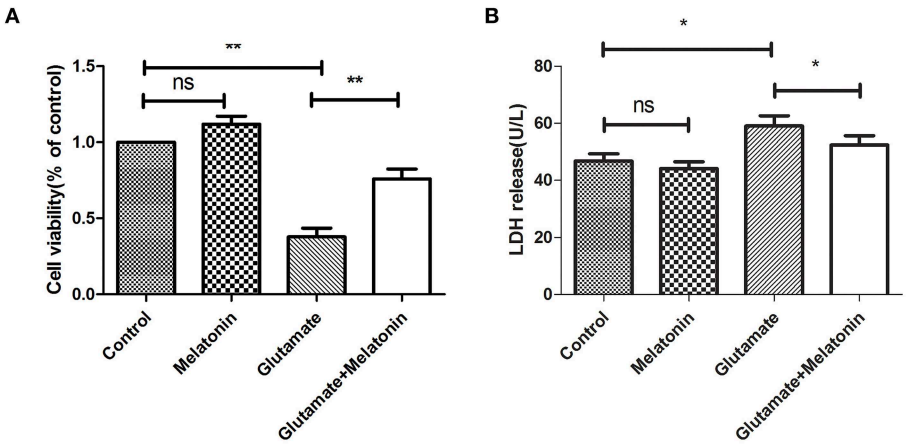


FIGURE 2 | Protective effect of melatonin on glutamate-induced injury in HT22 cells. **(A)** Effect of melatonin on cell survival rate: $F = 27.242$, $P < 0.001$; control group vs. glutamate injury group: $P < 0.001$, glutamate injury group vs. melatonin pretreatment group: $P = 0.005$; **(B)** Effect of melatonin on the release of LDH: $F = 11.553$, $P < 0.001$; control group vs. glutamate injury group: $P = 0.011$, glutamate injury group vs. melatonin pretreatment group: $P = 0.039$; $^{*}P < 0.05$, $^{**}P < 0.01$. ns, not significant ($n = 6$/group).

Effect of Melatonin on Glutamate-Induced Oxidative Stress in HT22 Cells

The levels of SOD, GSH, GSSG, and GSH/GSSG in each group were measured. SOD activity, GSH concentration and the GSH/GSSG ratio in the glutamate injury group decreased sharply, and GSH concentration increased significantly (**Figures 3A,B,D**). The differences between the glutamate injury group, the control group and the melatonin pretreatment group

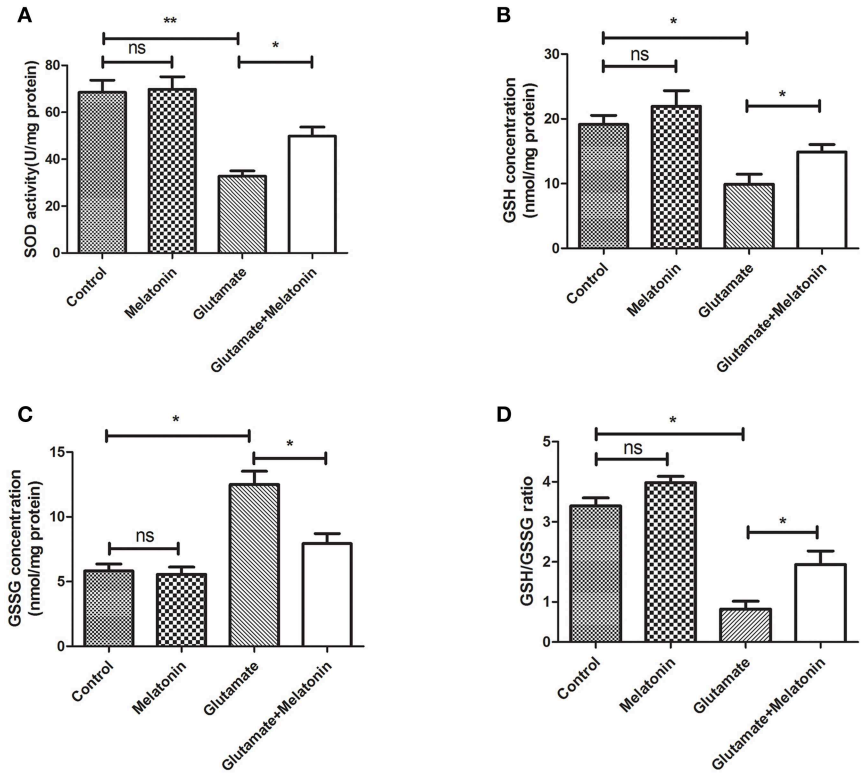


FIGURE 3 | Effect of melatonin on glutamate-induced oxidation of HT22 cells. **(A)** Effect of melatonin on the activity of SOD in cells: $F = 19.937$, $P < 0.001$; control group vs. glutamate injury group: $P < 0.001$, glutamate injury group vs. melatonin pretreatment group: $P = 0.034$; **(B)** Effect of melatonin on the intracellular GSH concentration: $F = 9.315$, $P < 0.001$; control group vs. glutamate injury group: $P = 0.011$, glutamate injury group vs. melatonin pretreatment group: $P = 0.044$; **(C)** Effect of melatonin on the intracellular GSSG concentration: $F = 5.921$, $P = 0.005$; control group vs. glutamate injury group: $P = 0.014$, glutamate injury group vs. melatonin pretreatment group: $P = 0.045$; **(D)** Effects of melatonin on GSH/GSSG ratio in cells: $F = 8.363$, $P = 0.001$; control group vs. glutamate injury group: $P = 0.011$, glutamate injury group vs. melatonin pretreatment group: $P = 0.039$; $^*P < 0.05$, $^{**}P < 0.01$. ns, not significant ($n = 6$/group).

were highly statistically significant (**Figure 3C**). These results suggest that melatonin reversed the changes in these oxidative indicators induced by glutamate and maintained normal cellular oxidative levels in cells.

Effect of Melatonin on Glutamate-Induced Mitochondrial Membrane Potential Changes Using Mito-Tracker

The fluorescence ratios of the FL-1 channel (green fluorescence) and FL-2 channel (red fluorescence) were measured using flow cytometry. The results showed that the number of red monosomic cells decreased in the glutamate injury group, and increased after melatonin intervention, which indicates that melatonin improved the glutamate-induced migration of the cell population (**Figure 4A**).

Changes of mitochondrial membrane potential in HT22 cells were observed under confocal microscopy. The red fluorescence intensity of Mito-Tracker Red CMXROS in the glutamate injury group declined greatly. Melatonin restored the red fluorescence intensity in the melatonin pretreatment group (**Figures 4B,C**). These results indicate that glutamate decreased mitochondrial membrane potential, and melatonin significantly maintained the normal level of mitochondrial membrane potential.

Effect of Melatonin on Glutamate-Induced Mitochondrial Membrane Potential Changes Using JC-1

When the mitochondrial membrane potential is relatively high, JC-1 aggregates in the matrix of mitochondria to form a polymer that produces red fluorescence. When the mitochondrial membrane potential is low, JC-1 cannot aggregate in the matrix of mitochondria, which produces green fluorescence. The decrease in mitochondrial membrane potential is a marker event in the early stage of apoptosis. Therefore, the change of JC-1 from red to green fluorescence is an indicator of early stage apoptosis. The results of flow cytometry showed that the number of green monosomic cells increased in the glutamate injury group, i.e., the mitochondrial membrane potential decreased, and the mitochondrial membrane potential increased in the melatonin pretreatment group (**Figure 5**). These results are consistent with the results of the mitochondrial membrane potential results using the mitochondrial fluorescence probe Mito-Tracker. These results indicate that melatonin reversed

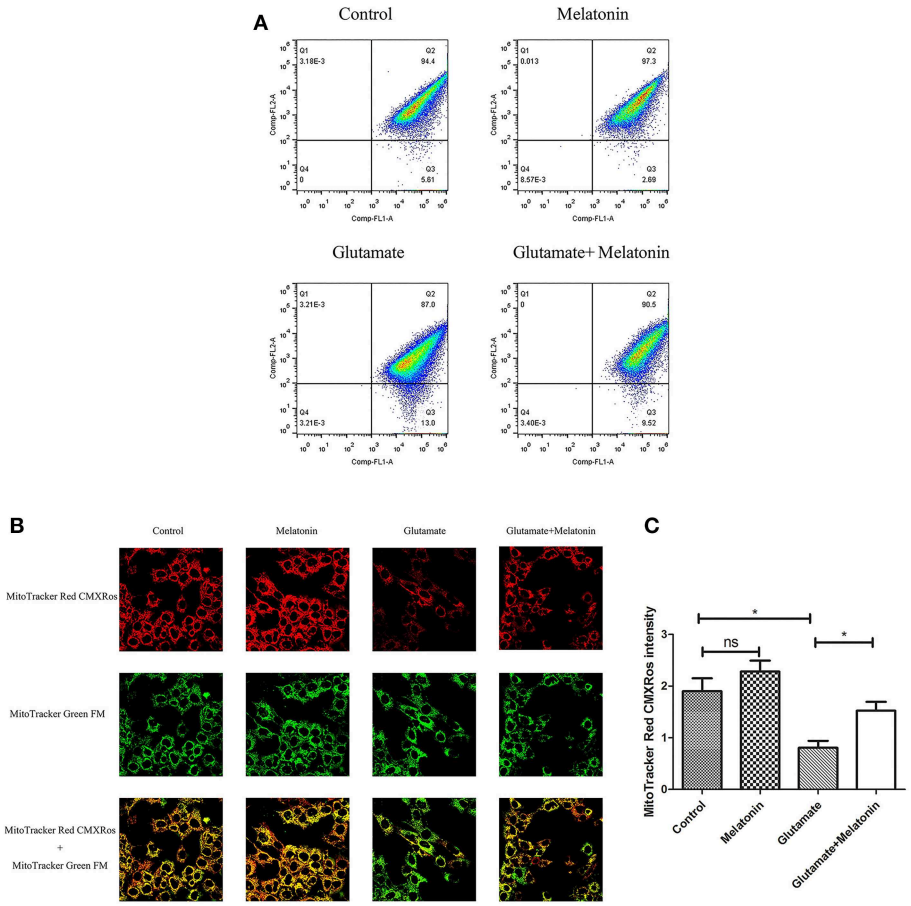


FIGURE 4 | Effects of melatonin on glutamate-induced mitochondrial membrane potential changes by Mito-Tracker. **(A)** Detection of cell mitochondrial membrane potential changes by flow cytometry; **(B)** Fluorescence intensity of Mito-Tracker Red CMXROS in cells; **(C)** Quantitative analysis of mean fluorescence intensity of Mito-Tracker Red CMXROS in cells: $F = 10.946$, $P < 0.001$; control group vs. glutamate injury group: $P = 0.011$, glutamate injury group vs. melatonin pretreatment group: $P = 0.035$; $^*P < 0.05$. ns, not significant ($n = 6$/group).

the glutamate-induced decrease in mitochondrial membrane potential and inhibited apoptosis.

Effect of Melatonin on Glutamate-Induced Mitochondrial ROS Accumulation in HT22 Cells

Flow cytometry revealed an increase in red monosomic cells in the glutamate injury group. Red monosomic cells decreased in the melatonin pretreatment group compared to the glutamate group (**Figure 6A**).

Confocal microscopy indicated significantly higher levels of mitochondrial ROS content in the glutamate injury group than the control group, which indicates that glutamate enhanced the production of ROS in mitochondria. Melatonin pretreatment reduced the accumulation of mitochondrial ROS content (**Figures 6B,C**). These results demonstrate that melatonin inhibited the glutamate-induced increase in ROS content in cells and protected the cells.

Effect of Melatonin on Glutamate-Induced Mitochondrial Autophagy in HT22 Cells

The effect of glutamate on intracellular mitophagy was investigated under confocal microscopy. The intracellular fluorescence intensity of Mtphagy Dye in the glutamate group was higher than the control group, and melatonin pretreatment slowed the glutamate-induced increase in the intracellular fluorescence intensity of Mtphagy Dye. These results indicate that melatonin inhibited the glutamate-induced increase of mitophagy in HT22 cells (**Figures 7A,B**).

Effects of Melatonin on Glutamate-Induced Changes in Mitochondrial Autophagy Protein Expression

The effect of melatonin on the expression of mitophagy-related proteins was determined using Western blot. The results showed a decrease in the expression of Bcl-2 in the glutamate injury group and a large increase in the expression of Beclin-1 and the ratio of Beclin-1/Bcl-2 in

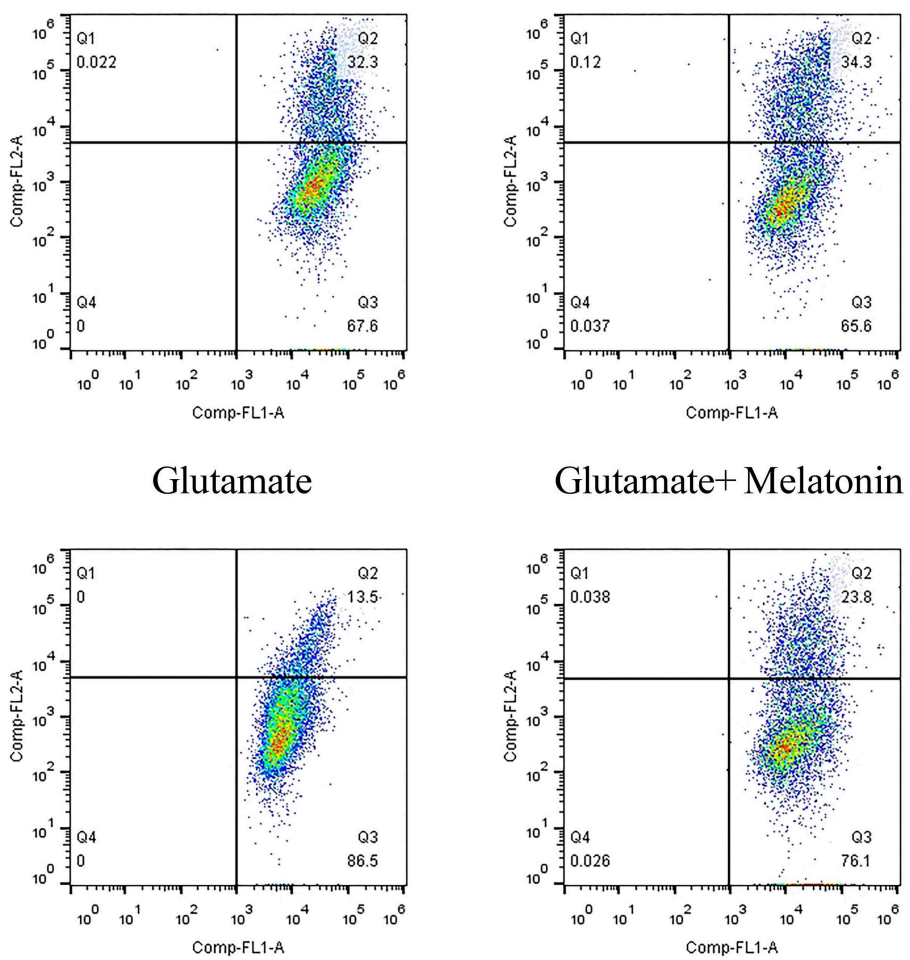


FIGURE 5 | Effect of melatonin on glutamate-induced mitochondrial membrane potential changes using JC-1.

the glutamate injury group compared to the control group. However, melatonin intervention reversed these effects, which indicates that melatonin inhibited glutamate-induced mitophagy (**Figure 8**, **Supplementary Figures 1**–**3**).

The Effect of Cyclosporine A (CsA) on Glutamate-Induced Mitochondrial Autophagy

Cyclosporine A (CsA) is a mitophagy inhibitor. The effect of mitochondrial autophagy on glutamate-induced apoptosis was determined using Cyclosporine A (CsA). Cells were divided into a glutamate group and cyclosporine intervention group. The results indicated that CsA intervention improved the glutamate-induced red fluorescence intensity of Mito-Tracker Red CMXROS (**Figures 9A,B**), reversed the glutamate-induced decline of mitochondrial membrane potential (**Figures 9C,J**), inhibited the glutamate-induced increase of mitochondrial ROS content in cells (**Figures 9D–F**). slowed the increase in Mtphagy Dye fluorescence intensity in mitochondria (**Figures 9G,H**) and decreased the glutamate-induced expression of mitophagy-related proteins (**Figure 9I**). These findings indicate that melatonin inhibited glutamate-induced apoptosis via inhibition of mitophagy, which protected the cells.

DISCUSSION

The current study demonstrated the following principal findings: (1) glutamate resulted in mitochondrial dysfunction induced by oxidative stress in cells, which manifested in the decline of superoxide dismutase, glutathione (GSH) and mitochondrial membrane potential and a rise in GSSG (oxidized glutathione) and mitochondrial reactive oxygen species; (2) glutamate resulted in mitophagy, which was reflected by the fluorescence intensity of Mitophagy-Tracker Red CMXROS and the protein levels of Beclin-1/Bcl-2; (3) treatment with

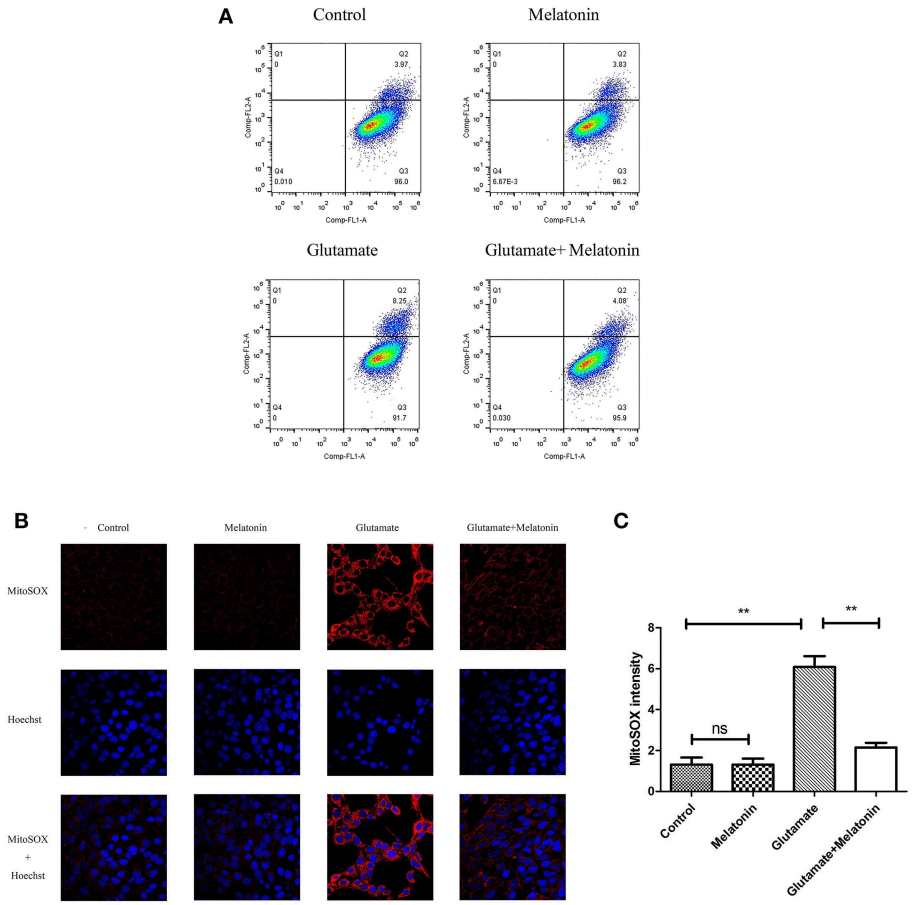


FIGURE 6 | Effect of melatonin on mitochondrial reactive oxygen species (ROS) accumulation in cells. **(A)** Detection of ROS content in mitochondria using flow cytometry; **(B)** Fluorescence intensity of ROS in mitochondria; **(C)** Quantitative analysis of average fluorescence intensity of ROS in mitochondria: $F = 24.903$, $P < 0.001$; control group vs. glutamate injury group: $P < 0.001$, glutamate injury group vs. melatonin pretreatment group: $P < 0.001$; $^{**}P < 0.01$. ns, not significant ($n = 6$/group).

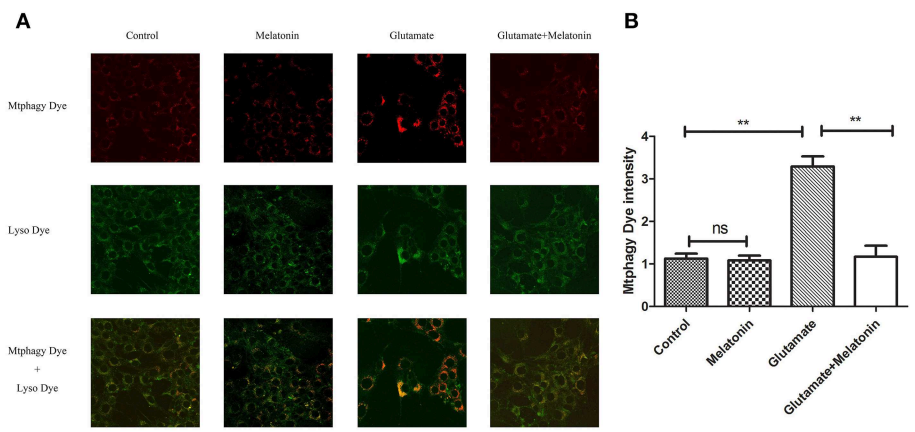


FIGURE 7 | Effect of melatonin on glutamate-induced mitophagy in HT22 cells. **(A)** Fluorescence intensity of mitochondrial Mtphagy Dye and Lyso Dye in cells; **(B)** Quantitative analyses of average fluorescence intensity of mitochondrial Mtphagy Dye in cells: $F = 26.214$, $P < 0.001$; control group vs. glutamate injury group: $P < 0.001$, glutamate injury group vs. melatonin pretreatment group: $P < 0.001$; $^{**}P < 0.01$. ns, not significant ($n = 6$/group).

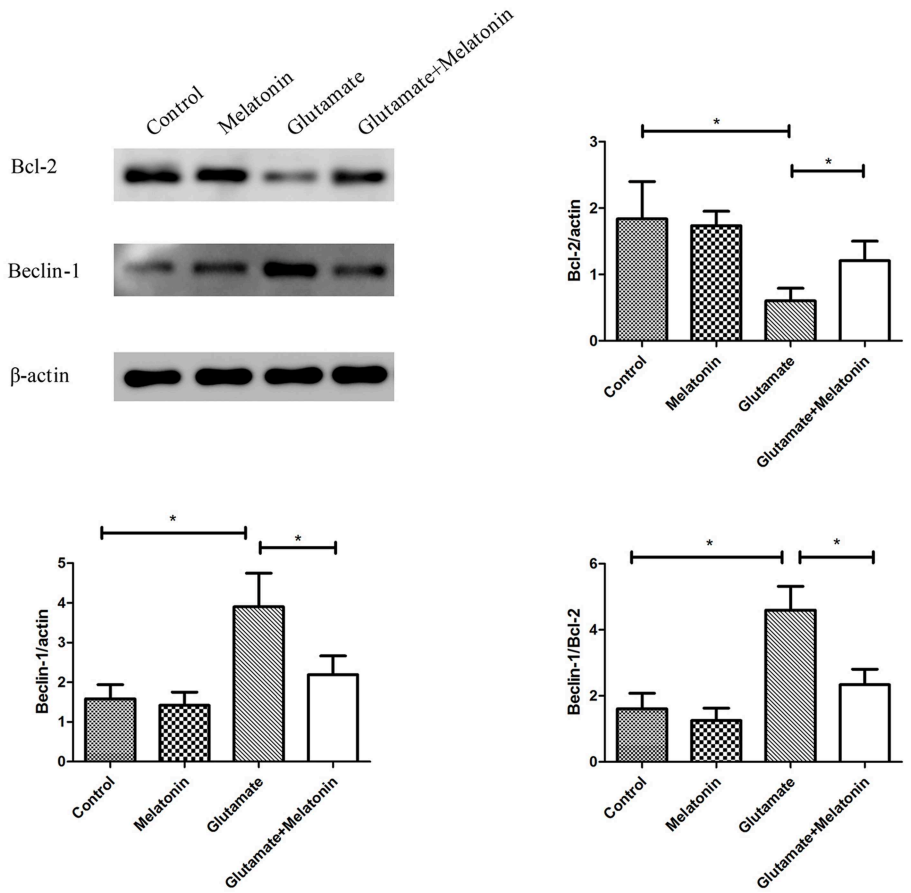


FIGURE 8 | Effect of melatonin on the glutamate-induced expression of mitochondrial autophagy. Bcl-2/actin: $F = 5.888$, $P = 0.005$; control group vs. glutamate injury group: $P = 0.010$, glutamate injury group vs. melatonin pretreatment group: $P = 0.013$; Beclin-1/actin: $F = 6.509$, $P = 0.003$; control group vs. glutamate injury group: $P = 0.011$, glutamate injury group vs. melatonin pretreatment group: $P = 0.044$; Beclin-1/Bcl-2: $F = 6.625$, $P = 0.003$; control group vs. glutamate injury group: $P = 0.010$, glutamate injury group vs. melatonin pretreatment group: $P = 0.029$; $^*P < 0.05$ ($n = 6$/group).

melatonin significantly restored these glutamate-induced abnormal changes; and (4) the mitophagy inhibitor CsA significantly corrected the glutamate-induced alterations, as measured by the fluorescence intensity of Mitophagy-Tracker Red CMXROS, mitochondrial ROS, and mitochondrial membrane potential changes. Our findings support the hypothesis that melatonin exerts neuroprotective effects against glutamate-induced excitotoxicity via a reduction in mitophagy-related oxidative stress and maintenance of mitochondrial function, involving the Beclin-1/Bcl-2 signaling pathway.

In this study, we used a very high glutamate concentration (5 mM). It should be pointed out that a 24 h 5 mM glutamate exposure is never going to happen *in vivo*. However, the present experiment was conducted *in vitro* model. There are many literatures that use 5 mM glutamate to treat HT22 cells to induce oxidative stress *in vitro* (42–45). In addition, our preliminary experiments treated cells with 1.25, 2.5, 5, and 10 mmol/L glutamate concentration for 24 h. The results confirmed that after 24 h of treatment with 5 mM glutamate, the cell viability was reduced to 50% or lower (data not shown). Therefore, this experiment continued to use an *in vitro* model of hippocampal neuronal damage using 5 mM glutamate used by the predecessors.

This experiment used HT22 cells, an immortalized neuronal cell line derived from the mouse hippocampus, which lacks a functional ionotropic glutamate receptor and is therefore commonly used to study non-receptor-mediated oxidation. HT22 cells have been used widely as an *in vitro* model to study the neurotoxic effects of glutamate (46, 47). It has been shown that glutamate induces HT22 mouse hippocampal cell death exclusively through the oxytotic pathway as these cells do not express functional ionotropic receptors (48). Especially, Pereira et al. (49) demonstrated that the neurotoxicity of glutamate in HT22 cells is due to the inhibition of cystine, which causes GSH consumption and oxidative stress. Especially, by using HT22 *in vitro* cell model, Herrera et al. (19) have showed that melatonin prevents cell death through a direct antioxidant effect specifically targeted

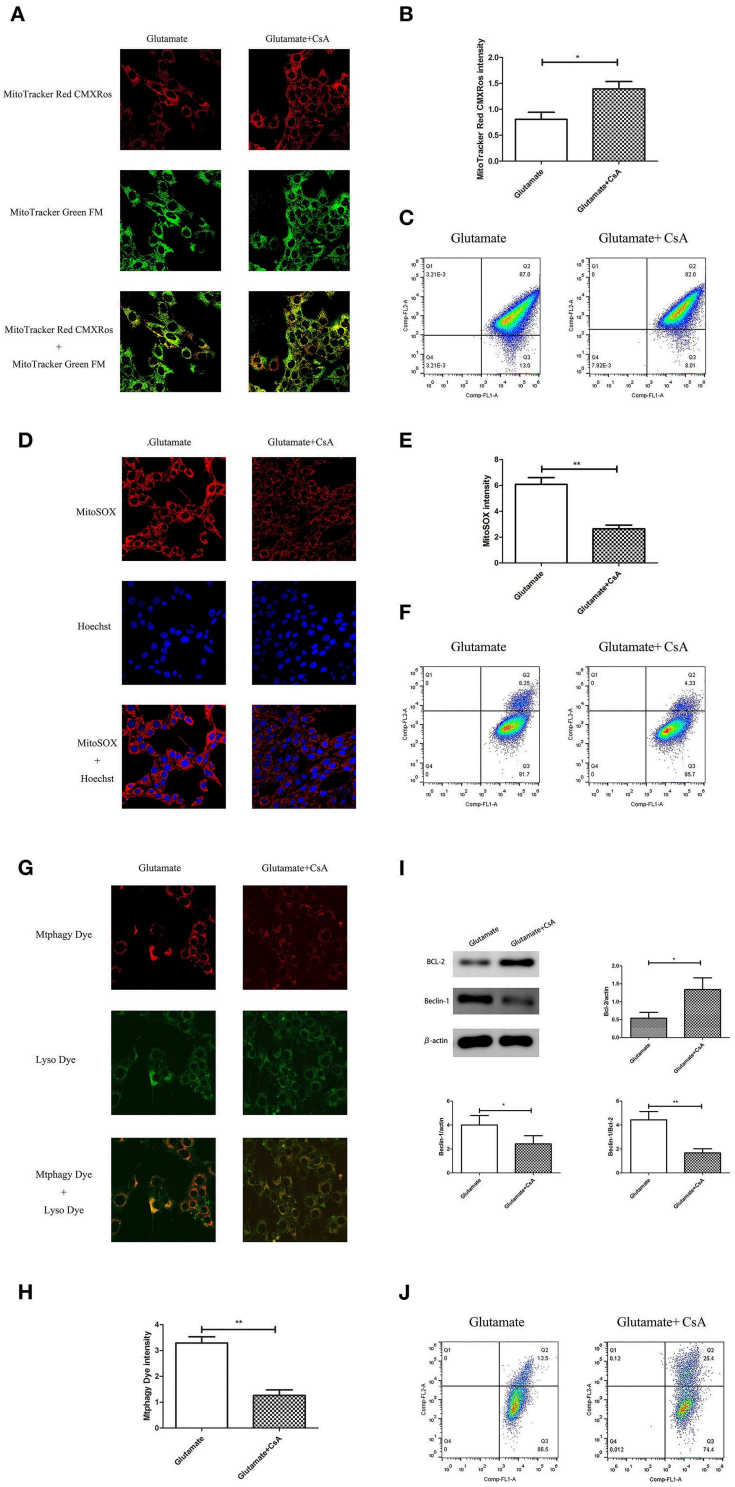
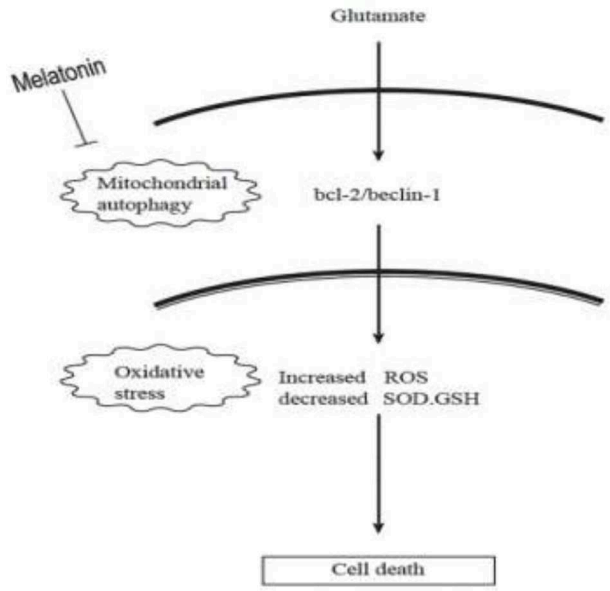


FIGURE 9 | Effect of cyclosporine A (CsA) on glutamate-induced mitochondrial autophagy. **(A)** Fluorescence intensity of Mito-Tracker Red CMXROS in cells; **(B)** Quantitative analyses of mean fluorescence intensity of Mito-Tracker Red CMXROS in cells: $t = 3.074$, $P = 0.012$; **(C)** Mitochondrial membrane potential was detected using flow cytometry and Mito-Tracker; **(D)** Fluorescence intensity of mitochondrial ROS; **(E)** Quantitative analysis of mean fluorescence intensity of mitochondrial ROS: $t = 4.511$, $P = 0.001$; **(F)** Detection of ROS content in cell mitochondria using flow cytometry; **(G)** Fluorescence intensity of mitochondrial Mtphagy Dye and Lyso Dye in cells; **(H)** Quantitative analyses of mean fluorescence intensity of mitochondrial Mtphagy Dye in cells: $t = 5.687$, $P < 0.001$; **(I)** Expression of mitochondrial autophagy-related proteins in cells: Bcl-2/actin: $t = 2.799$, $P = 0.019$; Beclin-1/actin: $t = 2.856$, $P = 0.017$; Beclin-1/Bcl-2: $t = 4.218$, $P = 0.002$; **(J)** Observation of mitochondrial membrane potential changes using JC-1. $^*P < 0.05$, $^{**}P < 0.01$ ($n = 6$/group).

at mitochondria. They found that none of the described transducers of melatonin signaling seems to be implicated in the neuroprotection provided by the indole. In addition, melatonin membrane receptors are not involved as well-known antagonists of these receptors were unable to prevent the neuroprotective effect. They found that none of the described transducers of melatonin signaling seems to be implicated in the neuroprotection provided by the indole. in addition, melatonin membrane receptors are not involved as well-known antagonists of these receptors were unable to prevent the neuroprotective effect.

In fact, melatonin has been shown to protect against a wide variety of neuronal insults and to have effects at the mitochondrial level (50), but little is known of the mechanisms implied in this effect. We recently demonstrated that exposure of HT22 cells to high doses of glutamate reduced cell survival, which was mediated by increased free radicals. We found that this mechanism involved the glutamate-induced inhibition of superoxide dismutase (SOD), glutathione (GSH) and mitochondrial membrane potential, and an increase in oxidized glutathione (GSSG) and mitochondrial ROS content (51). Here, we used MitoSOX Red Reagent and a Gallios flow cytometer to determinate mitochondrial reactive oxygen species (ROS) and the mitochondrial fluorescent probe Mito-Tracker to detect mitochondrial membrane potential. We provide additional evidence to support the findings that oxidative stress-related ROS accumulation and mitochondrial dysfunction are involved in glutamate toxicity in HT22 cells. Our present results and Takagi's findings that the decrease in mitochondrial membrane potential observed after ischemia/reperfusion injury triggered mitophagy (52) demonstrated, for the first time, that melatonin therapy reduced mitophagy-related oxidative stress and preserved mitochondria function following glutamate-induced excitotoxicity.

Autophagy is a process by which cells degrade proteins and organelles via a lysosome-dependent pathway. The pathway that degrades mitochondria via autophagy is called mitochondrial autophagy (mitophagy), which is the specific process of autophagic elimination of its own mitochondria and an important regulatory mechanism for cells to maintain homeostasis (53). Mitophagy exerts a dual function; it promotes cell survival or death depending on the cellular context and microenvironment (54). The degradation of mitochondria via the mitophagy pathway is critical for cell survival under physiological conditions (55). However, hyperactivated mitophagy impairs mitochondrial function. For example, Cesarini et al. (55) showed that melatonin supplementation reduced cell death and restored mitochondrial function via autophagy regulation in hippocampal HT22 cells from the effects of serum deprivation. Feng et al. (56) found that the myocardium was protected from ischemia/reperfusion injury via a reduction in mitochondrial dysfunction and mitophagy. The Chinese herbal extract Xiaoxuming Decoction reduced mitochondrial activation and maintained mitochondrial function via reducing the expression of LC3, Beclin-1, and Lamp1 (57). Notably, Nopparat et al. (22) demonstrated that melatonin protected the SK-N-SH neuronal dopaminergic cell line against methamphetamine-induced autophagy via inhibition of the dissociation of the Bcl-2/Beclin-1 complex. Bcl-2 plays a role in the negative regulation of autophagy by blocking Beclin-1. Here, we further validated this view by using the mitophagy inhibitor cyclosporin A (CsA). Mitochondria is considered to be the main source of ROS, and mitochondrial dysfunction can lead to impaired intracellular calcium homeostasis, induce accumulation of reactive oxygen species, and cause oxidative stress. Cyclosporin A is a specific inhibitor of mitochondrial permeability transition, which inhibits mitochondrial depolarization and mitophagy by interfering with the interaction of cyclophilin D and mitochondrial permeability transition pores. The current results showed for the first time that after the use of mitophagy inhibitor CsA, the expression of Bcl-2 was increased, the expression of Beclin-1 was decreased, the mitochondrial damage caused by glutamate was alleviated, as well as the reduced amount of released ROS, which was in accordance with the results obtained after using melatonin. We thus speculate that Bcl-2/Beclin-1 mediated mitophagy may underlying melatonin' anti-oxidative stress and neuroprotective effects induced by glutamate in an *in vitro* HT22 hippocampal cell model. The following is a graphical summary of our findings:



In conclusion, our findings provide new insight into the complexity of the neuroprotective role of melatonin, particularly on neuronal mitochondrial function via the mitophagy pathway after glutamate-induced neuronal excitotoxicity in HT22 cells. These findings have potential translational medical value and provide new experimental evidence for the use of melatonin as an adjunct in the treatment of epilepsy.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

HN was the designer and dissertation writer of this study. DW, MJ, and DZ were the operators of this experiment and were responsible for the statistical analysis of the data.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00550/full#supplementary-material

Supplementary Figure 1 | The full-length Western blot image of β-actin protein expression in each group.

Supplementary Figure 2 | The full-length Western blot image of BCL-2 protein expression in each group.

Supplementary Figure 3 | The full-length Western blot image of Beclin-1 protein expression in each group.

REFERENCES

1. Mosley M. Neonatal seizures. *Pediatr Rev.* (2010) 31:127–8. doi: 10.1542/pir.31-3-127
2. Castelhano AS, Cassane Gdos S, Scorza FA, Cysneiros RM. Altered anxiety-related and abnormal social behaviors in rats exposed to early life seizures. *Front Behav Neurosci.* (2013) 7:36. doi: 10.3389/fnbeh.2013.00036
3. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, et al. Selective head cooling with Mild systemic hypothermia after neonatal encephalopathy:multicentre randomised trial. *Lancet.* (2005) 365:663–70. doi: 10.1016/S0140-6736(05)17946-X
4. Mitra S, Bale G, Mathieson S, Uria-Avellanal C, Meek J, Tachtsidis I, et al. Changes in cerebral oxidative metabolism during neonatal seizures following hypoxic-ischemic brain injury. *Front Pediatr.* (2016) 4:83. doi: 10.3389/fped.2016.00083
5. Kharod P, Mishra D, Juneja M. Drug-resistant epilepsy in Indian children at a tertiary-care public hospital. *Childs Nerv Syst.* (2019) 35:775–8. doi: 10.1007/s00381-019-04084-5
6. Sanchez RM, Jensen FE. Maturational aspects of epilepsy mechanisms and consequences for the immature brain. *Epilepsia.* (2001) 42:577–85. doi: 10.1046/j.1528-1157.2001.12000.x
7. Brigo F, Igwe SC, Del FA. Melatonin as add-on treatment for epilepsy. *Cochrane Database Syst Rev.* (2016) 2016:CD006967. doi: 10.1002/14651858.CD006967.pub4
8. Çiçek NP, Kamaşak T, Serin M, Okten A, Alver A, Cansu A. The effects of valproate and topiramate use on serum insulin, leptin, neuropeptide Y and ghrelin levels in epileptic children. *Seizure.* (2018) 58:90–5. doi: 10.1016/j.seizure.2018.03.013
9. Agar E. The role of cannabinoids and leptin in neurological diseases. *Acta Neurol Scand.* (2015) 132:371–80. doi: 10.1111/ane.12411
10. Biagini G, Locatelli V, Torsello A. Ghrelin anticonvulsive properties: is it a matter of desacylation? *Epilepsia.* (2012) 53:1277–8. doi: 10.1111/j.1528-1167.2012.03546.x
11. Ling Y, Wang DD, Sun YX, Zhao DJ, Ni H. Neuro-behavioral status and the hippocampal expression of metabolic associated genes in wild-type rat following a ketogenic diet. *Front Neurol.* (2019) 10:65. doi: 10.3389/fneur.2019.00065
12. Giordano C, Marchiò M, Timofeeva E, Biagini G. Neuroactive peptides as putative mediators of antiepileptic ketogenic diets. *Front Neurol.* (2014) 5:63. doi: 10.3389/fneur.2014.00063
13. Fenoglio-Simeone KA, Wilke JC, Milligan HL, Allen CN, Rho JM, Maganti RK. Ketogenic diet treatment abolishes seizure periodicity and improves diurnal rhythmicity in epileptic Kcna-1null mice. *Epilepsia.* (2009) 50:2027–34. doi: 10.1111/j.1528-1167.2009.02163.x
14. Rocha AKAA, de Lima E, Amaral F, Peres R, Cipolla-Neto J, Amado D. Altered MT1 and MT2 melatonin receptors expression in the hippocampus of pilocarpine-induced epileptic rats. *Epilepsy Behav.* (2017) 71 (Pt A):23–34. doi: 10.1016/j.yebeh.2017.01.020
15. Bruni O, Alonso-Alconada D, Besag F, Biran V, Braam W, Cortese S, et al. Current role of melatonin in pediatric neurology: clinicalrecommendations. *Eur J Paediatr Neurol.* (2015) 19:122–33. doi: 10.1016/j.ejpn.2014.12.007
16. Lo SF, Muti P, Blandino G, Strano S. Melatonin and hippo pathway: is there existing cross-talk? *Int J Mol Sci.* (2017) 18:E1913. doi: 10.3390/ijms18091913
17. Sanchez-Barcelo EJ, Rueda N, Mediavilla MD, Martinez-Cue C, Reiter RJ. Clinical uses of melatonin in neurological diseases and mental and behavioural disorders. *Curr Med Chem.* (2017) 24:3851–78. doi: 10.2174/0929867324666170718105557
18. Quiros I, Mayo JC, Garcia-Suarez O, Hevia D, Martin V, Rodríguez C, et al. Melatonin prevents glucocorticoid inhibition of cell proliferation and toxicity in hippocampal cells by reducing glucocorticoidreceptor nuclear translocation. *J Steroid Biochem Mol Biol.* (2008) 110:116–24. doi: 10.1016/j.jsbmb.2008.02.009
19. Herrera F, Martin V, García-Santos G, Rodriguez-Blanco J, Antolín I, Rodriguez C. Melatonin prevents glutamate-induced oxytosis in the HT22 mouse hippocampal cell line through an anti-oxidant effect specifically targeting mitochondria. *J Neurochem.* (2007) 100:736–46. doi: 10.1111/j.1471-4159.2006.04228.x
20. Lezoualc'h F, Skutella T, Widmann M, Behl C. Melatonin prevents oxidative stress-induced celldeath in hippocampal cells. *Neuroreport.* (1996) 7:2071–7. doi: 10.1097/00001756-199609020-00003
21. Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B. Melatonin as amitochondriatargeted antioxidant: one of evolution's best ideas. *Cell Mol Life Sci.* (2017) 74:3863–81. doi: 10.1007/s00018-017-2609-7
22. Nopparat C, Porter JE, Ebadi M, Govitrapong P. The mechanism for the neuroprotective effectof melatonin against methamphetamine-induced autophagy. *J Pineal Res.* (2010) 49:382–9. doi: 10.1111/j.1600-079X.2010.00805.x
23. Prieto-Domínguez N, Méndez-Blanco C, Carbajo-Pescador S, Fondevila F, García-Palomo A, González-Gallego J, et al. Melatonin enhances sorafenib actions inhuman hepatocarcinoma cells by inhibiting mTORC1/p70S6K/HIF-1α and hypoxia-mediated mitophagy. *Oncotarget.* (2017) 8:91402–14. doi: 10.18632/oncotarget.20592
24. Ni H, Sun Q, Tian T, Feng X, Sun BL. Long-term expression of metabolism-associated genes in the rat hippocampus following recurrent neonatal seizures and its regulation by melatonin. *Mol Med Rep.* (2015) 12:2727–34. doi: 10.3892/mmr.2015.3691
25. Ni H, Sun Q, Tian T, Feng X, Sun BL. Prophylactic treatment with melatonin before recurrentneonatal seizures: effects on long-term neurobehavioral changes and the underlying expressionof metabolism-related genes in rat hippocampus and cerebral cortex. *Pharmacol Biochem Behav.* (2015) 133:25–30. doi: 10.1016/j.pbb.2015.03.012
26. Song JH, Kang KS, Choi YK. Protective effect of casuarinin against glutamate-induced apoptosis in HT22 cells through inhibition of oxidative stress-mediated MAPK phosphorylation. *Bioorg Med Chem Lett.* (2017) 27:5109–13. doi: 10.1016/j.bmcl.2017.10.075
27. Zhang J, Feng J, Ma D, Wang F, Wang Y, Li C, et al. Neuroprotective mitochondrial remodeling by AKAP121/PKA protects HT22 cell from glutamate-induced oxidative stress. *Mol Neurobiol.* (2019) 56:5586–607. doi: 10.1007/s12035-018-1464-3
28. Cheng A, Lu Y, Huang Q, Zuo Z. Attenuating oxygen-glucose deprivation-caused autophagosome accumulation may be involved in sevoflurane

postconditioning-induced protection in humanneuron-like cells. *Eur J Pharmacol.* (2019) 849:84–95. doi: 10.1016/j.ejphar.2019.01.051
29. Gao F, Wang B, Chang T, Li M, Fang W, Li ZH, et al. The iron prochelator BHAPI attenuates glutamate-induced oxidative stress via Wnt-β/Catenin Pathway in HT22 cells. *Brain Res Bull.* (2018) 139:285–91. doi: 10.1016/j.brainresbull.2018.03.011
30. Iwayama K, Kimura J, Mishima A, Kusakabe A, Ohtaki KI, Tampo Y, et al. Low concentrations of clarithromycin upregulate cellular antioxidant enzymes and phosphorylation of extracellular signal-regulated kinase in human small airway epithelial cells. *J Pharmaceut Health Care Sci.* (2018) 4:2–14. doi: 10.1186/s40780-018-0120-4
31. Das T, Simone M, Ibugo AI, Witting PK, Manefield M, Manos J. Glutathione enhances antibiotic efficiency and effectiveness of DNase I in disrupting *Pseudomonas aeruginosa* biofilms while also inhibiting pyocyanin activity, thus facilitating restoration of cell enzymatic activity, confluence and viability. *Front Microbiol.* (2017) 8:2429-. doi: 10.3389/fmicb.2017.02429
32. Murera D, Arbogast F, Arnold J, Bouis D, Muller S, Gros F. CD4 T cell autophagy is integral to memory maintenance. *Sci Rep.* (2018) 8:5951. doi: 10.1038/s41598-018-23993-0
33. Alakhova DY, Rapoport NY, Batrakova EV, Timoshin AA, Li S, Nicholls D, et al. Differential metabolic responses to pluronic in MDR and non-MDR cells: a novel pathway for chemosensitization of drug resistant cancers. *J Control Release.* (2010) 142:89–100. doi: 10.1016/j.jconrel.2009.09.026
34. He F, Zeng Y, Wu X, Ji Y, He X, Andrus T, et al. Endogenous HIV-1 Vpr-mediated apoptosis and proteome alteration of human T-cell leukemia virus-1 transformed C8166 cells. *Apoptosis.* (2009) 14:1212–26. doi: 10.1007/s10495-009-0380-4
35. Josey BJ, Inks ES, Wen X, Chou CJ. Structure–activity relationship study of vitamin K derivatives yields highly potent neuroprotective agents. *J Med Chem.* (2013) 56:1007–22. doi: 10.1021/jm301485d
36. Liu R, Wu CX, Zhou D, Yang F, Tian S, Zang L, et al. Pinocembrin protects against β-amyloid-induced toxcity in neurons through inhibiting receptor for advanced glycation endproducts (RAGE)-independent signaling pathways and regulating mitochondrion-mediated apoptosis. *BMC Med.* (2012) 10:105. doi: 10.1186/1741-7015-10-105
37. Ip WK, Medzhitov R. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. *Nat Commun.* (2015) 6:6931. doi: 10.1038/ncomms7931
38. Kameyama K, Motoyama K, Tanaka N, Yamashita Y, Higashi T, Arima H. Induction of mitophagy-mediated antitumor activity with folate-appended methyl-β-cyclodextrin. *Int J Nanomed.* (2017) 12:3433–46. doi: 10.2147/IJN.S133482
39. Chen Z, Wang B, Yu F, Chen Q, Tian Y, Ma S, et al. The roles of mitochondriain radiation-induced autophagic cell death in cervical cancer cells. *Tumor Biol.* (2016) 37:1–9. doi: 10.1007/s13277-015-4190-8
40. Zhang L, Zhang Z, Wang J, Lv D, Zhu T, Wang F, et al. Melatonin regulates the activities of ovary and delays the fertility decline in female animals via MT1/AM-PK pathway. *J Pineal Res.* (2018) 66:e12550. doi: 10.1111/jpi.12550
41. Wang Y, Zeng S. Melatonin promotes ubiquitination of phosphorylated pro-apoptotic protein Bcl-2-interacting mediator of cell death-extra long (BimEL) in porcine granulosa cells. *Int J Mol Sci.* (2018) 19:3431. doi: 10.3390/ijms19113431
42. Schulz M, Gonzaga LV, de Souza V, Farina M, Vitali L, Micke GA, et al. Neuroprotective effect of juçara (Euterpe edulis Martius) fruits extracts against glutamate-induced oxytosis in HT22 hippocampal cells. *Food Res Int.* (2019) 120:114–23. doi: 10.1016/j.foodres.2019.02.030
43. Nguyen HQ, Zada S, Lai TH, Pham TM, Hwang JS, Ahmed M, et al. Calpain-dependent Beclin1 cleavage stimulates senescence-associated cell death in HT22 hippocampal cells under the oxidative stress conditions. *Neurosci Lett.* (2019) 701:106–11. doi: 10.1016/j.neulet.2019.02.036
44. Choi HJ, Kang KS, Fukui M, Zhu BT. Critical role of the JNK-p53-GADD45α apoptotic cascade in mediating oxidative cytotoxicity in hippocampal neurons. *Br J Pharmacol.* (2011) 162:175–92. doi: 10.1111/j.1476-5381.2010.01041.x
45. Kim DH, Kim DW, Jung BH, Lee JH, Lee H, Hwang GS, et al. Ginsenoside Rb2 suppresses the glutamate-mediated oxidative stress and neuronal cell death in HT22 cells. *J Ginseng Res.* (2019) 43:326–34. doi: 10.1016/j.jgr.2018.12.002
46. Tan S, Sagara Y, Liu Y, Maher P, Schubert D. The regulation of reactive oxygen species production during programmed cell death. *J Cell Biol.* (1998) 141:1423–32. doi: 10.1083/jcb.141.6.1423
47. Xu X, Chua CC, Kong J, Kostrzewa RM, Kumaraguru U, Hamdy RC, et al. Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in HT-22 cells. *J Neurochem.* (2007) 103:2004–14. doi: 10.1111/j.1471-4159.2007.04884.x
48. Maher P, Davis JB. The role of monoamine metabolism in glutamate toxicity. *J Neurosci.* (1996) 16:6394–401. doi: 10.1523/JNEUROSCI.16-20-06394.1996
49. Pereira CM, Oliveira CR. Glutamate toxicity on a PC12 cell line involves glutathione (GSH) depletion and oxidative stress. *Free Radic Biol Med.* (1997) 23:637–47. doi: 10.1016/S0891-5849(97)00020-8
50. León J, Acuña-Castroviejo D, Escames G, Tan DX, Reiter RJ. Melatonin mitigates mitochondrial malfunction. *J Pineal Res.* (2005) 38:1–9. doi: 10.1111/j.1600-079X.2004.00181.x
51. Jin MF, Ni H, Li LL. Leptin maintained zinc homeostasis against glutamate-induced excitotoxicity by preventing mitophagy-mediated mitochondrial activation in HT22 hippocampal neuronalcells. *Front Neurol.* (2018) 9:322. doi: 10.3389/fneur.2018.00322
52. Takagi H, Matsui Y, Sadoshima J. The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart. *Antioxid Redox Signal.* (2007) 9:1373–81. doi: 10.1089/ars.2007.1689
53. Batlevi Y, La Spada AR. Mitochondrial autophagy in neural function, neurodegenerative disease,neuron cell death, and aging. *Neurobiol Dis.* (2011) 43:46–51. doi: 10.1016/j.nbd.2010.09.009
54. Kulikov AV, Luchkina EA, Gogvadze V, Zhivotovsky B. Mitophagy: link to cancer development and therapy. *Biochem Biophys Res Commun.* (2017) 482:432–9. doi: 10.1016/j.bbrc.2016.10.088
55. Cesarini E, Cerioni L, Canonico B, Di Sario G, Guidarelli A, Lattanzi D, et al. Melatonin protects hippocampal HT22 cells from the effects of serum deprivation specifically targeting mitochondria. *PLoS ONE.* (2018) 13:e0203001. doi: 10.1371/journal.pone.0203001
56. Feng Y, Madungwe NB, da Cruz Junho CV, Bopassa JC. Activation of G protein-coupled oestrogen receptor 1 at the onset of reperfusion protects the myocardium against ischemia/reperfusion injury by reducing mitochondrial dysfunction and mitophagy. *Br J Pharmacol.* (2017) 174:4329–44. doi: 10.1111/bph.14033
57. Lan R, Zhang Y, Wu T, Ma YZ, Wang BQ, Zheng HZ, et al. Xiao-Xu-Ming decoction reduced mitophagy activation and improved mitochondrial functionin cerebral ischemia and reperfusion injury. *Behav Neurol.* (2018) 2018:4147502. doi: 10.1155/2018/4147502

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Novel Personalized Dietary Treatment for Autism Based on the Gut-Immune-Endocrine-Brain Axis

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Autism spectrum disorder (ASD) is a neurodevelopmental condition manifesting with impaired social interaction and communication, and restricted and repetitive behaviors and interests. In this perspective article, a more comprehensive approach than the gut-brain axis, hereby termed the "gut-immune-endocrine-brain" axis, is taken, based on which a personalized treatment plan for ASD is presented. ASD has no known etiology or cure, making desperate parents willing to try any treatment that worked for an individual with ASD, without much regard for its effectiveness, safety or side effects. This has been the case for restrictive dietary interventions as gluten-free/casein-free and ketogenic diets and recently, probiotics have emerged as the new such fad. One of the concerns about these dietary and probiotic treatments is their non-specificity: they may not be effective for all individuals with ASD, not all probiotic strains may have the beneficial qualities advertised indiscriminately for probiotics, and strains conferring benefits in one condition may not be probiotic in another. Not all children with ASD show immune reactivity to dietary proteins in wheat and milk, and wheat and milk may not be the only dietary elements to which reactivity is exhibited, where dietary aquaporins that resemble human aquaporins may elicit antibody reactivity in genetically susceptible individuals, which may include individuals with ASD. These observations are utilized to formulate a three-step plan to create effective, targeted, personalized treatments with as few side effects as possible, enabled by a systems approach connecting the various findings for dietary, immune, and neuroautoimmune reactivity in individuals with ASD.

Keywords: autism, autism spectrum disorder, diet, personalized, immune, endocrine

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Autism spectrum disorder (ASD) is a neurodevelopmental difference where affected individuals manifest impaired social interaction and communication, and restricted and repetitive behaviors and interests (1). Large cohort twin studies show that both genetic and shared and non-shared environmental factors contribute to ASD (2, 3), though a clear etiology is not known.

For devastating neurological conditions like ASD, it has been suggested that any simple lifestyle choice that could prevent or arrest neuroautoimmune reactivity should be investigated, such as the controllable choice of diet (4). There have been years of discussions about the administration of gluten-free/casein-free (GF/CF) diets and ketogenic diet (KD) and more recently of probiotics for ASD. The problem with these dietary recommendations is that they are made at a general level for all individuals with ASD.

This non-personalized approach that consider all individuals on the spectrum to have the same constellation of metabolic, endocrine, and physiological alterations is one of the reasons underlying

the inconclusive evidence for their effectiveness, where some studies show improvement in ASD symptoms after GF/CF diet administration (5–9), and others do not (10–12). Similarly, though KD was shown to improve sociability and communication and decrease self-directed repetitive behavior in a genetic mouse model of ASD (13), in a human trial, some participants were not able to tolerate the KD and of the remaining group, only 60% showed improvement in ASD symptoms, which ranged from minor to significant improvement (14). These findings suggest that these diets may not be effective for all individuals with ASD and some may even experience discomforting outcomes that make them discontinue the regimen, which is the issue that this perspective article aims to address.

This article presents a novel, personalized dietary treatment regimen that will (1) avoid the difficulty and the potentially harmful effects of limiting certain nutrients unnecessarily and (2) create a targeted treatment for the individual immunological and endocrine profile of each patient of this disorder for which there is yet no cure.

This perspective does not undermine the possibility that GF/CF diet and KD may benefit some individuals with ASD, as was shown in a study that found both diets to result in significant improvement in ASD symptoms in a group of 15 participants, and KD to yield better cognition and sociability results compared to GF/CF diet (15). Instead, it argues that these generalized dietary plans of GF/CF diet and KD do not benefit all individuals with ASD given the different physiological profiles of each individual, and that as certain negative effects of these diets have started to emerge with recent investigations (16), they should not be recommended to all individuals with ASD without an initial screening and should be replaced by individualized dietary plans that fit their specific profile the best.

Such an idea to make alterations to these traditional diets have recently been embraced by other researchers as well. One trial tested a modified ketogenic gluten-free diet with the addition of MCT supplement and found certain improvements on core autism symptoms except for restricted and repetitive behaviors (17). Another trial tested a gluten-free, casein-free, soy-free diet with the addition of special vitamin/mineral supplements including essential fatty acids and digestive enzymes and observed improvements in ASD symptoms of 67 children and adults with ASD, yet some participants also experienced sickness, intestinal symptoms, mild nausea, loose stools, facial rash, worsening behaviors, increased aggression, and increased spinning behavior (18). These findings attest to the necessity of the perspective presented here that a simple modification of GF/CF and ketogenic diets with the addition of dietary supplements may not be sufficient and a profiling of each patient and the creation of personal diets may be what is needed to avoid these negative experiences and ensure the maximal benefit and satisfaction from dietary treatments.

Upon observing that the GF/CF diet resulted in greater improvements in children with ASD who had gastrointestinal symptoms, food allergies, and food sensitivities, Pennesi and Klein (19) suggested that gastrointestinal and immune factors may differentiate diet responders from diet non-responders. We build on this perspective by taking a personal approach to these immunological parameters, and aim to provide a system to discover the particular dietary regimen to which each individual with ASD will respond the most.

GUT-IMMUNE-ENDOCRINE-BRAIN AXIS MODEL FOR AUTISM

Throughout the years, external agents have been implicated in ASD and investigated mainly via comparisons of their levels in the body systems of or utilization by individuals with ASD and general population, such as pesticides (20, 21), toxic metals (22–24), and antibiotics (25, 26). These past propositions have remained at a speculative level owing to an absence of a delineated mechanism for their effect at the time. They recently gained traction with the discovery that these toxicants selectively target ASD genes (27) and the understanding of the different metabolic, signaling, immune-inflammatory functions of the gut microbiota on muscles, liver, and the brain (28), resulting in an increasing research focus on the involvement of the gut-brain axis in various neurological disorders including ASD (29).

With such a "paradigm shift in neuroscience" (29), previous findings including but not limited to the following started being investigated under the umbrella of the gut-brain axis: differences in urine peptides (30), intestinal permeability (31), gastrointestinal problems in individuals with ASD [which are not only experienced more often but also correlate with ASD severity (32)], and improvements in ASD behavior due to dietary changes. By suggesting a connection between the gut and the brain in ASD (33), this axis offers an explanatory model to link such diverse findings from individuals with ASD.

Though much speculation and theoretical perspectives have been offered about the involvement of the gut-brain axis in ASD in the past decade, recently emerging causal evidence is what brings a more solid grounding to this approach. Firstly, transplantation of gut microbiota from human donors with ASD induced core ASD behaviors in mice, whose brains showed alternative splicing of ASD-related genes (34). This finding supports not only a causal role for microbiota in the emergence of ASD symptoms but also a connection between the microbiota in the gut and ASD risk genes in the brain. Secondly, this evidenced connection between the gut and the brain was further clarified by another recent study. It found that probiotic rescue of social deficits in ASD mouse models happened via the vagus nerve and oxytocinergic and dopaminergic signaling in the brain (35). Thirdly, probiotic treatment rescued social deficits in genetic, environmental, and idiopathic mouse models of ASD (35, 36), and restored synaptic, in addition to social, deficits induced by maternal high-fat diet in mice offspring (37). These findings implicate probiotics as a promising treatment for many ASD cases with different etiologies. These are exciting developments, as the initial findings from 6 years ago of probiotic rescue of ASD symptoms (38) did not reveal the mechanism of action for this improvement, and recent evidence for probiotic effectiveness in different etiological ASD models and specific pathways for their

effects bring scientific credence to the potential therapeutic value of gut-brain investigations in ASD.

More recently, another dimension was added to this axis given the aberrant immune responses and inflammatory profiles widely reported in ASD (39, 40), yielding the "gut-immune-brain" axis (41–43). The only three papers referencing this axis in relation to ASD consider inflammation and immune reaction in a systematic fashion and in general terms, where one presents a systematic review of gut-immune-brain mechanisms in ASD (41), the other investigates the role of sex in this communication (42), the latter suggests amino acids as a potential treatment to reduce inflammation and alter gut microbiota composition (43). I believe that a more specific approach to immunity that considers to which compounds that immune reaction is given in each individual with ASD is warranted, and this idea forms the basis of the treatment proposed in this paper.

Given the ability of the microbiota to produce psychoactive chemicals that are hormones or have hormonal qualities and considering the gut microbiota as a "unique collective endocrine organ," Obrenovich et al. (44) have recently put forth the term "microbiota-gut-brain-endocrine interactome" based on the concept of an interactome denoting the complete molecular interactions in a biochemical system. Though they have created this term to explain the co-metabolism in human hosts in general, it is applicable to ASD well. Here, I wish to combine this terminology with the immune domain and conceive a "gut-immune-endocrine-brain interactome" on which I base my personalized treatment approach.

The bidirectional signaling between the gut and the brain is believed to happen through four main signaling pathways, which are the neural pathway including the vagus nerve enabling the gut microbiota to influence the brain and central nervous system efferent neurons that influence the gut; endocrine pathway involving enteroendocrine signaling stimulated by bacterial byproducts and hypothalamic-pituitary-adrenal (HPA) axis activation modulating the gut microbiota; neurotransmitters including serotonin of which about 95% is produced in the gut and those released by sympathetic activation and/or anti-inflammatory reflexes that influence the gut; and immune signaling from the reactions of the gut defensive barrier to externally derived pathogens and internal agents (45–47). While within the gut-brain axis, immune and endocrine functions are considered simply as pathways enabling communication between the gut and the brain, the new naming used here of the gut-immune-endocrine-brain axis additionally considers the influences of immune and endocrine mechanisms on the brain and neurological pathology.

PROBIOTICS

Probiotics, live microorganisms that provide a health benefit to the host when given in adequate amounts (48), are enjoying popularity as a potential treatment avenue for ASD, supported by initial hopeful findings of normalizations in gut microbiota alterations, improvements in gastrointestinal symptoms, and decreases in autism symptoms and severity upon probiotic administration in individuals with ASD (49–51).

In a previous paper, I have noted that the holistic physiological effects of dietary interventions are mostly unknown and though GF/CF diet and KD may benefit some subgroups of individuals with ASD, they have certain negative gut microbial, gastrointestinal, and metabolic effects. So, I suggested probiotics as a safer alternative than these restrictive diets since they assist gluten digestion, counteract gluten's harmful effects on gut permeability, reduce gut inflammation, increase gut integrity, and improve gastrointestinal and ASD symptoms (16). I concluded by suggesting the possibility that specific strains or combinations of probiotics may work better for individuals with ASD with specific co-morbid conditions, such as dietary protein intolerance. Nonetheless, while discussing the potential mechanisms of effect via which probiotics can serve similar benefits as restrictive diets without their harmful effects, I have not specified the probiotic strains that possess the specific features I talk about. Therefore, I may have fallen into the same fallacy that I have criticized above of considering probiotics in general terms, which I endeavor to rectify herein.

In reaction to misrepresentations about probiotics in the media, industry, and scientific field and generalized statements overlooking their limitations, a recent appeal was made to strictly observe the scientific definition and avoid generalizations from studies of single probiotic products (52). These authors reiterate the requirements to call an organism probiotic, such as being alive in adequate numbers, identified genetically and classified with latest terminology, and proven effective with appropriately sized and designed studies (52). They also note the importance of defining the basis for population stratification when designing individualized therapies (52), which is done in the present paper with clear screening procedures.

Similarly, a different group set out to show how such glorifications of probiotics as panaceas are indeed too good to be true. They found that probiotics create an individualized effect on gut bacteria and mucosal community structure, and based on these findings, they call for the development of new personalized probiotic approaches (53). This parallels the views of another group who note the dramatic increase in scientific, public, and industrial interest in probiotics and prebiotics as potential management agents for gut microbiota, and suggest that this field has the potential to create a new route for personalized medicine (54).

Following these appeals for personalized approaches and for avoiding generalized, non-specific references to probiotics, I present a novel, personalized dietary treatment based on the gut-immune-endocrine-brain axis for autism spectrum disorder.

NOVEL, PERSONALIZED DIETARY TREATMENT FOR ASD

Background

Dietary proteins and interventions have been proposed to affect ASD symptoms through the gut-brain axis.

Panksepp (55) observed that behavior induced by low doses of narcotics resembled ASD behaviors, and proposed an excessive or unusual activity in endogenous brain opioid systems of children with ASD. Subsequently, an opioid-excess theory of

autism was proposed. According to this theory, in individuals with ASD, peptides with opioid activity are formed from dietary sources, especially from those containing gluten and casein. These peptides pass through an abnormally permeable gut membrane and enter the central nervous system to influence neurotransmission and other physiological symptoms (56).

The underlying microbiota-gut-brain axis mechanisms of GF/CF diets have also been related to the premises of the opioid-excess theory (57). The increased endogenous opioids in the body fluids of individuals with ASD have been connected to altered activity in ASD of the DPP4 enzyme, which is able to decrease endogenous and exogenous opioid peptide levels in the blood (58). These excessive amounts of opioid peptides are suggested to pass the blood-brain barrier, bind to opioid receptors, and result in ASD symptoms. As gluten and casein are the precursors for these opioid peptides, removing them is proposed to improve ASD symptoms by positively influencing neurotransmission in the brain either directly or indirectly (58).

However, it is not clear whether this mechanism is at work in all individuals with ASD. This idea is supported by a randomized, double-blind, placebo-controlled trial administering a digestive enzyme supplement, which did not find any significant clinical improvement of ASD symptoms (59). This suggests that not all individuals with ASD may have abnormal digestion of gluten and casein leading to opioid peptides. Similarly, children who responded to a GF/CF diet were observed to give a heightened immune response to cow milk protein and/or gliadin (60). So, it is possible that not all individuals with ASD may have digestive problems with or immune reactivity to cow milk protein and gliadin. Therefore, individual measurements should be taken before recommending a GF/CF diet, which may not provide any behavioral benefits for some individuals with ASD and even be another negative factor aggravating social isolation (12).

Besides the elevated inflammatory reaction to dietary proteins observed in some individuals with ASD, it was also found that a subgroup of children with ASD produce antibodies against gliadin peptides and Purkinje cells (61). This finding is important for two reasons. First, it supports the existence a subgroup, and not all measured individuals with ASD, to have an immune reaction to gluten and gliadin, and it does so via another measurement besides pro-inflammatory cytokines, that of antibodies. Such antibody production may be another reason why GF/CF diets resulted in behavioral improvements for some ASD patients but not all of them. This finding attests once more to the need for individualized dietary treatment regimens for ASD. Secondly, the finding of a significant percentage of children with ASD showing elevated antibodies against not only gliadin but also cerebellar peptides suggests that the aberrant immunoreactivity in some individuals with ASD may not be limited to dietary proteins. Though the authors of this study suggest that these responses may be responsible for some neurological symptoms in ASD, the mechanism behind this and how such autoimmunoreactivity relates to aberrant immune reactions to dietary elements was not revealed until very recently.

Though it was not specifically tested in individuals with ASD, the very recent investigation into the similarities between dietary and human aquaporins may connect findings about dietary protein reactivity and autoimmunity in ASD. Aquaporins are membrane channel proteins that are found in plants and humans, including the gut barrier and the blood-brain barrier. Lambert et al. (4) showed that because of the shared structural homology between plant and human aquaporins, antibody immune reactions against food aquaporins can lead to neuroautoimmune reactivity. An already impaired intestinal barrier such as that suggested for ASD or a compromised gastrointestinal barrier due to reactivity with aquaporins in the gut barrier would allow antigenic material from foods and gut bacteria into the blood. Excessive antibody formation to these products may then trigger systemic inflammation and autoimmune reactivity, which may in turn affect the blood-brain barrier integrity, allowing circulating antibodies to enter the brain and target neurological tissues that resemble the antibody's target food antigen (4). As authors suggest that this environmentally induced neuroautoimmunity begins with genetic susceptibility for neurological disorders, this may be one model to connect genetic susceptibility and environmental triggers in ASD and the pathway underlying the previously mentioned finding of elevated antibodies against gluten and cerebellar cells in a group of children with ASD.

From Evidence to Treatment

Two suggestions come from abovementioned researchers that can help guide the creation of personalized treatments for ASD. Jyonouchi et al. (60), upon discovering that the subgroup of ASD children who clinically responded to GF/CF diet had an increased production of proinflammatory cytokine TNF-α with cow milk protein and/or gliadin, suggest that these responses to dietary proteins may be a simple, objective marker to assess the presence of dietary protein intolerance in children with ASD. Lambert et al. (4) propose that practitioners working with patients with neurological conditions should test for food aquaporin reactivity, and if they obtain a positive result, should remove the offending foods, which may ameliorate the patient's clinical condition.

These dietary recommendations may nicely combine with prebiotic and probiotic components. One group who found increased mycotoxin levels in individuals with ASD and especially of the mycotoxin Ochratoxin A (OTA) suggested a personalized diet coupled with probiotics for OTA-positive individuals with ASD (62). Another group combined an exclusion diet with prebiotics. Prebiotics are non-digestible food ingredients that are fermented by probiotics, resulting in health benefits to the host by selectively stimulating these beneficial bacteria and in the production of short-chain fatty acids (SCFAs) that can diffuse into the blood, enabling a distal effect on numerous body organs (63). As the administration of prebiotics and an exclusion diet resulted in improvements in social behavior, gut microbiota, and metabolites in children with ASD, the authors propose that multiple interventions, such as their combination, may be more relevant to improve both physiological and psychological traits in ASD (64).

Taking these novel findings and recommendations by researchers into account, I present a personalized dietary treatment regimen based on the gut-immune-endocrine-brain axis and the specific immune reactivity and gut microbial profile of each individual on the autism spectrum.

This proposed personalized treatment comprises the following three steps (**Figure 1**).

Step 1: Screening

(a) Screen for inflammatory response to milk and wheat proteins [see procedure by Jyonouchi et al. (60)]
(b) Screen for reactivity to dietary aquaporins including but not limited to milk and wheat [see procedure by Lambert et al. (4)]
(c) Perform fecal or another mapping of gut bacterial profile.

This preliminary proposition focused on milk and gluten proteins, since the currently available evidence suggests them to be the peptides against which children with ASD show higher levels of immunoglobulin antibodies (61, 65). In children with ASD, the antibodies against milk and gliadin peptides of gluten react with cerebellar peptides, which is not the case for antibodies for corn and soy, pointing to the significant antigenic cross-reactivity of gluten and milk with cerebellar antigens (61, 65). If future studies reveal similar antigenic cross-reactivity effects or increased immunoglobulin levels in individuals with ASD for other dietary proteins, they can also be included in the screening process.

Step 2: Implementation

Create a patient-specific dietary restriction plan based on inflammatory and aquaporin reactivity results from the screening.

Complement with prebiotic supplements [the most common prebiotics are Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and trans-galacto-oligosaccharides (TOS) (63)] and targeted probiotic strains that address the microbial imbalance in the gut [see procedures by Tomova et al. (49) and Shaaban et al. (50)].

Though two studies normalized altered gut microbiota in individuals with ASD using probiotic supplements, a complete mapping of the effects of these used and other probiotics should be done before administration or recommendation. Through the ingestion of appropriate probiotic strains or prebiotic growth substrates for the beneficial bacteria (63), gut microbial imbalance in individuals with ASD can be restored.

Here, the focus should be on correcting altered levels of gut microbial strains that produce critical SCFAs, such as butyrate, which is recently implicated in the mechanism of non-allergic gluten/wheat sensitivity via its effects on a chain reaction of events in the gut (66). The results of future investigations along these lines in individuals with ASD may necessitate the addition of screening for the levels of important SCFA-producing gut bacteria to Step 1 in the forthcoming refinements of the present proposal.

When creating the probiotic and prebiotic cocktail, attention should be paid to the recently published reiteration that in order to be considered probiotic, microbial strain designation and at least one human study are needed (52). Additionally, if a strain is shown to be effective for a certain function, such as improving the gut barrier, then that strain is expected to confer the same benefit when administered in the presence of other strains, at its previously tested dose (52). This notion supports the utilization of more than one probiotic strain in this cocktail depending on the specific needs of the individual.

Step 3: Evaluation

Test for the gut microbial, gut and blood-brain barrier-related, and inflammatory changes in individuals with ASD via measurements before and after treatment.

Observe differences in repetitive/restrictive behaviors, communication, and sociability in individuals with ASD using

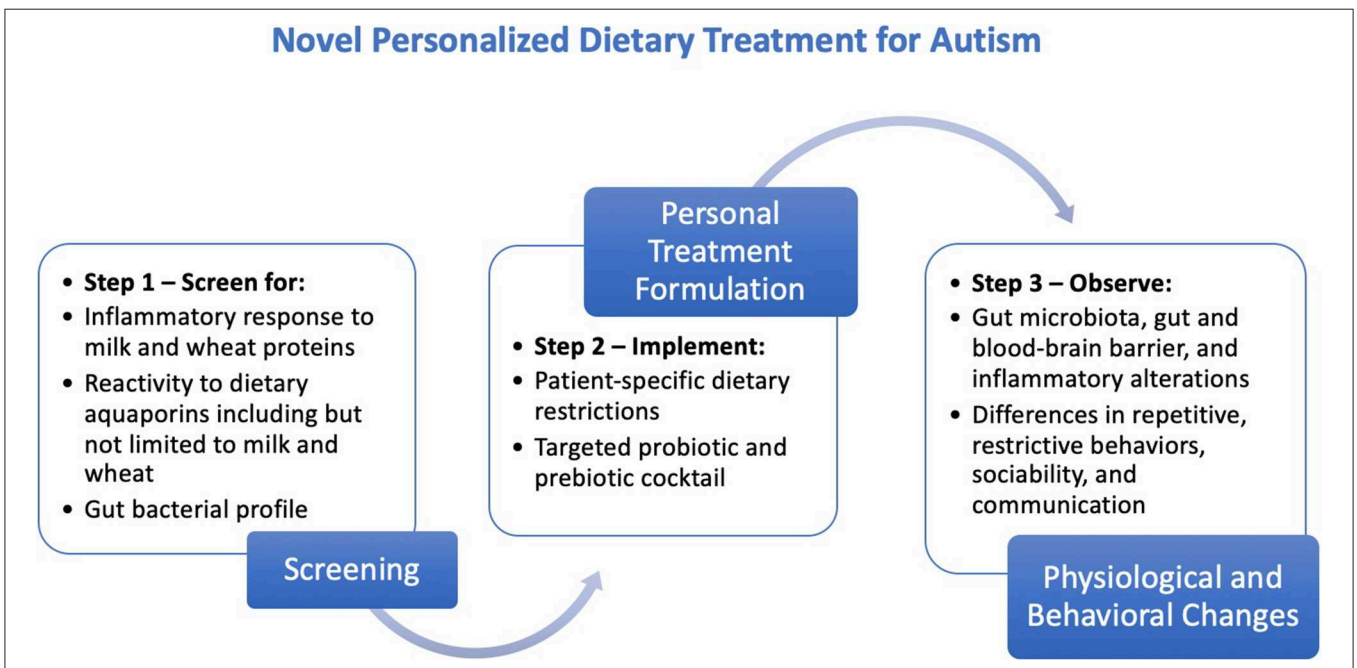

FIGURE 1 | Steps for the creation, implementation, and evaluation of the proposed personalized dietary treatment regimen for individuals with ASD.

measurements as Childhood Autism Rating Scale (CARS), Autism Diagnostic Observation Schedule (ADOS), and Autism Treatment Evaluation Checklist (ATEC).

It should be noted that this is a preliminary description of this novel personalized dietary treatment approach to ASD. Its aim lies more in the dissemination of this perspective than in the creation of a solid protocol, which will hopefully be the next step. Following investigations will evaluate the effectiveness and feasibility of such personalized screenings and the resulting dietary regimens for individuals with ASD of all ages, both sexes, and different levels of condition severity.

CONCLUSION

This perspective article translates the current emphasis on the gut-brain axis and appeal for personalized interventions into a feasible step-by-step application to create personalized dietary treatments for individuals with autism. By following a more integrated gut-immune-endocrine-brain axis model, it explains why and how to formulate an individualized dietary plan that matches the specific inflammatory and aquaporin reactivity responses of each individual with ASD. This way, individuals with ASD can avoid the harmful gut microbial, gastrointestinal, and metabolic effects of GF/CF and ketogenic diets delineated previously (16) and receive targeted treatments for their specific physiological profiles. More investigations into the full spectrum of effect of particular probiotics and prebiotics will help flesh out a protocol to include specific recommendations for individuals based on their personal needs. This next step of nutritional neuropsychopharmacology (67) is what will enable the formulation of patient-specific treatment regimens with utmost effectiveness and minimal side effects for individuals with ASD.

AUTHOR CONTRIBUTIONS

CD conceptualized and wrote the manuscript.

REFERENCES

1. American Psychiatric Association. *Desk Reference to the Diagnostic Criteria From DSM-5*. Washington, DC: American Psychiatric Publishing (2013).
2. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. Genetic heritability and shared environmental factors among twin pairs with autism. *Arch Gen Psychiatry*. (2011) 68:1095–102. doi: 10.1001/archgenpsychiatry.2011.76
3. Sandin S, Lichtenstein P, Kuja-Halkola R, Larsson H, Hultman CM, Reichenberg A. The familial risk of autism. *JAMA*. (2014) 311:1770–7. doi: 10.1001/jama.2014.4144
4. Lambert J, Mejia S, Vojdani A. Plant and human aquaporins: pathogenesis from gut to brain. *Immunol Res*. (2019) 67:12–20. doi: 10.1007/s12026-018-9046-z
5. Knivsberg AM, Reichelt KL, Nødland M, Høien T. Autistic syndromes and diet: a follow-up study. *J Curriculum Stud*. (1995) 39:223–36. doi: 10.1080/0031383950390304
6. Knivsberg AM, Reichelt KL, Høien T, Nødland M. A randomised, controlled study of dietary intervention in autistic syndromes. *Nutr Neurosci*. (2002) 5:251–61. doi: 10.1080/10284150290028945
7. Reichelt KL, Knivsberg AM, Lind G, Nødland M. Probable etiology and possible treatment of childhood autism. *Brain Dysfunction*. (1991) 4:308–19.
8. Whiteley P, Rodgers J, Savery D, Shattock P. A gluten-free diet as an intervention for autism and associated spectrum disorders: preliminary findings. *Autism*. (1999) 3:45–65. doi: 10.1177/1362361399003001005
9. Whiteley P, Haracopos D, Knivsberg AM, Reichelt KL, Parlar S, Jacobsen J, et al. The ScanBrit randomised, controlled, single-blind study of a gluten-and casein-free dietary intervention for children with autism spectrum disorders. *Nutr Neurosci*. (2010) 13:87–100. doi: 10.1179/147683010X12611460763922
10. Elder JH, Shankar M, Shuster J, Theriaque D, Burns S, Sherrill L. The gluten-free, casein-free diet in autism: results of a preliminary double blind clinical trial. *J Autism Dev Disord*. (2006) 36:413–20. doi: 10.1007/s10803-006-0079-0
11. Hyman SL, Stewart PA, Foley J, Peck R, Morris DD, Wang H, et al. The gluten-free/casein-free diet: a double-blind challenge trial in children with autism. *J Autism Dev Disord*. (2016) 46:205–20. doi: 10.1007/s10803-015-2564-9
12. Sponheim E. Gluten-free diet in infantile autism. A therapeutic trial. *Tidsskr Nor Laegeforen*. (1991) 111:704–7.
13. Ruskin DN, Svedova J, Cote JL, Sandau U, Rho JM, Kawamura M Jr, et al. Ketogenic diet improves core symptoms of autism in BTBR mice. *PLoS ONE*. (2013) 8:e65021. doi: 10.1371/journal.pone.0065021
14. Evangeliou A, Vlachonikolis I, Mihailidou H, Spilioti M, Skarpalezou A, Makaronas N, et al. Application of a ketogenic diet in children with autistic behavior: pilot study. *J Child Neurol*. (2003) 18:113–8. doi: 10.1177/0883073803018 0020501
15. El-Rashidy O, El-Baz F, El-Gendy Y, Khalaf R, Reda D, Saad K. Ketogenic diet versus gluten free casein free diet in autistic children: a case-control study. *Metab Brain Dis*. (2017) 32:1935–41. doi: 10.1007/s11011-017-0088-z
16. Doenyas C. Dietary interventions for autism spectrum disorder: new perspectives from the gut-brain axis. *Physiol Behav*. (2018) 194:577–82. doi: 10.1016/j.physbeh.2018.07.014
17. Lee RW, Corley MJ, Pang A, Arakaki G, Abbott L, Nishimoto M, et al. A modified ketogenic gluten-free diet with MCT improves behavior in children with autism spectrum disorder. *Physiol Behav*. (2018) 188:205–11. doi: 10.1016/j.physbeh.2018.02.006
18. Adams J, Audhya T, Geis E, Gehn E, Fimbres V, Pollard E, et al. Comprehensive nutritional and dietary intervention for autism spectrum disorder—a randomized, controlled 12-month trial. *Nutrients*. (2018) 10:369. doi: 10.3390/nu10030369
19. Pennesi CM, Klein LC. Effectiveness of the gluten-free, casein-free diet for children diagnosed with autism spectrum disorder: based on parental report. *Nutr Neurosci*. (2012) 15:85–91. doi: 10.1179/1476830512Y.0000000003
20. Edelson SB, Cantor DS. Autism: xenobiotic influences. *Toxicol Ind Health*. (1998) 14:799–811. doi: 10.1177/074823379801400603
21. Roberts EM, English PB, Grether JK, Windham GC, Somberg L, Wolff C. Maternal residence near agricultural pesticide applications and autism spectrum disorders among children in the California Central Valley. *Environ Health Perspect*. (2007) 115:1482–9. doi: 10.1289/ehp.10168
22. Adams JB, Baral M, Geis E, Mitchell J, Ingram J, Hensley A, et al. The severity of autism is associated with toxic metal body burden and red blood cell glutathione levels. *J Toxicol*. (2009) 2009:532640. doi: 10.1155/2009/532640
23. Fido A, Al-Saad S. Toxic trace elements in the hair of children with autism. *Autism*. (2005) 9:290–8. doi: 10.1177/1362361305053255

24. Geier DA, Kern JK, Garver CR, Adams JB, Audhya T, Nataf R, et al. Biomarkers of environmental toxicity and susceptibility in autism. *J Neurol Sci*. (2009) 280:101–8. doi: 10.1016/j.jns.2008.08.021
25. Fallon, J. (2005). Could one of the most widely prescribed antibiotics amoxicillin/clavulanate "augmentin^{TM}" be a risk factor for autism? *Med Hypotheses*. 64, 312–315. doi: 10.1016/j.mehy.2004.06.023
26. Kern JK, Grannemann BD, Trivedi MH, Adams JB. Sulfhydryl-reactive metals in autism. *J Toxicol Environ Health*. (2007) 70:715–21. doi: 10.1080/15287390601188060
27. Carter CJ, Blizard RA. Autism genes are selectively targeted by environmental pollutants including pesticides, heavy metals, bisphenol A, phthalates and many others in food, cosmetics or household products. *Neurochem Int*. (2016) 101:83–109. doi: 10.1016/j.neuint.2016.10.011
28. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. *Science*. (2012) 336:1262–7. doi: 10.1126/science.1223813
29. Mayer EA, Knight R, Mazmanian SK, Cryan JF, Tillisch K. Gut microbes and the brain: paradigm shift in neuroscience. *J Neurosci*. (2014) 34:15490–6. doi: 10.1523/JNEUROSCI.3299-14.2014
30. Reichelt KL, Knivsberg AM. Can the pathophysiology of autism be explained by the nature of the discovered urine peptides? *Nutr Neurosci*. (2003) 6:19–28. doi: 10.1080/1028415021000042839
31. d'Eufemia P, Celli M, Finocchiaro R, Pacifico L, Viozzi L, Zaccagnini M, et al. Abnormal intestinal permeability in children with autism. *Acta Paediatr*. (1996) 85:1076–9. doi: 10.1111/j.1651-2227.1996.tb14220.x
32. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism-comparisons to typical children and correlation with autism severity. *BMC Gastroenterol*. (2011) 11:1–13. doi: 10.1186/1471-230X-11-22
33. Reichelt KL, Knivsberg AM. The possibility and probability of a gut-to-brain connection in autism. *Ann Clin Psychiatry*. (2009) 21:205–11. Available online at: https://www.researchgate.net/profile/Karl_Reichelt/publication/232530717_Probable_etiology_and_possible_treatment_of_childhood_autism/links/558ba78108ae02c9d1f964ea/Probable-etiology-and-possible-treatment-of-childhood-autism.pdf
34. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. *Cell*. (2019) 177:1600–18. doi: 10.1016/j.cell.2019.05.004
35. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder. *Neuron*. (2019) 101:246–59. doi: 10.1016/j.neuron.2018.11.018
36. Tabouy L, Getselter D, Ziv O, Karpuj M, Tabouy T, Lukic I, et al. Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. *Brain Behav Immunity*. (2018) 73:310–9. doi: 10.1016/j.bbi.2018.05.015
37. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell*. (2016) 165:1762–75. doi: 10.1016/j.cell.2016.06.001
38. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*. (2013) 155:1451–63. doi: 10.1016/j.cell.2013.11.024
39. Ashwood P, Wills S, Van de Water J. (2006). The immune response in autism: a new frontier for autism research. *J Leukoc Biol*. 80, 1–15. doi: 10.1189/jlb.1205707
40. Stigler KA, Sweeten TL, Posey DJ, McDougle CJ. Autism and immune factors: a comprehensive review. *Res Autism Spectr Disord*. (2009) 3:840–60. doi: 10.1016/j.rasd.2009.01.007
41. Azhari A, Azizan F, Esposito G. A systematic review of gut-immune-brain mechanisms in Autism Spectrum Disorder. *Dev Psychobiol*. (2019) 61:752–71. doi: 10.1002/dev.21803
42. Kopec AM, Fiorentino MR, Bilbo SD. Gut-immune-brain dysfunction in autism: importance of sex. *Brain Res*. (2018) 1693:214–7. doi: 10.1016/j.brainres.2018.01.009
43. Van Sadelhoff J, Perez-Pardo P, Wu J, Garssen J, Van Bergenhenegouwen J, Hogenkamp A, et al. The gut-immune-brain axis in autism spectrum disorders; a focus on amino acids. *Front Endocrinol*. (2019) 10:247. doi: 10.3389/fendo.2019.00247
44. Obrenovich M, Rai H, Chittoor Mana TS, Shola D, McCloskey B, Sass C, et al. Dietary co-metabolism within the microbiota-gut-brain-endocrine metabolic interactome. *BAO Microbiol*. (2017) 2:022. Available online at: https://www.researchgate.net/profile/Mark_Obrenovich/publication/314091205_Dietary_Co-Metabolism_within_the_Microbiota-Gut-Brain-Endocrine_Metabolic_Interactome/links/58b4c675aca2725b541c3e3a/Dietary-Co-Metabolism-within-the-Microbiota-Gut-Brain-Endocrine-Metabolic-Interactome.pdf
45. Anadure RK, Shankar S, Prasad AS. *The Gut-Brain Axis. Medicine Update 2019*. Association of Physicians India (2019).
46. Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G. The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. *Front Integr Neurosci*. (2013) 7:70. doi: 10.3389/fnint.2013.00070
47. Fülling C, Dinan TG, Cryan JF. Gut microbe to brain signaling: what happens in vagus.… *Neuron*. (2019) 101:998–1002. doi: 10.1016/j.neuron.2019.02.008
48. FAO/WHO. (2002). *Guidelines for the Evaluation of Probiotics in Food*. Available online at: https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed July 17, 2019).
49. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, et al. Gastrointestinal microbiota in children with autism in Slovakia. *Physiol Behav*. (2015) 138:179–87. doi: 10.1016/j.physbeh.2014.10.033
50. Shaaban SY, El Gendy YG, Mehanna NS, El-Senousy WM, El-Feki HS, Saad K, et al. The role of probiotics in children with autism spectrum disorder: a prospective, open-label study. *Nutr Neurosci*. (2018) 21:676–81. doi: 10.1080/1028415X.2017.1347746
51. Sanctuary MR, Kain JN, Chen SY, Kalanetra K, Lemay DG, Rose DR, et al. Pilot study of probiotic/colostrum supplementation on gut function in children with autism and gastrointestinal symptoms. *PLoS ONE*. (2019) 14:e0210064. doi: 10.1371/journal.pone.0210064
52. Reid G, Gadir AA, Dhir R. Probiotics: reiterating what they are and what they are not. *Front Microbiol*. (2019) 10:424. doi: 10.3389/fmicb.2019.00424
53. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell*. (2018) 174:1388–405. doi: 10.1016/j.cell.2018.08.041
54. Vyas U, Ranganathan N. Probiotics, prebiotics, and synbiotics: gut and beyond. *Gastroenterol Res Pract*. (2012) 2012:872716. doi: 10.1155/2012/872716
55. Panksepp J. A neurochemical theory of autism. *Trends Neurosci*. (1979) 2:174–7. doi: 10.1016/0166-2236(79)90071-7
56. Whiteley P, Shattock P. Biochemical aspects in autism spectrum disorders: updating the opioid-excess theory and presenting new opportunities for biomedical intervention. *Expert Opin Ther Targets*. (2002) 6:175–83. doi: 10.1517/14728222.6.2.175
57. Whiteley P, Shattock P, Carr K, Hooper M, Todd L. How could a gluten- and casein-free diet ameliorate symptoms associated with autism spectrum conditions? *Autism Insights*. (2010) 2010:39–53. doi: 10.4137/AUI.S6275
58. Ciéslinska A, Kostyra E, Savelkoul HFJ. Treating autism spectrum disorder with gluten-free and casein-free diet: the underlying microbiota-gut-brain axis mechanisms. *HSOA J Clin Immunol Immunother*. (2017) 3, 1–11. doi: 10.24966/CIIT-8844/100009
59. Munasinghe SA, Oliff C, Finn J, Wray JA. Digestive enzyme supplementation for autism spectrum disorders: a double-blind randomized controlled trial. *J Autism Dev Disord*. (2010) 40:1131–8. doi: 10.1007/s10803-010-0974-2
60. Jyonouchi H, Sun S, Itokazu N. Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder. *Neuropsychobiology*. (2002) 46:76–84. doi: 10.1159/000065416
61. Vojdani A, O'Bryan T, Green JA, McCandless J, Woeller KN, Vojdani E, et al. Immune response to dietary proteins, gliadin and cerebellar

peptides in children with autism. *Nutr Neurosci.* (2004) 7:151–61. doi: 10.1080/10284150400004155
62. De Santis B, Brera C, Mezzelani A, Soricelli S, Ciceri F, Moretti G, et al. Role of mycotoxins in the pathobiology of autism: a first evidence. *Nut Neurosci.* (2019) 22:132–44. doi: 10.1080/1028415X.2017.1357793
63. Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, et al. Prebiotics: definition, types, sources, mechanisms, and clinical applications. *Foods*. (2019) 8:92. doi: 10.3390/foods8030092
64. Grimaldi R, Gibson GR, Vulevic J, Giallourou N, Castro-Mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). *Microbiome*. (2018) 6:133. doi: 10.1186/s40168-018-0523-3
65. Vojdani A, Campbell AW, Anyanwu E, Kashanian A, Bock K, Vojdani E. Antibodies to neuron-specific antigens in children with autism: possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae* and Streptococcus group A. *J Neuroimmunol.* (2002) 129:168–77. doi: 10.1016/S0165-5728(02)00180-7
66. Leccioli V, Oliveri M, Romeo M, Berretta M, Rossi P. A new proposal for the pathogenic mechanism of non-coeliac/non-allergic gluten/wheat sensitivity: piecing together the puzzle of recent scientific evidence. *Nutrients.* (2017) 9:1203. doi: 10.3390/nu9111203
67. Provensi G, Schmidt SD, Boehme M, Bastiaanssen TF, Rani B, Costa A, et al. Preventing adolescent stress-induced cognitive and microbiome changes by diet. *Proc Natl Acad Sci USA*. (2019) 116:9644–51. doi: 10.1073/pnas.1820832116

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Long-Term Effects of Zinc Deficiency and Zinc Supplementation on Developmental Seizure-Induced Brain Damage and the Underlying GPR39/ZnT-3 and MBP Expression in the Hippocampus

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We previously illustrated that long-term upregulated expression of ZnT-3 in the hippocampus of rats that underwent neonatal seizures was restored by pretreatment with a ketogenic diet. It was recently demonstrated that upregulated expression of ZnT-3 was associated with increased concentrations of intracellular free zinc ions in an *in vitro* model of glutamate-induced hippocampal neuronal excitotoxic damage. However, there is still a lack of research on the effects of different concentrations of zinc in the diet on developmental convulsive brain injury. The aim of this study was to investigate the effects of different zinc concentrations in the diet on long-term neurobehavioral and seizure thresholds following lithium chloride-pilocarpine-induced developmental seizures. Sprague-Dawley rats (postnatal day 27, P27) were randomly assigned to one of six dietary groups for 4 weeks: normal zinc control group (Control group, 44 mg/kg Zn), Zn-deficient control group (ZD group, 2.7 mg/kg Zn), Zn supplemented control group (ZS group, 246 mg/kg Zn), pilocarpine-induced seizure plus regular zinc diet group (SE group, 44 mg/kg Zn), seizure plus low-zinc diet group (SE + ZD group, 2.7 mg/kg Zn), and seizure plus high-zinc diet group (SE + ZS group, 246 mg/kg Zn). Novel object recognition and passive avoidance tests were performed on rats at P42 and P56. After routine seizure threshold detection and Timm staining procedures at P57, expression of GPR39, ZnT-3, and MBP were detected in the hippocampus by Western blot analysis. The results revealed that the Zinc-deficient diet for 4 weeks aggravated the long-term adverse effects of developmental seizures, evidenced by weight, cognition, seizure threshold and serum zinc concentrations, which were paralleled by expression changes in hippocampal GPR39 and ZnT-3. In contrast, zinc supplementation for 4 weeks significantly improved damage-related changes described above and rescued the abnormal expression of GPR39, ZnT-3, and MBP in the hippocampus. Similar alterations between the expression pattern of MBP and aberrant sprouting of mossy fibers in the hippocampus may indicate that sprouting is a secondary pathological change caused by developmental brain damage

rather than the cause of epileptogenesis. Up-regulation of MBP protein levels in the high zinc diet-treated seizure group as well as the corresponding improvement of cognitive impairment and reduced hippocampal mossy fiber regenerative sprouting, may represent a compensatory mechanism for neuronal membrane damage and repair.

Keywords: zinc diet, developmental seizure, GPR39, ZnT-3, MBP, plasma membrane integrity

INTRODUCTION

Developmental seizure-induced brain damage is often accompanied by cognitive deficits, memory disorders, emotional abnormalities, etc. (Rzezak et al., 2017; Vidaurre and Twanow, 2017; De Toffol et al., 2018), which has serious consequences on families and society. At present, 20 to 30% of patients with epilepsy do not experience relief after reasonable standard drug treatment, which is known as refractory epilepsy (López González et al., 2015). Temporal lobe epilepsy is one of the most common forms of refractory epilepsy and is caused by changes in molecular networks, and structure and function after brain injury, leading to a pathological environment that ultimately promotes excitability and produces repeated spontaneous seizures. This process is known as epileptogenesis (Jehi and Vezzani, 2014). The mechanism of epileptogenesis may be multifactorial and is not yet fully understood, limiting the development of new drugs. Therefore, exploring the pathophysiological mechanisms of epileptogenesis is critical to the development of new, safe, and effective drugs.

An imbalance between neuronal excitation and inhibition defines the mechanism of epilepsy (Staley, 2015), and zinc ions are involved in convulsive excitotoxic neuronal injury. Zinc ions interact with various targets that mediate neuronal excitation or inhibition. For example, using zinc ion chelating agent (TPEN) to induce zinc deficiency in an *in vitro* neuronal cell model, zinc deficiency was shown to cause decreased cell viability and increased rates of apoptotic. These changes are reversed by zinc supplementation (Tian et al., 2018).

Zinc transporter 3 (ZnT-3) knockout mice are more sensitive to seizures caused by kainic acid injection due to their lack of synaptic zinc ions (Mcallister and Dyck, 2017), indicating that a deficiency in synaptic zinc ions reduces seizure thresholds. Ketogenic diet (KD) is a nutritional treatment that is beneficial in epilepsy refractory to antiepileptic drugs. It was previously shown that the mechanism(s) of KD's action involve altered zinc metabolism, as KD rescues seizure-induced elevated ZnT-3 expression in the hippocampus (Tian et al., 2015). We recently demonstrated that glutamate stimulation of HT22 hippocampal neurons significantly increases intracellular zinc ion concentrations, which is positively correlated with mitophagy levels and mitochondrial dysfunction (Jin et al., 2018). These studies highlight the possibility that zinc ion signaling is a novel target for inhibiting epileptogenesis.

At present, there are few studies using *in vivo* models to investigate the effects of zinc intervention on epilepsy, and the results are often contradictory due to the type of epilepsy, the dose of zinc intervention and the route of administration. Kumar et al. (2015) once investigated the effect of zinc ions on acute seizures. They found that oral administration of 2, 20, or 200 mg/kg zinc sulfate for 2 weeks did not affect acute seizures induced by maximum electroconvulsive shock; however, 2 mg/kg zinc administration significantly reduced seizure duration and increased the latency of seizures induced by pentylenetetrazol (PTZ). In addition, 200 mg/kg zinc sulfate intervention significantly reduced the number of ignited animals and reduced the seizure severity score. In contrast, Baraka et al. (2012) reported the opposite results. They found that intraperitoneal injection of zinc sulfate at 60 mg/kg for 3 weeks increased the severity of pilocarpine-induced seizures. Therefore, it is necessary to further study the role of zinc in epilepsy and its underlying molecular mechanisms using *in vivo* animal models.

Assessing the effects of different concentrations of zinc diet on developmental seizure-induced brain damage may be an important step in elucidating the role of zinc in epilepsy. Based on the *in vivo* animal model of developmental seizures induced by lithium chloride-pilocarpine, this study explored the long-term effects of zinc deficiency and zinc supplementation on developmental seizure-induced brain damage, focusing on the parameters of cognition, seizure threshold, hippocampal regenerative mossy fiber sprouting and expression of ZnT-3 and GPR39 in hippocampus to further reveal the relationship between zinc and epileptogenesis and provide new insights for the prevention and treatment of epilepsy. G protein-coupled receptor 39 (GPR39) is a metabotropic zinc-specific receptor (Kovacs et al., 2014). GPR39 knockout enhances susceptibility to kainic acid-induced seizures and increases seizure duration (Gilad et al., 2015). In addition, we assessed expression of myelin basic protein (MBP) because it plays a key role in controlling neuronal membrane integrity and axonal regeneration (Snaidero et al., 2017).

MATERIALS AND METHODS

Animal Preparation

Postnatal day 27 (P27) male Sprague-Dawley rats (n = 145) were obtained from the Zhao Yan (Suzhou) New Drug Research Center, Co., Ltd. [Animal License No. SCXK (Su) 2018-0006], China. Animals were treated in accordance with the guidelines set by the National Institutes of Health (Bethesda, MD, United States) for the humane treatment of animals and the Declaration of Helsinki. This study was approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Soochow University. The program was approved by the Medical Ethics Committee of Soochow University Children's Hospital. Adequate measures were taken to minimize pain and

the number of animals used. One hundred forty-five 27-day-old SD rats were randomly divided into two groups, a control group (n = 36) and a model group (n = 109). The control group was randomly divided into 3 subgroups with 12 rats each: normal zinc control group (Control group, 44 mg/kg Zn), Zn-deficient control group (ZD group, 2.7 mg/kg Zn), Zn supplemented control group (ZS group, 246 mg/kg Zn). The remaining 109 rats were used for the seizure model. Rats with successful modeling were randomly assigned to a subgroup of the seizure groups: pilocarpine-induced seizure plus regular zinc diet group (SE group, 44 mg/kg Zn), seizure plus low zinc diet group (SE + ZD group, 2.7 mg/kg Zn), seizure plus high zinc diet group (SE + ZS group, 246 mg/kg Zn).

Induction of Status Epilepticus (Torolira et al., 2016)

Rats in each seizure group were injected with lithium chloride (127 mg/kg, i.p.) at P27. Twenty-four hours later (P28), scopolamine methyl chloride hydrobromide (1 mg/kg, i.p.) was injected to reduce peripheral cholinergic response. Thirty minutes later, pilocarpine (320 mg/kg, i.p.) was injected. Then, behavioral changes were observed. According to Racine classification (Racine, 1972), the model is considered to be successful and included in the study if the seizure degree reaches level IV or above; otherwise, the animal is excluded. Seventy-nine rats achieved grade IV and above in our experiments. Eleven animals died due to generalized tonic seizures, and the remaining 68 were randomly assigned to the SE group (n = 23), the SE + ZD group (n = 23), and the SE + ZS group (n = 22). During the subsequent feeding process, 3, 2, and 2 rats died in the SE, SE + ZD, and SE + ZS groups, respectively. In the six groups (Control, ZD, ZS, SE, SE + ZD, and SE + ZS), 12, 12, 12, 20, 21, and 20 rats survived, respectively. In each group, 10 rats (picric acid marker) were randomly labeled for weight measurement and behavioral testing. The remaining animals in each group were reserved for proteomics testing and were not included in the present study.

Dietary Intervention

During the modeling period (P27, P28), each group was fed a normal zinc diet. After convulsion, Control and SE groups continued to receive a normal zinc diet (44 mg/kg zinc) for 28 days. ZD and SE + ZD groups were fed a low zinc diet (2.7 mg/kg zinc) for 28 days. ZS and SE + ZS groups were fed a high zinc diet (246 mg/kg zinc) for 28 days.

Weight Monitoring

The body weight of rats in each group (labeling with picric acid) was recorded every 6 days.

Novel Object Recognition Test (Jung et al., 2019)

Each rat was placed into a 50 cm × 50 cm × 50 cm uncovered test chamber for 10 min the day before the test as an introduction to the environment.

During the training phase, two identical objects are placed in two opposite parallel corners of the box, allowing each rat to probe both objects for 10 min.

For the test phase, one of the objects was replaced by another new object after a 1 h interval, and rats were allowed to explore again for 10 min. Exploring is defined as pointing the nose at an object and/or contacting the object with the nose at a distance of no more than 2 cm. Experiments were performed in a quiet environment to the extent that was possible, and the test chamber was wiped clean with 75% ethanol after each experiment. Rats have a natural tendency to explore novelty and usually spend more time exploring new objects than familiar ones that are remembered. The time that the rat spends exploring familiar objects and novel objects was recorded, and cognitive index was calculated (the time spent exploring the novel object divided by total exploration time).

Determination of Serum Zinc Concentration

Blood samples were collected from the tail vein of mice in each group at P57. Blood samples were centrifuged at 3000 r/min for 10 min, and supernatants were transferred into labeled EP tubes. According to the instructions of the zinc assay kit, 12 μL sample (serum, with zinc standard solution and deionized water used as blanks), and 200 μL reagent I was added to a 37°C water bath for 5 min. Absorbance A1 was read at 570 nm, and then reagent II was added to a 37°C water bath for 5 min. Absorbance A1 was read at 570 nm, and absorbance A2 was read after adding reagent II in a water bath for 5 min. ΔA = A2–A1. Sample concentration = (ΔA sample)/(ΔA standard) * Standard concentration.

Establishment of a "Two-Hit" Model and Determination of Seizure Threshold (Ni et al., 2018)

After lithium chloride-pilocarpine induced seizures, animals from all six groups were injected with penicillin (5.1 × 106 U/kg/d, i.p.) at P57. The time to the first seizure after penicillin injection was recorded as seizure latency (min) (seizure threshold). The observation time was 1 h.

Timm Staining

At the end of the seizure threshold test on P57, four rats from each group were randomly selected and each given an i.p. injection of chloral hydrate at a dose of 1 ml/100 g followed by regular Timm staining (n = 4/each group, P57) (Ni et al., 2009). Briefly, animals were perfused through the heart with 0.4% sodium sulfide (100 ml) and then 4% PFA in PBS (100 ml). The brains were equilibrated sequentially with 30% sucrose at 4°C for further analysis. 30 μm-thick coronal brain sections were stained in the solutions consisted of 30% gum arabic, 3.825% citric acid, 3.525% sodium citrate, 3.525% hydroquinone, and 25.5% silver nitrate. The slides were incubated at 26°C for 70 min. The person who scored the Timm staining was blind to the experimental grouping.

Western Blot Analysis

The remaining rats in each group were perfused with 4% chloral hydrate (1 ml/100 g, P57). Hippocampi were placed in a precooled labeled EP tube and then quickly stored at −80°C. Four rat tissues were taken from each group for protein extraction, and the remaining tissues were frozen for other purposes. Detailed steps of the Western blotting method have been previously described (Ni et al., 2018).

Briefly, polyvinylidene fluoride membrane blots were incubated with one of the following antibodies: goat anti-ZnT3 (1:1000, Santa Cruz), rabbit anti-GPR39 polyclonal antibody (1:1000, Biorbyt), rat anti- MBP monoclonal antibody (1:2000, Abcam), mouse anti-GAPDH monoclonal antibody (1:5000, Proteintech) or rabbit anti-β-tubulin polyclonal antibody (1:5000, Proteintech) in TBST contain 5% non-fat dry milk overnight at 4°C. Membranes were then incubated with secondary antibodies for approximately 2 h. ECL chemiluminescence kit A and B were mixed in equal volume, and PVDF membranes were immersed in the luminescent liquid for approximately 2 min, and then the film was placed in an automatic developing device for exposure (LAS 4010, GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Grayscale values of each band were analyzed using ImageJ image processing software.

Statistical Analysis

Body weight was analyzed by two-way, repeated-measures ANOVA (treatment as a between subject factor and training day as a within subject factor). Serum zinc concentration, seizure threshold, Timm staining, neurobehavioral data and protein levels were analyzed using one-way ANOVA with *post hoc* tests. Data are presented as the mean ± SD. Statistical significance was considered $P < 0.05$.

RESULTS

Weight Monitoring

As shown in **Figure 1**, during establishment of the seizure model (P27), there were no significant differences in body weight between rats in each group. At P42, the body weight of SE, SE + ZD, and SE + ZS groups was statistically decreased compared to Control, ZD and ZS groups, respectively ($P < 0.05$). At P56, the body weight of SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups was significantly decreased compared to Control, ZD, ZS, Control, and SE groups, respectively ($P < 0.05$).

Novel Object Recognition Test

At P42, there was a significant difference in the recognition index of each group ($P < 0.05$). Further pairwise comparison revealed that recognition indexes were statistically reduced in SE, SE + ZD, and SE + ZS groups compared to the corresponding control, ZD and ZS groups, respectively ($P < 0.05$). At P56, there was a significant difference in the recognition index of each group ($P < 0.05$). Further pairwise comparison indicated that recognition indexes were statistically reduced in SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups compared to the corresponding control, ZD, ZS, control, and SE groups, respectively ($P < 0.05$, **Figure 2**).

Determination of Serum Zinc Concentration

There were statistical differences in serum zinc concentration among the six groups at both P43 and P57 ($P < 0.05$). At P43, serum zinc concentrations in the SE, SE + ZD, and SE + ZS groups were obviously lower than corresponding groups of Control, ZD, and ZS groups, respectively ($P < 0.05$). At P57, there were significantly lower serum zinc concentrations

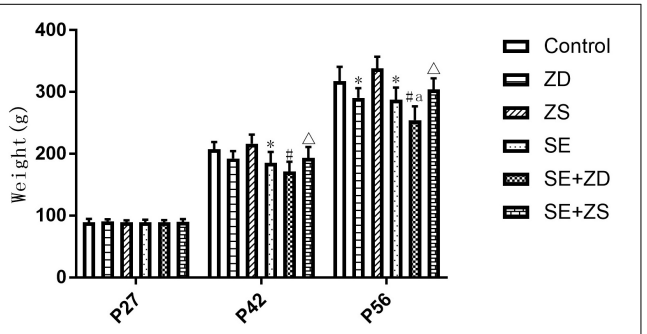

FIGURE 1 | Comparison of body weights at different ages (g, $n = 10$/group). There were no significant differences between rats in each group at P27. At P42, the body weight of lithium chloride-pilocarpine-treated injured rats of SE, SE + ZD, and SE + ZS groups was significantly decreased compared to Control, ZD and ZS groups, respectively. At P56, the body weight of lithium chloride-pilocarpine-treated rats of SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups was significantly decreased compared to Control, ZD, ZS, Control, and SE groups, respectively (two-way repeated-measures ANOVA, $^*P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ compared to ZS group, a $P < 0.05$ compared to SE group).

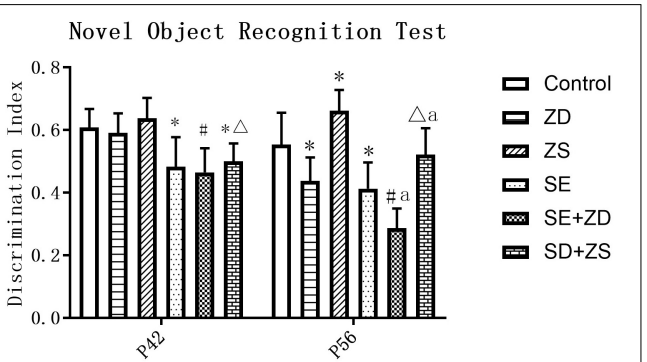

FIGURE 2 | Novel object recognition test. At P42, the recognition indexes were significantly reduced in the pilocarpine-treated rats of SE, SE + ZD, and SE + ZS groups compared to the corresponding control, ZD, ZS, and control groups, respectively. At P56, the recognition indexes were significantly reduced in SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups compared to the corresponding control, ZD, ZS, control and SE groups, respectively (one-way ANOVA with *post hoc* tests, $^*P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ compared to ZS group, a $P < 0.05$ compared to SE group $n = 10$/group).

in SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups compared to corresponding Control, ZD, ZS, Control, and SE groups, respectively ($P < 0.05$). In addition, at P57, serum zinc concentrations in ZS and SE + ZS groups were statistically increased compared to corresponding Control and SE groups, respectively ($P < 0.05$, **Figure 3**).

Seizure Threshold

We compared seizure susceptibility by exposing the rats to penicillin. The time to the first seizure after penicillin injection was recorded as the seizure latency (min) (seizure threshold). Seizure onset is obviously earlier in penicillin-treated rats in SE, SE + ZD, SE + ZS groups than in non-penicillin-treated rats in Control, ZD, ZS groups, respectively. In addition, there were strongly decreased seizure thresholds in ZD and SE + ZD groups compared with corresponding Control and SE groups, respectively. Furthermore, there were significantly increased seizure thresholds in ZS and SE + ZS groups compared with corresponding Control and SE groups, respectively ($P < 0.05$, **Figure 4**).

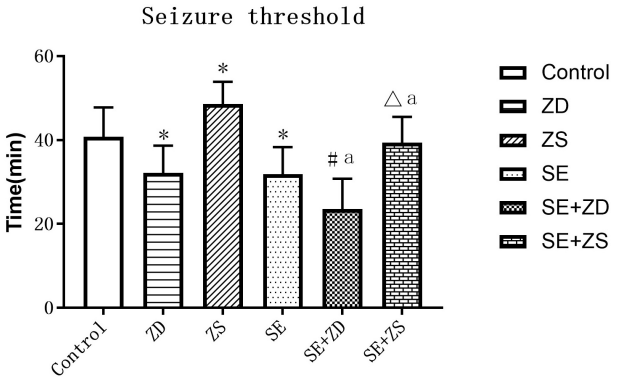

FIGURE 4 | Seizure threshold analysis (min). There were significantly decreased seizure thresholds in rats of SE, SE + ZD, SE + ZS, ZD, Control, SE + ZD, and SE groups compared with corresponding Control, ZD, ZS, Control, ZS, SE, and SE + ZS groups, respectively (one-way ANOVA with *post hoc* tests, $*P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ compared to ZS group, a $P < 0.05$ compared to SE group).

Timm Staining

There were significant differences in the sprouting scores of mossy fibers in the hippocampal CA3 and DG subfields among the six groups ($P < 0.05$). The Timm particle scores in the hippocampal CA3 and DG subfields were obviously increased in SE, SE + ZD, and SE + ZS groups compared to corresponding Control, ZD, and ZS groups, respectively ($P < 0.05$). Compared to the SE group, Timm particle scores in hippocampal CA3 and DG areas of the SE + ZD group were significantly increased ($P < 0.05$). Timm particle scores of hippocampal CA3 and DG in the SE + ZS group were still higher than in Control, while scores were lower than in the SE group ($P < 0.05$). There were no significant differences in Timm particle scores between ZD and Control groups or between ZS and Control groups (**Figure 5**).

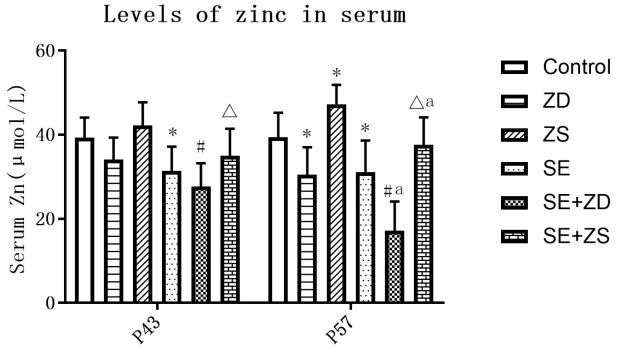

FIGURE 3 | Determination of serum zinc concentration (μmol/L). At P43, serum zinc concentrations of rats in the pilocarpine-treated SE, SE + ZD, and SE + ZS groups were significantly lower than corresponding groups of Control, ZD and ZS groups, respectively. At P57, there were significantly lower serum zinc concentrations in SE, SE + ZD, SE + ZS, ZD, and SE + ZD groups compared to corresponding Control, ZD, ZS, Control, and SE groups, respectively. In addition, at P57, serum zinc concentrations in ZS and SE + ZS groups were significantly increased compared to corresponding Control and SE groups, respectively (one-way ANOVA with *post hoc* tests, $*P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ to ZS group, a $P < 0.05$ compared to SE group).

Western Blot Analysis

As shown in **Figure 6A** (GPR39) and **Figure 6B** (ZnT3), expression of GPR39 and ZnT3 were markedly lower in ZD and SE groups compared to the control group ($P < 0.05$). In contrast, expression of GPR39 and ZnT3 were significantly higher in the ZS group than in the control group ($P < 0.05$). Expression of GPR39 and ZnT3 were obviously lower in the SE + ZD group compared to the ZD and SE groups ($P < 0.05$). Furthermore, expression of GPR39 and ZnT3 were significantly upregulated in the SE + ZS group compared to the SE group but were obviously decreased compared to the ZS group ($P < 0.05$).

There was markedly downregulated expression of MBP in penicillin-treated SE, SE + ZD, and SE + ZS groups compared to corresponding control, ZD and ZS groups, respectively. Compared with the SE group, MBP expression was further decreased in the SE + ZD group ($P < 0.05$), and compared to the SE group, MBP expression was significantly increased in the SE + ZS group ($P < 0.05$) (**Figure 6C**).

DISCUSSION

This study investigated the effects of different concentrations of dietary zinc on long-term cognition, seizure threshold, MF sprouting and gene expression in the hippocampus following lithium chloride-pilocarpine-induced developmental seizures. There are three main findings in this study. First, a zinc-deficient diet for 4 weeks aggravates seizure-induced weight loss, cognitive impairment and decreased concentration of serum zinc as well as seizure threshold, while increasing aberrant mossy fiber sprouting, which were in parallel with reduced

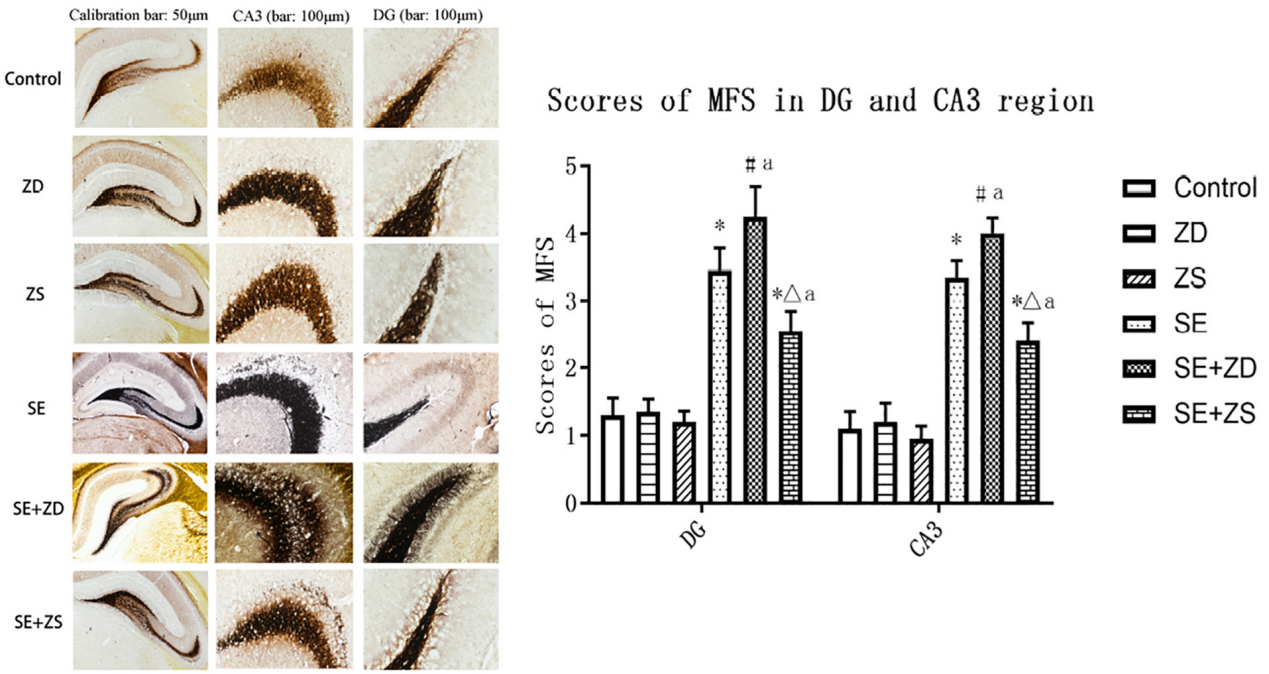

FIGURE 5 | Mossy fiber sprouting in CA3 and dentate gyrus subfields by Timm staining. Developmental seizures significantly increased the Timm particle scores in the hippocampal CA3 and DG subfields of rats in SE, SE + ZD, and SE + ZS groups compared to corresponding Control, ZD and ZS groups, respectively. Moreover, the Timm particle scores were significantly higher in SE + ZD group compared to the SE group. Further, Timm particle scores were significantly lower in the SE + ZS group than in the SE group (one-way ANOVA with *post hoc* tests, $^{*}P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ compared to ZS group, a $P < 0.05$ compared to SE group, $n = 4$/group).

expression of GPR39, ZnT -3 and MBP in the hippocampus. Second, a zinc supplemented diet for 4 weeks significantly improves the abovementioned injury-related changes caused by developmental seizures and may correct the abnormal expression of GPR39, ZnT-3, and MBP in the hippocampus. Third, the expression pattern of MBP in hippocampus coincided with aberrant sprouting of mossy fibers. In addition, low-zinc or high-zinc diet intervention for 2 weeks exerted no significant effect on body weight, cognition, or serum zinc concentrations.

Epidemiological data indicate that a large number of diseases affecting one-third of the world's population is attributed to zinc deficiency, and a lack of bioavailable zinc intake in the diet is the most common cause of zinc deficiency (Abbaspour et al., 2015). Karweina et al. (2015) showed that high doses of zinc oxide may lead to imbalanced zinc homeostasis in animals and changes in ZIP4 methylation. Zinc deficiency and zinc supplementation also affect homocysteine levels and related enzyme expression in rats (Jing et al., 2015). Rats fed rice with fortified zinc oxide exhibited significantly increased body weight (Della Lucia et al., 2014). Our current results are consistent with these studies. Herein, three different dietary Zn levels were used in growing rats according to previous studies (Nagatomo et al., 1998; Takeda et al., 2006b). Serum zinc concentrations were measured at P43 and P57. Results showed that at P57 following 4 weeks of differential diet treatments, there were significantly lower serum zinc concentration in the ZD group and significantly higher serum zinc concentration in ZS groups compared to the control group. Correspondingly, low-zinc and high-zinc diet interventions for 4 weeks had significant effects on body weight and cognition in control groups. These results indicate that long-term zinc deficiency or high zinc in the diet causes decreased or increased blood zinc concentrations, potentially affecting growth and development. However, our study also showed that different doses of zinc in the diet for 2 weeks had no significant effects on body weight or cognition in normal rats and showed no significant differences in serum zinc concentration in ZD and ZS groups compared to the normal zinc diet control group at P43. This indicates that under normal physiological conditions, the body has a regulatory mechanism for blood zinc concentration, which is not easily imbalanced by dietary zinc deficiency.

There are many studies on the effects of zinc loading or deprivation on seizure thresholds. The earliest report came from the Barry-Sterman team. They found that zinc loading delayed kindled seizure induction, while zinc deprivation accelerated kindling (Barry-Sterman et al., 1986). Subsequently, using seizure-susceptible EL mice, Fukahori and Itoh (1990) found that zinc deficiency causes increased seizure susceptibility, while reducing by zinc loading, but an adequate diet had no effect. However, using the same epilepsy-susceptible EL mice, a study by Nagatomo et al. (1998) denied this conclusion. They showed that convulsive seizures in EL mice fed a Zn-deficient diet for 4 weeks were more effectively inhibited than in mice receiving a high zinc or sufficient zinc diet for the same period. However,

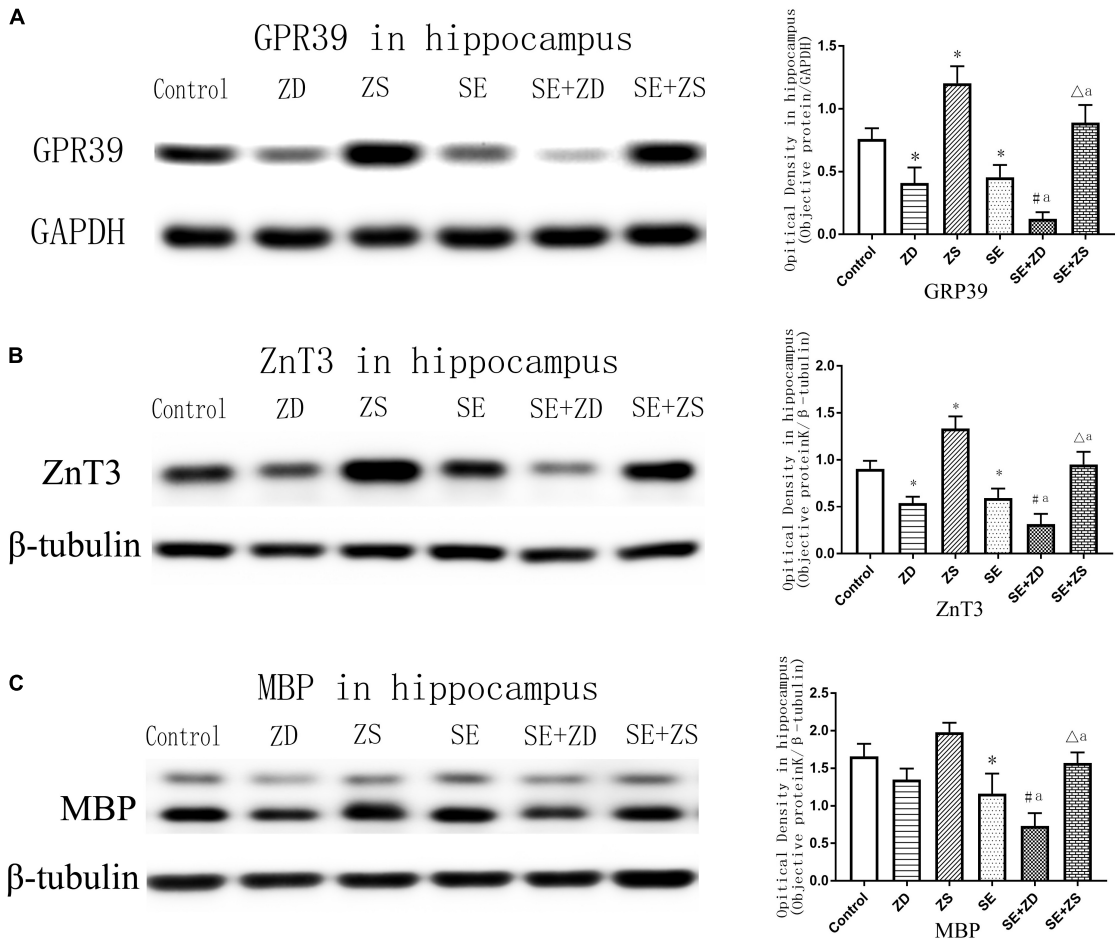

FIGURE 6 | Protein levels of GPR39 **(A)**, ZnT-3 **(B)**, and MBP **(C)** in the hippocampus (one-way ANOVA with *post hoc* tests, $*P < 0.05$, compared to Control group, $\#P < 0.05$ compared to ZD group, $\Delta P < 0.05$ compared to ZS group, a $P < 0.05$ compared to SE group, $n = 4$/group).

later studies by Takeda et al. (2003a, 2005, 2006a, 2009) reinforced the idea that young mice fed a zinc-deficient diet for 4 weeks experienced reduced convulsive threshold. This is in accordance with our present findings. Herein, we also found that zinc-deficient diets for 4 weeks reduced seizure threshold, while the threshold increased after 4 weeks of a zinc supplemented diet. The underlying mechanisms whereby this occurs have not been clearly defined, but parallel changes in cognitive deficits and aberrant sprouting of hippocampal mossy fibers provide valuable clues.

Here, we compared the different effects of zinc loading or deprivation on cognitive function through the novel object recognition test. The novel object recognition test is a popular method for detecting rodent non-spatial memory neurobiology. The principle of this test is that rodents have a natural tendency to explore new items. The memory capacity and degree of damage are assessed by quantitatively calculating the amount of time the rodent spends exploring presented objects. Here, we showed that at P42 following 2 weeks zinc loading or deprivation, there was no significant difference in the recognition index of each group among the non-penicillin-treated control, ZD and ZS groups. On the other hand, however, the recognition indexes were significantly reduced in penicillin-treated SE, SE + ZD, and SE + ZS groups compared to the corresponding control, ZD and ZS groups, respectively. This suggests that developmental seizures can cause long-term cognitive impairment, which is consistent with previous studies (Ni et al., 2009, 2018). The results also showed that 2-week dietary treatment with different zinc concentrations had no significant intervention effect on cognitive impairment. Notably, at P56 following 4 weeks zinc loading or deprivation, although there was still a significant reduction in the recognition index of penicillin-treated SE and SE + ZD groups compared to the corresponding control and ZD groups, but the recognition index in rats of the zinc loading-treated SE + ZS group increased significantly compared to the normal diet-treated SE group, which indicated that the 4-week zinc loading diet can significantly improve long-term cognitive impairment caused by developmental seizures. We hypothesize that this neuroprotective effect of zinc loading diet may be related to the regulation of hippocampal zinc metabolism homeostasis. The rodent hippocampus plays an important role in the memory of the encoded object, and

temporary or permanent damage to the hippocampus will destroy the memory of the object (Cohen and Stackman, 2015). The hippocampus is susceptible to zinc deficiency in the brain. In particular, zinc deficiency reduces the reactive zinc pool, as revealed by hippocampal histochemical Timm staining, which is closely related to high susceptibility to seizures (Takeda et al., 2003b, 2006b; Takeda and Tamano, 2009). Zn(2+) coexists with glutamate at the end of hippocampal mossy fiber and regulates its release, thereby affecting synaptic function (Besser et al., 2009). The incidence of tonic-clonic convulsion was markedly increased after i.p. injection of zinc chelators Clioquinol (CQ) and TPEN in adult Noda epileptic rats (Takeda et al., 2013). Combined with the finding in this study that zinc loading diet intervention markedly improved the regenerative mossy fiber sprouting in hippocampus induced by developmental seizures as revealed by Timm staining, we speculate that one of the mechanisms for this neuroprotection may be related to the improvement of zinc transporter-mediated mossy fiber spouting in hippocampus.

In this study, we examined expression of zinc metabolism-related genes. GPR39 is a rhodopsin-like G protein-coupled receptor consisting of 435 amino acid residues and is classified as a member of the ghrelin/neurotensin receptor subfamily according to its amino acid sequence. In addition to its expression in the gastrointestinal tract, liver, fat, and pancreas, GPR39 is also widely expressed in the hippocampus, which is also a zinc-enriched region, suggesting the neurotransmitter role of zinc may be related to GPR39 (Khan, 2016). It was initially thought that obestatin was a natural ligand for GPR39, and later Holst et al. (2007) suggested that Zn2+ may be a "physiological regulator" of GPR39. Synaptically released Zn(2+) triggers metabotropic activity in the CA3 region of cultured hippocampal neurons, which was attenuated by knockdown of GPR39 expression (Besser et al., 2009). Our present findings indicate that a zinc-deficient diet can further lead to decreased expression of GPR39 in the hippocampus, which in turn aggravates cognitive impairment. In contrast, we demonstrated for the first time that a zinc supplemented diet significantly improves cognitive dysfunction and up-regulates expression of GPR39 in the hippocampus, suggesting a protective effect of high-zinc intervention on developmental seizure-induced brain damage. In combination with clinical studies, which indicate that serum zinc levels in untreated epileptic children and in children with systemic refractory epilepsy were significantly lower than in healthy controls (Wojciak et al., 2007; Seven et al., 2013; Kheradmand et al., 2014; Saad et al., 2014), our results suggest that a zinc supplemented diet may have potential translational medical value for the intervention of developmental convulsive brain damage.

Currently, only two studies from Siegward and Kumar have found that a zinc supplemented diet suppresses convulsive seizures and reduce seizure thresholds (Elsas et al., 2009; Kumar et al., 2015). In addition, a study by Contestabile et al. (2016) found that 4 weeks of zinc supplementation strongly impairs consolidation of hippocampal-dependent memory in wild type adult rats through contextual fear conditioning and inhibitory avoidance testing. This is consistent with our present results. Herein, we also showed that the recognition indexes were significantly reduced in ZD groups compared to the control group. Moreover, our study demonstrated that a zinc supplemented diet attenuates cognitive impairment caused by developmental seizures, as shown by the comparison between SE and SE + ZS groups. This is in accordance with a study from Cope et al. (2011) who revealed that supplemental zinc prevents cognitive and behavioral deficits associated with traumatic brain injury (TBI).

Of particular interest, we found that the intervention effect of a zinc supplemented diet on cognitive impairment caused by developmental seizures was consistent with the effect on hippocampal regenerative mossy fiber sprouting and was consistent with expression of MBP. The mechanism for this phenomenon is still unclear. However, the finding that hippocampal regenerative mossy fiber sprouting and MBP are both brain damage indicators rather than the cause of brain damage may help explain the phenomenon of synchronization between the two indicators in the pathophysiological process of brain injury and repair. MBP is one of the most abundant structural proteins in oligodendrocyte myelin and is essential for cognitive function. MBP is negatively correlated with the severity of seizure-induced brain injury and has been recognized as a biomarker for brain damage. MBP is reduced in epileptic foci in patients with refractory epilepsy, suggesting that this change interferes with the transmission of nerve impulses (Marchi et al., 2010). Mesial temporal lobe epilepsy (MTLE) is usually associated with cognitive deficits. Proteomics analysis by Persike et al. (2018) demonstrated up-regulated protein level of MBP in MTLE patients, suggesting a compensatory mechanism due to epilepsy-related nerve damage. Song et al. (2018) observed the damage of myelin microstructure and the decrease of MBP caused by lithium-pilocarpine-induced state convulsion in immature rats by transmission electron microscopy and Western blot analysis, this damage was improved after down-regulating Lingo-1 expression. Meanwhile, it is recently noted that the seizure-induced hippocampal mossy fiber sprouting may not be a potential therapeutic target for epilepsy, but only a pathological hallmark change caused by convulsive brain injury (Cavarsan et al., 2018; Koyama and Ikegaya, 2018). Further, MBP is also reported to induce the proliferation of cultured Schwann cells and astrocytes and thus improve the integrity of the neuronal plasma membrane (Tzeng et al., 1999; Snaidero et al., 2017). Based on these findings, we speculate that similar alterations between the expression pattern of MBP and aberrant sprouting of mossy fibers in the hippocampus may indicate that sprouting is a secondary pathological change caused by developmental brain damage rather than the cause of epileptogenesis. Up-regulation of MBP protein levels in the high zinc diet-treated seizure group (SE + ZS), as well as the corresponding improvement of cognitive impairment and reduced hippocampal mossy fiber regenerative sprouting, may represent a compensatory mechanism for neuronal membrane damage and repair.

It is worth noting that expression patterns of ZnT-3 observed in this study were not entirely consistent with observed changes in hippocampal mossy fiber sprouting. However, this does not

negate the traditional notion that ZnT-3 is a sprouting marker. Since we tested expression levels of ZnT-3 protein in the entire hippocampus, we did not selectively detect expression of ZnT-3 in the mossy fiber pathway. Local anatomic-specific pathway expression changes using immunohistochemistry techniques merit further investigation.

CONCLUSION

A Zinc-deficient diet for 4 weeks aggravates long-term adverse effects of developmental seizures, including parameters of weight, cognition, seizure threshold and serum zinc concentration, which paralleled expression changes observed in hippocampal GPR39 and ZnT-3. In contrast, zinc supplementation for 4 weeks significantly improved the above described damage-related changes, attenuating the abnormal expression of GPR39, ZnT-3, and MBP in the hippocampus. Similar alterations between the expression pattern of MBP and aberrant sprouting of mossy fibers in the hippocampus may indicate that sprouting is a secondary pathological change caused by developmental brain damage rather than the cause of epileptogenesis. Up-regulation of MBP protein levels in the high zinc diet-treated seizure group (SE + ZS), as well as the corresponding improvement of cognitive impairment and reduced hippocampal mossy fiber regenerative sprouting, may represent a compensatory mechanism for neuronal membrane damage and repair.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

Animal Subjects: The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Soochow University. The program was approved by the Medical Ethics Committee of Soochow University Children's Hospital.

AUTHOR CONTRIBUTIONS

HN designed the study, analyzed the data, and wrote the manuscript. N-NC, D-JZ, Y-XS, and D-DW were the operators of the experiment and were responsible for the statistical analysis of data.

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REFERENCES

Abbaspour, N., Amini, M., Hurrell, R., and Schulin, R. (2015). Modeling zinc intake for intervention and scenario analysis. *Food Nutr. Bull.* 36, 89–101. doi: 10.1177/0379572115586399

Baraka, A. M., Hassab El Nabi, W., and El Ghotni, S. (2012). Investigating the role of zinc in a rat model of epilepsy. *CNS Neurosci. Ther.* 18, 327–333. doi: 10.1111/j.1755-5949.2011.00252.x

Barry-Sterman, M., Shouse, M. N., Fairchild, M. D., and Belsito, O. (1986). Kindled seizure induction alters and is altered by zinc absorption. *Brain Res.* 383, 382–386. doi: 10.1016/0006-8993(86)90045-4

Besser, L., Chorin, E., Sekler, I., Silverman, W. F., Atkin, S., Russell, J. T., et al. (2009). Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. *J. Neurosci.* 29, 2890–2901. doi: 10.1523/JNEUROSCI.5093-08.2009

Cavarsan, C. F., Malheiros, J., Hamani, C., Najm, I., and Covolan, L. (2018). Is mossy fiber sprouting a potential therapeutic target for epilepsy? *Front. Neurol.* 9:1023. doi: 10.3389/fneur.2018.01023

Cohen, S. J., and Stackman, R. W. Jr. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav. Brain Res.* 285, 105–117. doi: 10.1016/j.bbr.2014.08.002

Contestabile, A., Peña-Altamira, E., Virgili, M., and Monti, B. (2016). Zinc supplementation in rats impairs hippocampal-dependent memory consolidation and dampens post-traumatic recollection of stressful event. *Eur. Neuropsychopharmacol.* 26, 1070–1082. doi: 10.1016/j.euroneuro.2015.12.041

Cope, E. C., Morris, D. R., Scrimgeour, A. G., VanLandingham, J. W., and Levenson, C. W. (2011). Zinc supplementation provides behavioral resiliency in a rat model of traumatic brain injury. *Physiol. Behav.* 104, 942–947. doi: 10.1016/j.physbeh.2011.06.007

De Toffol, B., Hingray, C., Biberon, J., and Ei-Hage, W. (2018). Psychiatric comorbidities in epilepsy. *Presse Med.* 47, 243–250. doi: 10.1016/j.lpm.2017.10.019

Della Lucia, C. M., Santos, L. L., Rodrigues, K. C., Rodrigues, V. C., Martino, H. S., and Sant'Ana, H. M. (2014). Bioavailability of zinc in Wistar rats fed with rice fortified with zinc oxide. *Nutrients* 6, 2279–2289. doi: 10.3390/nu6062279

Elsas, S. M., Hazany, S., Gregory, W. L., and Mody, I. (2009). Hippocampal zinc infusion delays the development of afterdischarges and seizures in a kindling model of epilepsy. *Epilepsia* 50, 870–879. doi: 10.1111/j.1528-1167.2008.01913.x

Fukahori, M., and Itoh, M. (1990). Effects of dietary zinc status on seizure susceptibility and hippocampal zinc content in the El (epilepsy) mouse. *Brain Res.* 529, 16–22. doi: 10.1016/0006-8993(90)90806-m

Gilad, D., Shorer, S., Ketzef, M., Friedman, A., Sekler, I., Aizenman, E., et al. (2015). Homeostatic regulation of KCC2 activity by the zinc receptor mZnR/GPR39 during seizures. *Neurobiol. Dis.* 81, 4–13. doi: 10.1016/j.nbd.2014.12.020

Holst, B., Egerod, K. L., Schild, E., Vickers, S. P., Cheetham, S., Gerlach, L. O., et al. (2007). GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* 148, 13–20. doi: 10.1210/en.2006-0933

Jehi, L. E., and Vezzani, A. (2014). Novel concepts in epileptogenesis and its prevention. *Neurotherapeutics* 11, 229–230. doi: 10.1007/s13311-014-0268-z

Jin, M. F., Ni, H., and Li, L. L. (2018). Leptin maintained zinc homeostasis against glutamate-induced excitotoxicity by preventing mitophagy-mediated mitochondrial activation in HT22 hippocampal neuronal cells. *Front. Neurol.* 9:322. doi: 10.3389/fneur.2018.00322

Jing, M., Rech, L., Wu, Y., Goltz, D., Taylor, C. G., and House, J. D. (2015). Effects of zinc deficiency and zinc supplementation on homocysteine levels and related enzyme expression in rats. *J. Trace Elem. Med. Biol.* 30, 77–82. doi: 10.1016/j.jtemb.2014.10.013

Jung, H. Y., Kwon, H. J., Kim, W., Nam, S. M., Kim, J. W., Hahn, K. R., et al. (2019). Phosphoglycerate mutase 1 promotes cell proliferation and neuroblast differentiation in the dentate gyrus by facilitating the phosphorylation of cAMP response element-binding protein. *Neurochem. Res.* 44, 1–10. doi: 10.1007/s11064-018-2678-5

Karweina, D., Kreuzer-Redmer, S., Müller, U., Franken, T., Pieper, R., Baron, U., et al. (2015). The zinc concentration in the diet and the length of the feeding period affect the methylation status of the ZIP4 Zinc transporter gene in piflets. *PLoS One* 10:e0143098. doi: 10.1371/journal.pone.0143098

Khan, M. Z. (2016). A possible significant role of zinc and GPR39 zinc sensing receptor in Alzheimer disease and epilepsy. *Biomed. Pharmacother.* 79, 263–272. doi: 10.1016/j.biopha.2016.02.026

Kheradmand, Z., Yarali, B., Zare, A., Pourpak, Z., Shams, S., and Ashrafi, M. R. (2014). Comparison of serum zinc and copper levels in children and adolescents with intractable and controlled epilepsy. *Iran J. Child. Neurol.* 8, 49–54.

Kovacs, Z., Schacht, T., Herrmann, A. K., Albrecht, P., Lefkimmiatis, K., and Methner, A. (2014). Protein kinase inhibitor beta enhances the constitutive activity of G-protein-coupled zinc receptor GPR39. *Biochem. J.* 462, 125–132. doi: 10.1042/BJ20131198

Koyama, R., and Ikegaya, Y. (2018). The molecular and cellular mechanisms of axon guidance in mossy fiber sprouting. *Front. Neurol.* 9:382. doi: 10.3389/fneur.2018.00382

Kumar, H., Katyal, J., and Gupta, Y. K. (2015). Low dose zinc supplementation beneficially affects seizure development in experimental seizure models in rats. *Biol. Trace Elem. Res.* 163, 208–216. doi: 10.1007/s12011-014-0181-7

López González, F. J., Rodríguez Osorio, X., Gil-Nagel Rein, A., Carreño Martínez, M., Serratosa Fernández, J., Villanueva Haba, V., et al. (2015). Drug-resistant epilepsy: definition and treatment alternatives. *Neurologia* 30, 439–446. doi: 10.1016/j.nrl.2014.04.012

Marchi, N., Teng, Q., Ghosh, C., Fan, Q., Nguyen, M. T., Desai, N. K., et al. (2010). Blood-brain barrier damage, but not parenchymal white blood cells, is a hallmark of seizure activity. *Brain Res.* 1353, 176–186. doi: 10.1016/j.brainres.2010.06.051

Mcallister, B. B., and Dyck, R. H. (2017). Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function. *Neurosci. Biobehav. Rev.* 80, 329–350. doi: 10.1016/j.neubiorev.2017.06.006

Nagatomo, I., Akasaki, Y., Uchida, M., Kuchiiwa, S., Nakagawa, S., and Takigawa, M. (1998). Influence of dietary zinc on convulsive seizures and hippocampal NADPH diaphorase-positive neurons in seizure susceptible EL mouse. *Brain Res.* 789, 213–220. doi: 10.1016/S0006-8993(97)01442-X

Ni, H., Chen, S. H., Li, L. L., and Jin, M. F. (2018). Leptin treatment prevents long-term abnormalities in cognition, seizure threshold, hippocampal mossy fiber sprouting and ZnT3/CB-D28k expression in a rat developmental "twist" seizure model. *Epilepsy Res.* 139, 164–170. doi: 10.1016/j.eplepsyres.2017.12.009

Ni, H., Jiang, Y. W., Tao, L. Y., Cen, J. N., and Wu, X. R. (2009). Effects of penicillin-induced developmental epilepticus on hippocampal regenerative sprouting, related gene expression and cognitive deficits in rats. *Toxicol. Lett.* 188, 161–166. doi: 10.1016/j.toxlet.2009.04.002

Persike, D. S., Marques-Carneiro, J. E., Stein, M. L. L., Yacubian, E. M. T., Centeno, R., Canzian, M., et al. (2018). Altered proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Pharmaceuticals* 11:95. doi: 10.3390/ph11040095

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* 32, 281–294. doi: 10.1016/0013-4694(72)90177-0

Rzezak, P., Lima, E. M., Gargaro, A. C., Coimbra, E., de Vincentiis, S., Velasco, T. R., et al. (2017). Everyday memory impairment in patients with temporal lobe epilepsy caused by hippocampal sclerosis. *Epilepsy Behav.* 69, 31–36. doi: 10.1016/j.yebeh.2017.01.008

Saad, K., Hammad, E., Hassan, A. F., and Badry, R. (2014). Trace element, oxidant, and antioxidant enzyme values in blood of children with refractory epilepsy. *Int. J. Neurosci.* 124, 181–186. doi: 10.3109/00207454.2013.831851

Seven, M., Basaran, S. Y., Cengiz, M., Unal, S., and Yuksel, A. (2013). Deficiency of selenium and zinc as a causative factor for idiopathic intractable epilepsy. *Epilepsy Res.* 104, 35–39. doi: 10.1016/j.eplepsyres.2012.09.013

Snaidero, N., Velte, C., Myllykoski, M., Raasakka, A., Ignatev, A., Werner, H. B., et al. (2017). Antagonistic functions of MBP and CNP establish cytosolic channels in CNS myelin. *Cell Rep.* 18, 314–323. doi: 10.1016/j.celrep.2016.12.053

Song, X. J., Han, W., He, R., Li, T. Y., Xie, L. L., Cheng, L., et al. (2018). Alterations of hippocampal myelin sheath and axon sprouting by status convulsion and regulating Lingo-1 expression with RNA interference in immature and adult rats. *Neurochem. Res.* 43, 721–735. doi: 10.1007/s11064-018-2474-2

Staley, K. (2015). Molecular mechanisms of epilepsy. *Nat. Neurosci.* 18, 367–372. doi: 10.1038/nn.3947

Takeda, A., Hirate, M., Tamano, H., Nisibaba, D., and Oku, N. (2003a). Susceptibility to kainate-induced seizures under dietary zinc deficiency. *J. Neurochem.* 85, 1575–1580. doi: 10.1046/j.1471-4159.2003.01803.x

Takeda, A., Hirate, M., Tamano, H., and Oku, N. (2003b). Release of glutamate and GABA in the hippocampus under zinc deficiency. *J. Neurosci. Res.* 72, 537–542. doi: 10.1002/jnr.10600

Takeda, A., Iida, M., Ando, M., Nakamura, M., Tamano, H., and Oku, N. (2013). Enhanced susceptibility to spontaneous seizures of noda epileptic rats by loss of synaptic zn(2+). *PLoS One* 8:e71372. doi: 10.1371/journal.pone.0071372

Takeda, A., Itoh, H., Hirate, M., and Oku, N. (2006a). Region-specific loss of zinc in the brain in pentylentetrazole-induced seizures and seizure susceptibility in zinc deficiency. *Epilepsy Res.* 70, 41–48. doi: 10.1016/j.eplepsyres.2006.03.002

Takeda, A., Itoh, H., Tamano, H., and Oku, N. (2006b). Responsiveness to kainate in young rats after 2-week zinc deprivation. *Biometals* 19, 565–572. doi: 10.1007/s10534-005-6145-9

Takeda, A., Itoh, H., Nagayoshi, A., and Oku, N. (2009). Abnormal Ca2+ mobilization in hippocampal slices of epileptic animals fed a zinc-deficient diet. *Epilepsy Res.* 83, 73–80. doi: 10.1016/j.eplepsyres.2008.09.009

Takeda, A., and Tamano, H. (2009). Insight into zinc signaling from dietary zinc deficiency. *Brain Res. Rev.* 62, 33–44. doi: 10.1016/j.brainresrev.2009.09.003

Takeda, A., Tamano, H., and Oku, N. (2005). Involvement of unusual glutamate release in kainate-induced seizures in zinc-deficient adult rats. *Epilepsy Res.* 66, 137–143. doi: 10.1016/j.eplepsyres.2005.07.011

Tian, K., Wang, Y. X., Li, L. X., and Liu, Y. Q. (2018). Neuronal death/apoptosis induced by intracellular zinc deficiency associated with changes in amino-acid neurotransmitters and glutamate receptor subtypes. *J. Inorg. Biochem.* 179, 54–59. doi: 10.1016/j.jinorgbio.2017.11.014

Tian, T., Ni, H., and Sun, B. L. (2015). Neurobehavioral deficits in a rat model of recurrent neonatal seizures are prevented by a ketogenic diet and correlate with Hippocampal Zinc/Lipid transporter signals. *Biol. Trace Elem. Res.* 167, 251–258. doi: 10.1007/s12011-015-0285-8

Torolira, D., Suchomelova, L., Wasterlain, C. G., and Niquet, J. (2016). Widespread neuronal injury in a model of cholinergic status epilepticus in postnatal day 7 rat pups. *Epilepsy Res.* 120, 47–54. doi: 10.1016/j.eplepsyres.2015.11.005

Tzeng, S. F., Deibler, G. E., and DeVries, G. H. (1999). Myelin basic protein and myelin basic protein peptides induce the proliferation of Schwann cells via ganglioside GM1 and the FGF receptor. *Neurochem. Res.* 24, 255–260.

Vidaurre, J., and Twanow, J. D. E. (2017). Attention deficit hyperactivity disorder and associated cognitive dysfunction in pediatric epilepsy. *Semin. Pediatr. Neurol.* 24, 282–291. doi: 10.1016/j.spen.2017.10.009

Wojciak, R. W., Mojs, E., Stanislawska-Kubiak, M., and Samborski, W. (2007). The serum zinc, copper, iron, and chromium concentrations in epileptic children. *Epilepsy Res.* 104, 40–44. doi: 10.1016/j.eplepsyres.2012.09.009

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REVIEW
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Emerging Roles of Long Non-coding RNAs in Chronic Neuropathic Pain

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Chronic neuropathic pain, a type of chronic and potentially disabling pain caused by a disease or injury of the somatosensory nervous system, spinal cord injury, or various chronic conditions, such as viral infections (e.g., post-herpetic neuralgia), autoimmune diseases, cancers, and metabolic disorders (e.g., diabetes mellitus), is one of the most intense types of chronic pain, which incurs a major socio-economic burden and is a serious public health issue, with an estimated prevalence of 7–10% in adults throughout the world. Presently, the available drug treatments (e.g., anticonvulsants acting at calcium channels, serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, opioids, topical lidocaine, etc.) for chronic neuropathic pain patients are still rare and have disappointing efficacy, which makes it difficult to relieve the patients' painful symptoms, and, at best, they only try to reduce the patients' ability to tolerate pain. Long non-coding RNAs (lncRNAs), a type of transcript of more than 200 nucleotides with no protein-coding or limited capacity, were identified to be abnormally expressed in the spinal cord, dorsal root ganglion, hippocampus, and prefrontal cortex under chronic neuropathic pain conditions. Moreover, a rapidly growing body of data has clearly pointed out that nearly 40% of lncRNAs exist specifically in the nervous system. Hence, it was speculated that these dysregulated lncRNAs might participate in the occurrence, development, and progression of chronic neuropathic pain. In other words, if we deeply delve into the potential roles of lncRNAs in the pathogenesis of chronic neuropathic pain, this may open up new strategies and directions for the development of novel targeted drugs to cure this refractory disorder. In this article, we primarily review the status of chronic neuropathic pain and provide a general overview of lncRNAs, the detailed roles of lncRNAs in the nervous system and its related diseases, and the abnormal expression of lncRNAs and their potential clinical applications in chronic neuropathic pain. We hope that through the above description, readers can gain a better understanding of the emerging roles of lncRNAs in chronic neuropathic pain.

Keywords: chronic neuropathic pain, long non-coding RNAs (lncRNAs), neuronal associated disorders, DRG neurons, glioma cells

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INTRODUCTION

Chronic Neuropathic Pain

An epidemiological survey has clearly revealed that nearly 19% of adult Europeans suffered from chronic pain of moderate to severe intensity, which not only seriously affected the physical and mental quality of their social and working lives but also represents a significant public health issue that can be costly to the healthcare system (Rapo-Pylkko et al., 2017). Among all the different types of chronic pain, neuropathic pain is relatively common, but difficult to treat and manage (Baron, 2006, 2009). Moreover, neuropathic pain occurs in approximately 7–10% of the general population; therefore, neuropathic pain remains one of the most serious public health problems (van Hecke et al., 2014). Traditionally, the International Association for the Study of Pain (IASP) proposed a clinical description for neuropathic pain, which is defined as "pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Scholz et al., 2019). However, this definition is broad, covering over 100 conditions, and has been controversial in recent years (National Institute of Neurological Disorders Stroke rt PASSG, 2005; Meacham et al., 2017). The main reason is that many scholars believe that the meaning of the term "dysfunction" is still relatively vague (Baron, 2009). Furthermore, it does not distinguish well between neuropathic pain and nociceptive pain (Vranken, 2012). Recently, a new precise definition of neuropathic pain has been put forward to avoid the above shortcomings, and to further beneficially support clinical and research purposes (Lauria et al., 2012; Treede et al., 2019). The revised definition is that "pain arose as a direct consequence of a lesion or disease affecting the somatosensory system," which definitely indicates that the peripheral or central somatosensory system must be involved (Scholz et al., 2019; Treede et al., 2019). According to the underlying etiology and the anatomical location of the specific lesion, clinical neuropathic pain is haply divided into four main types, namely, painful peripheral neuropathies, central pain syndromes, complex painful neuropathic disorders, and mixed-pain syndromes (Baron, 2009; Nijs et al., 2016). Painful peripheral neuropathies are caused by ischemic, traumatic, inflammatory, infectious, metabolic, or compressive damage to the peripheral nervous system (PNS), such as polyneuritis, phantom limb pain, diabetic peripheral neuralgia, post-herpetic neuralgia, etc. (Beran, 2015; Watson and Dyck, 2015). Central pain syndromes result from the dysfunction of or damage to the central nervous system (CNS) pain conduction pathway, mainly involving cerebrovascular disease, spinal cord trauma, etc. (Petzke, 2010). Complex painful neuropathic disorders are also called "complex regional pain syndromes (CRPS)," which may result in trauma and affect the limbs (Baron and Wasner, 2001; Borchers and Gershwin, 2014). In addition, mixed-pain syndromes are the combination of nociceptive and neuropathic pain (Baron, 2009; Picelli et al., 2016).

Generally, patients with neuropathic pain may show distinct negative and positive sensory symptoms or signs in the nerve distribution area (Baron, 2006). Firstly, the negative sensory symptoms predominately contain hypoesthesia, pallhypoesthesia, hypoalgesia, thermohypoesthesia, etc. (Baron, 2006; Shaygan et al., 2014). Secondly, the positive sensory symptoms primarily include paresthesia, paroxysmal pain, superficial pain, and evoked pain. This would stimulate induced hypersensitivity and pain, such as light-pressure, gentle static pressure, and sharp pinprick (Baron, 2006; Soler et al., 2017). However, in a clinical setting, patients often were diagnosed as hyperesthesias that are mainly referred to as paresthesia and hyperalgesia (Zimmermann, 2001). The two types of hyperesthesia need to be clearly distinguished. Paresthesia (also known as mechanodynamic painful paresthesia) is defined as the non-noxious stimuli (e.g., light touch, warm, cool), and normally would not cause painful sensation in the normal nerve areas, but it could cause strongly painful sensation in the damaged nerve areas (Baron, 2009; Arle et al., 2016). Nevertheless, hyperalgesia is defined as an increased pain sensitivity or painful summation to mildly noxious stimuli (Baron, 2009; Jensen and Finnerup, 2014). To date, pharmacotherapy is the main therapeutic strategy in neuropathic pain patients. However, these drugs and agents lack specificity and efficiency, and could reduce the pain to a more tolerable level (Teixeira, 2009; Helfert et al., 2015). Thus, it is urgently needed to further explore the potential molecular mechanisms of the neuropathic pain development, which may provide a rational solution for clinical treatment and novel drug discovery in neuropathic pain.

Long Non-coding RNAs (lncRNAs)

With the development of larger-scale sequencing techniques and bioinformatics methods, the Encyclopedia of DNA Elements (ENCODE) project, the most comprehensive effort yet for surveying transcription in human cells, has clearly uncovered that only approximately 2% of the whole human genome encodes functional protein-coding genes, while the remaining genome is actively transcribed into a diversity of RNAs that were defined as non-coding RNAs (ncRNAs) and were previously believed to be non-functional "dark matter" (Qu and Fang, 2013). However, an accumulating body of evidence in recent years reported that, similar to protein-coding genes, ncRNAs are also involved in a wide variety of biological processes, such as cell proliferation, cellular structure integrity, cellular development, differentiation, stem cell pluripotency, signaling transductions, genomic imprinting, the maintenance of genomic integrity, reprogramming, heat shock response, etc. (Nicolas, 2017; Sherstyuk et al., 2018). Therefore, it was no surprise that abnormal expression profiles of ncRNAs were implicated in the initiation, development, and progression processes of many human diseases, including cardiovascular syndromes, diabetes, autoimmune diseases, nervous system disorders, cancers, and so on (Huang et al., 2013). However, among all classifications of ncRNAs, lncRNAs, a heterogeneous group of transcripts that are greater than 200 nucleotides (nt) in length, account for more than 80% of ncRNAs (Jarroux et al., 2017). Because lncRNAs occupy the largest class of ncRNAs and represent the most prevalent and functionally diverse class of ncRNAs, they attract interest in the scientific community in the past decades (Roberts et al., 2014).

Since Brannan and his colleagues reported the first lncRNA H19 in 1990, thousands of lncRNAs have been identified in a large diversity of species, including mammalian animals, plants, yeast, prokaryotes, and even viruses, by a large number of scientists (Jarroux et al., 2017). It is reported that the lncRNA is bidirectional transcribed, depending on their proximity to the nearest protein-coding transcripts. It can be grouped into five main categories: sense lncRNAs (lncRNAs initiate inside or 5' of a protein-coding gene, then are transcribed in the same direction as protein-coding genes, and ultimately overlap at least one protein-coding exon), antisense lncRNAs (lncRNAs initiate inside or 3' of a protein-coding gene, then are transcribed in the opposite direction of protein-coding genes, and ultimately overlap at least one protein-coding exon), bidirectional lncRNAs (the expression of the lncRNA and its neighboring protein-coding genes on the opposite strand is initiated in close genomic proximity), intronic lncRNAs (the entire sequence of the lncRNA falls within the intron of a protein-coding gene), and intergenic lncRNAs (also termed large intervening ncRNAs or lincRNAs, in which the entire sequence of the lncRNA falls between two protein-coding genes as a distinct unit) (**Figure 1A**; Ma et al., 2013; St Laurent et al., 2015). In addition, an increasing number of studies have found that lncRNAs play versatile roles in many aspects of gene regulation through four functional mechanisms: signaling and acting as a decoy, guide, and scaffolding molecule (**Figure 1B**; Akhade et al., 2017; Delas and Hannon, 2017). First, as a signaling molecule, lncRNAs are usually transcribed in a specific time and space, and their transcripts can integrate developmental cues, interpret cell status, or respond to multiple stimuli and further regulate the expression of other genes. Therefore, lncRNAs are seen as an important marker in a biological event (Wang and Chang, 2011). Second, as a decoy molecule, lncRNA can block the binding of transcriptional repressors to their homologous target gene promoters, as well as protein molecules, chromatin modifications, etc., and thereby lncRNAs could ultimately inhibit their functions (Ma et al., 2012). Third, as a guide molecule, lncRNAs are able to directly bind to the protein molecules and then guide the protein complexes containing the above-described protein-bound molecules to be precisely localized on a specific target (Wang and Chang, 2011). On the other hand, lncRNA can also recruit more transcription-related and epigenetic-related regulatory factors widely and further guide their localizations (Goff and Rinn, 2015). Finally, as a scaffold molecule, lncRNAs have many different structural domains that can combine different effector molecules to achieve the assembly of macromolecules at the same time. In fact, lncRNA is a complex assembly center platform, which can recruit many related molecules together to further activate or inhibit the expression of other genes (Bonasio et al., 2010; Ma et al., 2012). It is extremely important for the transmission of many biological signals, intermolecular interactions, and the precise regulation of the specificity and dynamics of the

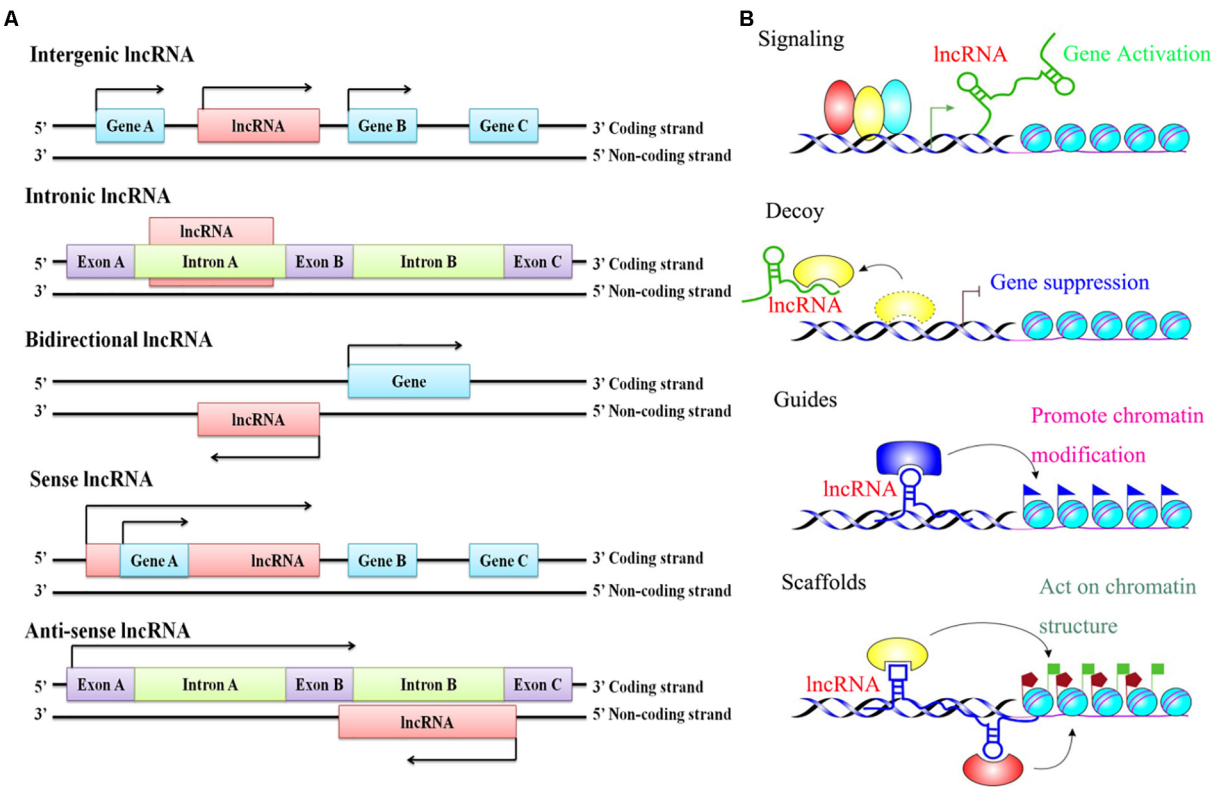

FIGURE 1 | The classification and functional mechanisms of lncRNAs. **(A)** Classification of lncRNAs according to the relative proximity to the nearest protein-coding transcripts; **(B)** Functions of lncRNAs in regulating target genes.

signal itself (Wang and Chang, 2011; Moran et al., 2012). Hence, based on the multiple functions of lncRNAs, researchers have gradually shifted their focus of research over the last few decades from previous studies on protein-coding gene-induced diseases to studies on lncRNA-induced diseases (Moran et al., 2012; Delas and Hannon, 2017).

THE ROLES OF lncRNAs IN THE NERVOUS SYSTEM AND ITS RELATED DISEASES

The human nervous system is the most highly evolved and sophisticated biological system that plays a leading role in the regulation of physiological and functional activities in the body (Herculano-Houzel, 2009; Herculano-Houzel et al., 2014). Generally, the functions and physiological processes of different organs and systems in the human body are not carried out in isolation, but they are closely connected, interacting and cooperating under the direct or indirect control of the nervous system, which makes the human body become a complete and unified organism to achieve and maintain normal life activities when the human body encounters interoceptive and environmental stimuli (Herculano-Houzel, 2012; Kohl and Dulac, 2018). It is well-known that the human nervous system is mainly composed of nerve tissues that contain two basic types of cells, neurons and glial cells, and is divided into two major parts, the CNS and the PNS (Garner and Mayford, 2012; Zimmerman et al., 2017). The CNS, containing the brain and the spinal cord, is where we receive sensory information, generate thoughts and emotions, and store memories (Kaucka and Adameyko, 2014; Engelhardt et al., 2017). The perfect presentation of CNS and PNS functions predominately depends on the dynamic neural networks organized by neuronal and glial cells (Guarino et al., 2017). However, the development of these cells requires a precise spatiotemporal regulation of stem/progenitor cell proliferation and differentiation at various levels, such as epigenetic, transcriptional, or post-transcriptional processes, and then forms appropriate connections with each other (Qureshi and Mehler, 2010). In recent years, emerging data have reported that an increasing number of lncRNAs were found to play vital roles in mediating the developmental complexity of the nervous system, and these abundant lncRNAs are precisely, dynamically, and specifically expressed in the brain via temporal and spatial expression patterns (Qureshi and Mehler, 2013). In fact, it is not difficult to understand that the development of the nervous system is actually a complex neural network formed by the tight gene regulation of its constituent cells (Garner and Mayford, 2012). Nevertheless, based on the explanation of the functions of lncRNAs in the above, it can be known that the regulatory mechanisms of lncRNAs may participate in the entire process of nervous system development (Wu et al., 2013). Therefore, not surprisingly, the dysregulation of or mutations in lncRNA gene loci might directly affect nervous system development, function, maintenance, and differentiation and be intimately associated with the molecular pathophysiology of a broad array of the most devastating neurological diseases, such as glioma, neuropathic pain, autism spectrum disorder (ASD), Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Asperger's syndrome, schizophrenia, developmental delay, amyotrophic lateral sclerosis (ALS), depression, autism, and others (Riva et al., 2016; Wan et al., 2017).

First, we explored the roles of lncRNA in neuronal development (Wu et al., 2013; Ramos et al., 2016). Several lines of evidence have demonstrated that the profiles of lncRNA expression present a dynamic change during the process of neuronal development (Rani et al., 2016). For example, Mercer et al. discovered that 1328 lncRNAs were identified in the mouse, whereas among these lncRNAs, 849 lncRNAs were located in the mouse brain with 623 lncRNAs exhibiting selective profiles for specific regions (e.g., olfactory bulb, hippocampus, cortex, or cerebellum), cell types (e.g., neurons, glia cells), and subcellular compartments (e.g., dendrite, axon, Nissl body, soma, etc.), which suggested that these lncRNAs might be involved in regulating specific processes through cell- type-, subcellular- compartment-, and developmental-stage-specific manners (Bussotti et al., 2016). Amaral et al. revealed that the lncRNA Sox2OT was expressed in regions of constitutive adult neurogenesis and mediated the expression of the Sox2 gene, which is a key transcription factor (TF) that is responsible for the neural induction and maintenance of neural stem and progenitor cells, indicating that the lncRNA Sox2OT might participate in neural cell fate decisions (Amaral et al., 2009). Other studies related to lncRNAs in neuronal development are shown in **Table 1**. Throughout the study of these lncRNAs in neurodevelopment, it is known that the methods of exploring the physiological functions of lncRNAs are basically the same. Researchers often use qRT-PCR to detect changes in the expression of lncRNAs in blood samples from patients with neurological diseases, in cell model samples, or in animal model samples. Moreover, a dual-luciferase reporter assay was applied to verify the target interaction between lncRNAs and their downstream target genes. In addition, CCK8, colony formation assay, and flow cytometry were utilized to assess the influences of lncRNA on neuronal development.

Second, we continue to investigate the effects of lncRNAs on neurological diseases (Riva et al., 2016). For instance, over-expressed lncRNA HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1), facilitates tumor growth and invasion through up-regulating HOXA1 and sequestering G9a/EZH2/Dnmts away from the HOXA1 gene in glioblastoma multiforme (Li Q. et al., 2018). LncRNA small nucleolar RNA host gene 12 (SNHG12) accelerates angiogenesis following ischemic stroke via a miR-150/VEGF pathway, which further clarifies the mechanism of angiogenesis after ischemic stroke and provides a target for the treatment of this disease (Zhao M. et al., 2018). The imbalance of lncRNA AK127244 might have a particular influence on language development and behavior or mood in neuropsychiatric disorders (Duong et al., 2015). Nuclear factor-k-gene binding (NF-kB) interacting lncRNA (NKILA) promotes the endoplasmic reticulum stress/autophagy pathway and suppresses the NF-kB pathway after intracerebral hemorrhage (Jia et al., 2018). Other studies linked to lncRNAs in neuronal-associated disorders are detailed in **Table 2**.

TABLE 1 | lncRNAs' roles in neuronal development.

lncRNAs	Location	Expression	Samples	Roles of lncRNAs in neuronal development	References
BC1 (Brain cytoplasmic RNA 1)	Chromosome 7	Down-regulated	Mice	Changes behavioral phenotypes including reduced exploration and increased anxiety	Lewejohann et al., 2004
BRN1B (also named Pantr2, POU domain, class 3, transcription factor 3 adjacent non-coding transcript 2)	Chromosome 1	Down-regulated	Developing mouse pups	Controls differentiation of delaminating neural progenitor cells	Sauvageau et al., 2013
DALI (DNMT1 associated lincRNA)	Chromosome 1	Up-regulated	Neuroblastoma cells	Drives the expression of an essential neuronal differentiation gene expression program	Chalei et al., 2014
EVF2 (also termed DLX6-AS1, DLX6 antisense RNA 1)	Chromosome 7	Down-regulated	Mice	Disrupts the excitatory to inhibitory neuron balance in the post-natal hippocampus and dentate gyrus	Bond et al., 2009
GOMAFU (also called MIAT, myocardial infarction associated transcript)	Chromosome 5	Down-regulated	Mice	Regulates splicing of several neuronal genes and increases amacrine cell and Muller glia differentiation	Rapicavoli et al., 2010; Briggs et al., 2015
KCNA2-AS (Potassium voltage-gated channel subfamily A member 2 antisense RNA)	–	Up-regulated	The PNS of rats	Implicate in the control of neuronal plasticity	Briggs et al., 2015
Nkx2.2AS (NKX2-2 antisense RNA)	20p11.22	Up-regulated	Neural stem cells (NSCs)	Enhances oligodendrocytic differentiation	Tochitani and Hayashizaki, 2008
NTAB (Nitrilotriacetate monooxygenase component B)	–	Up-regulated	Developing and adult rat brain	Involve regulation of RNA transport or translation in neuronal processes	French et al., 2001
PAUPAR (PAX6 upstream antisense RNA)	11p13	Up-regulated	Neuroblastoma cells	Regulates a transcriptional program that influences the cell-cycle profile and differentiation	Vance et al., 2014
PNKY (A neural-specific lncRNA)	Chromosome 6	Down-regulated	Dividing NSCs of mouse and human brain	Controls the balance between self-renewal and neuronal differentiation	Ramos et al., 2015
RMST (Rhabdomyosarcoma 2 associated transcript)	12q23.1; 12q21	Up-regulated	Human brain, Human embryonic stem cells (hESCs)	Required for neural differentiation	Ng et al., 2013
SIX3OS (SIX homeobox 3, opposite strand 1)	Chromosome 17	Up-regulated	Mice	Controls the specification of photoreceptors, bipolar cells, and Muller glia and regulates retinal development	Rapicavoli et al., 2011
TUG1 (Taurine up-regulated 1)	Chromosome 11	Up-regulated	Mice	Involved in retinal development	Young et al., 2005
TUNA (Tcl1 upstream neuron-associated lincRNA)	Chromosome 12	Up-regulated	Mice embryonic stem cells (mESCs)	Controls pluripotency and neural lineage commitment	Lin et al., 2014

THE ABNORMAL EXPRESSION OF lncRNAs AND THEIR POTENTIAL CLINICAL APPLICATIONS IN CHRONIC NEUROPATHIC PAIN

Chronic neuropathic pain generally resulting from injury to or disease of the nervous system has always been very difficult in clinical treatment, mainly because almost all patients with chronic neuropathic pain are notoriously resistant to the actions of currently available analgesics, such as non-steroidal anti-inflammatory drugs and opioids, which could exert effective therapeutic roles in nociceptive pain (Jackson, 2006). Pharmacological (e.g., tricyclic antidepressants, anticonvulsants, selective serotonin norepinephrine reuptake inhibitors, and topical lidocaine), non-pharmacological (e.g., behavioral, cognitive, integrative, and physical approaches), and interventional (e.g., neuroablative and neuromodulation techniques) therapies are currently used in the treatment of chronic neuropathic pain. These treatments cannot completely control the symptoms of patients with chronic neuropathic pain, which may be associated with the diversity of pathophysiological situations, different pain etiologies, genetic predispositions, etc. (Gilron et al., 2015). Thus, chronic neuropathic pain remains a distressing and debilitating disease, there is still no panacea for the treatment of chronic neuropathic pain in the clinic, and it is imperative to further develop chronic neuropathic pain drugs (Jensen and Finnerup, 2007; Xu et al., 2016). In recent years, a large number of studies have revealed that many prominent and well-characterized mechanisms were observed in chronic neuropathic pain, including (1) ongoing ectopic activity in peripheral nerves, which resulted in excitation–inhibition imbalance; (2) the aberrant production and release of anti-inflammatory or proinflammatory cytokines at the site of injury, which triggers peripheral sensitization or central sensitization; (3) the impairment of endogenous inhibitory mechanisms of

TABLE 2 | lncRNAs' roles in neuronal associated disorders.

lncRNAs	Location	Expression	Samples	Neuronal associated disorders	Roles of lncRNAs in neuronal associated disorders	References
ANRIL (Antisense non-coding RNA in the INK4 locus)	9p21.3	Up-regulated	Patients	Intracranial aneurysms	ANRIL may become a molecular marker of intracranial aneurysms in the future	Che, 2017
AK042766	–	Up-regulated	Mice	Restless Legs Syndrome (RLS)	AK042766 may regulate the expression of the Meis1 gene during the pathogenesis of Restless Legs Syndrome (RLS)	Ponjavic et al., 2009
BC200 (also termed BCYRN1, brain cytoplasmic RNA 1)	2p21	Up-regulated	Patients	AD	BC200 is involved in the synaptic and neural network dysfunction that is found in both early and later stages of AD	Li H. et al., 2018
BACE1-AS (Beta-secretase 1 antisense RNA)	11q23.3	Down-regulated	Patients, mice	AD	BACE1-AS drives overproduction of toxic AB-42 peptides, which then feedback to further induce BACE1-AS overexpression, accelerating amyloid accumulation and finally leading to the generation of AD	Faghihi et al., 2008
DISC2 (Disrupted in schizophrenia 2)	1q42.2	Down-regulated	Patients	Neuropsychiatric disorders	DISC2 has been implicated in the development of neuropsychiatric disorders, such as autism spectrum disorder	Williams et al., 2009
H19 (Human homolog 19)	11p15.5	Up-regulated	Patients, human glioma cell lines	CNS tumors	H19 deregulation may be relevant for CNS tumors, such as glioma	Muller et al., 2000
GOMAFU	Chromosome 5	Down-regulated	Mice, human pluripotent-cell-derived neurons	Schizophrenia	GOMAFU may be involved in driving this aberrant splicing of DISC1 and ERRB4 in schizophrenia	Barry, 2014; Briggs et al., 2015
KCNA2-AS	–	Up-regulated	Rat	Neuropathic pain	KCNA2-AS appears to be a key driver of neuropathic pain symptoms	Briggs et al., 2015; Li et al., 2019
M21981	–	Up-regulated	Mice	MS	M21981 are involved in abnormal CD8+ T-cell differentiation and activation in the pathophysiology of MS	Hafler et al., 2007; Qureshi et al., 2010
MEG3 (Maternally expressed gene 3)	14q32.2	Up-regulated	Glioma cells U251	Glioma	MEG3 inhibits proliferation and migration but induces autophagy by regulation of Sirt7 and PI3K/AKT/mTOR pathway in glioma cells	Xu et al., 2018
MSNP1AS (Moesin pseudogene 1 antisense RNA)	5p14.1	Up-regulated	Patients	ASD	MSNP1AS may regulate MSN protein by binding to and stabilizing MSN mRNA, and that this mechanism may causally connect SNP variants in the MSNP1AS locus to ASD pathogenesis	Kerin et al., 2012; DeWitt et al., 2016
REST/CoREST-regulated lncRNAs	–	Down-regulated	Patients	HD	The potential disruption of REST-regulated lncRNA expression in HD may lead to additional disturbances in lncRNA-mediated chromatin and transcriptional regulatory processes through a feed-forward mechanism	Johnson, 2012
UCH1LAS (Ubiquitin C-terminal hydrolase L1 antisense RNA)	4p14	Up-regulated	Patients	PD	UCH1LAS involves in regulating pathways related to the development of PD	Kraus et al., 2017
XCI (X chromosome inactivation)	Chromosome X	Up-regulated	Patients	CNS tumors	Perturbations in XCI expression are associated with CNS tumors	Qureshi et al., 2010

TABLE 3 | Comprehensive analysis of aberrant lncRNA expression profiles in chronic neuropathic-related conditions.

lncRNAs	Expression levels	Localization of lncRNAs expression	Samples	Potential regulatory mechanism of dysregulated lncRNAs	References
BC168687	Up-regulated	DRG neurons	Streptozotocin-induced diabetic rats	lncRNA BC168687 may participate in the pathogenesis of diabetic neuropathic pain mediated by P2 × 7 receptor.	Liu C. et al., 2017
CCAT1 (Colon cancer-associated transcript-1)	Down-regulated	DRG neurons, spinal dorsal horn, hippocampus and anterior cingulated cortex	Bilateral sciatic nerve CCI rats	lncRNA CCAT1 overexpression could alleviate the pain thresholds and promote the expression of serum and glucocorticoid regulated protein kinase 3 (SGK3) through sponging miR-155.	Dou et al., 2017
DGCR5 (DiGeorge syndrome critical region gene 5)	Down-regulated	DRG neurons	CCI rats	DGCR5 overexpression was able to alleviate neuropathic pain development including mechanical and thermal hyperalgesia through sponging miR-330-3p and regulating PDCD4 in CCI rat models.	Peng et al., 2019
KCNA2-AS (Potassium voltage-gated channel subfamily A member 2 antisense RNA)	Up-regulated	DRG neurons	Rat, mouse, monkey and human	Overexpression of KCNA2-AS down-regulated Kcna2, reduced total voltage-gated potassium current, increased excitability in DRG neurons, and produced neuropathic pain symptoms, but blocking KCNA2-AS reversed nerve injury-induced down-regulation of DRG Kcna2 and attenuated development and maintenance of neuropathic pain.	Li et al., 2019
LINC00657	Up-regulated	DRG neurons	CCI rats	LINC00657 suppressed neuropathic pain-related symptoms, such as mechanical and thermal hyperalgesia, by modulating miR-136/ZEB1 axis.	Shen et al., 2019
MALAT1 (metastasis-associated lung adenocarcinoma transcript 1)	Down-regulated	DRG neurons	CCI rats	Neuropathic pain behaviors such as mechanical and thermal hyperalgesia were reduced by the inhibition of MALAT1, while the loss of MALAT1 was able to depress the neuroinflammation process via the inhibition of COX-2, interleukin-1β, and interleukin-6 accompanied by miR-206/ZEB2 axis.	Chen et al., 2019
MRAK009713	Up-regulated	DRG neurons	CCI rats	MRAK009713 is a novel positive regulator of neuropathic pain in rats through regulating the expression and function of the P2 × 3 receptor.	Li et al., 2017
NEAT1 (Nuclear paraspeckle assembly transcript 1)	Up-regulated	Spinal cord	CCI rats	NEAT1 contributes to neuropathic pain development through targeting miR-381/HMGB1 axis in CCI rat models.	Xia et al., 2018
NON-RATT021972	Up-regulated	DRG neurons	Type 2 diabetes mellitus rats	NON-RATT021972 siRNA treatment suppressed the up-regulated expression and activation of the P2 × 3 receptor and reduced the hyperalgesia potentiated by the pro-inflammatory cytokine TNF-α in Type 2 diabetes mellitus rats.	Peng et al., 2017
PKIA-AS1 (PKIA antisense RNA 1)	Up-regulated	Spinal cord	Spinal nerve ligation model rats	Overexpression of PKIA-AS1 was sufficient to induce neuropathic pain-like symptoms in uninjured rats by directly regulating the expression and function of CDK6, which is essential for the initiation and maintenance of neuroinflammation and neuropathic pain.	Hu et al., 2019
SCN9A NAT (SCN9A natural antisense transcript)	Down-regulated	DRG neurons	Painful diabetic rats	SCN9A NAT correlates with the emergence of pain-related behaviors characteristic of painful diabetic neuropathy.	Li et al., 2015
T-UCRs (Transcribed ultraconserved regions)	Up-regulated	Spinal cord	Spinal nerve ligation-induced neuropathic pain in mice	T-UCR involved in the pathogenesis of neuropathic pain.	Jiang et al., 2016
uc.48+	Up-regulated	DRG neurons	Diabetic rats	The siRNA treatment of lncRNA uc.48+ may alleviate the diabetic neuropathic pain by inhibiting the excitatory transmission mediated by the P2 × 3 receptor in DRG.	Wang et al., 2016
XIST (X inactive specific transcript)	Up-regulated	Spinal cord	CCI rats	XIST accelerates neuropathic pain progression through regulation of miR-137/TNFAIP1, miR-154/STAT3, or miR-150/ZEB1 axis in CCI rat models.	Wei et al., 2018; Yan et al., 2018; Zhao Y. et al., 2018

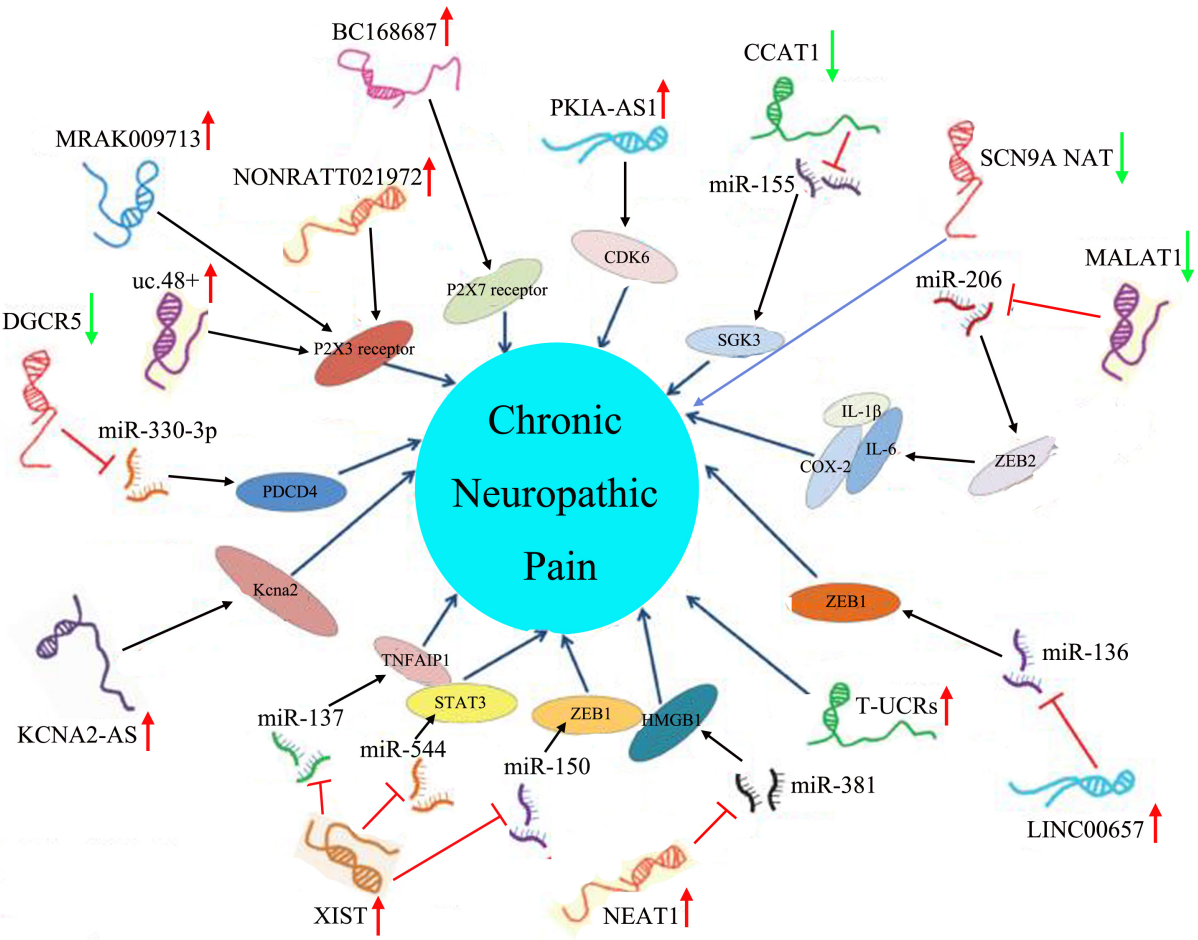

FIGURE 2 | Aberrant lncRNA expression and their downstream molecular pathways in chronic neuropathic pain. The red arrow represented an up-regulated trend of lncRNAs, and the green arrow represented a down-regulated trend of lncRNAs.

nociception sensitivity-induced microglial sensitivity after nerve injury, which enhanced the post-injury pain sensitivity; and (4) microglial activation after nerve injury, which contributed to the pathogenesis of pain hypersensitivity (Gilron et al., 2015). Therefore, in the full understanding of the pathogenesis of chronic neuropathic pain, we can develop corresponding new therapeutic drugs for chronic neuropathic pain patients according to their relevant mechanisms (Gilron et al., 2006; Vranken, 2012). Furthermore, most extensive evidence has definitely pointed out that there were many dysregulated lncRNAs expressed in damaged nerves, primary sensory dorsal root ganglion neurons, spinal cord dorsal roots, the prefrontal cortex, the post-synaptic dorsal horn, and even in higher-order neurons up to the cortical level after peripheral nerve injury; therefore, it was speculated that these dysregulated lncRNAs might be involved in the pathogenesis of chronic neuropathic pain (Li et al., 2019). Furthermore, this speculation was also demonstrated by a large number of researchers. For instance, Liu et al. constructed a mouse model of spared nerve injury-induced neuropathic pain and found, by microarray technology, that there were 22,213 abnormally expressed lncRNAs, which might be involved in the activities of cytokines (IL-17A and IL-17F) and chemokines (CCL-2, CCL-5, and CCL-7), in the spinal cord, suggesting that differentially expressed lncRNAs played a pivotal role in the progression of neuropathic pain (Liu Z. et al., 2017). Additionally, Jiang et al. identified 511 differentially expressed (>2-fold change) lncRNAs (366 up-regulated and 145 down-regulated) in the spinal cord of mice following spinal nerve ligation-induced neuropathic pain, and these lncRNAs might be related to Toll-like receptor signaling, cytokine–cytokine receptor interaction, and peroxisome proliferator-activated receptor signaling pathways, which indicated that abnormally expressed lncRNAs might be implicated in the pathogenesis of chronic neuropathic pain (Jiang et al., 2015). With the deepening of lncRNA research, scientists are no longer limited to the study of lncRNA expression profiles of chronic neuropathic pain, and they have begun to specifically explore the biological function of these differentially expressed lncRNAs in chronic neuropathic pain (Wu et al., 2019). Thus, the amassing literature referring to lncRNAs in chronic neuropathic pain is presented in detail in **Table 3** and **Figure 2**.

It should be pointed out that all the current biological functions related to lncRNAs in chronic neuropathic pain are based on cellular or animal models, and research on the human body can be limited only to their expression levels, but not their biological functions (Kumar et al., 2018). The major reason is that chronic neuropathic pain is usually prone to irreversible damage in the human body, which is difficult for us to control (Gierthmuhlen and Baron, 2016). Hence, animal models of chronic neuropathic pain can be mainly grouped into four categories, including nerve injury models [such as spinal cord injury, excitotoxins, spinal hemi-section, thalamic syndrome, sciatic nerve chronic constriction injury (CCI), spinal nerve ligation, spared nerve injury, sciatic inflammatory neuritis, etc.], drug-induced chronic neuropathic pain models (such as anti-cancer-agent-induced neuropathy, vincristine-induced neuropathic pain, cisplatin-induced neuropathic pain, etc.), disease-induced neuropathy models (such as diabetes-induced neuropathy, the post-herpetic neuralgia model, human-immunodeficiency-virus-induced neuropathy, etc.), and miscellaneous models [such as chronic ethanol consumption/withdrawal-induced neuropathy, pyridoxine (vitamin B6)-induced neuropathy, inherited-induced neuropathies, etc.] (Kumar et al., 2018). Based on these models, whether the roles of lncRNAs in chronic neuropathic pain can be well reflected in the human body still needs to be further explored.

CONCLUSION AND FUTURE DIRECTIONS

Chronic neuropathic pain is one of the most challenging and refractory neurological diseases in a great number of patients (Gierthmuhlen and Baron, 2016). In the clinic, chronic neuropathic pain patients are often characterized by symptoms of spasticity, muscle weakness, dysesthesia, spontaneous pain, allodynia, hyperalgesia, poor proprioception, and so on (Gilron et al., 2015). Currently, in response to these symptoms of chronic neuropathic pain, there are still no clinically effective drugs for their treatment (Gilron et al., 2006). Previous studies have precisely revealed that the available analgesics, such as non-steroidal anti-inflammatory drugs and opioids, which could effectively improve the clinical signs or symptoms of nociceptive pain, did not produce an adequate effect on chronic neuropathic pain patients (Vranken, 2012). Emerging lines of evidence have also uncovered that, despite a mass of pharmacological studies performed in chronic neuropathic pain, these newly developed treatment strategies, including pharmacological, non-pharmacological, and interventional methods, remain unable to adequately alleviate the patients' pain or reduce the pain only to a range that the patients can tolerate (Jackson, 2006). Moreover, on the other hand, epidemiological surveys have reported that the prevalence of chronic neuropathic pain in the gross population is approximately 7–10% around the world (Zimmermann, 2001; van Hecke et al., 2014). Therefore, chronic neuropathic pain represents a worldwide challenging issue that is a socio-economic burden not only for patients and caregivers but also for the healthcare system (Baron, 2009). Currently, an increasing number of clinicians believe that patients with chronic neuropathic pain have not been treated well because the pathogenesis of the disease is not completely understood (Vranken, 2012). In-depth studies of the pathogenesis of chronic neuropathic pain may provide a solid theoretical basis for the development of new drugs or agents. lncRNAs, belonging to a family of RNAs characterized as transcripts more than 200 nucleotides in length, were discovered to participate in the cellular and molecular pathological processes of multiple nervous system diseases, including chronic neuropathic pain, by regulating the expression of neuronal-function-related genes (Qureshi et al., 2010; Wu et al., 2019). Hence, many researchers have turned their attention to the study of lncRNAs, with the thought that unveiling the underlying mechanism of how lncRNAs are involved in the occurrence, development and progression of chronic neuropathic pain may offer novel avenues to prevent and treat this refractory disease (Qureshi and Mehler, 2013).

In recent years, small-molecule targeted therapies using miRNAs or lncRNAs have been carried out in many disease areas. These molecules attracted so much attention due to their biology functions in regulating protein-coding genes at different levels, including epigenetic level, transcription level, post-transcriptional level, etc. In addition, their expressions not only have temporal and spatial specificity, but also cell and tissue specificity. Thus, these biological characteristics enable drug developers to find more accurate targets when developing new drugs or agents. Nonetheless, it is undeniable that there are still many challenges in the application of small molecules in the treatment of chronic neuropathic pain, for example, the transformation between experiment research and clinical application. Given that chronic neuropathic pain can cause irreversible damage to the human body, almost all models for chronic neuropathic pain research are based on cell or animal experiments. However, there are some differences between *in vitro* experiments and *in vivo* experiments, and these differences are unpredictable. Therefore, how the results obtained by small-molecule targeted therapy in *in vitro* experiments can be transformed and applied to human body is still a big problem.

AUTHOR CONTRIBUTIONS

XJ and YZ proposed the concept, collected the literatures, and edited the manuscript. WW drafted the manuscript.

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REFERENCES

Akhade, V. S., Pal, D., and Kanduri, C. (2017). Long noncoding RNA: genome organization and mechanism of action. *Adv. Exp. Med. Biol.* 1008, 47–74. doi: 10.1007/978-981-10-5203-3_2

Amaral, P. P., Neyt, C., Wilkins, S. J., Askarian-Amiri, M. E., Sunkin, S. M., Perkins, A. C., et al. (2009). Complex architecture and regulated expression of the Sox2ot locus during vertebrate development. *RNA* 15, 2013–2027. doi: 10.1261/rna.1705309

Arle, J. E., Mei, L., Carlson, K. W., and Shils, J. L. (2016). High-frequency stimulation of dorsal column axons: potential underlying mechanism of paresthesia-free neuropathic pain relief. *Neuromodulation* 19, 385–397. doi: 10.1111/ner.12436

Baron, R. (2006). Mechanisms of disease: neuropathic pain–a clinical perspective. *Nat. Clin. Pract. Neurol.* 2, 95–106. doi: 10.1038/ncpneuro0113

Baron, R. (2009). Neuropathic pain: a clinical perspective. *Handb. Exp. Pharmacol.* 194, 3–30. doi: 10.1007/978-3-540-79090-7_1

Baron, R., and Wasner, G. (2001). Complex regional pain syndromes. *Curr. Pain Headache Rep.* 5, 114–123.

Barry, G. (2014). Integrating the roles of long and small non-coding RNA in brain function and disease. *Mol. Psychiatry* 19, 410–416. doi: 10.1038/mp.2013.196

Beran, R. (2015). Paraesthesia and peripheral neuropathy. *Aust. Fam. Physician* 44, 92–95.

Bonasio, R., Tu, S., and Reinberg, D. (2010). Molecular signals of epigenetic states. *Science* 330, 612–616. doi: 10.1126/science.1191078

Bond, A. M., Vangompel, M. J., Sametsky, E. A., Clark, M. F., Savage, J. C., Disterhoft, J. F., et al. (2009). Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat. Neurosci.* 12, 1020–1027. doi: 10.1038/nn.2371

Borchers, A. T., and Gershwin, M. E. (2014). Complex regional pain syndrome: a comprehensive and critical review. *Autoimmun. Rev.* 13, 242–265. doi: 10.1016/j.autrev.2013.10.006

Briggs, J. A., Wolvetang, E. J., Mattick, J. S., Rinn, J. L., and Barry, G. (2015). Mechanisms of long non-coding rnas in mammalian nervous system development. *Plast. Dis. Evol. Neuron* 88, 861–877. doi: 10.1016/j.neuron.2015.09.045

Bussotti, G., Leonardi, T., Clark, M. B., Mercer, T. R., Crawford, J., Malquori, L., et al. (2016). Improved definition of the mouse transcriptome via targeted RNA sequencing. *Genome Res.* 26, 705–716. doi: 10.1101/gr.199760.115

Chalei, V., Sansom, S. N., Kong, L., Lee, S., Montiel, J. F., Vance, K. W., et al. (2014). The long non-coding RNA Dali is an epigenetic regulator of neural differentiation. *Elife* 3:e04530.

Che, J. (2017). Molecular mechanisms of the intracranial aneurysms and their association with the long noncoding ribonucleic acid ANRIL-A review of literature. *Neurol. India* 65, 718–728.

Chen, Z. L., Liu, J. Y., Wang, F., and Jing, X. (2019). Suppression of MALAT1 ameliorates chronic constriction injury-induced neuropathic pain in rats via modulating miR-206 and ZEB2. *J. Cell Physiol.* [Epub ahead of print].

Delas, M. J., and Hannon, G. J. (2017). lncRNAs in development and disease: from functions to mechanisms. *Open Biol.* 7:170121. doi: 10.1098/rsob.170121

DeWitt, J. J., Hecht, P. M., Grepo, N., Wilkinson, B., Evgrafov, O. V., Morris, K. V., et al. (2016). Transcriptional gene silencing of the autism-associated long noncoding RNA MSNP1AS in human neural progenitor cells. *Dev. Neurosci.* 38, 375–383. doi: 10.1159/000453258

Dou, L., Lin, H., Wang, K., Zhu, G., Zou, X., Chang, E., et al. (2017). Long non-coding RNA CCAT1 modulates neuropathic pain progression through sponging miR-155. *Oncotarget* 8, 89949–89957.

Duong, L. T., Hoeffding, L. K., Petersen, K. B., Knudsen, C. D., Thygesen, J. H., Klitten, L. L., et al. (2015). Two rare deletions upstream of the NRXN1 gene (2p16.3) affecting the non-coding mRNA AK127244 segregate with diverse psychopathological phenotypes in a family. *Eur. J. Med. Genet.* 58, 650–653. doi: 10.1016/j.ejmg.2015.11.004

Engelhardt, B., Vajkoczy, P., and Weller, R. O. (2017). The movers and shapers in immune privilege of the CNS. *Nat. Immunol.* 18, 123–131. doi: 10.1038/ni.3666

Faghihi, M. A., Modarresi, F., Khalil, A. M., Wood, D. E., Sahagan, B. G., Morgan, T. E., et al. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat. Med.* 14, 723–730. doi: 10.1038/nm1784

French, P. J., Bliss, T. V., and O'Connor, V. (2001). Ntab, a novel non-coding RNA abundantly expressed in rat brain. *Neuroscience* 108, 207–215. doi: 10.1016/s0306-4522(01)00408-0

Garner, A., and Mayford, M. (2012). New approaches to neural circuits in behavior. *Learn. Mem.* 19, 385–390. doi: 10.1101/lm.025049.111

Gierthmuhlen, J., and Baron, R. (2016). Neuropathic pain. *Semin. Neurol.* 36, 462–468.

Gilron, I., Baron, R., and Jensen, T. (2015). Neuropathic pain: principles of diagnosis and treatment. *Mayo Clin. Proc.* 90, 532–545. doi: 10.1016/j.mayocp.2015.01.018

Gilron, I., Watson, C. P., Cahill, C. M., and Moulin, D. E. (2006). Neuropathic pain: a practical guide for the clinician. *CMAJ* 175, 265–275. doi: 10.1503/cmaj.060146

Goff, L. A., and Rinn, J. L. (2015). Linking RNA biology to lncRNAs. *Genome Res.* 25, 1456–1465. doi: 10.1101/gr.191122.115

Guarino, D., Nannipieri, M., Iervasi, G., Taddei, S., and Bruno, R. M. (2017). The Role of the Autonomic Nervous System in the Pathophysiology of Obesity. *Front. Physiol.* 8:665. doi: 10.3389/fphys.2017.00665

Hafler, D. A., Compston, A., Sawcer, S., Lander, E. S., Daly, M. J., et al. (2007). Risk alleles for multiple sclerosis identified by a genomewide study. *N. Engl. J. Med.* 357, 851–862. doi: 10.1056/nejmoa073493

Helfert, S. M., Reimer, M., Hoper, J., and Baron, R. (2015). Individualized pharmacological treatment of neuropathic pain. *Clin. Pharmacol. Ther.* 97, 135–142. doi: 10.1002/cpt.19

Herculano-Houzel, S. (2009). The human brain in numbers: a linearly scaled-up primate brain. *Front. Hum. Neurosci.* 3:31. doi: 10.3389/neuro.09.031.2009

Herculano-Houzel, S. (2012). Neuronal scaling rules for primate brains: the primate advantage. *Prog. Brain Res.* 195, 325–340. doi: 10.1016/b978-0-444-53860-4.00015-5

Herculano-Houzel, S., Avelino-de-Souza, K., Neves, K., Porfirio, J., Messeder, D., Mattos Feijo, L., et al. (2014). The elephant brain in numbers. *Front. Neuroanat.* 8:46. doi: 10.3389/fnana.2014.00046

Hu, J. Z., Rong, Z. J., Li, M., Li, P., Jiang, L. Y., Luo, Z. X., et al. (2019). Silencing of lncRNA PKIA-AS1 Attenuates Spinal Nerve Ligation-Induced Neuropathic Pain Through Epigenetic Downregulation of CDK6 Expression. *Front. Cell Neurosci.* 13:50. doi: 10.3389/fncel.2019.00050

Huang, Y., Wang, J. P., Yu, X. L., Wang, Z. V., Xu, T. S., and Cheng, X. C. (2013). Non-coding RNAs and diseases. *Mol. Biol.* 47, 531–543.

Jackson, K. C. II (2006). Pharmacotherapy for neuropathic pain. *Pain Pract.* 6, 27–33. doi: 10.1111/j.1533-2500.2006.00055.x

Jarroux, J., Morillon, A., and Pinskaya, M. (2017). History. Discovery, and classification of lncRNAs. *Adv. Exp. Med. Biol.* 1008, 1–46. doi: 10.1007/978-981-10-5203-3_1

Jensen, T. S., and Finnerup, N. B. (2007). Management of neuropathic pain. *Curr. Opin. Support. Palliat. Care* 1, 126–131.

Jensen, T. S., and Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol.* 13, 924–935. doi: 10.1016/s1474-4422(14)70102-4

Jia, J., Zhang, M., Li, Q., Zhou, Q., and Jiang, Y. (2018). Long noncoding ribonucleic acid NKILA induces the endoplasmic reticulum stress/autophagy pathway and inhibits the nuclear factor-k-gene binding pathway in rats after intracerebral hemorrhage. *J. Cell Physiol.* 233, 8839–8849. doi: 10.1002/jcp.26798

Jiang, B. C., Sun, W. X., He, L. N., Cao, D. L., Zhang, Z. J., and Gao, Y. J. (2015). Identification of lncRNA expression profile in the spinal cord of mice following spinal nerve ligation-induced neuropathic pain. *Mol. Pain* 11:43.

Jiang, B. C., Yang, T., He, L. N., Tao, Y. X., and Gao, Y. J. (2016). Altered T-UCRs expression profile in the spinal cord of mice with neuropathic pain. *Transl. Perioper. Pain Med.* 1, 1–10.

Johnson, R. (2012). Long non-coding RNAs in Huntington's disease neurodegeneration. *Neurobiol. Dis.* 46, 245–254. doi: 10.1016/j.nbd.2011.12.006

Kaucka, M., and Adameyko, I. (2014). Non-canonical functions of the peripheral nerve. *Exp. Cell Res.* 321, 17–24. doi: 10.1016/j.yexcr.2013.10.004

Kerin, T., Ramanathan, A., Rivas, K., Grepo, N., Coetzee, G. A., and Campbell, D. B. (2012). A noncoding RNA antisense to moesin at 5p14.1 in autism. *Sci. Transl. Med.* 4:128ra40. doi: 10.1126/scitranslmed.3003479

Kohl, J., and Dulac, C. (2018). Neural control of parental behaviors. *Curr. Opin. Neurobiol.* 49, 116–122. doi: 10.1016/j.conb.2018.02.002

Kraus, T. F. J., Haider, M., Spanner, J., Steinmaurer, M., Dietinger, V., and Kretzschmar, H. A. (2017). Altered long noncoding rna expression precedes the course of parkinson's disease-a preliminary report. *Mol. Neurobiol.* 54, 2869–2877. doi: 10.1007/s12035-016-9854-x

Kumar, A., Kaur, H., and Singh, A. (2018). Neuropathic Pain models caused by damage to central or peripheral nervous system. *Pharmacol. Rep.* 70, 206–216. doi: 10.1016/j.pharep.2017.09.009

Lauria, G., Faber, C. G., Merkies, I. S., and Waxman, S. G. (2012). Diagnosis of neuropathic pain: challenges and possibilities. *Expert Opin. Med. Diagn.* 6, 89–93. doi: 10.1517/17530059.2012.661712

Lewejohann, L., Skryabin, B. V., Sachser, N., Prehn, C., Heiduschka, P., Thanos, S., et al. (2004). Role of a neuronal small non-messenger RNA: behavioural alterations in BC1 RNA-deleted mice. *Behav. Brain Res.* 154, 273–289. doi: 10.1016/j.bbr.2004.02.015

Li, G., Jiang, H., Zheng, C., Zhu, G., Xu, Y., Sheng, X., et al. (2017). Long noncoding RNA MRAK009713 is a novel regulator of neuropathic pain in rats. *Pain* 158, 2042–2052. doi: 10.1097/j.pain.0000000000001013

Li, H., Zheng, L., Jiang, A., Mo, Y., and Gong, Q. (2018). Identification of the biological affection of long noncoding RNA BC200 in Alzheimer's disease. *Neuroreport* 29, 1061–1067. doi: 10.1097/wnr.0000000000001057

Li, Q., Dong, C., Cui, J., Wang, Y., and Hong, X. (2018). Over-expressed lncRNA HOTAIRM1 promotes tumor growth and invasion through up-regulating HOXA1 and sequestering G9a/EZH2/Dnmts away from the HOXA1 gene in glioblastoma multiforme. *J. Exp. Clin. Cancer Res.* 37:265.

Li, Q. S., Cheng, P., Favis, R., Wickenden, A., Romano, G., and Wang, H. (2015). SCN9A variants may be implicated in neuropathic pain associated with diabetic peripheral neuropathy and pain severity. *Clin. J. Pain* 31, 976–982. doi: 10.1097/ajp.0000000000000205

Li, Z., Li, X., Chen, X., Li, S., Ho, I. H. T., Liu, X., et al. (2019). Emerging roles of long non-coding RNAs in neuropathic pain. *Cell Prolif.* 52:e12528. doi: 10.1111/cpr.12528

Lin, N., Chang, K. Y., Li, Z., Gates, K., Rana, Z. A., Dang, J., et al. (2014). An evolutionarily conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. *Mol. Cell* 53, 1005–1019. doi: 10.1016/j.molcel.2014.01.021

Liu, C., Tao, J., Wu, H., Yang, Y., Chen, Q., Deng, Z., et al. (2017). Effects of LncRNA BC168687 siRNA on diabetic neuropathic pain mediated by P2X7 receptor on SGCs in DRG of rats. *Biomed. Res. Int.* 2017:7831251.

Liu, Z., Liang, Y., Wang, H., Lu, Z., Chen, J., Huang, Q., et al. (2017). LncRNA expression in the spinal cord modulated by minocycline in a mouse model of spared nerve injury. *J. Pain Res.* 10, 2503–2514. doi: 10.2147/jpr.s147055

Ma, H., Hao, Y., Dong, X., Gong, Q., Chen, J., Zhang, J., et al. (2012). Molecular mechanisms and function prediction of long noncoding RNA. *ScientificWorldJournal.*2012:541786.

Ma, L., Bajic, V. B., and Zhang, Z. (2013). On the classification of long non-coding RNAs. *RNA Biol.* 10, 925–933.

Meacham, K., Shepherd, A., Mohapatra, D. P., and Haroutounian, S. (2017). Neuropathic pain: central vs. Peripheral mechanisms. *Curr. Pain Headache Rep.* 21:28.

Moran, V. A., Perera, R. J., and Khalil, A. M. (2012). Emerging functional and mechanistic paradigms of mammalian long non-coding RNAs. *Nucleic Acids Res.* 40, 6391–6400. doi: 10.1093/nar/gks296

Muller, S., Zirkel, D., Westphal, M., and Zumkeller, W. (2000). Genomic imprinting of IGF2 and H19 in human meningiomas. *Eur. J. Cancer.* 36, 651–655. doi: 10.1016/s0959-8049(99)00328-7

National Institute of Neurological Disorders Stroke rt PASSG, (2005). Recombinant tissue plasminogen activator for minor strokes: the national institute of neurological disorders and stroke rt-PA stroke study experience. *Ann. Emerg. Med.* 46, 243–252. doi: 10.1016/j.annemergmed.2005.02.013

Ng, S. Y., Bogu, G. K., Soh, B. S., and Stanton, L. W. (2013). The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. *Mol. Cell* 51, 349–359. doi: 10.1016/j.molcel.2013.07.017

Nicolas, F. E. (2017). Role of ncRNAs in development. Diagnosis and treatment of human cancer. *Recent Pat Anticancer Drug Discov.* 12, 128–135. doi: 10.2174/1574892812666170105113415

Nijs, J., Leysen, L., Adriaenssens, N., Aguilar Ferrandiz, M. E., Devoogdt, N., Tassenoy, A., et al. (2016). Pain following cancer treatment: guidelines for the clinical classification of predominant neuropathic, nociceptive and central sensitization pain. *Acta Oncol.* 55, 659–663. doi: 10.3109/0284186x.2016.1167958

Peng, C., Zhang, C., Su, Z., and Lin, D. (2019). DGCR5 attenuates neuropathic pain through sponging miR-330-3p and regulating PDCD4 in CCI rat models. *J. Cell Physiol.* 234, 7292–7300. doi: 10.1002/jcp.27487

Peng, H., Zou, L., Xie, J., Wu, H., Wu, B., Zhu, G., et al. (2017). lncRNA NONRATT021972 siRNA decreases diabetic neuropathic pain mediated by the P2X3 receptor in dorsal root ganglia. *Mol. Neurobiol.* 54, 511–523. doi: 10.1007/s12035-015-9632-1

Petzke, F. (2010). CNS processing of pain in functional somatic syndromes. *Schmerz* 24, 146–155.

Picelli, A., Buzzi, M. G., Cisari, C., Gandolfi, M., Porru, D., Bonadiman, S., et al. (2016). Headache, low back pain, other nociceptive and mixed pain conditions in neurorehabilitation. Evidence and recommendations from the Italian consensus conference on pain in neurorehabilitation. *Eur. J. Phys. Rehabil. Med.* 52, 867–880.

Ponjavic, J., Oliver, P. L., Lunter, G., and Ponting, C. P. (2009). Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. *PLoS Genet.* 5:e1000617. doi: 10.1371/journal.pgen.1000617

Qu, H., and Fang, X. (2013). A brief review on the human encyclopedia of DNA Elements (ENCODE) project. *Genomics Proteomics Bioinform.* 11, 135–141. doi: 10.1016/j.gpb.2013.05.001

Qureshi, I. A., Mattick, J. S., and Mehler, M. F. (2010). Long non-coding RNAs in nervous system function and disease. *Brain Res.* 1338, 20–35. doi: 10.1016/j.brainres.2010.03.110

Qureshi, I. A., and Mehler, M. F. (2010). Impact of nuclear organization and dynamics on epigenetic regulation in the central nervous system: implications for neurological disease states. *Ann. N. Y. Acad. Sci.* 1204(Suppl.), E20–E37.

Qureshi, I. A., and Mehler, M. F. (2013). Long non-coding RNAs: novel targets for nervous system disease diagnosis and therapy. *Neurotherapeutics* 10, 632–646. doi: 10.1007/s13311-013-0199-0

Ramos, A. D., Andersen, R. E., Liu, S. J., Nowakowski, T. J., Hong, S. J., Gertz, C., et al. (2015). The long noncoding RNA Pnky regulates neuronal differentiation of embryonic and postnatal neural stem cells. *Cell Stem Cell* 16, 439–447. doi: 10.1016/j.stem.2015.02.007

Ramos, A. D., Attenello, F. J., and Lim, D. A. (2016). Uncovering the roles of long noncoding RNAs in neural development and glioma progression. *Neurosci. Lett.* 625, 70–79. doi: 10.1016/j.neulet.2015.12.025

Rani, N., Nowakowski, T. J., Zhou, H., Godshalk, S. E., Lisi, V., Kriegstein, A. R., et al. (2016). A Primate lncRNA mediates notch signaling during neuronal development by sequestering miRNA. *Neuron* 90, 1174–1188. doi: 10.1016/j.neuron.2016.05.005

Rapicavoli, N. A., Poth, E. M., and Blackshaw, S. (2010). The long noncoding RNA RNCR2 directs mouse retinal cell specification. *BMC Dev. Biol.* 10:49. doi: 10.1186/1471-213X-10-49

Rapicavoli, N. A., Poth, E. M., Zhu, H., and Blackshaw, S. (2011). The long noncoding RNA Six3OS acts in trans to regulate retinal development by modulating Six3 activity. *Neural Dev.* 6:32. doi: 10.1186/1749-8104-6-32

Rapo-Pylkko, S., Haanpaa, M., and Liira, H. (2017). A one-year follow-up study of chronic pain in community-dwelling older adults with and without neuropathic pain. *BMC Geriatr.* 17:152. doi: 10.1186/s12877-017-0537-x

Riva, P., Ratti, A., and Venturin, M. (2016). The long non-coding RNAs in neurodegenerative diseases: novel mechanisms of pathogenesis. *Curr. Alzheimer Res.* 13, 1219–1231. doi: 10.2174/1567205013666160622112234

Roberts, T. C., Morris, K. V., and Weinberg, M. S. (2014). Perspectives on the mechanism of transcriptional regulation by long non-coding RNAs. *Epigenetics* 9, 13–20. doi: 10.4161/epi.26700

Sauvageau, M., Goff, L. A., Lodato, S., Bonev, B., Groff, A. F., Gerhardinger, C., et al. (2013). Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *eLife* 2:e01749.

Scholz, J., Finnerup, N. B., Attal, N., Aziz, Q., Baron, R., Bennett, M. I., et al. (2019). The IASP classification of chronic pain for ICD-11: chronic neuropathic pain. *Pain* 160, 53–59. doi: 10.1097/j.pain.0000000000001365

Shaygan, M., Boger, A., and Kroner-Herwig, B. (2014). Neuropathic sensory symptoms: association with pain and psychological factors. *Neuropsychiatr. Dis. Treat.* 10, 897–906.

Shen, F., Zheng, H., Zhou, L., Li, W., Zhang, Y., and Xu, X. (2019). LINC00657 expedites neuropathic pain development by modulating miR-136/ZEB1 axis in a rat model. *J. Cell Biochem.* 120, 1000–1010. doi: 10.1002/jcb.27466

Sherstyuk, V. V., Medvedev, S. P., and Zakian, S. M. (2018). Noncoding RNAs in the regulation of pluripotency and reprogramming. *Stem Cell Rev.* 14, 58–70. doi: 10.1007/s12015-017-9782-9

Soler, M. D., Morina, D., Rodriguez, N., Sauri, J., Vidal, J., Navarro, A., et al. (2017). Sensory symptom profiles of patients with neuropathic pain after spinal cord injury. *Clin. J. Pain* 33, 827–834. doi: 10.1097/ajp.0000000000000467

St Laurent, G., Wahlestedt, C., and Kapranov, P. (2015). The Landscape of long noncoding RNA classification. *Trends Genet.* 31, 239–251. doi: 10.1016/j.tig.2015.03.007

Teixeira, M. J. (2009). Challenges in the treatment of neuropathic pain. *Drugs Today* 45(Suppl. C), 1–5.

Tochitani, S., and Hayashizaki, Y. (2008). Nkx2.2 antisense RNA overexpression enhanced oligodendrocytic differentiation. *Biochem. Biophys. Res. Commun.* 372, 691–696. doi: 10.1016/j.bbrc.2008.05.127

Treede, R. D., Rief, W., Barke, A., Aziz, Q., Bennett, M. I., Benoliel, R., et al. (2019). Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). *Pain* 160, 19–27.

van Hecke, O., Austin, S. K., Khan, R. A., Smith, B. H., and Torrance, N. (2014). Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain* 155, 654–662. doi: 10.1016/j.pain.2013.11.013

Vance, K. W., Sansom, S. N., Lee, S., Chalei, V., Kong, L., Cooper, S. E., et al. (2014). The long non-coding RNA Paupar regulates the expression of both local and distal genes. *EMBO J.* 33, 296–311. doi: 10.1002/embj.201386225

Vranken, J. H. (2012). Elucidation of pathophysiology and treatment of neuropathic pain. *Cent. Nerv. Syst. Agents Med. Chem.* 12, 304–314. doi: 10.2174/187152412803760645

Wan, P., Su, W., and Zhuo, Y. (2017). The role of long noncoding RNAs in neurodegenerative diseases. *Mol. Neurobiol.* 54, 2012–2021. doi: 10.1007/s12035-016-9793-6

Wang, K. C., and Chang, H. Y. (2011). Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 43, 904–914. doi: 10.1016/j.molcel.2011.08.018

Wang, S., Xu, H., Zou, L., Xie, J., Wu, H., Wu, B., et al. (2016). LncRNA uc.48+ is involved in diabetic neuropathic pain mediated by the P2X3 receptor in the dorsal root ganglia. *Purinergic Signal.* 12, 139–148. doi: 10.1007/s11302-015-9488-x

Watson, J. C., and Dyck, P. J. (2015). Peripheral neuropathy: a practical approach to diagnosis and symptom management. *Mayo Clin. Proc.* 90, 940–951. doi: 10.1016/j.mayocp.2015.05.004

Wei, M., Li, L., Zhang, Y., Zhang, Z. J., Liu, H. L., and Bao, H. G. (2018). LncRNA X inactive specific transcript contributes to neuropathic pain development by sponging miR-154-5p via inducing toll-like receptor 5 in CCI rat models. *J. Cell Biochem*. [Epub ahead of print].

Williams, J. M., Beck, T. F., Pearson, D. M., Proud, M. B., Cheung, S. W., and Scott, D. A. (2009). A 1q42 deletion involving DISC1. DISC2, and TSNAX in an autism spectrum disorder. *Am. J. Med. Genet. A* 149A, 1758–1762. doi: 10.1002/ajmg.a.32941

Wu, P., Zuo, X., Deng, H., Liu, X., Liu, L., and Ji, A. (2013). Roles of long noncoding RNAs in brain development, functional diversification and neurodegenerative diseases. *Brain Res. Bull.* 97, 69–80. doi: 10.1016/j.brainresbull.2013.06.001

Wu, S., Bono, J., and Tao, Y. X. (2019). Long noncoding RNA (lncRNA): a target in neuropathic pain. *Expert Opin. Ther. Targets* 23, 15–20. doi: 10.1080/14728222.2019.1550075

Xia, L. X., Ke, C., and Lu, J. M. (2018). NEAT1 contributes to neuropathic pain development through targeting miR-381/HMGB1 axis in CCI rat models. *J. Cell Physiol.* 233, 7103–7111. doi: 10.1002/jcp.26526

Xu, D. H., Chi, G. N., Zhao, C. H., and Li, D. Y. (2018). Long noncoding RNA MEG3 inhibits proliferation and migration but induces autophagy by regulation of Sirt7 and PI3K/AKT/mTOR pathway in glioma cells. *J. Cell Biochem*. [Epub ahead of print].

Xu, L., Zhang, Y., and Huang, Y. (2016). Advances in the treatment of neuropathic pain. *Adv. Exp. Med. Biol.* 904, 117–129.

Yan, X. T., Lu, J. M., Wang, Y., Cheng, X. L., He, X. H., Zheng, W. Z., et al. (2018). XIST accelerates neuropathic pain progression through regulation of miR-150 and ZEB1 in CCI rat models. *J. Cell Physiol.* 233, 6098–6106.

Young, T. L., Matsuda, T., and Cepko, C. L. (2005). The noncoding RNA taurine upregulated gene 1 is required for differentiation of the murine retina. *Curr. Biol.* 15, 501–512. doi: 10.1016/j.cub.2005.02.027

Zhao, M., Wang, J., Xi, X., Tan, N., and Zhang, L. (2018). SNHG12 promotes angiogenesis following ischemic stroke via regulating miR-150/VEGF pathway. *Neuroscience* 390, 231–240. doi: 10.1016/j.neuroscience.2018.08.029

Zhao, Y., Li, S., Xia, N., Shi, Y., and Zhao, C. M. (2018). Effects of XIST/miR-137 axis on neuropathic pain by targeting TNFAIP1 in a rat model. *J. Cell Physiol.* 233, 4307–4316. doi: 10.1002/jcp.26254

Zimmerman, C. A., Leib, D. E., and Knight, Z. A. (2017). Neural circuits underlying thirst and fluid homeostasis. *Nat. Rev. Neurosci.* 18, 459–469. doi: 10.1038/nrn.2017.71

Zimmermann, M. (2001). Pathobiology of neuropathic pain. *Eur. J. Pharmacol.* 429, 23–37. doi: 10.1016/s0014-2999(01)01303-6

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Acute Phase Serum Leptin, Adiponectin, Interleukin-6, and Visfatin Are Altered in Chinese Children With Febrile Seizures: A Cross-Sectional Study

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Adipokines, including leptin, visfatin, adiponectin, and interleukin-6 (IL)-6, play multiple roles in the pathophysiology of epilepsy and febrile seizures (FS). We aimed to investigate the associations among plasma adipokines, mainly leptin, visfatin, adiponectin, or IL-6, and the prognosis of FS. This prospective cross-sectional study was conducted from January 2017 to December 2018 at the Wuxi Second People' Hospital China. The levels of serum leptin, visfatin, adiponectin, and IL-6 in 55 children with FS (FS group) were compared with 42 febrile children without seizure (FC group) and 48 healthy children (HC group) in an acute phase. The correlation with clinical indicators was determined by logistic regression analysis. Serum adiponectin and IL-6 levels were significantly higher in the FS group than in the FC and HC groups ($p < 0.05$), but there was no statistical difference between the FC and HC groups. In addition, logistic regression analysis showed that high concentrations of adiponectin and IL-6 were significantly associated with the occurrence of FS. For leptin and visfatin, they were significantly lower in the FS and FC groups than in the normal control group, but there was no statistical difference between the FS and FC groups. Our results suggest that higher plasma levels of IL-6 and adiponectin may serve as an additional biomarker in the early treatment or follow-up of the FS children.

Keywords: febrile seizures, adiponectin, IL-6, leptin, visfatin

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INTRODUCTION

Febrile seizures (FS) are one of the most common clinical diseases in pediatric neurology. It occurs between 6 months and 6 years of age and occurs in ∼2–5% of children in the United States and Western Europe, ∼5–14% in Asian countries, and 3–5% in China (1–3). According to the age, frequency, duration, and type of seizures, FS is divided into simple febrile seizures (SFS) and complex febrile seizures (CFS). SFS accounts for 70–75% of seizures, while CFS accounts for 9–35%. Approximately 30–50% of cases may reoccur after the first attack, and the risk of FS turning into epilepsy is 4–5 times that of the general population (4). Repeated FS can lead to brain injuries, such as motor dysfunction, language disability, behavioral cognitive impairment, and higher risk of epilepsy (4, 5).

Therefore, early diagnosis of children with FS is of great significance for early intervention and reduction of neurological sequelae and is an urgent task for pediatric clinics.

With the widespread application of technologies, such as molecular biology, in medicine, some biomarkers for predicting or diagnosing FS have attracted attention. Imuekemhe et al. in 1989 and 1996 found that lactic acid in the serum and cerebrospinal fluid of children with FS was significantly increased (6). Wellmann et al. found that the serum copeptin and Von Willebrand factor (VWF) of children with FS were significantly higher than those of the control group (3, 7). The levels of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and anti-inflammatory cytokines, such as interleukin-4 (IL-4), in the peripheral blood of children with FS also exhibited significant changes, suggesting that these inflammatory molecules may play an important role in the pathogenesis of FS (8–10). These observations suggest that serum levels of some bioactive substances have a specific relation with FS, but so far, none has been clinically recognized and applied. Thus, further research is recommended.

At present, research on the role of adipokines in the pathogenesis of FS is receiving attention. Multiple studies have examined changes in serum adipokine levels in children with FS (11–13). However, they have variable results, and there are issues with the studies, such as small numbers of cases, young age, and lack of correlation analysis with clinical indicators. In particular, as far as we know, there are few studies in China to assess the changes of peripheral blood adipokines in children with FS; this may be related to the low education level and low income of parents of children with FS in many areas, which leads to parents' unwillingness to cooperate with the investigation. The present study was completed in Wuxi, in the Jiangnan region, the most developed industrial city in China. Citizens in this area have higher education levels and income, and generally have medical insurance as a guarantee, and the nutritional status of their children is better than that of children in other regions. These combined factors enabled the children's parents to undertake the financial expenses of the investigation, thus ensuring the successful completion of the study.

The purpose of the study is: (1) to look at changes in serum biomarkers and (2) to provide useful data on additional biomarkers during early treatment or follow-up of the FS children.

MATERIALS AND METHODS

This was a prospective cross-sectional study performed at the Wuxi Second People' Hospital China from January 2017 to December 2018. Fifty-five children with an FS diagnosed in the same hospital were enrolled in the study. The age of the patients ranged from 9 months to 8 years (mean 34 months). Diagnosis of FS followed the criteria established by American Academy of Pediatrics in 2011 (14). The electroencephalogram (EEG) was normal for all patients or showed mild non-specific abnormalities. FS were divided into two groups, namely, the simple FS (SFS) group ($n = 42$), defined as those with primary generalized seizures that lasted for <15 min and did not recur within 24 h, and the complex FS (CFS) group ($n = 13$), defined as those with seizures that were focal, prolonged (≥15 min), and/or recurrent within 24 h (14). The diseases in the FS group include acute upper respiratory tract infection, acute bronchitis, herpes angina, and acute laryngitis. The study protocol was approved by the Human Ethics Committee of the hospital, and informed consent was obtained from the parents of children.

Children with epilepsy, hereditary metabolic disease, congenital malformation, intracranial space-occupying lesions, intracranial infection, intellectual disability, and other brain injuries were excluded. Patients with diseases known to affect adipocytokines, such as diabetes, genetic syndromes (such as glycogen accumulation disease, mucopolysaccharidosis, Gaucher disease, and other diseases of sugar or lipid metabolism disorders), obese patients, and gastrointestinal diseases, such as diarrhea, were also excluded.

Ninety healthy children of comparable age and sex, without a history of febrile or afebrile seizures, were enrolled as a control group. Children with original heart, brain, endocrine, and other basic diseases were excluded. We subdivided our control children into two groups.

The FC group ($n = 42$): children hospitalized at our pediatric department with fever due to infection, except for central nervous system infection. Diseases in the FC group included acute upper respiratory tract infections, acute bronchitis, acute laryngitis, and bronchopneumonia. Because the children in the FS group were all suffering from respiratory diseases, the children in the FC group were also selected children with respiratory diseases, excluding children with gastrointestinal diseases, such as diarrhea. Children with otitis media, diarrhea, skin rash, electrolyte disturbance, and other complications were also excluded.

In addition, hyponatremia-induced convulsions mostly occur in moderate (125–130 mmol/L) to severely low sodium levels (<125 mmol/L), so this study excluded children with serum sodium <130 mmol/L in both the FS and FC groups.

HC group ($n = 48$): children who attended pediatric outpatient clinics for routine physical examinations with no history of illness for 1 month before and after the physical examination.

All patients and controls had appropriate medical history and received comprehensive clinical and detailed neurological examinations. Laboratory studies were performed on all children, including complete blood count (CBC), C-reactive protein (CRP), and body temperature (in Celsius) at admission.

Venous blood samples (5 ml, non-fasting status) were obtained from the patient within 3 h after the seizure. Two-milliliter samples were collected for CBC, CRP, glucose, and sodium detection. Electrolytes such as potassium, calcium, magnesium, and chlorine were also tested. Because the cases of electrolyte disturbance were excluded, the blood electrolytes of the enrolled cases were within the normal range, so no statistical analysis was done. The remaining blood samples were centrifuged, and the serum was stored in EP tubes below −80°C without anticoagulation treatment until use. Control samples

TABLE 1 | Comparison of clinical and laboratory findings of patient and control group[e].

	FS group ($n = 55$)	Fc group ($n = 42$)	Hc group ($n = 48$)	*P*-value
Age (months)	35.38 ± 21.19	42.52 ± 21.40	41.35 ± 24.50	>0.05*
Sex (male/female)[d]	32/23	22/20	26/22	>0.05
BT (°C) on admission	39.39 ± 0.65[b]	39.34 ± 0.57[c]	36.69 ± 0.27[a]	<0.05*
WBC ($\times 10^9$/L)	6.00 (2.70–21.65)	7.94 (2.10–31.86)	6.29 (3.70–9.54)	>0.05#
N (%)	48.06 ± 25.25	42.74 ± 20.18	43.15 ± 14.99	>0.05*
HB (g/L)	119.75 ± 9.54[a]	124.76 ± 7.86[b]	125.42 ± 10.19[c]	<0.05*
PLT ($\times 10^9$/L)	200.56 ± 70.95[a]	256.86 ± 111.47[b]	251.44 ± 91.16[c]	<0.05*
CRP (mg/L)	2.00 (0.50–90.30)	1.75 (0.50–15.80)	1.65 (0.50–7.10)	>0.05#
Glucose (mmol/L)	5.50 (3.48–8.61)	5.33 (4.77–8.64)	5.25 (4.59–8.64)	>0.05#
Sodium (mmol/L)	137 (130–142)[a]	138 (133–149)[b]	138 (134–149)[c]	<0.05#

FS, febrile seizures; FC, febrile control; HC, healthy control; WBC, white blood cell; HB, hemoglobin; N, neutrophil; PLT, platelet; CRP, C-reactive protein.

*#The P-value is for Kruskal-Wallis test; *The P-value is for One-way ANOVA test.*

a,b,con means refer to significant difference between means when K-W or One-way ANOVA test refers to significance by multiple comparison analysis (bc, non-significant; ab, ac, significant).

dChi-square test.

eData shown mean ± standard deviation and median (min–max).

were collected immediately from the vein and similarly stored and analyzed.

Serum levels of leptin were assessed using the solid phase sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ELISA kit, Invitrogen, California, USA). The intra-assay coefficient of variation (CV) value of leptin was 3.9%, and the inter-assay coefficient of variation was 5.3%.

Serum adiponectin levels were measured using ELISA technology (eBioscience, California, USA). The intra-assay CV value was 4.2% and the inter-assay CV was 3.1% for adiponectin.

The serum levels of visfatin were detected with a commercially available ELISA kit (LifeSpan Biosciences, Seattle, USA). The intra-assay CV value was <10%, and the inter-assay CV was <15% for visfatin.

The serum IL-6 concentration was assessed according to the manufacturer's instructions by quantitative sandwich enzyme immunoassay technology (ELISA kit provided by Multi sciences, Hangzhou, China). The intra-assay CV value was 5.0% and the inter-assay CV was 4.6% for IL-6.

Statistical Analysis

The statistical evaluation was conducted using the SPSS software version 17.0 (SPSS Inc. Chicago, Illinois, USA). Categorical variables are shown as frequencies and compared with the chi-squared test, and continuous data were expressed as mean ± standard deviation (normal distribution) or median (min–max) (non-normal distribution) to better meet the statistics principle. Test selection was based on evaluating the variables for normal distribution using the Kolmogorov–Smirnov test. An independent *t*-test and Mann-Whitney *U*-test were used to analyze data between two groups. One-way analysis of variance (ANOVA) test followed by the least significant difference (LSD) and Kruskal-Wallis test followed by Nemenyi test were carried out to compare more than two independent groups (FS, FC, and HC). Spearman's correlation was used to test the correlation analysis. Binomial logistic regression analysis was used to define the association between febrile seizures (as the dependent variable) and estimated plasma adipocytokines levels (as the independent variables). Statistical significance was set at $p < 0.05$.

RESULTS

Table 1 shows the comparison of selected clinical and laboratory data between the FS group, the FC group, and the control group. The study included 55 FS patients (mean age 2.9 ± 1.8 years)−13 of whom had complex FS (CFS) (mean age 3.6 ± 2.3)−42 FC patients (mean age 3.5 ± 1.8), and 48 healthy children who served as controls (HC, mean age 3.4 ± 2.0). The FS, FC, and HC groups were found to be comparable with regard to the age, the ratios of sex, white blood cell (WBC) count, neutrophil ratio, CRP, and serum glucose ($p > 0.05$). However, hemoglobin, the platelet count and serum sodium level were found to be significantly lower in the FS group than in the FC and HC groups ($p < 0.05$).

Table 2 shows the changes in serum levels of leptin, adiponectin, interleukin-6, and visfatin in the three groups. Serum leptin and visfatin levels in the FS and FC groups were significantly lower than those in the HC group ($P < 0.01$), but there was no difference between the FS and FC groups. The levels of serum adiponectin and IL-6 in FS children were significantly higher than those in the FC and HC groups ($P < 0.01$), but there was no difference between FC and HC groups.

When we compared the SFS with the CFS group, we found that the platelet count of the CFS group was significantly lower than that of the SFS group. There was no significant difference between other clinical data and laboratory findings in the two groups (**Table 3**).

All children with FS were followed up until November 2019, and 22 cases of recurrence were found, of which 17 relapsed in the SFS group and five relapsed in the CFS group. There were no significant differences in serum leptin, visfatin, adiponectin, or IL-6 between the relapsed and non-relapsed groups (**Table 4**).

TABLE 2 | Comparison of adipocytokines between the patient group and control groups[a].

	FS group ($n = 55$)	Fc group ($n = 42$)	Hc group ($n = 48$)	*P*-value (among groups)	*P*-value (FS and FC)	*p*-value (FS and HC)	*p*-value (FC and HC)
Leptin (pg/ml)	539.75 (9.41–2030.08)	393.93 (181.51–1545.95)	1447.87 (19.23–2948.69)	<0.05[#]	0.65[##]	<0.001[##]	<0.001[##]
Visfatin (pg/ml)	2.90 (0.04–13.50)	2.62 (0.02–9.82)	7.06 (1.25–22.84)	<0.05[#]	1[##]	<0.001[##]	<0.001[##]
Adiponectin (μg/ml)	13.09 ± 4.38	8.20 ± 2.53	7.19 ± 2.39	<0.05*	<0.001**	<0.001**	0.149**
IL-6 (pg/ml)	8.96 (4.73–38.74)	5.17 (4.61–46.09)	5.11 (4.60–31.03)	<0.05[#]	<0.001[##]	<0.001[##]	0.58[##]

FS, febrile seizures; FC, febrile control; HC, healthy control; IL-6, Interleukin 6.
*[#]The P-value is for Kruskal-Wallis test; *The P-value is for One-way ANOVA test; [##]The P-value is for Nemenyi test; **The P-value is for LSD test.*
[a]Data shown mean ± standard deviation and median (min–max).

TABLE 3 | Comparison of clinical and laboratory findings between SFS group and CFS group[b].

Characteristics	SFS group ($n = 42$)	CFS group ($n = 13$)	*P*-value
Age (months)	33.00 ± 18.35	43.08 ± 28.10	>0.05[#]
Sex (male/female)[a]	27/15	5/8	>0.05
BT (°C) on admission	39.31 ± 0.63	39.48 ± 0.72	>0.05[#]
Laboratory findings			
WBC ($\times 10^9$/L)	7.51 ± 3.68	7.90 ± 5.11	>0.05[#]
N (%)	46.67 ± 25.36	52.55 ± 25.35	>0.05[#]
HB (g/L)	120.19 ± 10.37	118.31 ± 6.26	>0.05[#]
PLT ($\times 10^9$/L)	211.67 ± 70.84	164.69 ± 60.65	<0.05[#]
CRP (g/L)	3.05 (0.50–60.30)	1.90 (0.50–90.30)	>0.05*
Glucose (mmol/L)	5.69 ± 1.34	5.34 ± 0.71	>0.05[#]
Sodium (mmol/L)	136.88 ± 2.69	135.77 ± 3.53	>0.05[#]
Leptin (pg/ml)	561.60 (9.41–2030.08)	274.48 (46.56–190.78)	>0.05*
Visfatin (pg/ml)	2.82 (0.04–13.50)	3.27 (0.56–8.65)	>0.05*
Adiponectin (μg/ml)	12.99 ± 4.59	13.40 ± 3.75	>0.05[#]
IL-6 (pg/ml)	9.34 (4.73–38.74)	8.50 (4.75–37.70)	>0.05*

SFS, Simple febrile seizures; CFS, Complex febrile seizures; BT, body temperature; WBC, white blood cell; HB, hemoglobin; N, neutrophil; PLT, platelet; CRP, C-reactive protein; IL-6, Interleukin 6.
*[#]The P-value is for independent t-test; *The P-value is for Mann-Whitney U-test.*
[a]Chi-square test.
[b]Data shown mean ± standard deviation and median (min–max).

TABLE 4 | Comparison of adipocytokines between the recurrent group and non-recurrent group[a].

	Recurrent group ($n = 22$)	Non-recurrent group ($n = 33$)	*P*-value
Leptin (pg/ml)	478.44 ± 386.41	696.36 ± 454.64	0.07[#]
Visfatin (pg/ml)	2.04 (0.56–8.65)	3.06 (0.04–13.50)	0.536*
Adiponectin (μg/ml)	13.22 ± 4.16	13.00 ± 4.58	0.852[#]
IL-6 (pg/ml)	10.00 (4.73–37.70)	8.38 (4.75–38.74)	0.536*

IL-6, Interleukin 6.
*[#]The P-value is for independent t-test; *The P-value is for Mann-Whitney U-test.*
[a]Data shown mean ± standard deviation and median (min–max).

TABLE 5 | Correlation analysis of adipokines in FS group.

Exercise parameter		*r*	*P*-value
Leptin			
	Visfatin	0.039	0.777
	Adiponectin	−0.159	0.247
	IL-6	0.179	0.192
Visfatin			
	Adiponectin	−0.039	0.779
	IL-6	0.11	0.423
Adiponectin	IL-6	−0.123	0.37

There was no correlation between leptin, adiponectin, visfatin, and IL-6 in the FS and FC groups. There was a weak negative correlation between leptin and adiponectin in the HC group (**Tables 5**–**7**). There is a slight negative correlation between serum sodium and FS (the correlation coefficient between serum sodium and FS is $r = -0.329$, $P < 0.001$). The detailed urine volume was not monitored, nor was urine sodium and creatinine detected, however, further studies are recommended. There is a positive correlation between body temperature and FS: $r = 0.46$, $P < 0.001$. In addition, no correlation was observed between seizures and age.

In the linear regression analysis, high serum adiponectin and IL-6 levels were significantly associated with the risk of febrile seizures among studied FS patients, but leptin and visfatin were not associated with FS. When binary logistic regression analysis was performed, the high serum adiponectin levels were the most significant risk factor associated with FS among studied children [odds ratio (OR): 1.669; 95% confidence interval (CI): 1.388–2.007; $P = 0.000$], and high serum IL-6 levels were also significantly associated with FS (OR: 1.079; 95% CI:1.022–1.139; $P = 0.006$) (**Table 8**).

In addition, we made a comparative analysis of serum indicators of children <3 years old and older than 3 years old. The group under 3-years-old included 74 children (mean age 1.8 ± 0.7 years), of which 33 were FS patients (mean age 1.8 ± 0.7

TABLE 6 | Correlation analysis of adipokines in FC group.

Exercise parameter		*r*	*P*-value
Leptin			
	Visfatin	−0.238	0.128
	Adiponectin	0.139	0.38
	IL-6	0.224	0.153
Visfatin			
	Adiponectin	0.094	0.555
	IL-6	−0.259	0.098
Adiponectin	IL-6	0.12	0.448

TABLE 7 | Correlation analysis of adipokines in HC group.

Exercise parameter		*r*	*P*-value
Leptin			
	Visfatin	0.182	0.217
	Adiponectin	−0.385	**0.007**
	IL-6	−0.082	0.581
Visfatin			
	Adiponectin	0.052	0.724
	IL-6	−0.193	0.188
Adiponectin	IL-6	0.236	0.106

IL-6, Interleukin 6.
P < 0.05 is marked in bold.

TABLE 8 | Binary logistic regression analysis of serum adiponectin and IL-6 levels as risk factors for febrile seizures among studied subjects.

Risk factor	Coefficient	*P*-value	Odds ratio	95% CI[a]
Adiponectin (μg/ml)	0.512	0.000	1.669	1.388–2.007
IL-6 (pg/ml)	0.076	0.006	1.079	1.022–1.139

IL-6, Interleukin 6.
[a]*CI indicates confidence interval.*

years), 18 were FC patients (mean age 1.9 ± 0.6 years), and 23 were healthy controls (mean Age 1.7 ± 0.8 years). The results of leptin, visfatin, and adiponectin in the group under 3-years-old were the same as the results of the whole group mentioned above. Serum IL-6 levels were significantly higher in children with FS compared to the HC group ($P < 0.01$), but there was no difference between either the FS and FC groups or the FC and HC groups (**Table 9**).

The 3+ year group included 71 children (mean age 4.9 ± 1.3 years), including 22 patients with FS (mean age 4.7 ± 1.4 years), 24 FC patients (mean age 4.8 ± 1.3 years), and 25 healthy controls (mean age: 5.1 ± 1.4 years). The results for visfatin, adiponectin, and IL-6 were the same as those for the entire group described above. The leptin levels in the FC group were significantly lower than those in the HC group ($P < 0.01$), but there was no difference between the FS group and the control group (**Table 10**).

DISCUSSION

This study measured blood routine, biochemical indicators, serum leptin, adiponectin, IL-6, and visfatin levels in children with FS, as well as in children of the same age with febrile illness without seizures (FC), and healthy controls (HCs). We also evaluated the correlation with clinical indicators by logistic regression analysis. The main findings of this study were that serum adiponectin and IL-6 levels were significantly higher in the FS group than in the FC and HC groups, while there was no statistical difference between the FC and HC groups.

FS mostly occurs in children from 6 months to 6 years old, but it can sometimes occur in children of an older age. These cases have a history of FS. In this study, there were three children with FS older than 6 years of age, all of whom had a history of FS before the age of 6, including two patients who had experienced FS twice and one patient who had experienced it five times. The three children were excluded from diseases, such as intracranial infection, space occupation, epilepsy, and genetic and metabolic abnormalities, and were diagnosed as FS after blood biochemistry, electroencephalography, cranial magnetic resonance imaging (MRI), and cerebrospinal fluid examination.

Comparison of clinical and laboratory findings of patient and control groups showed that Hb, platelet counts, and blood sodium levels were significantly lower in the FS group than those in the FC and HC groups. It has been shown that iron deficiency anemia may be related to the increased risk of children suffering from FS and is one of the risk factors for FS (15). Similarly, studies have found that the reduction of peripheral blood platelet counts in children with FS may be related to the release of a large number of inflammatory mediators caused by platelet activation caused by infection (16, 17). In addition, previous studies have found that children with FS are prone to hyponatremia (18, 19), which may be the result of FS, but it remains unclear whether it can predict the onset of FS (20, 21). Whether serum Hb level, platelet count, and sodium level can help predict the prognosis of FS is worthy of further study.

Analysis of the four adipokines showed that some of the results were similar to previous studies (8, 11–13), but this study also has new findings. A study by Turkish Güven et al. (11) found that the levels of adiponectin and IL-6 in children under the age of 3 in the FS group were significantly higher than those in the HC group ($p < 0.05$), which is consistent with the results of the present study, but there was no statistical difference in serum adiponectin and IL-6 levels between the FS and FC groups in Güven's study. However, in the current study, the adiponectin level in the FS group was also significantly higher than that in the FC group, and there was no statistical difference between the FC and HC groups, while in the Güven study, the adiponectin level in the FS group was not statistically different from the FC group. The main reason for this difference may be the sample size. In Güven's study, the sample size was relatively small, with FS = 33, FC = 26, and HC = 29 for each group. In this study, the sample sizes were FS = 55, FC = 42, and HC = 48. Other factors, such as antipyretic treatment and their relationship to the measured proteins, may also influence

TABLE 9 | Comparison of adipocytokines between the patient group and control groups under 3[a].

	FS group ($n = 33$)	Fc group ($n = 18$)	Hc group ($n = 23$)	*P*-value (among groups)	*P*-value (FS and FC)	*p*-value (FS and HC)	*p*-value (FC and HC)
Leptin (pg/ml)	539.75 (46.56–2030.08)	389.31 (181.51–1545.95)	1507.06 (101.43–2896.33)	<0.05[#]	0.96[##]	<0.001[##]	<0.001[##]
Visfatin (pg/ml)	2.68 (0.04–8.65)	2.78 (0.02–9.26)	6.84 (1.56–22.84)	<0.05[#]	1[##]	<0.001[##]	<0.001[##]
Adiponectin (μg/ml)	13.58 ± 4.77	8.50 ± 2.88	6.99 ± 2.54	<0.05*	<0.001**	<0.001**	0.237**
IL-6 (pg/ml)	8.50 (4.77–38.52)	5.50 (4.63–46.09)	5.16 (4.62–31.03)	<0.05[#]	0.06[##]	<0.001[##]	0.71[##]

FS, febrile seizures; FC, febrile control; HC, healthy control; IL-6, Interleukin 6.
*[#]The P-value is for Kruskal-Wallis test; *The P-value is for One-way ANOVA test; [##]The P-value is for Nemenyi test; **The P-value is for LSD test.*
[a]Data shown mean ± standard deviation and median (min–max).

TABLE 10 | Comparison of adipocytokines between the patient group and control groups over 3[a].

	FS group ($n = 22$)	FC group ($n = 24$)	Hc group ($n = 25$)	*P*-value (among groups)	*P*-value (FS and FC)	*p*-value (FS and HC)	*p*-value (FC and HC)
Leptin (pg/ml)	628.01 (9.41–1381.17)	476.26 (212.30–1002.90)	1155.75 (19.23–2948.69)	<0.05[#]	1.45[##]	0.06[##]	<0.001[##]
Visfatin (pg/ml)	3.27 (0.56–13.50)	2.49 (0.92–9.82)	7.38 (1.25–22.35)	<0.05[#]	0.99[##]	0.02[##]	0.01[##]
Adiponectin (μg/ml)	12.35 ± 3.70	7.98 ± 2.27	7.36 ± 2.28	<0.05*	<0.001**	<0.001**	0.446**
IL-6 (pg/ml)	10.48 (4.73–38.74)	4.82 (4.61–29.82)	5.11 (4.60–19.31)	<0.05[#]	0.01[##]	<0.001[##]	0.82[##]

FS, febrile seizures; FC, febrile control; HC, healthy control; IL-6, Interleukin 6.
*[#]The P-value is for Kruskal-Wallis test; *The P-value is for One-way ANOVA test; [##]The P-value is for Nemenyi test;**The P-value is for LSD test.*
[a]Data shown mean ± standard deviation and median (min–max).

the results, which merits further investigation. For example, in this study, children in the FS group and the FC group used ibuprofen to reduce fever, and animal experiments suggest that ibuprofen can reduce the concentration of serum leptin and IL-6 (22). In Güven's study, information about antipyretics is not provided.

In addition, it should be noted that the age of previous studies (8, 11–13) is <3-years-old. In this study, the age range is large, and there is no difference in the number of children over 3 years old and children under 3 years old. Therefore, we chose 3-years-old as the cut-off value between the groups. This not only enables better matching and comparisons with previous similar work, but also further expands previous related research. Our results from children over 3 years of age showed that many indicators were consistent with those <3 years old. For example, serum adiponectin levels in the FS group were significantly higher than those in the FC and HC groups, while there was no significant difference between the FC and HC groups. In addition, the level of IL-6 in the FS group was higher than that in the HC groups, but there was no difference in IL-6 levels between the FC and the HC groups. Logistic regression analysis further showed that high concentrations of adiponectin and IL-6 are related to the occurrence of FS. High serum adiponectin levels are the biggest risk factors for FS. High IL-6 levels are also the cause of FS risk factors, while leptin and visfatin were not related to the occurrence of FS. Therefore, it is reasonable to speculate that adiponectin and IL-6 levels can be used to predict the occurrence of FS.

Adiponectin is secreted by adipocytes and its basic function is to improve insulin sensitivity and fat oxidation (23). Later, widely expressed adiponectin receptors were found in the brain (24), and low concentrations of adiponectin were also detected in the cerebrospinal fluid (25), suggesting that adiponectin is involved in the regulation of brain metabolism and function. It is worth noting that adiponectin may have a neuroprotective effect in epilepsy. Adiponectin deficiency in mice on a high-fat diet led to increased seizure severity and hippocampal pathological changes (26). Neuronal damage caused by seizures is related to blood-brain barrier (BBB) leakage. Adiponectin retains the integrity of BBB and has neuroprotective effects in animal models of seizures caused by KA (27), suggesting that adiponectin plays an anticonvulsant role by protecting the integrity of vascular endothelial cells. Further research found that the protective effect of adiponectin on vascular endothelial cells may be related to its anti-inflammatory effect. Adiponectin can significantly inhibit the production of tumor necrosis factor-α (TNF-α) in macrophages (28). Adiponectin is protective against ischemic brain injury by modulating inflammatory pathways and endothelial function, and a low level of plasma adiponectin is associated with increased mortality after ischemic stroke (29, 30). Adiponectin affects the risk of dementia and

its pathophysiology through its anti-inflammatory and anti-atherosclerotic effects (31). Notably, both adiponectin and IL-6 are closely related to epilepsy. Previous studies have found that in patients with refractory epilepsy, serum adiponectin levels are lower than healthy controls, and serum IL-6 levels are higher (32). A recent meta-analysis showed an association between IL-6 (572,174,597) polymorphisms and susceptibility to FS. T alleles and TT genotypes may be associated with an increased risk of FS (33). Therefore, elevated serum adiponectin levels in children with FS may play an anti-inflammatory role and reduce IL-6-mediated inflammatory responses, thereby reducing brain damage caused by FS. This potential mechanism of neuroprotection may be related to the regulation of the BBB integrity.

There is limited information about the correlation between levels of leptin and visfatin with FS. Leptin is mainly secreted by white adipose tissue and regulates energy homeostasis by inhibiting food intake and reducing weight (34). In addition to its role in mammalian metabolism, circulating leptin can cross the BBB and act as a neurotrophic factor through its receptors, thereby regulating neural plasticity and cognitive function (35–37). Moreover, leptin has been shown to regulate both innate and adaptive immune responses in both normal and pathological conditions (38, 39). Leptin is an acute phase reactant and is considered as an inflammatory mediator, which contributes to the thermal changes in systemic inflammation (40). Previous studies have reported both anti-seizure and pro-seizure properties of leptin (41–44). Visfatin (nicotinamide phosphoribosyltransferase, NAMPT) is an enzyme which catalyzes the biosynthesis of nicotinamide adenine dinucleotide (NAD+) in mammals. Adipose-secreted visfatin serves as a neuroendocrine factor and has a strong impact on modulating brain functions (45). Visfatin protects neurons against ischemia-induced injury (46).

The earliest article on the relationship between FS and leptin published by Korean scholars reported that there was no difference in serum leptin levels between the FS group and the normal control. Güven et al. (11) later found that the leptin levels in the FS and FC groups were higher than those in the normal control group, while the study by Seham (12) showed that the leptin levels in the FS group were lower than those in the normal control group, but the leptin levels in the FC group were significantly higher than in the HC group. Here, we found that the leptin and visfatin levels in the FS and FC groups were significantly lower than those in the normal control group, but there was no statistical difference between the FS and FC groups. To the best of our knowledge, this is the first study to measure serum visfatin levels in children with FS. The reasons for these inconsistencies may be related to differences in sampling age, blood collection time, and primary diseases that cause fever. A multicenter follow-up study of the role of leptin and visfatin in children with FS and fever is recommended.

In addition, no significant difference was found between the SFS group and the CFS group, whether it was leptin, visfatin, or adiponectin and IL-6. There was also no difference between the relapsed and non-relapsed groups. Therefore, these factors cannot be used to predict CFS.

This study has some limitations. The blood samples were taken after the FS, meaning the FS itself might have induced the observed changes (in which case the diagnostic role of the proteins is negligible). It should be noted, however, that it is currently clinically impossible to accurately determine the exact time of a seizure in order to collect a blood sample before the seizure begins. Therefore, it is currently common practice to collect blood samples immediately after a seizure. For example, in an article published in 2020 by Costea et al. (47), the authors analyzed the predictive value of plasma biochemical marker molecules within half an hour after FS, which has a certain reference value for assisting the prognosis of seizure recurrence in children with FS.

In conclusion, this study suggests that higher plasma levels of IL-6 and adiponectin could serve as an additional biomarker in the early treatment or follow-up of FS children, which merits further investigation in large-scale multicenter studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Committee of the Wuxi Second People' Hospital China. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HN was the designer and dissertation writer of this study. JC, MJ, LT, and YL were the operators of this experiment and were responsible for the statistical analysis of the data. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Patel N, Ram D, Swiderska N, Mewasingh LD, Newton RW, Offringa M. Febrile seizures. *BMJ*. (2015) 351:h4240. doi: 10.1136/bmj.h4240
2. Leung AK, Hon KL, Leung TN. Febrile seizures: an overview. *Drugs Context*. (2018) 7:212536. doi: 10.7573/dic.212536
3. Pechmann A, Wellmann S, Stoecklin B, Krüger M, Zieger B. Increased von Willebrand factor parameters in children with febrile seizures. *PLoS ONE*. (2019) 14:e0210004. doi: 10.1371/journal.pone.0210004
4. Vestergaard M, Pedersen CB, Sidenius P, Olsen J, Christensen J. The long-term risk of epilepsy after febrile seizures in susceptible subgroups. *Am J Epidemiol*. (2007) 165:911–8. doi: 10.1093/aje/kwk086
5. Gibbs S, Chattopadhyaya B, Desgent S, elsemme AA, Hébert D, Tremblay L, et al. Long—term consequences of a prolonged febrile seizure in a dual pathology model. *Neurobiol Dis*. (2011) 43:312–21. doi: 10.1016/j.nbd.2011.02.013
6. Imuekemhe SO, Obi JO, Sykes RM, Alakija W. Cerebrospinal fluid/serum lactic acid in Nigerian children with febrile convulsions. *Ann Trop Paediatr*. (1996) 16:181–4. doi: 10.1080/02724936.1996.11747823
7. Stöcklin B, Fouzas S, Wellmann S, Cayir S, Skendaj R, Ramser M, et al. Copeptin as a serum biomarker of febrile seizures. *PLoS ONE*. (2015) 10:e0124663. doi: 10.1371/journal.pone.0124663
8. Gupta S, Aggarwal A, Faridi MM, Rai G, Das S, Kotru M. Serum Interleukin-6 Levels in Children with Febrile Seizures. *Indian Pediatr*. (2018) 55:411–3. doi: 10.1007/s13312-018-1284-6
9. Ha J, Choi J, Kwon A, Kim K, Kim SJ, Bae SH, et al. Interleukin-4 and tumor necrosis factor-alpha levels in children with febrile seizures. *Seizure*. (2018) 58:156–62. doi: 10.1016/j.seizure.2018.04.004
10. Sahin S, Uysal S, Yentür SP, Kaçar A. Reduced cerebrospinal fluid levels of interleukin-10 in children with febrile seizures. *Seizure*. (2019) 65:94–7. doi: 10.1016/j.seizure.2019.01.008
11. Güven AS, Icagasioglu FD, Duksal F, Sancakdar E, Alaygut D, Uysal EB, et al. Serum adiponectin, leptin, and interleukin 6 levels as adipocytokines in children with febrile seizures: the role of adipose tissue in febrile seizures. *Hum Exp Toxicol*. (2015) 34:878–83. doi: 10.1177/0960327114561662
12. Azab SF, Abdalhady MA, Almalky MAA, Amin EK, Sarhan DT, Elhindawy EM, et al. Serum and CSF adiponectin, leptin, and interleukin 6 levels as adipocytokines in Egyptian children with febrile seizures: a cross-sectional study. *Ital J Pediatr*. (2016) 42:38. doi: 10.1186/s13052-016-0250-y
13. Khoshdel A, Parvin N, Abbasi M. Selenium and leptin levels in febrile seizure: a case-control study in children. *Korean J Pediatr*. (2013) 56:80–5. doi: 10.3345/kjp.2013.56.2.80
14. Duffner PK, Berman PH, Baumann RJ, Fisher PG, Green JL, Schneider S, et al. Neurodiagnostic evaluation of the child with a simple febrile seizure. Subcommittee on Febrile Seizures; American Academy of Pediatrics. Collaborators (7). *Pediatrics*. (2011) 127:389–94. doi: 10.1542/peds.2010-3318
15. Kwak BO, Kim K, Kim S-N, Lee R. Relationship between iron deficiency anemia and febrile seizures in children: a systematic review and meta-analysis. *Seizure*. (2017) 52:27–34. doi: 10.1016/j.seizure.2017.09.009
16. Gontko–Romanowska K, Zaba Z, Panieński P, Steinborn B, Szemień M, Łukasik–Głebocka M, et al. The assessment of laboratory parameters in children with fever and febrile seizures. *Brain Behav*. (2017) 7:e00720. doi: 10.1002/brb3.720
17. Liu Z, Li X, Zhang M, Huang X, Bai J, Pan Z, et al. The role of mean platelet volume/platelet count ratio and neutrophil to lymphocyte ratio on the risk of febrile seizure. *Sci Rep*. (2018) 8:15123. doi: 10.1038/s41598-018-33373-3
18. Kiviranta T, Airaksinen EM. Low sodium levels in serum are associated with subsequent febrile seizures. *Acta Paediatr*. (1995) 84:1372–4. doi: 10.1111/j.1651-2227.1995.tb13571.x
19. Kiviranta T, Tuomisto L, Airaksinen EM. Osmolality and electrolytes in cerebrospinal fluid and serum of febrile children with and without seizures. *Eur J Pediatr*. (1996) 155:120–5. doi: 10.1007/BF02075764
20. Thoman JE, Duffner PK, Shucard JL. Do serum sodium levels predict febrile seizure recurrence within 24 hours? *Pediatr Neurol*. (2004) 31:342–4. doi: 10.1016/j.pediatrneurol.2004.05.013
21. Maksikharin A, Prommalikit O. Serum sodium levels do not predict recurrence of febrile seizures within 24 hours. *Paediatr Int Child Health*. (2015) 35:44–6. doi: 10.1179/2046905514Y.0000000159
22. Zivna H, Zivny P, Palicka V. Serum leptin concentrations after surgery in young rats. *Nutrition*. (2002) 18:643–6. doi: 10.1016/S0899-9007(02)00741-4
23. Ahima RS. Metabolic actions of adipocyte hormones: focus on adiponectin. *Obesity*. (2006) 14(Suppl 1):9S−15S. doi: 10.1038/oby.2006.276
24. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. (2003) 423:762–9. doi: 10.1038/nature01705
25. Kusminski CM, McTernan PG, Schraw T, Kos K, O'Hare JP, Ahima R, et al. Adiponectin complexes in human cerebrospinal fluid: distinct complex distribution from serum. *Diabetologia*. (2007) 50:634–42. doi: 10.1007/s00125-006-0577-9
26. Lee EB, Warmann G, Dhir R, Ahima RS. Metabolic dysfunction associated with adiponectin deficiency enhances kainic acid-induced seizure severity. *J Neurosci*. (2011) 31:14361–6. doi: 10.1523/JNEUROSCI.3171-11.2011
27. Jeon BT, Shin HJ, Kim JB, Kim YK, Lee DH, Kim KH, et al. Adiponectin protects hippocampal neurons against kainic acid-induced excitotoxicity. *Brain Res Rev*. (2009) 61:81–8. doi: 10.1016/j.brainresrev.2009.05.002
28. Díez JJ, Iglesias P. The role of the morel adipocyte derived hormone adiponectin in human disease. *Eur J Endocrinol*. (2003) 148:293–300. doi: 10.1530/eje.0.1480293
29. Nishimura M, Izumiya Y, Higuchi A, Shibata R, Qiu J, Kudo C, et al. Adiponectin prevents cerebral ischemic injury through endothelial nitric oxide synthase dependent mechanisms. *Circulation*. (2008) 117:216–23. doi: 10.1161/CIRCULATIONAHA.107.725044
30. Chen B, Liao WQ, Xu N, Xu H, Wen JY, Yu CA, et al. Adiponectin protects against cerebral ischemia-reperfusion injury through anti-inflammatory action. *Brain Res*. (2009) 1273:129–37. doi: 10.1016/j.brainres.2009.04.002
31. Chen R, Shu Y, Zeng Y. Links between adiponectin and dementia: from risk factors to pathophysiology. *Front Aging Neurosci*. (2020) 11:356. doi: 10.3389/fnagi.2019.00356
32. Ethemoglu O, Ay H, Koyuncu I, Gönel A. Comparison of cytokines and prooxidants/antioxidants markers among adults with refractory versus well-controlled epilepsy: a cross-sectional study. *Seizure*. (2018) 60:105–9. doi: 10.1016/j.seizure.2018.06.009
33. Chen Q, Li M, Zhang X, Zhang X, Zhong R, Lin W. Association between interleukin-6 gene polymorphisms and febrile seizure risk: a meta-analysis. *Medicine*. (2019) 98:e17167. doi: 10.1097/MD.0000000000017167
34. Brunner L, Nick HP, Cumin F, Chiesi M, Baum HP, Whitebread S, et al. Leptin is a physiologically important regulator of food intake. *Int J Obes Relat Metab Disord*. (1997) 21:1152–60. doi: 10.1038/sj.ijo.0800529
35. Banks WA, Clever CM, Farrell CL. Partial saturation and regional variation in the blood-to-brain transport of leptin in normal weight mice. *Am J Physiol Endocrinol Metab*. (2000) 278:E1158–65. doi: 10.1152/ajpendo.2000.278.6.E1158
36. Van Doorn C, Macht VA, Grillo CA, Reagan LP. Leptin resistance and hippocampal behavioral deficits. *Physiol Behav*. (2017) 176:207–13. doi: 10.1016/j.physbeh.2017.03.002
37. Funahashi H, Yada T, Suzuki R, Shioda S. Distribution, function, and properties of leptin receptors in the brain. *Int Rev Cytol*. (2003) 224:1–27. doi: 10.1016/S0074-7696(05)24001-9
38. Francisco V, Pino J, Campos-Cabaleiro V, Ruiz-Fernández C, Mera A, Gonzalez-Gay MA, et al. Obesity, fat mass and immune system: role for leptin. *Front Physiol*. (2018) 9:640. doi: 10.3389/fphys.2018.00640
39. Conde J, Scotece M, Gómez R, Gómez-Reino JJ, Lago F, Gualillo O. At the crossroad between immunity and metabolism: focus on leptin. *Expert Rev Clin Immunol*. (2010) 6:801–8. doi: 10.1586/eci.10.48
40. Maruna P, Gürlich R, Frasko R. Leptin–a new acute phase reactan. *Vnitr Lek*. (2001) 47:478–83.
41. Lambrechts DA, Brandt-Wouters E, Verschuure P, Vles HS, Majoie MJ. A prospective study on changes in blood levels of cholecystokinin-8 and leptin in patients with refractory epilepsy treated with the ketogenic diet. *Epilepsy Res*. (2016) 127:87–92. doi: 10.1016/j.eplepsyres.2016.08.014
42. Li LL, Li YC, Zhao DJ, Jin MF, Ni H. Leptin-regulated autophagy plays a role in long-term neurobehavioral injury after neonatal seizures and the regulation of zinc/cPLA2 and CaMK II signaling in cerebral cortex. *Epilepsy Res*. (2018) 146:103–11. doi: 10.1016/j.eplepsyres.2018.07.023

43. Arslan G, Alici SK, Ayyildiz M, Agar E. The role of CB1-receptors in the proconvulsant effect of leptin on penicillin-induced epileptiform activity in rats. *CNS Neurosci Ther*. (2013) 19:222–8. doi: 10.1111/cns.12075
44. Lynch JJ, Shek EW, Castagné V, Mittelstadt SW. The proconvulsant effects of leptin on glutamate receptor-mediated seizures in mice. *Brain Res Bull*. (2010) 82:99–103. doi: 10.1016/j.brainresbull.2010.02.003
45. Lee TH, Cheng KK, Hoo RL, Siu PM, Yau SY. The novel perspectives of adipokines on brain health. *Int J Mol Sci*. (2019) 20:5638. doi: 10.3390/ijms20225638
46. Jing Z, Xing J, Chen X, Stetler RA, Weng Z, Gan Y, et al. Neuronal NAMPT is released after cerebral ischemia and protects against white matter injury. *J Cereb Blood Flow Metab*. (2014) 34:1613–21. doi: 10.1038/jcbfm.2014.119
47. Costea RM, Maniu I, Dobrota L, Neamtu B. Stress hyperglycemia as predictive factor of recurrence in children with febrile seizures. *Brain Sci*. (2020) 10:131. doi: 10.3390/brainsci1003 0131

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Proteomics for Studying the Effects of Ketogenic Diet Against Lithium Chloride/Pilocarpine Induced Epilepsy in Rats

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The ketogenic diet (KD) demonstrates antiepileptogenic and neuroprotective efficacy, but the precise mechanisms are unclear. Here we explored the mechanism through systematic proteomics analysis of the lithium chloride-pilocarpine rat model. Sprague-Dawley rats (postnatal day 21, P21) were randomly divided into control (Ctr), seizure (SE), and KD treatment after seizure (SE + KD) groups. Tandem mass tag (TMT) labeling and liquid chromatography-tandem mass spectroscopy (LC-MS/MS) were utilized to assess changes in protein abundance in the hippocampus. A total of 5,564 proteins were identified, of which 110 showed a significant change in abundance between the SE and Ctr groups (18 upregulated and 92 downregulated), 278 between SE + KD and SE groups (218 upregulated and 60 downregulated), and 180 between Ctr and SE + KD groups (121 upregulated and 59 downregulated) (all $p < 0.05$). Seventy-nine proteins showing a significant change in abundance between SE and Ctr groups were reciprocally regulated in the SD + KD group compared to the SE group (i.e., the seizure-induced change was reversed by KD). Of these, five (dystrobrevin, centromere protein V, oxysterol-binding protein, tetraspanin-2, and progesterone receptor membrane component 2) were verified by parallel reaction monitoring. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that proteins of the synaptic vesicle cycle pathway were enriched both among proteins differing in abundance between SE and Ctr groups as well as between SE + KD and SE groups. This comprehensive proteomics analyze of KD-treated epilepsy by quantitative proteomics revealed novel molecular mechanisms of KD antiepileptogenic efficacy and potential treatment targets.

Keywords: ketogenic diet, antiepileptogenic, proteomics, hippocampus, rat-brain

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INTRODUCTION

Epilepsy is a chronic disease characterized clinically by recurrent and unpredictable seizures (Fisher et al., 2005) due to uncontrolled neuronal hyperactivity. A recent large-scale epidemiological survey of 196 countries and regions around the world found that there were 45.9 million people with epilepsy in 2016, with highest incidence in children aged 5 to 9 years (Beghi et al., 2019). Severe

status epilepticus or recurrent seizures can cause cognitive decline, impair quality of life, and increase the risks of injury and sudden death (Nashef et al., 1995). The most common treatments for epilepsy are oral antiepileptic drugs (AEDs). However, about 30% of children are resistant to currently available AEDs (Pluta and Jablonski, 2011).

The ketogenic diet (KD) is a high fat, low carbohydrate regime widely considered an effective non-drug treatment for epilepsy with documented anticonvulsant, antiepileptogenic, and neuroprotective effects on clinically refractory epilepsy and animal models of epilepsy (Lusardi et al., 2015; Simeone et al., 2018; Karimzadeh et al., 2019). Multiple therapeutic mechanisms have been proposed for KD-induced antiepileptogenesis, including increased adenosine and decreased DNA methylation, reduced mTORC1 activity, and blockade of histone deacetylases (Koene et al., 2019; Boison and Rho, 2020). Thus, it is critical to comprehensively assess the molecular changes associated with the KD in epilepsy. Moreover, the KD is often unpalatable, especially to children, and must be sustained for years, resulting in poor compliance. In addition, constipation and weight loss are common adverse effects (Cai et al., 2017). In several clinical studies, the KD was also found to influence mood. Although most of these studies reported positive effects (Halyburton et al., 2007; McClernon et al., 2007; Dm et al., 2016), some reported no effects or even negative effects on mood (Lambrechts et al., 2013; Iacovides et al., 2019). These inconsistences may be related to the type of disease before KD treatment, the number of subjects, and the duration of KD compliance, necessitating larger-scale, multiple-center studies to assess the influence of the KD on mood in specific diseases. Death during KD treatment has also been reported secondary to severe infection and malnutrition (Kang et al., 2004; Suo et al., 2013). Therefore, a better understanding of the therapeutic mechanisms may improve clinical application and reveal new targets for clinical anti-epileptic treatment.

Previous studies on the antiepileptogenic efficacy of the KD focused mainly on changes in the expression of specific preselected proteins or genes, while few have used gene chips to objectively explore larger-scale gene expression changes associated with KD treatment of epilepsy (Bough et al., 2006; Jeong et al., 2010). Modern proteomics techniques can reveal similarities and differences in protein expression at the individual, pathway, and network levels under various physiological and pathological states, thus providing a more comprehensive understanding of disease pathology and progression (Atamna et al., 2002). Such proteomics studies have examined the pathogenesis of epilepsy (Walker et al., 2016; Sadeghi et al., 2017), but not the mechanisms underlying the antiepileptogenic action of KD. At present, the main technologies used in proteomics research are two-dimensional gel electrophoresis and mass spectrometry (MS). The former is technically demanding, is not amenable to automation, and has limited separation capacity, especially for low abundance and hydrophobic proteins. Alternatively, mass spectrometry is suitable for high-throughput analysis by automation and can discriminate proteins of similar size and isoelectric point.

Therefore, we conducted the first proteomics analysis of the antiepileptogenic response to KD in the rat lithium chloride-pilocarpine-induced epileptic model using MS-based tandem mass tag (TMT) quantitative proteomics.

MATERIALS AND METHODS

Animals and Treatment

Animal Preparation

Postnatal day 21 (P21) Sprague-Dawley rats (n = 45) were obtained from JOINN Laboratories, Co. Ltd. (Suzhou, China) [License no. SCXK(SU) 2018-0006]. Animals were treated in accordance with the guidelines set by the National Institutes of Health (Bethesda, MD, United States) for the humane treatment of animals. Animal experiments were approved by the Animal Experimental Ethics Committee of Suzhou University. All rats were raised under a 12 h:12 h light: dark cycle with free access to drinking water and the indicated diet (normal or KD). Animals were protected from bright lights and excessive noise during housing. Rats were first randomly divided into a control group (Ctr, n = 10) and seizure model group (n = 35). Rats exhibiting status epilepticus following lithium chloride-pilocarpine treatment (detailed below) were then randomly assigned to the normal diet group (SE) or KD diet group (SE + KD).

Induction of Status Epilepticus

Status epilepticus was induced by lithium chloride-pilocarpine in accordance with our previous study (Chen et al., 2019). Briefly, 35 rats were injected intraperitoneally with 127 mg/kg lithium chloride (Sigma-Aldrich, United States) at P21 and 24 h later (P22) with 1 mg/kg scopolamine hydrobromide (TargetMol, United States) to reduce the peripheral cholinergic response to pilocarpine. Thirty minutes later, 320 mg/kg pilocarpine (Sigma-Aldrich, United States) was injected and response scored according to the Racine scale (Racine, 1972) as follows: (0) no abnormality; (1) mouth and facial movements; (2) head nodding; (3) unilateral forelimb clonus; (4) rearing with bilateral forelimb clonus; and (5) rearing and falling. Animals were selected for further study only if the seizure degree reached level IV or above (n = 28). The onset of status epilepticus was characterized by initial immobility and chewing followed by repetitive clonic activity of the trunk and limbs, repeated rearing with forelimb clonus and falling interspersed with immobility, chewing, and myoclonic jerks singularly or in series. Acute status epilepticus was stopped after 60 min by intraperitoneal administration of 300 mg/kg chloral hydrate (Sigma-Aldrich, United States). Five rats died due to generalized tonic seizures. Animals surviving status epilepticus were randomly divided into the normal diet SE group (n = 12) and SE + KD (n = 11) group. There were no differences in seizure duration and severity between groups. In each group, 10 rats were randomly labeled for weight and blood ketone measurements. After weight and blood ketone were measured, six rats in each group were randomly labeled for proteomics testing and parallel reaction monitoring (PRM) verification. Control group rats received the same treatments

TABLE 1 | Composition of normal and KDs.

Component	Normal diet (%)	KD (%)
Carbohydrate (starch)	50	–
Protein (casein)	20	20
Soybean oil	4.5	10
Lard	–	42
Butter	–	17
Sunflower seed oil	–	1
Fiber	5	5.5
AIN-76 mineral mixture	8	3.5
AIN-76 vitamin mixture	2.3	1
Energetic (kcal/g)	4	7.1
Ketogenic ratio	0.37:1	3.5:1

and evaluations but were injected intraperitoneally with 0.9% saline solution instead of pilocarpine. No epileptic seizures were observed in any Ctr group rat.

Dietary Intervention

During the modeling period (P21–P22), all groups were fed a normal diet. After vehicle treatment or status epilepticus induction, Ctr and SE groups continued to receive a normal diet for 28 days (4.5% fat, 20% protein and 50% carbohydrate), while the SE + KD group was fed the KD for 28 days (70% fat, 20% protein, and no carbohydrate). The KD formula was reported in detail previously (Ni et al., 2016). Further detail contents of the diets are shown in **Table 1**. Both diets were obtained from the Chinese Academy of Sciences, Shanghai Experimental Animal Center (Shanghai, China).

Weight and Blood Ketone Monitoring

Body weight and blood ketones were recorded at P49. After the rats were anesthetized, blood samples were collected from the tail vein and blood ketone levels measured using a Keto-detector (Beijing Yicheng Bioelectronics Technology, Co., Ltd., China).

Protein Extraction and Digestion

Samples of hippocampus were extracted, flash frozen to −80°C, ground into powder over liquid nitrogen, and transferred to 5-mL centrifuge tubes. Four volumes of pyrolysis buffer containing 8M urea and 1% protease inhibitor mixture (Calbiochem, San Diego, CA, United States) were added and the mixture sonicated three times on ice at high intensity using a Scientz ultrasonic system (Scientz, Ningbo, China). After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was transferred into another centrifuge tube, and the sediment at the bottom was discarded. The supernatant protein concentration was measured by the BCA kit (Beyotime, China). Supernatant proteins were then digested in trypsin (Promega, Madison, WI, United States) as described (Chen et al., 2018).

Tandem Mass Tag (TMT) Labeling

After protein digestion, peptides were desalinated on a chromatographic X C18 SPE column (Phenomenex, Torrance, CA, United States), vacuum-dried, dissolved in 0.5M TEAB (Sigma-Aldrich), and labeled according to the operation instructions of the 9-plex TMT kit (Thermo Fisher Scientific).

High-Performance Liquid Chromatography (HPLC) Fractionation

Labeled peptides were fractionated into 60 samples over 60 min by high pH reverse-phase HPLC using an Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length) and 8–32% acetonitrile (pH 9.0) gradient. Peptides were combined into 14 fractions and dried by vacuum centrifugation for mass spectroscopy.

Liquid Chromatography-Tandem Mass Spectroscopy (LC-MS/MS)

Peptides were dissolved in 0.1% formic acid (solvent A) and loaded directly onto a homemade reversed-phase analytical column (15-cm length, 75 μm inner diameter). Samples were then eluted at 350 nL/min using a mobile phase consisting of 0.1% formic acid in 98% acetonitrile solvent B under the control of an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific). The elution protocol was as follows: 9–26% solvent B for 40 min, 26–35% solvent B for 14 min, 35–80% solvent B for 3 min, and holding at 80% for the last 3 min. Eluted peptides were then subjected to nanoelectrospray ionization (NSI) followed by tandem mass spectrometry (MS/MS) using the Q ExactiveTM Plus system (Thermo Fisher Scientific) coupled to the UPLC. The electrostatic voltage applied was 2.1 kV and the m/z scan range was 400 to 1500. Both intact peptides and fragments were detected in the Orbitrap at resolutions of 70,000 and 35,000 FWHM, respectively. Peptides were then selected for MS/MS using a normalized collision energy (NCE) setting of 28. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1×10^4 in the MS survey scan with 30.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

Database Searches

The MS/MS data were processed using Maxquant (v.1.5.2.8) and searched against the Rat_Protemoe_1905 database (29,947 sequences). A reverse decoy database was used to calculate the false positive rate caused by random matching. Trypsin/P was specified as the cleavage enzyme allowing for up to two missing cleavages. The minimum peptide length was set at seven and the maximum number of peptide modifications at five. The mass tolerance for precursor ions was set to 20 ppm for the first search and to 5 ppm for the main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as the fixed modification, and acetylation and oxidation on Met were specified as variable modifications. False discovery rate (FDR) was adjusted to $< 1\%$.

Parallel Reaction Monitoring (PRM)

We used LC-PRMMS analysis to verify protein expression levels derived from TMT analysis. Peptides remaining from proteomics analyses (above) were dissolved in 0.1% formic acid (solvent

A), loaded directly onto a homemade reversed-phase analytical column, and eluted at a constant flow rate of 500 nL/min using the following mobile phase protocol control by an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific): 6 to 25% solvent B (0.1% formic acid in 98% acetonitrile) over 40 min, 25 to 35% solvent B over 12 min, 35 to 80% over 4 min, then holding at 80% for the last 4 min. The peptides were subjected to NSI followed by tandem mass spectrometry (MS/MS) using the Q ExactiveTM Plus system (Thermo Fisher Scientific) coupled to the UPLC. The electrospray voltage applied was 2.0 kV, m/z scan range was 360 to 1080 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for 20 MS/MS scans on the Orbitrap at a resolution of 17,500 using a data-independent procedure. AGC was set at 3E6 for full MS and 1E5 for MS/MS. The maximum injection time was set at 50 ms for full MS and 110 ms for MS/MS. The isolation window for MS/MS was set at 1.6 m/z. The NCE was 27% with high energy collision dissociation (HCD). The resulting MS data were processed using Skyline (v.3.6). Peptide settings were as follows: enzyme was set as trypsin [KR/P], max missed cleavage as 0, peptide length as 7–25, and fixed modification as alkylation on Cys. The transition settings were as follows: precursor charges were set as 2, 3, ion charges as 1, and ion as b, y. The product ions were set from ion 3 to last ion, and the ion match tolerance was set as 0.02 Da.

Bioinformatics Analysis

Gene Ontology (GO) Annotation

Gene Ontology is a major bioinformatics initiative to unify gene and gene product attributes across all species. The GO annotations for this study were derived from the UniProt-GOA database[1]. First, identified protein IDs were converted to UniProt IDs and then mapped to GO IDs. For identified proteins not annotated by the UniProt-GOA database, InterProScan was used to annotate GO function based on protein sequence alignment. Proteins were classified by GO annotation based on three categories: biological process, cellular component, and molecular function.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Annotation

Proteins were then annotated to KEGG pathways using the online service tools KEGG automatic annotation server (KAAS) and KEGG Mapper.

GO and KEGG Pathway Functional Enrichment

A two-tailed Fisher's exact test was used to test the enrichment of identified proteins against all proteins in GO and KEGG databases, with a corrected $p < 0.05$ considered significant.

Statistical Analysis

Body weights and blood ketones were compared among groups by one-way analysis of variance (ANOVA) with the indicated *post hoc* tests for pair-wise comparisons. A $p < 0.05$ was considered significant for all tests. GraphPad Prism version 5.0 was used for all data processing.

[1]http://www.ebi.ac.uk/GOA/

RESULTS

Effects of the Ketogenic Diet on Appearance

Rats receiving the KD diet following status epilepticus induction (SE + KD group) gained substantially less weight after the 28 days observation period than both seizure-induced rats fed a regular diet (SE group, $p < 0.01$) and control rats (Ctr group, $p < 0.01$) (**Figure 1A**). Most SE + KD rats developed constipation and oily fur but otherwise were active and showed no evidence of infectious or respiratory complications, and none of them died.

Blood Ketones

As shown in **Figure 1B**, blood ketone levels were significantly higher in the SE + KD group than Ctr and SE groups ($p < 0.01$), but did not differ between Ctr and SE groups ($p > 0.05$).

LC-MS/MS

The abundances of hippocampal proteins were compared among Ctr, SE, and SE + KD groups using LC-MS/MS to identify those showing differential abundance caused by KD (**Figure 2**). A total of 238264.0 secondary spectrograms were obtained by mass spectrometry, and 82,100 spectrograms were available for analysis. A total of 41,645 peptide segments were identified, among which 38,097 were specific segments. In total, 5,564 proteins were identified, of which 4,740 were quantifiable. The screening criteria for differential abundance of proteins were fold-change > 1.2 (upregulated) or < 0.83 (downregulated) and $p < 0.05$. According to these criteria, 110 proteins exhibited a significant change in abundance between the SE and Ctr groups (18 upregulated and 92 downregulated), 180 between SE and SE + KD groups (121 upregulated and 59 downregulated), and 278 between SE + KD and Ctr groups (218 upregulated and 60 downregulated). Detailed data are provided in **Supplementary Table S1**. Optimized screening criteria were then applied for those proteins showing reciprocal abundance changes between SE vs. Ctr and SE + KD vs. SE groups. In total, 79 proteins met this condition (**Supplementary Table S2**), of which 72 were downregulated in the SE group compared to the Ctr group but upregulated in the SE + KD group compared to the SE group (i.e., downregulation induced by seizure was reversed by KD). The five showing the largest fold-changes were Hmgb3 protein, cyclic nucleotide-gated channel beta 3, aldose reductase-related protein 1-like, complexin 3, and solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 6. The other seven proteins showing reciprocal regulation were upregulated in the SE group compared to the Ctr group but downregulated in the SE + KD group compared to the SE group. The five proteins showing the largest fold changes among these seven were round spermatid basic protein 1, uncharacterized protein M0R9L6, cyclin dependent kinase inhibitor, reproductive homeobox on X chromosome 12, and IQ motif containing GTPase activating protein 1 (Predicted) isoform CRA.b.

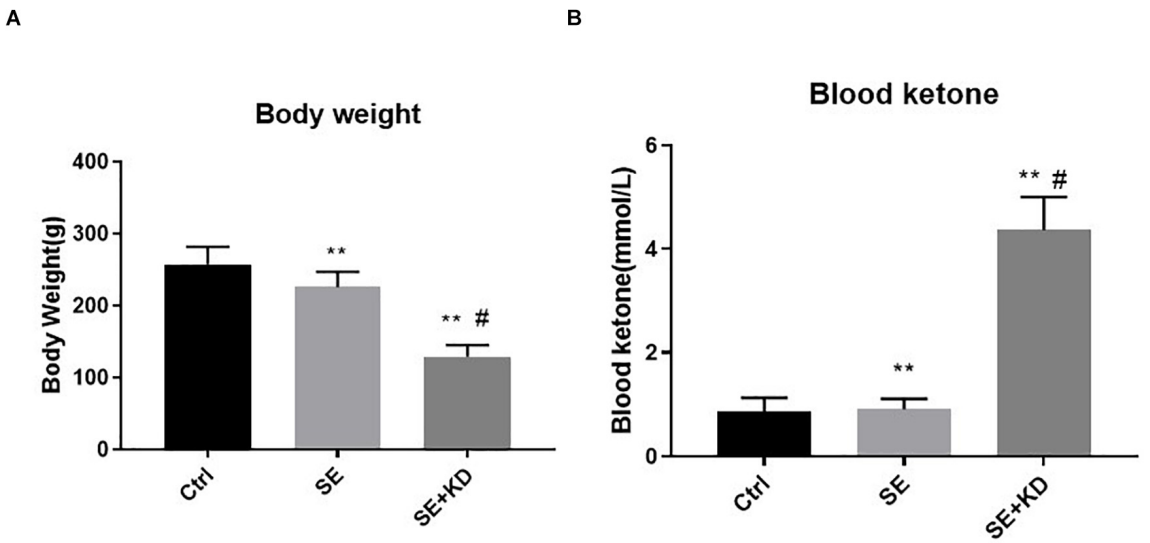

FIGURE 1 | Comparison of body weight **(A)** and blood ketones **(B)** among control (Ctr), seizure (SE), and seizure with ketogenic diet (SE + KD) groups at P49 (n = 10 rats/group). Body weights were significantly reduced in SE and SE + KD groups compared to the Ctr group, and significantly lower in the SE + KD group compared to the SE group. Blood ketone level was significantly higher in the SE + KD group compared to Ctr and SE groups, but did not differ between Ctr and SE groups. $^{**}p < 0.01$compared to Ctr group, $\#p < 0.01$ compared with SE group.

PRM Verification

To further verify the results of MS, five of these 79 reciprocally regulated proteins (dystrobrevin, centromere protein V, oxysterol-binding protein, tetraspanin-2, and progesterone receptor membrane component 2) were selected for PRM analysis. The screening criteria for PRM were based on the following principles: (1) proteins with potential biological function and significance; (2) proteins with a peptide fragment of no less than 1; (3) proteins associated with epilepsy but not reported or reported in only a few previous proteomic studies. Seven target peptide fragments of these five proteins were analyzed by Skyline, and the distributions of fragment ion peak areas are presented in **Supplementary Figures S3–S9**. The mean relative abundances of the target peptide fragments in each sample group are shown in **Table 2**. Differences in abundance of relative target proteins among sample groups were further calculated based on abundance of the corresponding peptide fragment (detailed data are provided in **Table 3**). Quantitative information on target peptide fragments was obtained from all nine samples. Compared to the Ctr group, the abundances of dystrobrevin, centromere protein V, oxysterol-binding protein, tetraspanin-2, and progesterone receptor membrane component 2 were downregulated in the SE group but upregulated in the SE + KD group, consistent with TMT results.

Bioinformatics Analysis

We use Bioinformatics tools to analyze the differential abundances of all proteins detected by MS.

GO Functional Annotation Analysis

The GO database is an international standardized functional classification system that comprehensively describes the characteristics of genes and their products. We performed GO functional annotation searches for all proteins identified in this study and then subjected those demonstrating differential abundance among groups to GO enrichment analysis using Fisher's exact test. According to secondary GO annotations, most of the 79 reciprocally regulated proteins can be classified into three major categories: "molecular interactions," "cell components," and "biological processes." The most common "molecular interaction" was "protein binding" (54 proteins, 65%), followed by "catalytic activity" (11 proteins), and "enzyme regulator" (seven proteins). The top three "cell components" classifications were "cell" (58 proteins), "organelle" (46 proteins), and "membrane" (29 proteins), while the top three "biological processes" classifications were "cellular process" (44 proteins), "single-organism process" (36 proteins), and "biological regulation" (32 proteins) (**Figure 3**). Additional classifications included "positive regulation of transferase activity," "post-transcriptional regulation of gene expression," "establishment of protein localization to organelle," and "other important biological processes." There were also significant group differences in expression of proteins with annotations "protein phosphatase binding," "phosphatase binding," "Ras GTPase binding," "small GTPase binding," "GTPase binding," and "other molecular function" as well as "cytosol," "macromolecular complex," "nucleus," "protein complex," "vesicle," and "other positioning proteins" (**Supplementary Figure S1**).

KEGG Pathway Analysis

Proteins interact within pathways and networks to perform specific biological functions and regulate pathophysiological processes. We used KEGG pathway analysis to reveal the biological pathways and relevant regulatory process involving

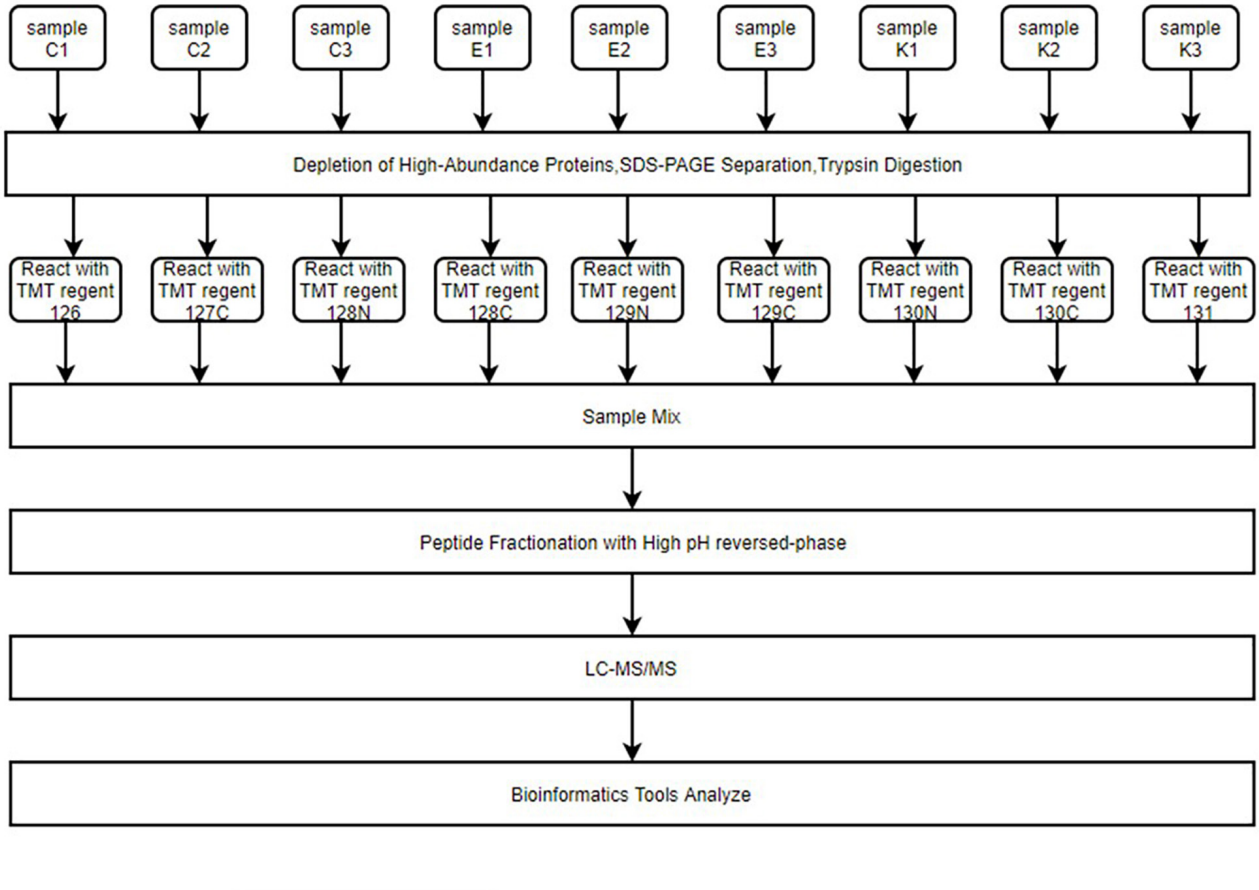

FIGURE 2 | The entire proteomics experimental process. Hippocampus samples were reacted with different isotope-labeling TMT regents after immunoaffinity depletion of high-abundance plasma proteins, SDS-PAGE separation, and FASP digestion. Samples were mixed and peptides fractured by high pH reverse-phase chromatography. Finally, LC–MS/MS was used for high-throughput screening of samples. Peptides were then analyzed for function using multiple bioinformatics tools.

TABLE 2 | Quantitative analysis of target peptide abundance.

Peptide	Protein accession	Protein	Protein gene	Ctr mean	SE mean	SE + KD_ mean	Ration SE/Ctr	Ration SE + KD/SE	Ration SE + KD/Ctr
LAAESSSSQPTQQR	D4A772	Dystrobrevin	Dtna	0.97	0.92	1.11	0.95	1.21	1.14
SGASGGLSGGESR	D4A9A3	Centromere protein V	CenpV	1.08	0.73	1.19	0.68	1.63	1.1
SNPGGFGIAPHCLDEGTVR	D4A9A3	Centromere protein V	CenpV	0.95	1.05	1.01	1.11	0.96	1.06
ASNQSQPLER	Q5BK47	Oxysterol-binding protein	Osbpl2	0.90	0.80	1.30	0.89	1.63	1.44
ESSEQVQPTCPK	Q9JJW1	Tetraspanin-2	Tspan2	0.92	0.82	1.28	0.89	1.53	1.36
GGDGSPGGAGATAAR	Q5XIU9	Progesterone receptor membrane component 2	Pgrmc2	0.99	0.73	1.26	0.74	1.75	1.29
DFSLEQLR	Q5XIU9	Progesterone receptor membrane component 2	Pgrmc2	0.91	1.09	1.00	1.19	0.92	1.10

hippocampal proteins differing in abundance among Ctr, SE, and SE + KD groups, especially those associated with epileptogenesis and the therapeutic mechanisms of KD. The proteins differing in abundance between SE and Ctr groups showed greatest enrichment in "PI3K-Akt signaling pathway," proteins differing in abundance between SE + KD and SE groups showed greatest enrichment in "vitamin digestion and absorption pathway," and proteins differing in abundance between SE + KD and Ctr groups showed greatest enrichment in "glycosaminoglycan degradation pathway" (**Supplementary Figure S2**). Proteins related to the synaptic vesicle cycle pathway were enriched not only among those differing in abundance between SE and Ctr groups but also among those differing in abundance between SE + KD and SE groups. Moreover, the abundances of complexin 3 and

TABLE 3 | Quantitative analysis of target protein abundance.

Protein	Ctr mean	SE mean	SE + KD mean	SE/Ctr ratio	SE + KD/Ctr ratio	SE + KD/SE ratio
Dystrobrevin	0.97	0.92	1.11	0.95	1.14	1.20
Centromere protein V	1.01	0.89	1.10	0.88	1.08	1.23
Oxysterol-binding protein	0.90	0.80	1.30	0.89	1.44	1.62
Tetraspanin-2	0.92	0.82	1.26	0.89	1.36	1.53
Progesterone receptor membrane component 2	0.95	0.91	1.14	0.95	1.19	1.25

solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 6 in the synaptic vesicle cycle pathway were reduced in the SE group compared to the Ctr group, and downregulation of both proteins was reversed by the KD (**Figures 4**, **5** and **Supplementary Tables S3**, **S4**).

DISCUSSION

In recent years, our team has conducted a series of studies on the neuroprotective and antiepileptogenic efficacies of KD in rats. Tian et al. (2015, 2016) found that chronic KD treatment reversed the adverse neurobehavioral, cognitive, and neurochemical changes in Sprague-Dawley rats subjected to recurrent neonatal seizures. Moreover, these studies utilized a novel "twist" seizure model to assess both spontaneous and induced seizures by coupling early-life flurothyl-induced neonatal seizures with later penicillin exposure, and demonstrated that KD could also increase seizure threshold to penicillin. Lusardi et al. (2015) used two epileptic models to examine the effect of KD on epileptogenesis, and found that 100% of all normal-fed rats demonstrated stage-3 seizures or higher after 15 pentylenetetrazol injections, whereas only 37% of KD-fed rats reached comparable seizure stages. They also found that normal-fed animals exhibited spontaneous seizures of progressively greater severity and frequency following pilocarpine induction, whereas KD-fed animals showed a prolonged reduction in seizure severity and frequency. Collectively, these studies demonstrated that KD can suppress epileptogenesis in rats. These findings and those of our previous study provide theoretical and technical support for the antiepileptogenic and neuroprotective effects of KD.

In the current study, we identified 79 proteins that were reciprocally regulated by KD (i.e., exhibiting upregulation in the SE group compared to the control group but downregulation in the SE + KD group compared to the SE group or vice versa). These reciprocal changes may be attributed to the antiepileptogenic effect of the KD. Furthermore, the same reciprocal changes in five proteins (dystrobrevin, tetraspanin-2, oxysterol-binding protein, progesterone receptor membrane component 2, and centromere protein V) were verified by PRM. Proteins differing in abundance between both Ctr and SE groups as well as SE + KD and SE groups were enriched in synaptic vesicle recycling pathway proteins according to KEGG pathway analysis, and two of these proteins, solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 6 and complexin 3, were reciprocally regulated. We suggest the following pathogenic processes to explain epileptogenesis and mitigation by the KD. The blood–brain barrier (BBB) was initially damaged by lithium chloride-pilocarpine-induced SE as indicated by abnormal abundance of α-dystrobrevin (Rigau et al., 2007). In turn, BBB disruption induced neuroinflammation as evidenced by tetraspan-2 upregulation, which led to dysfunctional lipid metabolism as evidenced by oxysterol-binding protein upregulation. Dysfunction of lipid metabolism induced mitochondrial dysfunction and deficient autophagy as indicated by the changes in abundance of progesterone receptor membrane component 2 and centromere protein V, respectively. Finally, defective autophagy resulted in accumulation of damaged mitochondria, triggering epilepsy and neuronal death. Alternatively, each of these pathogenic processes was reversed by KD. In addition, KD upregulated the abundance of solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 6 and complexin 3, both of which are neuroprotective (Ono et al., 1998; Van Liefferinge et al., 2015).

The dystrobrevins (DBs) α-DB and β-DB are cytosolic proteins encoded by the DTNA and DTNB genes, respectively. Alpha-DB in astrocyte end-feet is an important regulator of BBB permeability. It was reported that the aquaporin-4 water channel and Kir4.1 potassium channel were downregulated in the brain of DTNA knockout mice, resulting in enhanced cerebral capillary permeability, gradual cerebral edema, and ultimate damage to neurovascular units (Lien et al., 2012). Damage to the BBB can induce astrocyte dysfunction, neuroinflammation, and epilepsy (Rempe et al., 2018; Swissa et al., 2019). Our results suggest that KD mitigates epilepsy development in part by restoring BBB function through increased α-DB abundance.

Tetraspan-2 (Tspan2) is a small transmembrane protein widely distributed in the central nervous system. Knockout of Tspan2 activates white matter astrocytes and microglia (de Monasterio-Schrader et al., 2013), suggesting that Tspan2 inhibits neuroinflammation, a central pathogenic process in epilepsy (Ngugi et al., 2013). During the development of epilepsy, astrocytes and microglia proliferate, activate, and release inflammatory factors, leading to abnormal neural network connections and aggravating neurotoxicity (Rana and Musto, 2018). In contrast, KD promotes neuroprotection and suppresses epileptogenesis by inhibiting this inflammatory response (Stafstrom and Rho, 2012; Simeone et al., 2018). In the current study, the abundance of Tspan2 was downregulated in the SE group compared to the Ctr group but upregulated after KD. Therefore, we speculate that KD also suppresses epileptogenesis by increasing Tspan2 and suppressing epilepsy-associated neuroinflammation.

There is a strong mutual interaction between cellular inflammation and lipid metabolism, as imbalanced lipid metabolism can result in inflammation (Sun et al., 2009), while inflammation can promote cellular lipid uptake and accumulation, and inhibit cholesterol efflux (Khovidhunkit et al.,

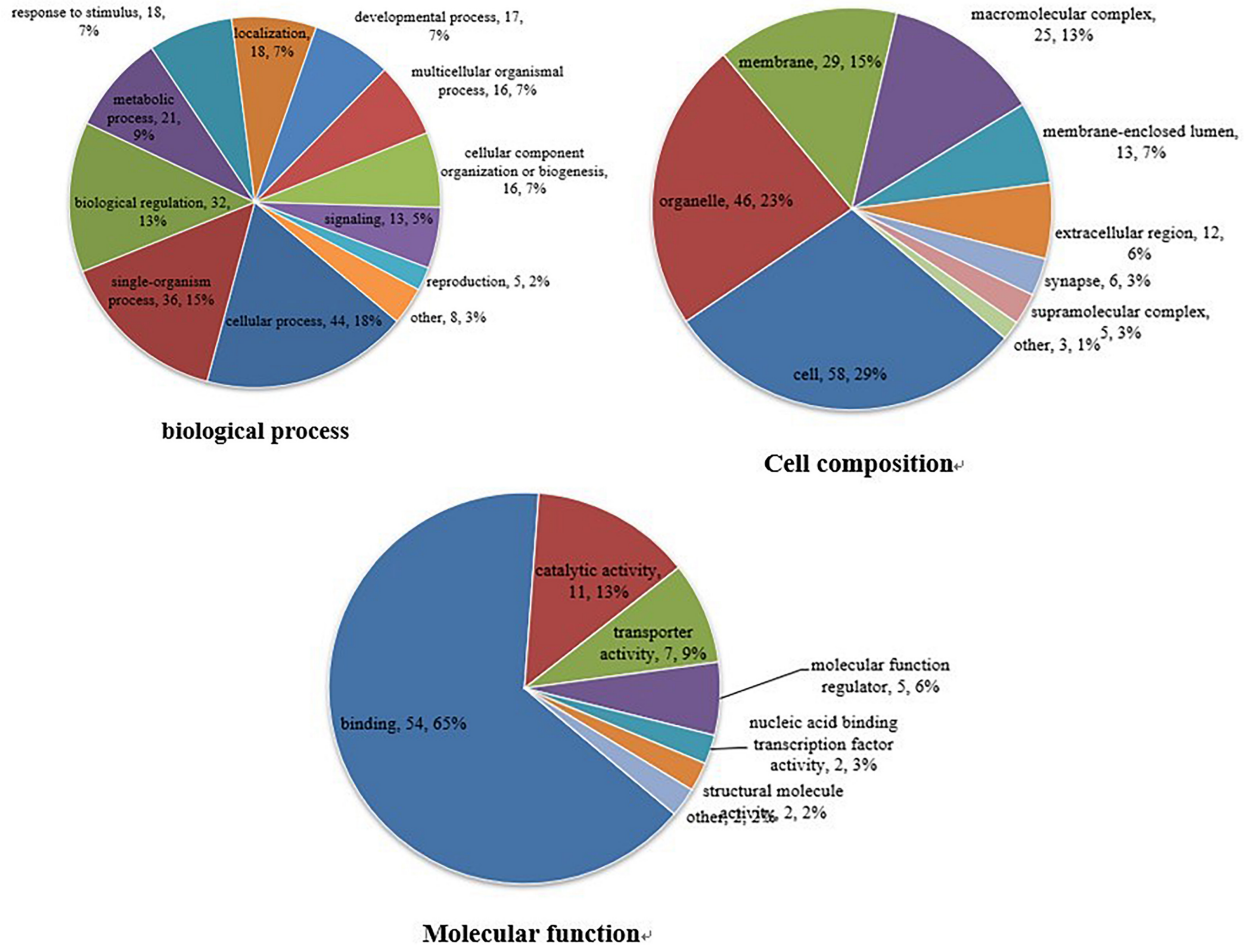

FIGURE 3 | Gene ontogeny (GO) annotation. Differentially abundant proteins were annotated according to molecular function, cell composition, and biological process. Differentially abundant proteins are mainly annotated as 'protein binding,' 'cell,' and 'cell process,' respectively, in terms of molecular function, cell composition, and biological process.

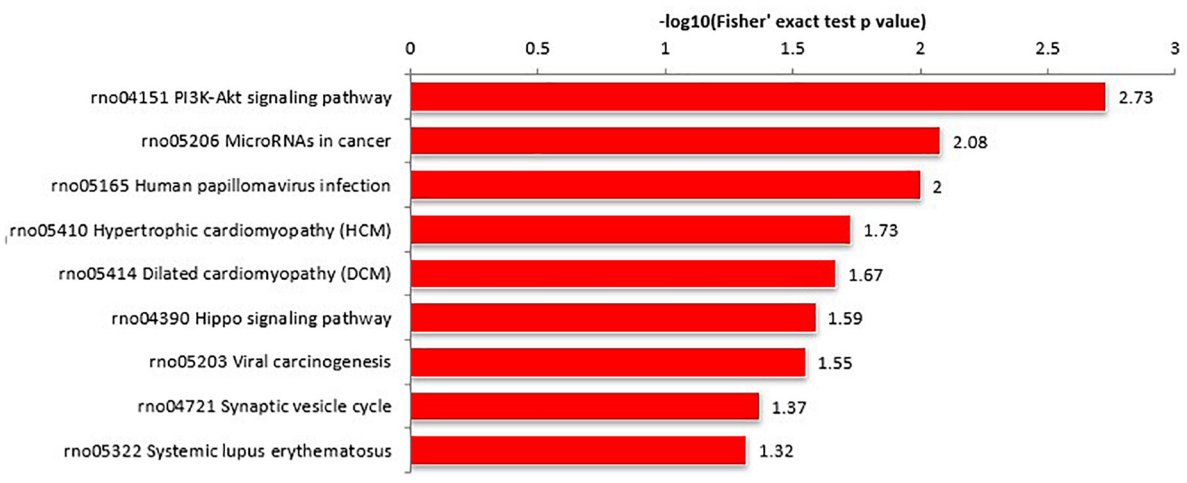

FIGURE 4 | KEGG pathway enrichment analysis of proteins differing in abundance between SE and Ctr groups. The 'PI3K-Akt signaling pathway' showed highest enrichment. These differentially abundant proteins were also enriched in 'synaptic vesicle cycle pathway.'

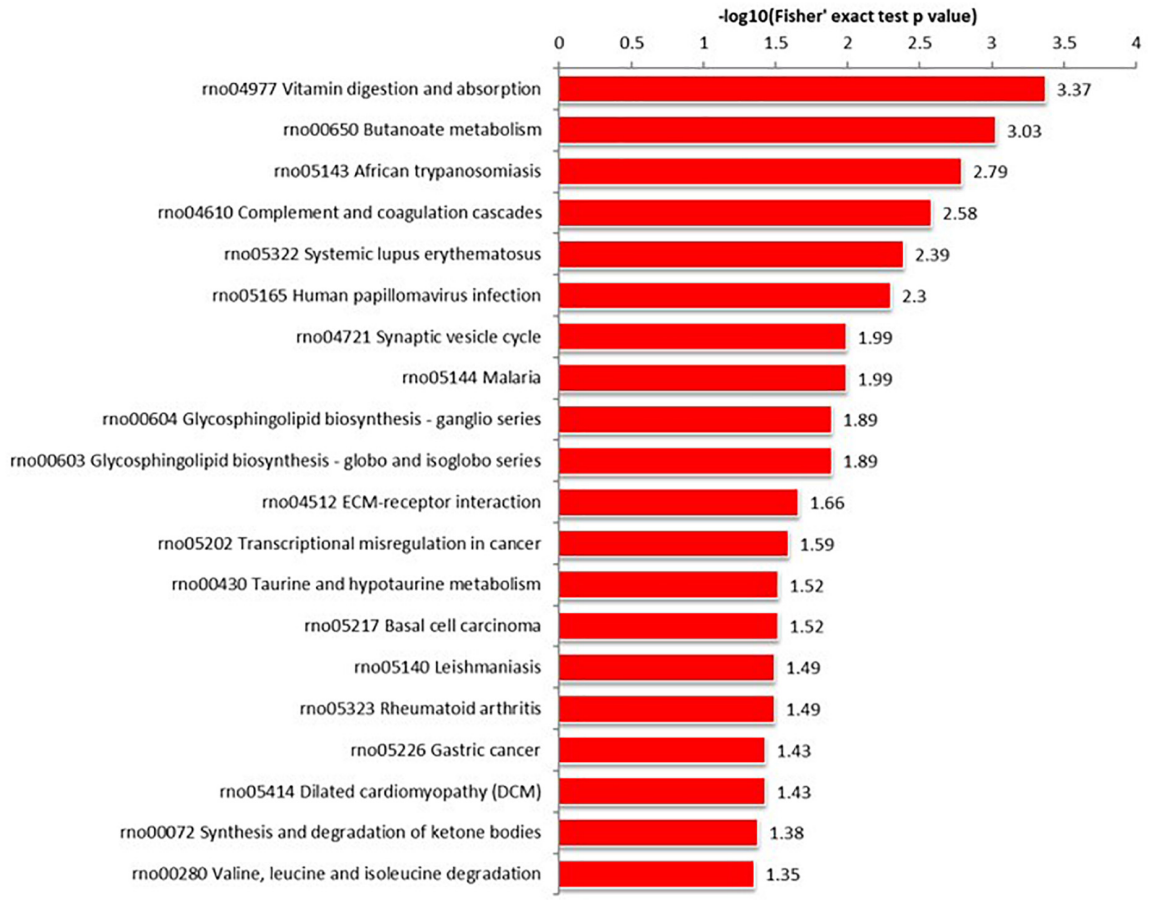

FIGURE 5 | KEGG pathway enrichment analysis of proteins differing in abundance between SE + KD and SE groups. Vitamin digestion and absorption pathway showed highest enrichment. Differentially abundant proteins were also enriched in 'synaptic vesicle cycle.'

2004; McGillicuddy et al., 2009). Oxysterol binding protein (Accession number: Q5BK47), also known as oxysterol binding protein-like 2 (OSBPL2), is a highly conserved transporter protein that controls cholesterol and PI (4,5) P2 levels in the plasma membrane (Wang et al., 2019b). In addition, OSBPL2 is involved in the synthesis of cholesterol and cholesterol ester. Knockout or silencing the OSBPL2 gene inhibited AMPK activity and increased intracellular cholesterol and cholesterol ester synthesis (Wang et al., 2019a; Zhang et al., 2019). Imbalanced cholesterol homeostasis is implicated in the pathogenesis of multiple disorders, including cardiovascular, cerebrovascular, and central nervous system diseases (Chistiakov et al., 2016; Xue-Shan et al., 2016; Puglisi and Yagci, 2019). Altered levels of cholesterol and certain oxysterols have been reported in the hippocampus of rats following kainic acid-induced epilepsy (Ong et al., 2003; Heverin et al., 2012). We found that levels of the lipid metabolism-related molecules ApoE, clusterin, and ACAT-1 were upregulated after flurothyl-induced recurrent seizures in neonatal rats, while KD reversed these changes as well as the cognitive and neurobehavioral abnormalities associated with seizures (Tian et al., 2015). Thus, KD may also protect against epilepsy and associated sequelae by normalizing lipid homeostasis. Indeed, the downregulation of OSBPL2 observed in the SE group compared to the Ctr group was reversed by KD, which may in turn reduce cellular cholesterol accumulation, thereby mitigating oxidative stress and mitochondrial damage (Wang et al., 2019a).

Accumulation of cholesterol is a major cause of mitochondrial dysfunction in different models and cells. In Alzheimer's disease and Niemann-Pick type C disease, mitochondrial cholesterol accumulation disrupts membrane physical properties and restricts the transport of glutathione into mitochondrial matrix, thus impairing mitochondrial function (Torres et al., 2019). In a mouse non-alcoholic fatty liver disease model, cholesterol overload contributed to a reduction in mitochondrial membrane potential and ATP content, and to significant alterations in mitochondrial dynamics (Dominguez-Perez et al., 2019). Kim et al. (2006) found that 7-ketocholesterol enhanced 1-methyl-4-phenylpyridinium-induced mitochondrial dysfunction and cell death in PC12 cells. Progesterone receptor membrane component 2 (PGRMC2) is a member of the progesterone membrane-related receptor (MAPR) family. It contains a heme-binding domain similar to cytochrome EB5 and a recent study (Galmozzi et al., 2019) found that deletion of PGMRC2 reduced

intracellular heme synthesis. Heme promotes neurogenesis as well as neuronal survival and growth. However, dysregulation of intracellular heme concentration can result in neurodegeneration and impaired neurological function (Gozzelino, 2016). Reduction of heme synthesis in primary rat hippocampal neurons using *n*-methyltropophyrin reduced mitochondrial complex IV, activated carbon monoxide synthetase, and altered amyloid precursor protein (APP)α and APPβ protein levels, suggesting that decreased heme contributes to the neuronal dysfunction of Alzheimer's disease (Atamna et al., 2002). In accord with these findings, blockade of heme biosynthesis by siRNA-mediated knockdown and n-methyltropophyrin IX treatment in differentiated SH-SY5Y neuroblastoma cells resulted in mitochondrial membrane depolarization, lower intracellular ATP production, APP aggregation, suppressed soluble (s)APPα secretion, and increased sAPPβ secretion (Gatta et al., 2009). Reduced intracellular heme was shown to disrupt mitochondrial function. Moreover, mitochondrial dysfunction is a common pathway for neurodegeneration (Rusek et al., 2019), so we speculate that decreased abundance of PGRMC2 in the SE group compared to the Ctr group is indicative of mitochondrial dysfunction, consistent with our previous study showing that flurothyl-induced seizures significantly depolarized mitochondrial membrane potential, reduced mitochondrial fusion protein 2 expression, and upregulated dynamic related protein 1 (drp1) in hippocampus (Liu et al., 2018). Conversely, KD upregulated PGRMC2, suggesting that KD also protects against neuronal death and epilepsy by sustaining mitochondrial function (Simeone et al., 2018; Rusek et al., 2019).

Intracellular cholesterol accumulation not only damages mitochondria, but also impairs autophagy by interfering with the fusion of autophagosomes with endosomal-lysosomal vesicles (Barbero-Camps et al., 2018). Autophagy defects reduce the capacity of cells to remove damaged organelles, protein aggregates, macromolecules, and other toxic substances, leading to dysfunction and death. Further, numerous studies have implicated autophagy defects in epilepsy. Knockout of ATG-7, a key molecule in the autophagy cascade, leads to spontaneous seizures in mice, implying that inhibition of autophagy is sufficient to induce epilepsy (Boya et al., 2013). We also reported that ratio of LC3 II/I was downregulated in the hippocampus of newborn rats subjected to repeated seizure induction using flurothyl, indicating reduced numbers of autophagosomes, while p62 was upregulated, indicating enhanced autophagic flux (Ni et al., 2016). Centromere protein V (CENPV) contributes to the maintenance of cell dynamics by stabilizing microtubules (Honda et al., 2009), and this process is critical for autophagy. The microtubule organizing center (MTOC) containing CENPV is critical for centripetal transport of autophagosomes from the cell periphery as well as for the fusion of autophagosomes and lysosomes (Kochl et al., 2006; Xu et al., 2014). In the present study, the abundance of CENPV was reduced in the SE group, suggesting impaired microtubule stability leading to disrupted autophagy. We suggest that the ability of KD to activate autophagic pathways and reduce brain injury in response to both pentylenetetrazol-induced seizures (Wang et al., 2018) and lithium chloride–pilocarpine-induced seizures is mediated by CENPV upregulation.

The synaptic vesicle cycle plays an important role in maintaining the structural and functional integrity of the presynaptic terminal. Disruption of synaptic vesicle recycling leading to defects in synaptic transmission may contribute to neurological disorders such as Alzheimer's disease and autism (Waites and Garner, 2011), and changes in synaptic vesicle recycling have also been observed in pilocarpine-induced status epilepticus model rats (Upreti et al., 2012). Further, KD can support synaptic vesicle recycling (Hrynevich et al., 2016), so we speculate that KD also prevents epileptogenesis by normalizing this pathway. In fact, synaptic vesicle recycling pathway proteins were enriched in both populations of proteins demonstrating differential abundance between groups (SE vs. Ctr and SE + KD vs. SE), and two proteins involved in the synaptic vesicle cycle, solute carrier family 17 member 6 and complexin 3, were reciprocally regulated (upregulated in the SE group and downregulated after KD). Thus, these proteins may be the targets of KD for preventing epileptogenesis.

Solute carrier family 17 (Sodium-dependent inorganic phosphate cotransporter), member 6, also known as vesicular glutamate transporter 2 (VGLUT2, encoded by Slc17a6) is a low affinity transporter of glutamate from the cytoplasm into synaptic vesicles (Bellocchio et al., 2000). Expression is lower in the hippocampus of patients with intractable epilepsy and hippocampal sclerosis (Van Liefferinge et al., 2015), consistent with findings of reduced abundance in the SE group. Lobo et al. (2011) found that high glutamic acid exposure reduced VGLUT2 expression by hippocampal neurons, resulting in substantial excitotoxicity. As KD reversed this decline, improved glutamate transport may also contribute to reduced epileptogenesis.

The complexins (Cplxs) are four small SNARE-related proteins (Cplx1–4) that regulate rapid calcium-triggered exocytosis of synaptic, and thus are important for maintaining synaptic neurotransmission (Hazell and Wang, 2005; Yi et al., 2006). Knockout of all Cplxs genes in mice significantly reduced the calcium-triggered release of glutamate and γ-aminobutyric acid from hippocampal and striatal neurons (Xue et al., 2008). Alternatively, injecting recombinant Cplx2 into Aplysia buccal ganglion neurons inhibited neurotransmitter release, while injecting Cplx2 antibody increased release (Ono et al., 1998). Mice harboring a mutant Cplx1 gene exhibited ataxia and sporadic convulsions (Reim et al., 2001). In the current study, the abundance of Cplx3 was decreased in the SE group and was restored by KD, suggesting that KD may mitigate epileptogenesis by reducing uncontrolled glutamate release, thereby restoring appropriate excitatory–inhibitory balance.

CONCLUSION

To our knowledge, this is the first study to comprehensively analyze the changes in protein abundance induced by the KD diet among epileptic model rats through quantitative proteomics. We identified several 100 proteins demonstrating differential

abundance among control, epilepsy, and epilepsy plus KD groups, of which 79 were reciprocally regulated by SE and KD. Five of these proteins were further verified by PRM. Subsets of these proteins are implicated in lipid metabolism, blood–brain barrier integrity, mitochondrial function, neuroinflammation, and autophagy. Other proteins regulated by both seizures and KD are involved in synaptic vesicle recycling. Collectively, these findings provide clues to the molecular mechanisms underlying the antiepileptogenic effects of KD and define multiple potential therapeutic targets. However, the precise molecular mechanisms of action require further verification. In future studies, we will focus on selected KD-sensitive target proteins and examine the phenotypic changes conferred by knockout and overexpression, identify proteins interacting with target proteins, observe the effects of target protein expression level changes on epilepsy-related pathophysiological processes, and examine if KD can preserve neural circuit integrity, normal behavior, and cognition in epileptic rats via changes in target protein expression.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal experiments were approved by the Animal Experimental Ethics Committee of Suzhou University.

AUTHOR CONTRIBUTIONS

HN designed the study. YZ and MJ performed the experiments. GS, YW, and YS analyzed the data and are responsible for the statistical analysis. YZ wrote the manuscript. All authors have reviewed and approved this version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.562853/full#supplementary-material

Supplementary Figure 1 | GO functional enrichment analysis of differentially abundant proteins.

Supplementary Figure 2 | KEGG pathway enrichment analysis of proteins differing in abundance between SE + KD and Ctr groups.

Supplementary Figure 3 | Distribution of fragment ion peak area: LAAESSSSQPTQQR (corresponding protein accession D4A772).

Supplementary Figure 4 | Distribution of fragment ion peak area: ASNQSQPLER (corresponding protein accession Q5BK47).

Supplementary Figure 5 | Distribution of fragment ion peak area: SGASGGLSGGESR (corresponding protein accession D4A9A3).

Supplementary Figure 6 | Distribution of fragment ion peak area: SNPGGFGIAPHCLDEGTVR (corresponding protein accession D4A9A3).

Supplementary Figure 7 | Distribution of fragment ion peak area: GGDGSPGGAGATAAR (corresponding protein accession Q5XIU9).

Supplementary Figure 8 | Distribution of fragment ion peak area: DFSLEQLR (corresponding protein accession Q5XIU9).

Supplementary Figure 9 | Distribution of fragment ion peak area: ESSEQVQPTCPK (corresponding protein accession Q9JJW1).

Supplementary Table 1 | Differential abundance of proteins among Ctr, SE, and SE + KD groups.

Supplementary Table 2 | Optimized differential abundance of proteins.

Supplementary Table 3 | KEGG pathway enrichment analysis of proteins differing in abundance between SE and Ctr groups.

Supplementary Table 4 | KEGG pathway enrichment analysis of proteins differing in abundance between SE + KD and SE groups.

REFERENCES

Atamna, H., Killilea, D. W., Killilea, A. N., and Ames, B. N. (2002). Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14807–14812. doi: 10.1073/pnas.192585799

Barbero-Camps, E., Roca-Agujetas, V., Bartolessis, I., de Dios, C., Fernandez-Checa, J. C., Mari, M., et al. (2018). Cholesterol impairs autophagy-mediated clearance of amyloid beta while promoting its secretion. *Autophagy* 14, 1129–1154. doi: 10.1080/15548627.2018.1438807

Beghi, E., Giussani, G., Nichols, E., Abd-Allah, F., Abdela, J., Abdelalim, A., et al. (2019). Global, regional, and national burden of epilepsy, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* 18, 357–375. doi: 10.1016/S1474-4422(18)30454-X

Bellocchio, E. E., Reimer, R. J., Fremeau, R. T. Jr., and Edwards, R. H. (2000). Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 289, 957–960. doi: 10.1126/science.289.5481.957

Boison, D., and Rho, J. M. (2020). Epigenetics and epilepsy prevention: the therapeutic potential of adenosine and metabolic therapies. *Neuropharmacology* 167:107741. doi: 10.1016/j.neuropharm.2019.107741

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., et al. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235. doi: 10.1002/ana.20899

Boya, P., Reggiori, F., and Codogno, P. (2013). Emerging regulation and functions of autophagy. *Nat. Cell Biol.* 15, 713–720. doi: 10.1038/ncb2788

Cai, Q. Y., Zhou, Z. J., Luo, R., Gan, J., Li, S. P., Mu, D. Z., et al. (2017). Safety and tolerability of the ketogenic diet used for the treatment of refractory childhood epilepsy: a systematic review of published prospective studies. *World J. Pediatr.* 13, 528–536. doi: 10.1007/s12519-017-0053-2

Chen, C. Y., Rao, S. S., Ren, L., Hu, X. K., Tan, Y. J., Hu, Y., et al. (2018). Exosomal DMBT1 from human urine-derived stem cells facilitates diabetic wound repair by promoting angiogenesis. *Theranostics* 8, 1607–1623. doi: 10.7150/thno.22958

Chen, N. N., Zhao, D. J., Sun, Y. X., Wang, D. D., and Ni, H. (2019). Long-term effects of zinc deficiency and zinc supplementation on developmental seizure-induced brain damage and the underlying GPR39/ZnT-3 and MBP Expression in the Hippocampus. *Front. Neurosci.* 13:920. doi: 10.3389/fnins.2019.00920

Chistiakov, D. A., Bobryshev, Y. V., and Orekhov, A. N. (2016). Macrophage-mediated cholesterol handling in atherosclerosis. *J. Cell. Mol. Med.* 20, 17–28. doi: 10.1111/jcmm.12689

de Monasterio-Schrader, P., Patzig, J., Mobius, W., Barrette, B., Wagner, T. L., Kusch, K., et al. (2013). Uncoupling of neuroinflammation from axonal degeneration in mice lacking the myelin protein tetraspanin-2. *Glia* 61, 1832–1847. doi: 10.1002/glia.22561

Dm, I. J., Postulart, D., Lambrechts, D., Majoie, M., de Kinderen, R. J. A., Hendriksen, J. G. M., et al. (2016). Cognitive and behavioral impact of the ketogenic diet in children and adolescents with refractory epilepsy: a randomized controlled trial. *Epilepsy Behav.* 60, 153–157. doi: 10.1016/j.yebeh.2016.04.033

Dominguez-Perez, M., Simoni-Nieves, A., Rosales, P., Nuno-Lambarri, N., Rosas-Lemus, M., Souza, V., et al. (2019). Cholesterol burden in the liver induces mitochondrial dynamic changes and resistance to apoptosis. *J. Cell. Physiol.* 234, 7213–7223. doi: 10.1002/jcp.27474

Fisher, R. S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., et al. (2005). Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 46, 470–472. doi: 10.1111/j.0013-9580.2005.66104.x

Galmozzi, A., Kok, B. P., Kim, A. S., Montenegro-Burke, J. R., Lee, J. Y., Spreafico, R., et al. (2019). PGRMC2 is an intracellular haem chaperone critical for adipocyte function. *Nature* 576, 138–142. doi: 10.1038/s41586-019-1774-2

Gatta, L. B., Vitali, M., Verardi, R., Arosio, P., and Finazzi, D. (2009). Inhibition of heme synthesis alters Amyloid Precursor Protein processing. *J. Neural Transm.* 116, 79–88. doi: 10.1007/s00702-008-0147-z

Gozzelino, R. (2016). The pathophysiology of heme in the brain. *Curr. Alzheimer Res.* 13, 174–184. doi: 10.2174/1567205012666150921103304

Halyburton, A. K., Brinkworth, G. D., Wilson, C. J., Noakes, M., Buckley, J. D., Keogh, J. B., et al. (2007). Low- and high-carbohydrate weight-loss diets have similar effects on mood but not cognitive performance. *Am. J. Clin. Nutr.* 86, 580–587. doi: 10.1093/ajcn/86.3.580

Hazell, A. S., and Wang, C. (2005). Downregulation of complexin I and complexin II in the medial thalamus is blocked by N-acetylcysteine in experimental Wernicke's encephalopathy. *J. Neurosci. Res.* 79, 200–207. doi: 10.1002/jnr.20278

Heverin, M., Engel, T., Meaney, S., Jimenez-Mateos, E. M., Al-Saudi, R., and Henshall, D. C. (2012). Bi-lateral changes to hippocampal cholesterol levels during epileptogenesis and in chronic epilepsy following focal-onset status epilepticus in mice. *Brain Res.* 1480, 81–90. doi: 10.1016/j.brainres.2012.08.018

Honda, Z., Suzuki, T., and Honda, H. (2009). Identification of CENP-V as a novel microtubule-associating molecule that activates Src family kinases through SH3 domain interaction. *Genes Cells* 14, 1383–1394. doi: 10.1111/j.1365-2443.2009.01355.x

Hrynevich, S. V., Waseem, T. V., Hebert, A., Pellerin, L., and Fedorovich, S. V. (2016). beta-Hydroxybutyrate supports synaptic vesicle cycling but reduces endocytosis and exocytosis in rat brain synaptosomes. *Neurochem. Int.* 93, 73–81. doi: 10.1016/j.neuint.2015.12.014

Iacovides, S., Goble, D., Paterson, B., and Meiring, R. M. (2019). Three consecutive weeks of nutritional ketosis has no effect on cognitive function, sleep, and mood compared with a high-carbohydrate, low-fat diet in healthy individuals: a randomized, crossover, controlled trial. *Am. J. Clin. Nutr.* 110, 349–357. doi: 10.1093/ajcn/nqz073

Jeong, H. J., Kim, H., Kim, Y. K., Park, S. K., Kang, D. W., and Yoon, D. (2010). The ketogenic diet suppresses the cathepsin E expression induced by kainic acid in the rat brain. *Yonsei Med. J.* 51, 653–660. doi: 10.3349/ymj.2010.51.5.653

Kang, H. C., Chung, D. E., Kim, D. W., and Kim, H. D. (2004). Early- and late-onset complications of the ketogenic diet for intractable epilepsy. *Epilepsia* 45, 1116–1123. doi: 10.1111/j.0013-9580.2004.10004.x

Karimzadeh, P., Moosavian, T., and Moosavian, H. R. (2019). Effects of a Formula-Based Ketogenic Diet on Refractory Epilepsy in 1 to 3 Year-Old Patients under Classic Ketogenic Diet. *Iran. J. Child Neurol.* 13, 83–90.

Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., et al. (2004). Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J. Lipid Res.* 45, 1169–1196. doi: 10.1194/jlr.R300019-JLR200

Kim, Y. J., Han, J. H., Han, E. S., and Lee, C. S. (2006). 7-Ketocholesterol enhances 1-methyl-4-phenylpyridinium-induced mitochondrial dysfunction and cell death in PC12 cells. *J. Neural Transm.* 113, 1877–1885. doi: 10.1007/s00702-006-0486-6

Kochl, R., Hu, X. W., Chan, E. Y., and Tooze, S. A. (2006). Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. *Traffic* 7, 129–145. doi: 10.1111/j.1600-0854.2005.00368.x

Koene, L. M. C., van Grondelle, S. E., Proietti Onori, M., Wallaard, I., Kooijman, N., van Oort, A., et al. (2019). Effects of antiepileptic drugs in a new TSC/mTOR-dependent epilepsy mouse model. *Ann. Clin. Transl. Neurol.* 6, 1273–1291. doi: 10.1002/acn3.50829

Lambrechts, D. A., Bovens, M. J., de la Parra, N. M., Hendriksen, J. G., Aldenkamp, A. P., and Majoie, M. J. (2013). Ketogenic diet effects on cognition, mood, and psychosocial adjustment in children. *Acta Neurol. Scand.* 127, 103–108. doi: 10.1111/j.1600-0404.2012.01686.x

Lien, C. F., Mohanta, S. K., Frontczak-Baniewicz, M., Swinny, J. D., Zablocka, B., and Gorecki, D. C. (2012). Absence of glial alpha-dystrobrevin causes abnormalities of the blood-brain barrier and progressive brain edema. *J. Biol. Chem.* 287, 41374–41385. doi: 10.1074/jbc.M112.400044

Liu, Y., Chen, J., Jin, M., Li, Z., Tian, T., Li, L., et al. (2018). Relationship between changes in mitochondrial function and hippocampal neuronal apoptosis after recurrent convulsion during developmental stage. *Exp. Ther. Med.* 16, 127–132. doi: 10.3892/etm.2018.6147

Lobo, A. C., Gomes, J. R., Catarino, T., Mele, M., Fernandez, P., Inacio, A. R., et al. (2011). Cleavage of the vesicular glutamate transporters under excitotoxic conditions. *Neurobiol. Dis.* 44, 292–303. doi: 10.1016/j.nbd.2011.07.010

Lusardi, T. A., Akula, K. K., Coffman, S. Q., Ruskin, D. N., Masino, S. A., and Boison, D. (2015). Ketogenic diet prevents epileptogenesis and disease progression in adult mice and rats. *Neuropharmacology* 99, 500–509. doi: 10.1016/j.neuropharm.2015.08.007

McClernon, F. J., Yancy, W. S. Jr., Eberstein, J. A., Atkins, R. C., and Westman, E. C. (2007). The effects of a low-carbohydrate ketogenic diet and a low-fat diet on mood, hunger, and other self-reported symptoms. *Obesity* 15, 182–187. doi: 10.1038/oby.2007.516

McGillicuddy, F. C., de la Llera Moya, M., Hinkle, C. C., Joshi, M. R., Chiquoine, E. H., Billheimer, J. T., et al. (2009). inflammation impairs reverse cholesterol transport *in vivo*. *Circulation* 119, 1135–1145. doi: 10.1161/CIRCULATIONAHA.108.810721

Nashef, L., Fish, D. R., Garner, S., Sander, J. W., and Shorvon, S. D. (1995). Sudden death in epilepsy: a study of incidence in a young cohort with epilepsy and learning difficulty. *Epilepsia* 36, 1187–1194. doi: 10.1111/j.1528-1157.1995.tb01061.x

Ngugi, A. K., Bottomley, C., Kleinschmidt, I., Wagner, R. G., Kakooza-Mwesige, A., Ae-Ngibise, K., et al. (2013). Prevalence of active convulsive epilepsy in sub-Saharan Africa and associated risk factors: cross-sectional and case-control studies. *Lancet Neurol.* 12, 253–263. doi: 10.1016/S1474-4422(13)70003-6

Ni, H., Zhao, D. J., and Tian, T. (2016). Ketogenic diet change cPLA2/clusterin and autophagy related gene expression and correlate with cognitive deficits and hippocampal MFs sprouting following neonatal seizures. *Epilepsy Res.* 120, 13–18. doi: 10.1016/j.eplepsyres.2015.11.021

Ong, W. Y., Goh, E. W., Lu, X. R., Farooqui, A. A., Patel, S. C., and Halliwell, B. (2003). Increase in cholesterol and cholesterol oxidation products, and role of cholesterol oxidation products in kainate-induced neuronal injury. *Brain Pathol.* 13, 250–262. doi: 10.1111/j.1750-3639.2003.tb00026.x

Ono, S., Baux, G., Sekiguchi, M., Fossier, P., Morel, N. F., Nihonmatsu, I., et al. (1998). Regulatory roles of complexins in neurotransmitter release from mature

presynaptic nerve terminals. *Eur. J. Neurosci.* 10, 2143–2152. doi: 10.1046/j.1460-9568.1998.00225.x

Pluta, R., and Jablonski, M. (2011). The ketogenic diet for epilepsy therapy in children: Quo vadis? *Nutrition* 27, 615–616. doi: 10.1016/j.nut.2010.12.015

Puglisi, A., and Yagci, Y. (2019). Cyclodextrin-based macromolecular systems as cholesterol-mopping therapeutic agents in niemann-pick disease type C. *Macromol. Rapid Commun.* 40:e1800557. doi: 10.1002/marc.201800557

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* 32, 281–294. doi: 10.1016/0013-4694(72)90177-0

Rana, A., and Musto, A. E. (2018). The role of inflammation in the development of epilepsy. *J. Neuroinflammation* 15:144. doi: 10.1186/s12974-018-1192-7

Reim, K., Mansour, M., Varoqueaux, F., McMahon, H. T., Sudhof, T. C., Brose, N., et al. (2001). Complexins regulate a late step in Ca2+-dependent neurotransmitter release. *Cell* 104, 71–81. doi: 10.1016/s0092-8674(01)00192-1

Rempe, R. G., Hartz, A. M. S., Soldner, E. L. B., Sokola, B. S., Alluri, S. R., Abner, E. L., et al. (2018). Matrix metalloproteinase-mediated blood-brain barrier dysfunction in epilepsy. *J. Neurosci.* 38, 4301–4315. doi: 10.1523/JNEUROSCI.2751-17.2018

Rigau, V., Morin, M., Rousset, M. C., de Bock, F., Lebrun, A., Coubes, P., et al. (2007). Angiogenesis is associated with blood-brain barrier permeability in temporal lobe epilepsy. *Brain* 130(Pt 7), 1942–1956. doi: 10.1093/brain/awm118

Rusek, M., Pluta, R., Ulamek-Koziol, M., and Czuczwar, S. J. (2019). Ketogenic diet in Alzheimer's disease. *Int. J. Mol. Sci.* 20:3892. doi: 10.3390/ijms20163892

Sadeghi, L., Rizvanov, A. A., Salafutdinov, I. I., Dabirmanesh, B., Sayyah, M., Fathollahi, Y., et al. (2017). Hippocampal asymmetry: differences in the left and right hippocampus proteome in the rat model of temporal lobe epilepsy. *J. Proteomics* 154, 22–29. doi: 10.1016/j.jprot.2016.11.023

Simeone, T. A., Simeone, K. A., Stafstrom, C. E., and Rho, J. M. (2018). Do ketone bodies mediate the anti-seizure effects of the ketogenic diet? *Neuropharmacology* 133, 233–241. doi: 10.1016/j.neuropharm.2018.01.011

Stafstrom, C. E., and Rho, J. M. (2012). The ketogenic diet as a treatment paradigm for diverse neurological disorders. *Front. Pharmacol.* 3:59. doi: 10.3389/fphar.2012.00059

Sun, Y., Ishibashi, M., Seimon, T., Lee, M., Sharma, S. M., Fitzgerald, K. A., et al. (2009). Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K. *Circ. Res.* 104, 455–465. doi: 10.1161/CIRCRESAHA.108.182568

Suo, C., Liao, J., Lu, X., Fang, K., Hu, Y., Chen, L., et al. (2013). Efficacy and safety of the ketogenic diet in Chinese children. *Seizure* 22, 174–178. doi: 10.1016/j.seizure.2012.11.014

Swissa, E., Serlin, Y., Vazana, U., Prager, O., and Friedman, A. (2019). Blood-brain barrier dysfunction in status epileptics: mechanisms and role in epileptogenesis. *Epilepsy Behav.* 101(Pt B), 106285. doi: 10.1016/j.yebeh.2019.04.038

Tian, T., Li, L. L., Zhang, S. Q., and Ni, H. (2016). Long-Term Effects of Ketogenic Diet on Subsequent Seizure-Induced Brain Injury During Early Adulthood: relationship of Seizure Thresholds to Zinc Transporter-Related Gene Expressions. *Biol. Trace Elem. Res.* 174, 369–376. doi: 10.1007/s12011-016-0730-3

Tian, T., Ni, H., and Sun, B. L. (2015). Neurobehavioral deficits in a rat model of recurrent neonatal seizures are prevented by a Ketogenic Diet and Correlate with Hippocampal Zinc/Lipid Transporter Signals. *Biol. Trace Elem. Res.* 167, 251–258. doi: 10.1007/s12011-015-0285-8

Torres, S., Garcia-Ruiz, C. M., and Fernandez-Checa, J. C. (2019). Mitochondrial cholesterol in Alzheimer's disease and niemann-pick type C disease. *Front. Neurol.* 10:1168. doi: 10.3389/fneur.2019.01168

Upreti, C., Otero, R., Partida, C., Skinner, F., Thakker, R., Pacheco, L. F., et al. (2012). Altered neurotransmitter release, vesicle recycling and presynaptic structure in the pilocarpine model of temporal lobe epilepsy. *Brain* 135(Pt 3), 869–885. doi: 10.1093/brain/awr341

Van Liefferinge, J., Jensen, C. J., Albertini, G., Bentea, E., Demuyser, T., Merckx, E., et al. (2015). Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis. *Neurosci. Lett.* 590, 184–188. doi: 10.1016/j.neulet.2015.01.080

Waites, C. L., and Garner, C. C. (2011). Presynaptic function in health and disease. *Trends Neurosci.* 34, 326–337. doi: 10.1016/j.tins.2011.03.004

Walker, A., Russmann, V., Deeg, C. A., von Toerne, C., Kleinwort, K. J. H., Szober, C., et al. (2016). Proteomic profiling of epileptogenesis in a rat model: focus on inflammation. *Brain Behav. Immun.* 53, 138–158. doi: 10.1016/j.bbi.2015.12.007

Wang, B. H., Hou, Q., Lu, Y. Q., Jia, M. M., Qiu, T., Wang, X. H., et al. (2018). Ketogenic diet attenuates neuronal injury via autophagy and mitochondrial pathways in pentylenetetrazol-kindled seizures. *Brain Res.* 1678, 106–115. doi: 10.1016/j.brainres.2017.10.009

Wang, H., Lin, C., Yao, J., Shi, H., Zhang, C., Wei, Q., et al. (2019a). Deletion of OSBPL2 in auditory cells increases cholesterol biosynthesis and drives reactive oxygen species production by inhibiting AMPK activity. *Cell Death Dis.* 10:627. doi: 10.1038/s41419-019-1858-9

Wang, H., Ma, Q., Qi, Y., Dong, J., Du, X., Rae, J., et al. (2019b). ORP2 delivers cholesterol to the plasma membrane in exchange for phosphatidylinositol 4, 5-Bisphosphate (PI(4,5)P2). *Mol. Cell* 73, 458–473.e7. doi: 10.1016/j.molcel.2018.11.014

Xu, M., Li, X. X., Chen, Y., Pitzer, A. L., Zhang, Y., and Li, P. L. (2014). Enhancement of dynein-mediated autophagosome trafficking and autophagy maturation by ROS in mouse coronary arterial myocytes. *J. Cell. Mol. Med.* 18, 2165–2175. doi: 10.1111/jcmm.12326

Xue, M., Stradomska, A., Chen, H., Brose, N., Zhang, W., Rosenmund, C., et al. (2008). Complexins facilitate neurotransmitter release at excitatory and inhibitory synapses in mammalian central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7875–7880. doi: 10.1073/pnas.0803012105

Xue-Shan, Z., Juan, P., Qi, W., Zhong, R., Li-Hong, P., Zhi-Han, T., et al. (2016). Imbalanced cholesterol metabolism in Alzheimer's disease. *Clin. Chim. Acta* 456, 107–114. doi: 10.1016/j.cca.2016.02.024

Yi, J. H., Hoover, R., McIntosh, T. K., and Hazell, A. S. (2006). Early, transient increase in complexin I and complexin II in the cerebral cortex following traumatic brain injury is attenuated by N-acetylcysteine. *J. Neurotrauma* 23, 86–96. doi: 10.1089/neu.2006.23.86

Zhang, C., Zhang, H., Zhang, M., Lin, C., Wang, H., Yao, J., et al. (2019). OSBPL2 deficiency upregulate SQLE expression increasing intracellular cholesterol and cholesteryl ester by AMPK/SP1 and SREBF2 signalling pathway. *Exp. Cell Res.* 383:111512. doi: 10.1016/j.yexcr.2019.111512

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Effects of Melatonin on Neurobehavior and Cognition in a Cerebral Palsy Model of *plppr5*–/– Mice

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Cerebral palsy (CP), a group of clinical syndromes caused by non-progressive brain damage in the developing fetus or infant, is one of the most common causes of lifelong physical disability in children in most countries. At present, many researchers believe that perinatal cerebral hypoxic ischemic injury or inflammatory injury are the main causes of cerebral palsy. Previous studies including our works confirmed that melatonin has a protective effect against convulsive brain damage during development and that it affects the expression of various molecules involved in processes such as metabolism, plasticity and signaling in the brain. Integral membrane protein *plppr5* is a new member of the plasticity-related protein family, which is specifically expressed in brain and spinal cord, and induces filopodia formation as well as neurite growth. It is highly expressed in the brain, especially in areas of high plasticity, such as the hippocampus. The signals are slightly lower in the cortex, the cerebellum, and in striatum. Noteworthy, during development *plppr5* mRNA is expressed in the spinal cord, i.e., in neuron rich regions such as in medial motor nuclei, suggesting that *plppr5* plays an important role in the regulation of neurons. However, the existing literature only states that *plppr5* is involved in the occurrence and stability of dendritic spines, and research on its possible involvement in neonatal ischemic hypoxic encephalopathy has not been previously reported. We used *plppr5* knockout (*plppr5*–/–) mice and their wild-type littermates to establish a model of hypoxicischemic brain injury (HI) to further explore the effects of melatonin on brain injury and the role of *plppr5* in this treatment in an HI model, which mainly focuses on cognition, exercise, learning, and memory. All the tests were performed at 3–4 weeks after HI. As for melatonin treatment, which was performed 5 min after HI injury and followed by every 24h. In these experiments, we found that there was a significant interaction between genotype and treatment in novel object recognition tests, surface righting reflex tests and forelimb suspension reflex tests, which represent learning and memory, motor function and coordination, and the forelimb grip of the mice, respectively. However, a significant main effect of genotype and treatment on performance in all behavioral tests were observed. Specifically, wild-type mice with HI injury performed better than *plppr5*–/– mice, regardless of treatment with melatonin or vehicle. Moreover, treatment with

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melatonin could improve behavior in the tests for wild-type mice with HI injury, but not for *plppr5*–/– mice. This study showed that *plppr5* knockout aggravated HI damage and partially weakened the neuroprotection of melatonin in some aspects (such as novel object recognition test and partial nerve reflexes), which deserves further study.

Keywords: cerebral palsy, hypoxic-ischemic, *plppr5*, melatonin, neurobehavior

INTRODUCTION

Cerebral palsy (CP) is one of the most common causes of lifelong physical disability in children in most countries (1–3). Its prevalence in live births is approximately 2.0‰–3.5‰ (4, 5). However, cerebral palsy is not a disease entity in the traditional sense but is instead a group of clinical syndromes caused by nonprogressive brain damage in developing fetuses or infants. At present, many researchers believe that perinatal cerebral hypoxic ischemic (HI) injury or inflammatory injury are the main causes of cerebral palsy (1, 6–8). Because a complex of symptoms including motor dysfunction are caused by cerebral palsy, it seriously affects the quality of life of patients and applies great pressure to the family and society. Therefore, the study of cerebral palsy is particularly important. Some previous experiments showed that the long-term behavioral performance of the neonatal ischemic hypoxia animal model (HI) is consistent with the characteristics of cerebral palsy, and the model has been widely recognized (8).

Members of the *plppr* (*PRG*) family (*PRG1-PRG5*) can mediate the regeneration process and lysophosphatidic acid (LPA) activity in neurons and are known to participate in neuronal plasticity (9, 10). *Plppr1* drives cell autonomous signaling pathways to participate in the regulation of spinal density and subsequent memory formation (11, 12). Our previous research on haloperazine-induced recurrent seizures in rats found that *PRG-1* mRNA and protein levels are significantly upregulated in the hippocampus and cerebral cortex in response to neonatal convulsions, and these levels are maintained over the long term (13). *Plppr2* induces collateral branch growth in axons (14). *Plppr3* induces neurites that are resistant to growth inhibitors associated with brain injury and can help restore function after spinal cord injury (15). The *Plppr4* protein is highly expressed during the development and regeneration of synapses, can regulate synaptic lysophosphatidic acid (LPA) levels and is associated with epilepsy and brain damage.

Plppr5 (*PRG5)* is a new member of the plasticity-related protein family. Integral membrane protein *plppr5* is specifically expressed in brain and spinal cord, and induces filopodia formation as well as neurite growth. *Plppr5* is highly expressed in the brain, especially in areas of high plasticity, such as the hippocampus. The signals are slightly lower in the cortex, the cerebellum, the stratum radiatum, and in striatum. Noteworthy, during development *plppr5* mRNA is expressed in the spinal cord, i.e., in neuron rich regions such as in medial motor nuclei (10, 16).

Autophagy inhibitor E64d pretreatment can improve convulsive brain injury in rats and downregulate the expression of *PRG-1, PRG-3, PRG-5*, cathepsin E and ApoE mRNA (17). Thomas Broggini suggested that *plppr5*, as an upstream molecule of ROCK, can overcome LPA and Nogo-A-induced neurite contraction caused by RhoA-ROCK-PIP5K kinase pathway activation (16). Recently, Tan et al. found that electroacupuncture stimulation may be involved in the neuroprotective effect of cerebral ischemia-reperfusion rats mediated through *plppr5/ NogoA-LPA/RhoA* signaling (18).

Multiple studies have shown that the use of neuroprotective drugs can improve the prognosis of cases of cerebral palsy. For example, magnesium sulfate taken before premature delivery can significantly reduce the risk of cerebral palsy within 2 years of birth (19). Erythropoietin can limit inflammation and decrease the death of cells in many animal studies of hypoxic-ischemic injury (20, 21). Melatonin (n-acetyl-5-methoxytryptamine, Mel) is a multitask molecule, that plays a role both as a chronobiological hormone (hormone of darkness), and a mediator of immunological responses (22, 23). In addition to the pineal gland secreting melatonin, an increasing number of studies have shown that extra-pineal tissues secrete melatonin as well (24). Meanwhile, Melatonin is synthesized "on demand" in response to innate immune responses or certain inflammatory conditions (23). Because of its high fat solubility, ease of passing through the blood-brain barrier and cell membranes, and low toxicity, it has gradually become a promising neuroprotective drug (25). It is already in the preclinical trial stage in the treatment of convulsive brain injury (26, 27).

Naskar's research showed that melatonin has been reported to restore lost striatal spines in the MPTP model of Parkinson's disease (28); Chakraborty reported that melatonin protects against behavioral dysfunctions and dendritic spine damage in 3-nitropropionic acid-induced rat model of Huntington's disease (29); Changes in dendritic complexity and synaptic plasticity are closely related to long-term neurological dysfunction after brain injury. In neurodevelopmental disorders and ischemic brain injury, a decrease in the number of dendritic branches and synaptic density can be found (30). So it is speculated that the two may have an internal connection. The neural plasticity signal pathway involved in *Plppr5* may be the target of the neuroprotective effect of melatonin, which is worthy of further study.

It is conceivable that the plasticity related protein family represented by *plppr5* may play a key role in recovering from brain injury. Neonatal hypoxic-ischemic encephalopathy is the most common brain injury in children, and moreover, neurodevelopment is very obvious during the development of the newborn. To the best of our knowledge, research on the role of *plppr5* in neonatal hypoxic ischemic encephalopathy has not yet been reported.

Therefore, in this study, we used *plppr5* gene knockout mice (*plppr5*–/–) and their wild-type littermates to establish a model of

ischemic hypoxic brain injury (HI) to further explore the effects of melatonin on brain injury and the possible role of *plppr5* in melatonin's protective effect against brain injury. These results will provide a stronger theoretical basis for the clinical use of melatonin in the treatment of ischemic hypoxic encephalopathy.

MATERIALS AND METHODS

Animal Preparation

The generation of *plppr5* knockout mice was conducted in the GemPharmatech Co, Ltd (formerly known as Nanjing Biomedicine Research Institute of Nanjing University, Nanjing, China) using CRISPR/Cas9 technology, and gRNA was designed and transcribed *in vitro*:

gRNA1:5'-ACCACTAGGCAGTAGAGACT-3',PAM : GGG;
gRNA2:5'-TGTGAGGACAATTGGCTCTA-3',PAM : AGG;
gRNA3:5'-GGGCTTTGTCGTGGGTGGCG-3',PAM : GGG;
gRNA4:5'-CACAGTCTCGTGGGAGGGCG-3',PAM : GGG.

Cas9 and gRNA were simultaneously injected into 200 fertilized mouse eggs. The Cas9 protein binds to the target site under the guidance of gRNA and causes DNA double-strand breaks, thereby achieving deletion of the base sequence of the target site. Final realization and gene knockout are shown in **Figure 1A**. A total of four positive F0 generation mice were obtained.

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Soochow University (ethical code:SUDA20201020A01). We took adequate measures to minimize animal suffering, and the sample size is based on the sample size used in our group's previous publications and other similar studies (31).

Experiments were performed on knockout mice backcrossed to C57BL/6 for over 15 generations at the time of experiment and several more generations in our own laboratory. The genotype of the transgenic mice was identified by PCR *via* genomic DNA prepared from tail biopsies using standard procedures. PCR Reaction Component was used as follows: 12.5μl of 2 × Taq Master Mix, Dye Plus, (Vazyme P112-03); 9.5μl of ddH_2O;1μl of each primer (10pmol/μl); 1μl of Template (≈100ng/μl). And the following thermal cycling was used: 95 °C 5 min; followed by 20 cycles of 98 °C 30 s, 65 °C (-0.5 °C/cycle) 30 s, 72 °C 45 s; followed by 20 cycles of 98 °C 30 s, 55 °C 30 s,72 °C 45 s; 72 °C 5 min; 10 °C hold. The following primers were used to amplify the PCR fragments **Figures 1B, C**:

①KO : XM709934-*Plppr5*-KO-TF2 : 5'-AGCTGGTGTTACATTACAGGCAG-3';
XM709934-*Plppr5*-KO-TR2 : 5'-GGGTTTCTTTCCACATAGTCACAG-3'.
②WT : XM709934-*Plppr5*-WT-TF1 : 5'-GTTCTCCAGTTCAATCATTGGG-3';
XM709934-*Plppr5*-WT-TR1 : 5'-ATGCTGTATCCCGTGCTTTCTG-3'.

①KO primers were designed to generate PCR fragments of 414 bp for the KO allele and 67739 bp for the WT allele, the latter of which was not amplified due to its large size. To distinguish

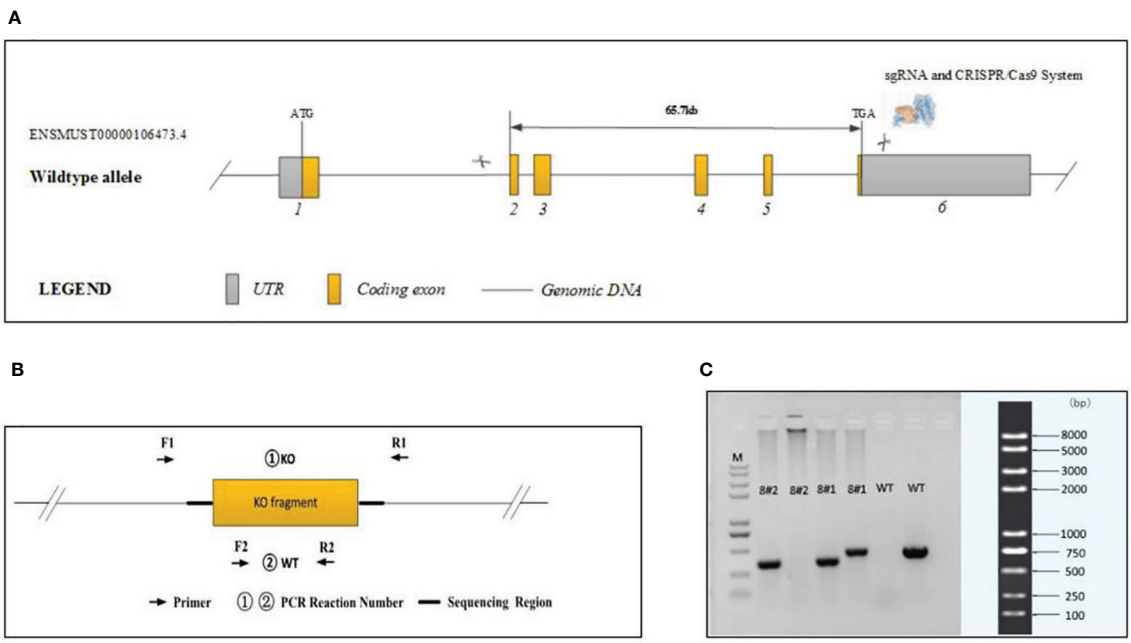

FIGURE 1 | Generation of *plppr5*–/– mice and confirmation of *plppr5* deficiency at the DNA level. **(A)** Gene sequence insertion; **(B)** PCR analysis; **(C)** Samples 8#2 and 8#1 correspond to the *plppr5*–/– and *plppr5*+/- mice, respectively. M represents the DNA ladder.

between the KO/WT and KO/KO mice, ②WT primers were designed to produce a 439 bp fragment for the WT allele only. A lot of experimental and clinical researches found that males show increase risk for brain-based developmental disorders including learning disabilities and cerebral palsy compared with females (32, 33). Therefore, in our experiment only male *plppr5*−/− mice and its wild-type littermates were used. We performed an experiment (**Figure 2**) during long-term follow-up.

Group Allocation

The mice used for behavioral testing come from different litters, and at least 1 or 2 mice for each treatment group in every litter: KO+Veh, KO+Mel, WT+Veh, and WT +Mel. Mice were weaned 21 days after birth, then they were group housed. Every group was divided into two cages with 6 mice per cage. Another three cages of mice of each genotype with the same hypoxic ischemic brain injury and treatment were used for TTC staining. Furthermore, in order to identify the success of the animal model, we designated another group as sham for every genotype (KO represents *plppr5*−/− mice with HI injury, WT means wild-type mice with HI injury, Mel means melatonin, Veh means vehicle.).

Animal Model of Hypoxia-Ischemia

The model of hypoxic-ischemic (HI) injury in newborn mouse pups was performed based on the well-established Rice Vannucci model (34). Briefly, C57/BL6 wild-type and plppr5 knockout postnatal day 10 (P10) pups (35) from the same parental generation were used. All of the pups were anesthetized with diethyl ether until they were fully anesthetized and unresponsive (36, 37). The left carotid artery was isolated, double ligated with a 5-0 surgical suture and cut between the ligations. Last, the skin was sutured back to close the incision. The entire procedure took no more than 6 min. The mice were allowed to recover for 1 h in their cages. The mice were then put in a 37°C chamber under a gas mixture of 8% oxygen and 92% nitrogen for 2 h. For the sham groups, sham animals received an incision but no ligation, and the pups were placed in a similar but not hypoxic environment. Finally, the pups were returned to their dams and kept in a standard environment. The operation was carried out in the afternoon for melatonin intervention would be in evening.

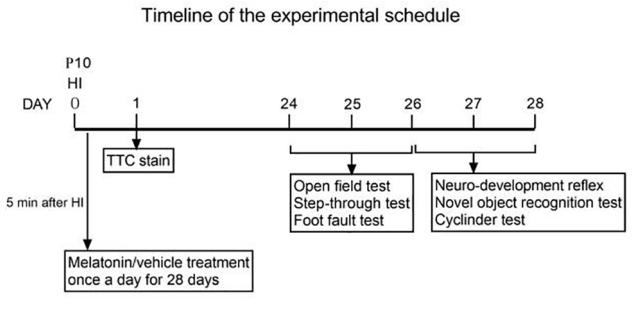

FIGURE 2 | Timeline of the experimental schedule.

TTC Staining

2,3,5-Triphenyltetrazolium chloride (TTC) is a redox indicator, which can be reduced by the mitochondrial enzyme succinate dehydrogenase to a fat-soluble compound. The normal brain tissue appears red, and the infarct areas are white (38). At 24 h after the HI insult, some pups were sacrificed, and the brains were extracted and then frozen at −20 °C for 10 min. Next, they were cut into ~2 mm sections coronally and then immersed in 1% TTC at 37 °C in a dark environment (10–20 min). Finally, the sections were immersed in a 4% paraformaldehyde solution overnight and photos were taken (39). Pictures of the stained slices were obtained and the infarct area was manually delineated using ImageJ. The degree of cerebral infarction is presented as the percentage of infarction volume to total brain volume.

Melatonin Intervention

Melatonin was purchased from Sigma (St. Louis, MO, USA). Melatonin was administered after the hypoxic-ischemic insult in groups KO+Mel and WT+Mel. The dose was intraperitoneally injected daily according to body weight (0.1 mg/10 g body weight per day) at 5 min after the surgery and once every 24 h for 4 weeks (27). Meanwhile, the other two groups were injected with vehicle of the same volume (40) and at the same time in the evening (18:00–20:00). The concentration of melatonin was 2 mg/ml (27).

Weight Monitoring

The body weight of the mice in each group was recorded every 4 days to estimate the physical development of the pups.

Behavioral Evaluation of Adolescent Mice

Behavioral testing was performed in a quiet room with non-distracting, homogenous lighting. In order to explore the long-term effect of melatonin on behavior, we choose the time-point of 4 weeks after HI injury to take these behavioral testing. Specifically, open field test, step-through test and foot fault test were performed during 24-26 days after HI injury (PND 34-36). Neurodevelopment reflex tests, novel object recognition were all evaluated during 27-28 days after HI injury (PND 37-38). Testers are unclear about the experiment grouping and handling. The details are as follows:

Neuro-Developmental Reflex

Negative geotaxis reflex: the negative geotaxis reflex is considered to reveal the functions of vestibular and proprioception of mice. The mice were blindfolded and placed on a 45° inclined plywood surface with the head towards the ground. We recorded the time it took for the mouse to switch from a head-down to a head-up position. The shorter the time, the better the mouse's reactivity was (41).

Cliff avoidance reflex: the cliff avoidance reflex is used to assess the ability of rodents to respond to adverse environments. The mouse was placed on the edge of a test bench with its forepaws and nose over the edge. We recorded the time from when the mouse turned away from the edge and completely reversed position. The shorter the time, the better the mouse's reactivity was (42).

Forelimb suspension reflex: a metal rod (diameter of 0.5 cm) was fixed at a height of 50 cm from the ground, and the mice were suspended with their forepaws grasping it. The time the animal remained on the bar was recorded. The longer the time, the better the mouse's reactivity was (43).

Surface righting reflex: each mouse was placed in a supine position and the time required to turn their body completely to the ground was recorded. Shorter times indicated faster reactions (41).

Novel Object Recognition Test

The novel object recognition test was performed in an open field test apparatus (40cm× 40cm× 30cm) under dim light in order to test the neurobiology of nonspatial memory in rodents. The entire test is divided into three stages: the first day is adaptation (T0) where the mouse is allowed to freely explore the open field for 5 min. Day 2 is training (T1), where the mouse is allowed to explore the arena with two identical triangular Lego toys and square Lego toys placed along the diagonal and glue them to the bottom of the box. The mice of each group were randomly placed in a test box with different Lego toys, and the mice were allowed to explore freely for 5 min. Testing (T2) takes place on Day 3: in the test phase after 24 h, one of the old objects is replaced with another shape object (that is, the same mouse faces a triangular toy and a square toy). Mice are born to like novelties; if the mice can recognize the familiar object, it will spend more time with the novel object. Object detection is defined as pointing the nose at the object from a distance of ≤ 2 cm, for instance, touching it with the forehead or nose to sniff or bite the object. The recognition index is used to indicate recognition memory, which is calculated as (Tnovel)/(Tnovel+Tfamiliar), as previously described (44).

Open Field Test

Mice were tested in the open field for general locomotor activity, anxiety, and willingness to explore. Animals were tested in an acrylic arena (72 × 72 × 50 cm). The floor of the arena was divided into 16 squares of 18 × 18 cm. To start each session, a mouse was placed in the center of the arena and allowed to explore for 5 min. After 24 h, each mouse was observed for 5 min. The arena was cleaned with 75% ethanol between trials to eliminate the smell left by the previous mouse. Observation indicators included time in the center area (defined as center duration time), the total grids traveled, rearing (defined as standing on the hindlimbs without touching the wall), and grooming of mice. The open score was calculated as (the total grids traveled + rearing) (45).

Step-Through Test (Passive Avoidance Response)

A DC pulse stimulus current was passed through the floor in a darkroom. The mice were first placed in a darkroom reaction box for 3 min, and the mice escaped to the bright room after the electric shock. At the beginning of the formal test, the mice were placed in the bright room with their backs facing away from the hole. The mice were shocked when they entered the dark room. The dark avoidance device automatically recorded the number of times the mice entered the dark room within 5 min, which is the number of errors and the time from when they first entered the dark room to avoid dark latency. We started the training experiment at 9:00 am on the first day and performed the test at 9:00 am the next day (24 h after the training experiment). We recorded the latency avoidance period and the number of errors of the mouse within 5 min (46, 47).

Foot Fault Test

The foot fault test was carried out to evaluate sensorimotor function after HI insult. The mice were allowed to walk on a grid, which is a homemade grid with a mesh size of 2 × 2 cm and a height of 50 cm above the ground. Before the test, every mouse was allowed to walk on it freely for 5 min, and 1 h later, the test was officially conducted. A wrong step was defined as limb fall or slip into the mires and the number of times the right forelimb and the right hindlimb were recorded separately (48).

Cylinder Test

The cylinder test was used to assess the asymmetry in the use of the forelimbs of the mice when subjected to ischemic damage. The mice were placed separately in a transparent cylinder (20 cm in diameter and 45 cm in height). The number of times the mice touched the wall of the cylinder over 5 min was used to calculate the asymmetry score of the mice after HI insult. The higher the score was, the more obvious the asymmetry of the limbs on both sides. Asymmetry of forelimb use and paw preference were calculated as (left (nonimpaired side) – right (impaired side))/(left + right) × 100% (49).

Determination of Seizure Threshold

Survivors of neonatal HI often develop brain injury and neurologic disabilities (e.g., cognitive deficits and epilepsy) in later life. All of the groups are injected with penicillin (5.1×10^6U/kg/d, i.p.) after behavioral evaluation. We recorded the time of the first seizure in the mouse and the seizure latency (min) (seizure threshold). The observation time was 90 min. According to the Racine classification, seizures are considered to occur if the seizure degree reaches level IV or above. Moreover, after the seizure started, the mouse was injected with 4% chloral hydrate immediately (50).

Timm Staining

Timm staining is based on the staining of Zn^{2+}-containing mossy fibers by a sulfide/silver stain as described previously. Briefly, the mice were deeply anesthetized and then fixed by transcardial perfusion with 0.9% NaCl followed by 0.3% Na_2S in 100 mM phosphate buffer (PB) and 4% paraformaldehyde in 100 mM phosphate buffer (PB). After perfusion, the brains were postfixed in 4% paraformaldehyde overnight at 4°C. Coronal sections were cut at 30μm on cryostat and stained for mossy fibers using Timm staining. The processing solutions were obtained from Sinopharm Chemical Reagent Co., Ltd., China and were as follows: 50% Arabic gumc, 15ml Citric acid buffer (3.825g citric acid,3.525g sodium citrate),45ml 5.67% hydroquinone, and 0.5ml 17% silver nitrate (51–53).Then stained for 40–60 min at 37°C. After rinsing, the sections were dehydrated in alcohol, cleared in xylene, and mounted on slides with

permount. Timm staining was analyzed at a magnification of 40 and 100 using an OLYMPUS PM20 automatic microscope (Olympus, Japan).

Statistical Analysis

The data were analyzed using SPSS 17.0 statistical analysis software (SPSS, Chicago, IL, USA).Behavioral test analysis was based on *a priori* performance criteria, that is, the double-blind principle was adopted, and the data statisticians did not understand the grouping of experimental animals. Two-way factorial analysis of variance (ANOVA) and Bonferroni *post hoc* tests were used for the statistical analyses of the variable behavioral test and TCC staining of infarct volume. For the analysis of weight data, three-way ANOVA with repeated measures was used with Bonferroni *post hoc* tests. To ensure there was complete equality of the variances of the differences between all variations in the related groups, an assumption of sphericity was conducted with Mauchly's test. If the assumption of sphericity was not met, the Greenhouse–Geisser correction was used. Normal distribution of the variables was assessed with the Shapiro-Wilk test for all variables in each treatment group. If the normal distribution was not satisfied, the data were transformed to a normal distribution. All data are presented as the mean ± standard deviation (SD). Throughout the study, *p values < 0.05 and **p values < 0.01 were considered significant and n.s., not significant. All graphs were created using GraphPad Prism version 8.0 software (GraphPad, San Diego, CA, USA).

RESULTS

TTC Staining

Infarct volume was used to evaluate brain damage at 24 h after HI injury. Three mice were subjected to TTC staining in each group. The results showed that for each genotype in the groups receiving melatonin or vehicle there was no significant interaction between genotype and treatment [$F_{(1,8)}$=0.225, P=0.648], but there was a significant main effect of genotype [$F_{(1,8)}$ =55.280, $P < 0.001$] and treatment [$F_{(1,8)}$ =29.354, $P < 0.05$] on infarct volume. Bonferroni *post hoc* testing revealed that the mice with melatonin-treatment had a decreased infarct volume relative to the vehicle-treated mice ($P< 0.001$) and that the wild-type mice with HI injury had a decreased volume relative to the knockout mice ($P< 0.001$) (**Table 1**, **Figures 3** and **4A**).

Weight Monitoring

Three-way ANOVA with repeated measures for each time and each genotype in all of the groups receiving the melatonin

TABLE 1 | Significant results for two-way analysis of variance.

		Treatment		Main Effect		Interaction Effect
		Veh (mean ± SD)	Mel (mean ± SD)	Genotype	Treatment	Genotype × Treatment
TCC staining						
Infarct volume	KO	0.252 ± 0.018	0.226 ± 0.006	F=55.28**	F=29.354*	F=0.225, P=0.648
	WT	0.202 ± 0.008	0.160 ± 0.013	p<0.001	P<0.05	
Neurodevelopmental reflex						
Negative geotaxis reflex test	KO	10.888 ± 1.711	8.623 ± 1.618	F=37.831**	F=28.716**	F=0.197, P=0.659
	WT	8.258 ± 1.504	5.583 ± 1.546	P<0.001	P<0.001	
Cliff avoidance reflex test	KO	10.476 ± 1.612	7.704 ± 2.541	F=41.204**	F=30.337**	F=0.004, P=0.950
	WT	7.251 ± 1.367	4.542 ± 0.979	P<0.001	P<0.001	
Open field test						
Center duration time	KO	4.829 ± 1.557	2.955 ± 2.000	F=13.280*	F=23.474**	F=0.305, P=0.583
	WT	3.292 ± 1.058	1.395 ± 0.586	P=0.001	P<0.001	
Locomotor score	KO	79.917 ± 30.330	137.417 ± 43.976	F=68.441**	F=30.950**	F=0.127, P=0.724
	WT	166.500 ± 16.839	256.583 ± 96.937	P<0.001	P<0.001	
Number of grooming	KO	5.083 ± 1.564	3.500 ± 1.446	F=21.084**	F=19.078**	F=0.013, P=0.911
	WT	3.417 ± 1.240	1.750 ± 0.754	P<0.001	P<0.001	
Step-through test						
Number of errors	KO	2.833 ± 0.718	2.083 ± 0.669	F=33.835**	F=16.755**	F=0.046, P=0.830
	WT	1.750 ± 0.754	0.917 ± 0.515	P<0.001	P<0.001	
Latency avoidance period	KO	27.423 ± 16.561	48.397 ± 32.748	F=57.605**	F=23.363**	F=2.865, P=0.098
	WT	76.167 ± 48.152	210.525 ± 56.346	P<0.001	P<0.001	
Foot-fault test						
Forelimb foot-fault test	KO	21.333 ± 2.708	16.333 ± 6.893	F=16.121**	F=17.195**	F=0.039, P=0.844
	WT	16.500 ± 3.289	11.000 ± 3.357	P<0.001	P<0.001	
Hindlimb foot-fault test	KO	18.917 ± 3.554	14.583 ± 4.719	F=25.385**	F=17.270**	F=0.110, P=0.742
	WT	13.583 ± 3.579	8.500 ± 3.729	P<0.001	P<0.001	
Cylinder test	KO	0.876 ± 0.189	0.613 ± 0.256	F=20.48**	F=17.239**	F=0.053, P=0.819
	WT	0.588 ± 0.282	0.295 ± 0.186	P<0.001	P<0.001	
Determination of Seizure Threshold	KO	20.078 ± 5.334	29.727 ± 7.286	F=52.722**	F=17.117**	F=0.078, P=0.782
	WT	36.552 ± 5.453	44.982 ± 8.624	P<0.001	P<0.001	

*Bonferroni's method is used for multiple comparisons with all possible pairwise differences of means. WT, Wild-type mice with HI injury; KO, knockout mice with HI injury; Mel, melatonin; Veh, vehicle; ns, Not Significant. *P < 0.05; **P < 0.001. [Two-way ANOVA, Bonferroni post hoc test (details are shown in text)].*

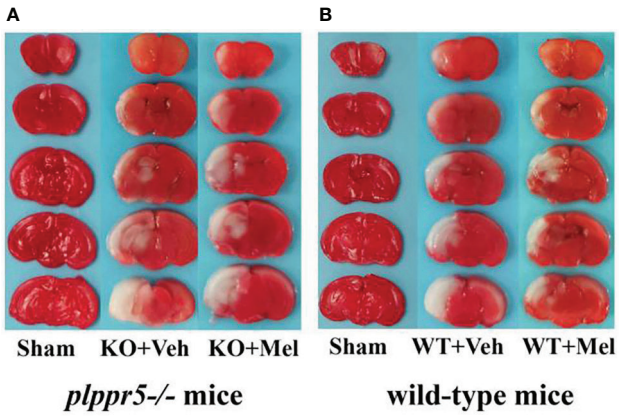

FIGURE 3 | Photomicrographs of TTC-stained coronal slices showing brain infarction 24 h after HI: **(A)** *plppr5*–/– mice; **(B)** Wild-type mice. WT, wild-type mice with HI injury; KO, knockout mice with HI injury; Mel, melatonin; Veh, vehicle.

treatment or vehicle revealed no significant interaction between genotype, treatment and time [$F_{(1.562,1.242)}$=1.258, P=0.305] on weight. A significant genotype × time [$F_{(2.299,25.288)}$=17.135, P<0.001] and treatment × time [$F_{(2.438,26.820)}$=3.811, P<0.05] interaction as well as a main effect of genotype [$F_{(1,11)}$=7.709, P<0.05], treatment [$F_{(1,11)}$=32.385, P<0.001], and time [$F_{(1,11)}$ =1671.417, P<0.001] were observed. Details are shown in **Table 2**. Bonferroni *post hoc* testing of the weight in each genotype group receiving melatonin treatment or vehicle indicated that all of the groups had a significant increase (P<0.05) in weight from P10 to P38 (**Figure 5**). Bonferroni *post hoc* testing also revealed an increased weight in WT mice receiving vehicle (WT+Veh) at time P38 (P<0.05) compared to the KO mice treated with vehicle (KO+Veh). Meanwhile, WT mice receiving melatonin (WT +Mel) had increased weight at time P22 (P<0.05), P26 (P<0.05), P30 (P<0.05), P34 (P<0.05), and P38 (P<0.05) compared to the KO mice treated with melatonin (KO+Mel). Bonferroni *post hoc* testing also showed melatonin increased body weight compared to the vehicle at the times P14 (P<0.05),

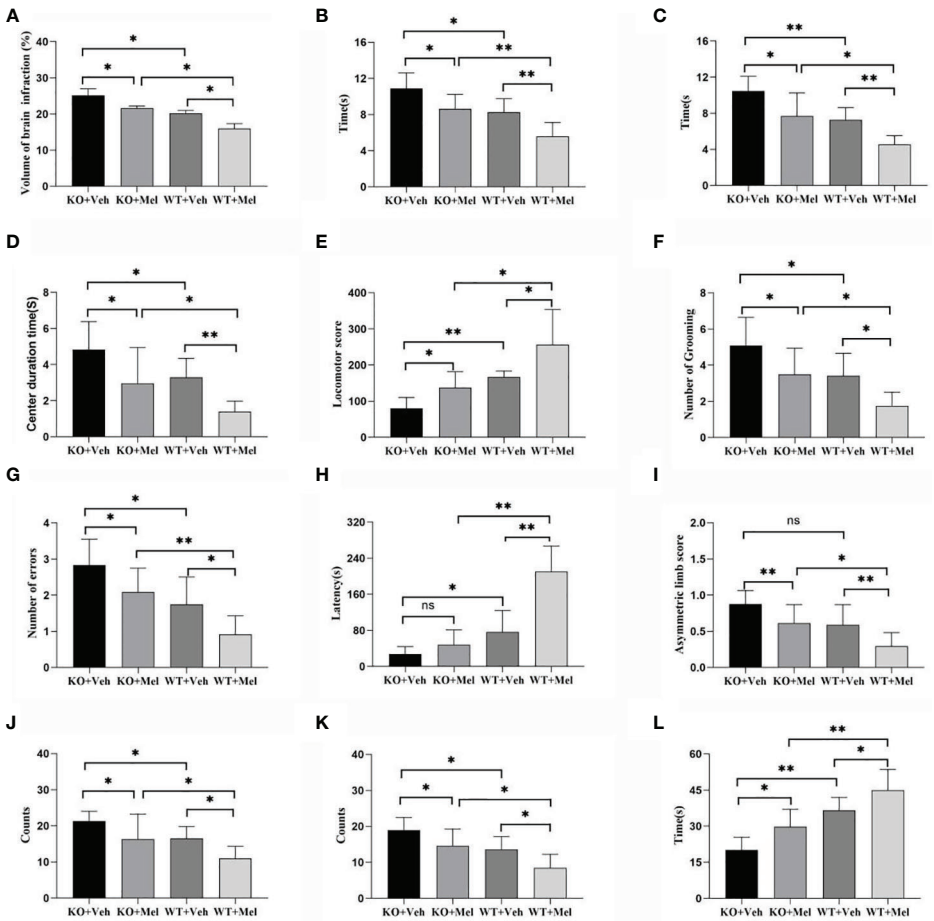

FIGURE 4 | Behavioral test in wild-type mice with HI injury treated with vehicle or melatonin as well as knockout mice with HI injury receiving vehicle and melatonin treatments as indicated. **(A)** TTC staining; **(B)** Negative geotaxis reflex test; **(C)** Cliff avoidance reflex test; **(D)** Open field test-center duration time; **(E)** Open field test-locomotor score; **(F)** Open field test-number of grooming; **(G)** Step through test-number of errors; **(H)** Step through test-latency avoidance period; **(I)** Cylinder test; **(J)** Foot fault test-forelimb foot fault test; **(K)** Foot fault test-hindlimb foot fault test; **(L)** Seizure threshold. [Two-way ANOVA, Bonferroni *post hoc* test (details are shown in text)].

TABLE 2 | Significant results for three-way analysis of variance.

	KO		WT	
	Veh(Mean ± SD)	Mel (Mean ± SD)	Veh (Mean ± SD)	Mel (mean ± SD)
P10	5.342 ± 1.281	5.993 ± 0.628	5.476 ± 0.839	5.973 ± 0.691
P14	5.422 ± 1.362	6.145 ± 0.855	5.576 ± 0.792	6.223 ± 0.844
P18	6.378 ± 1.149	6.941 ± 1.206	6.608 ± 0.849	7.45 ± 0.894
P22	7.526 ± 1.370	7.668 ± 1.108	8.463 ± 0.849	9.289 ± 1.151
P26	9.438 ± 1.479	9.515 ± 1.108	10.436 ± 0.611	11.091 ± 1.222
P30	11.516 ± 1.769	11.605 ± 1.126	12.583 ± 0.595	13.414 ± 1.341
P34	13.334 ± 1.554	13.673 ± 1.092	14.48 ± 0.652	15.763 ± 1.012
P38	15.178 ± 1.375	16.504 ± 0.938	16.615 ± 0.593	18.069 ± 1.012
genotype	F = 7.709*		P = 0.018	
treatment	F = 32.385**		P < 0.001	
time	F = 1671.417**		P < 0.001	
genotype × treatment	F = 0.610		P = 0.451	
genotype × time	F=17.135**		P < 0.001	
treatment × time	F=3.811*		P = 0.028	
genotype × treatment × time	F=1.258		P = 0.305	

*Bonferroni's method is used for multiple comparisons with all possible pairwise differences of means. WT, Wild-type mice with HI injury; KO, knockout mice with HI injury; Mel, melatonin; Veh, vehicle; ns, Not Significant ; *P < 0.05; **P < 0.001. [Three-way ANOVA, Bonferroni post hoc test (details are shown in text)].*

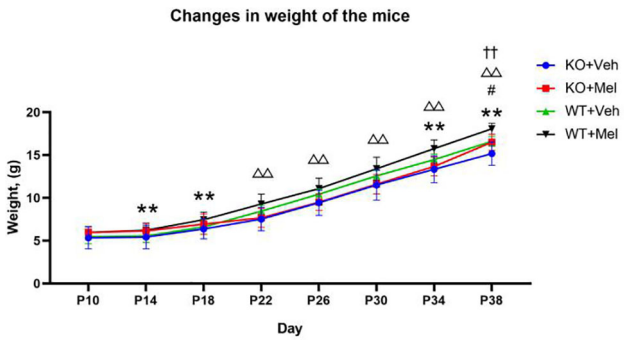

FIGURE 5 | Changes in weight of the mice: **P < 0.001 for WT+Mel vs. WT; △△P < 0.001 for KO+Mel vs. WT+Mel; #P < 0.05 for KO+Mel vs. KO; ††P < 0.001 for KO vs. WT. [Three-way ANOVA, Bonferroni *post hoc* test (details are shown in text)].

P18 (P<0.05), P34 (P<0.05), and P38 (P<0.001) in WT mice. Similarly, melatonin increased weight compared to the vehicle at the time P38 (P<0.05) in KO mice.

Neurodevelopmental Reflex

Negative Geotaxis Reflex and Cliff Avoidance Reflex Tests

Two-way ANOVA did not reveal significant genotype and treatment interaction effects on the performance in the negative geotaxis reflex test [$F_{(1,44)}$=0.197, P=0.659] and cliff avoidance reflex test [$F_{(1,44)}$=0.004, P=0.950]. However, a significant main effect of genotype [$F_{(1,44)}$=37.831, P<0.001; $F_{(1,44)}$=41.204, P<0.001] and treatment [$F_{(1,44)}$=28.716, P<0.001; $F_{(1,44)}$=30.337, P<0.001] was found for the negative geotaxis reflex test and cliff avoidance reflex test, respectively. Bonferroni *post hoc* testing of both behavioral tests revealed that the mice with melatonin-treatment performed better than the vehicle-treated mice, and the wild-type groups performed better than knockout groups (the P-values were all less than 0.001) (**Table 1**, **Figures 4B, C**).

Surface Righting Reflex Text

Two-way ANOVA for each genotype in all of the groups receiving the melatonin treatment or vehicle indicated a significant genotype × treatment interaction [$F_{(1,44)}$=5.247, P<0.05] as well as a significant main effect of genotype [$F_{(1,44)}$=49.422, P<0.001] and treatment [$F_{(1,44)}$=5.247, P<0.05] on the surface righting reflex test. Bonferroni *post hoc* tests revealed no significant differences between the KO with HI injury group treated with melatonin (KO+Mel) and the vehicle group (KO+Veh) (P>0.05). However, a significant decrease in surface righting reflex time was observed in the wild-type with HI injury group treated with melatonin (WT+Mel) compared to the vehicle group (WT+Veh) (P<0.001). In addition, whether treated with melatonin or vehicle, wild-type mice with HI injury had a decreased time of surface righting reflex than the knockout mice [P<0.001, P<0.001, respectively] (**Table 3**, **Figure 6B**).

Forelimb Suspension Reflex Test

Two-way ANOVA for each genotype in all of the groups receiving the melatonin treatment or vehicle indicated a significant genotype × treatment interaction [$F_{(1,44)}$=4.206, P<0.05] as well as a significant main effect of genotype [$F_{(1,44)}$= 36.721, P<0.001] and treatment [$F_{(1,44)}$=4.297, P<0.05] on the forelimb suspension reflex test. Bonferroni *post hoc* tests revealed no significant differences between the KO with HI injury group treated with melatonin (KO+Mel) and the vehicle group (KO+Veh) (P=0.978). However, a significant increase in forelimb suspension reflex time was observed in the wild-type with HI injury group treated with melatonin (WT+Mel)

TABLE 3 | Significant results for two-way analysis of variance.

		Treatment		Main Effect		Interaction Effect
		Veh (mean ± SD)	Mel (mean ± SD)	Genotype	Treatment	Genotype × Treatment
Forelimb suspension reflex test	KO	3.543 ± 0.987	3.558 ± 1.464	F=36.721**	F=4.297*	F=4.206*, P=0.046
	WT	6.021 ± 2.332	8.568 ± 3.125	P<0.001	P=0.044	
Surface righting reflex text	KO	0.269 ± 0.037	0.266 ± 0.043	F=49.422**	F=5.247*	F=5.247*, P=0.027
	WT	0.219 ± 0.037	0.171 ± 0.027	P<0.001	P=0.027	
Novel object recognition test	KO	0.205 ± 0.096	0.344 ± 0.217	F=67.604**	F=32.52**	F=6.204*, P=0.017
	WT	0.453 ± 0.138	0.808 ± 0.122	P<0.001	P<0.001	

*WT, Wild-type mice with HI injury; KO, knockout mice with HI injury; Mel, melatonin; Veh, vehicle; *P < 0.05; **P < 0.001. [Two-way ANOVA, Bonferroni post hoc test (details are shown in text)].*

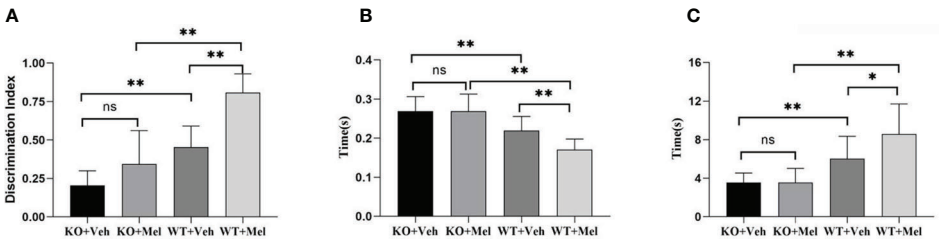

FIGURE 6 | Behavioral test in wild-type mice with HI injury treated with vehicle or melatonin as well as knockout mice with HI injury receiving vehicle and melatonin treatments as indicated. **(A)** Novel object recognition test; **(B)** Surface righting reflex test; **(C)** Forelimb suspension reflex test. [Two-way ANOVA, Bonferroni *post hoc* test (details are shown in text)].

compared to the vehicle group (WT+Veh) (P<0.05). In addition, whether treated with melatonin or vehicle, wild-type mice with HI injury had an increased time of forelimb suspension reflex than knockout mice [P<0.001, P<0.001, respectively]. (**Table 3**, **Figure 6C**).

Novel Object Recognition Test

Two-way ANOVA for each genotype in all of the groups receiving the melatonin treatment or vehicle indicated a significant genotype × treatment interaction [$F_{(1,44)}$=6.204, P=0.017] as well as a significant main effect of genotype [$F_{(1,44)}$= 67.604, P<0.001] and treatment [$F_{(1,44)}$=32.520, P<0.001] on the novel object recognition test. Bonferroni *post hoc* tests revealed no significant differences between knockout with HI injury group treated with melatonin (KO+Mel) and the vehicle group (KO+ Veh) (P=0.054). However, a significant increase in novel object cognition index was observed in the wild-type with HI injury group treated with melatonin (WT+Mel) compared to the vehicle group (WT+Veh) (P<0.001). In addition, whether treated with melatonin or vehicle, wild-type mice with HI injury performed better than knockout mice [P<0.001, P<0.001, respectively] (**Table 3**, **Figure 6A**).

Open Field Test

Center Duration Time

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.305, P=0.583] for the center duration time. Main effects of genotype [$F_{(1,44)}$=13.280, P=0.001] and treatment [$F_{(1,44)}$=23.474, P<0.001] on the center duration time were found. Bonferroni *post hoc* testing of the center duration time revealed that the mice with melatonin treatment had a decreased center duration time than vehicle-treated mice (P< 0.001) and the wild-type mice with HI injury spent less time in the center than the knockout mice (P< 0.001) (**Table 1**, **Figure 4D**).

Locomotor Score

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.127, P=0.724] for the locomotor score. Main effects of genotype [$F_{(1,44)}$=68.441, P<0.001] and treatment [$F_{(1,44)}$=30.950, P<0.001] were found on the locomotor score. Bonferroni *post hoc* testing of the locomotor score revealed that the mice with melatonin-treatment performed better than those who received vehicle-treatment (P< 0.001), and the wild-type groups performed better than the knockout groups (P< 0.001) (**Table 1**, **Figure 4E**).

Number of Grooming

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.013, P=0.911] for the number of grooming. Main effects of genotype [$F_{(1,44)}$=21.084, P<0.001] and treatment [$F_{(1,44)}$=19.078, P<0.001] on the number of grooming were found. Bonferroni *post hoc* testing of the number of grooming revealed that the mice with melatonin-treatment had decreased numbers of grooming compared with the vehicle-treated mice (P< 0.001), and the wild-type groups had a decreased number of grooming compared with the knockout groups (P< 0.001) (**Table 1**, **Figure 4F**).

Step-Through Test

Number of Errors

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.046, P=0.830] for the number of errors. Main effects of genotype [$F_{(1,44)}$=33.835, P<0.001] and treatment [$F_{(1,44)}$=16.755, P<0.001] on the number of errors were found. Bonferroni *post hoc* testing on the number of errors revealed that the mice with melatonin-treatment had a decreased number of errors compared with the vehicle-treated mice (P< 0.001) and that the wild-type groups had a decreased number of errors compared with the knockout groups (P< 0.001) (**Table 1**, **Figure 4G**).

Latency Avoidance Period

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=2.865, P=0.098] for the latency avoidance period. Main effects of genotype [$F_{(1,44)}$=57.605, P<0.001] and treatment [$F_{(1,44)}$=23.363, P<0.001] on the latency avoidance period were found. Bonferroni *post hoc* testing of the latency avoidance period revealed that the mice with melatonin-treatment had an extended latency avoidance period compared with the vehicle-treated mice (P< 0.001), and the wild-type groups had an extended latency avoidance period relative to the knockout groups (P< 0.001) (**Table 1**, **Figure 4H**).

Foot Fault Test

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.039, P=0.844] for the forelimb foot-fault test or for the hindlimbs [$F_{(1,44)}$=0.110, P=0.742]. Main effects of genotype [$F_{(1,44)}$=16.121, P<0.001] and treatment [$F_{(1,44)}$=17.195, P<0.001] on the forelimb foot-fault test were found. Main effects of genotype [$F_{(1,44)}$=25.385, P<0.001] and treatment [$F_{(1,44)}$= 17.270, P<0.001] on the hindlimb foot-fault test were found as well. Bonferroni *post hoc* testing of the forelimb foot-fault test revealed that the mice with melatonin-treatment performed better than the vehicle-treated mice (P< 0.001), and the wild-type groups performed better than the knockout groups (P< 0.001). There was a statistically significant difference for the forelimb foot-fault test (P< 0.001) (**Table 1**, **Figures 4J, K**).

Cylinder Test

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.053, P=0.819] for the cylinder test. Main effects of genotype [$F_{(1,44)}$=20.480, P<0.001] and treatment [$F_{(1,44)}$=17.239, P<0.001] on the cylinder test were found. Bonferroni *post hoc* testing of the cylinder test revealed that the mice with melatonin-treatment performed better than the vehicle-treated mice (P< 0.001) and that the wild-type groups performed better than the knockout groups (P< 0.001) (**Table 1**, **Figure 4I**).

Determination of Seizure Threshold

Two-way ANOVA showed no significant interaction between genotype and treatment [$F_{(1,44)}$=0.078, P=0.782] for the determination of seizure threshold. Main effects of genotype [$F_{(1,44)}$=52.722, P<0.001] and treatment [$F_{(1,44)}$=17.117, P<0.001] on the determination of seizure threshold were found. Bonferroni *post hoc* testing of the determination of seizure threshold revealed that the mice with melatonin-treatment were higher than that of the vehicle-treated mice (P< 0.001) and the wild-type groups were higher than the knockout groups (P< 0.001) (**Table 1**, **Figure 4L**).

Timm Staining

As can be seen from the results of Timm staining, the hippocampus in the four groups were all atrophy (all groups were given hypoxic-ischemic treatment), especially in the KO+Veh group, we can hardly see a clear hippocampus structure; However, hippocampal atrophy was alleviated when mice were treated with melatonin for 4 weeks especially in the WT+Mel and KO+Mel groups compared with the corresponding control groups (WT+Veh and KO+Veh). The results show that plppr5 knockout aggravated hippocampal atrophy caused by HI injury, while melatonin treatment significantly alleviate the atrophy.

In terms of hippocampal mossy fiber distribution and germination indicators, plppr5 knockout significantly inhibited the growth of mossy fiber, which was demonstrated in the comparison of KO+Veh and WT+Veh group without melatonin treatment, and in KO+Veh group, we can see rare and discontinuous moss fiber coloration only in CA3 but not DG (dentate gyrus) but no sprouting in both area. However, in WT+Veh group, moss fiber coloration can be seen in both CA3 and DG area, and it is more obvious in CA3.

The comparison of the two groups of KO+Mel and WT+Mel showed that the distribution of moss fibers in the DG and CA3 is clear. Meanwhile, there is no sprouting in the KO+Mel group, while the DG area of the WT+Mel group has obvious sprouting. Therefore, we can speculate that plppr5 knockout inhibited the axon regeneration of hippocampal granulosa cells (**Figure 7**).

DISCUSSION

The key findings of the current study are that *plppr5* is related to the protective effect of melatonin, and the protective effect of melatonin on some neurobehaviors and cognition may be realized through the *plppr5* pathway. Meanwhile, the results show that *plppr5* knockout aggravated hippocampal atrophy caused by HI injury, while melatonin treatment significantly alleviate the atrophy.

Nerve development is unique to the early stage. It begins during embryogenesis and continues through fetal development and the neonatal period. Childhood and adolescence are important periods for learning, memory and emotional responses development. The establishment of memory and emotional responses is initiated in this period. Several risk factors, including perinatal hypoxic-ischemia (HI), can cause severe sequelae, such as chronic behavior disorders, social disability, impaired execution, and cerebral palsy in preterm infants (54). Clinical studies have confirmed that its effects on quality of life can persist through adulthood (55).

In this study, the behavioral changes in mice in the experiment simulated cerebral palsy in children, such as

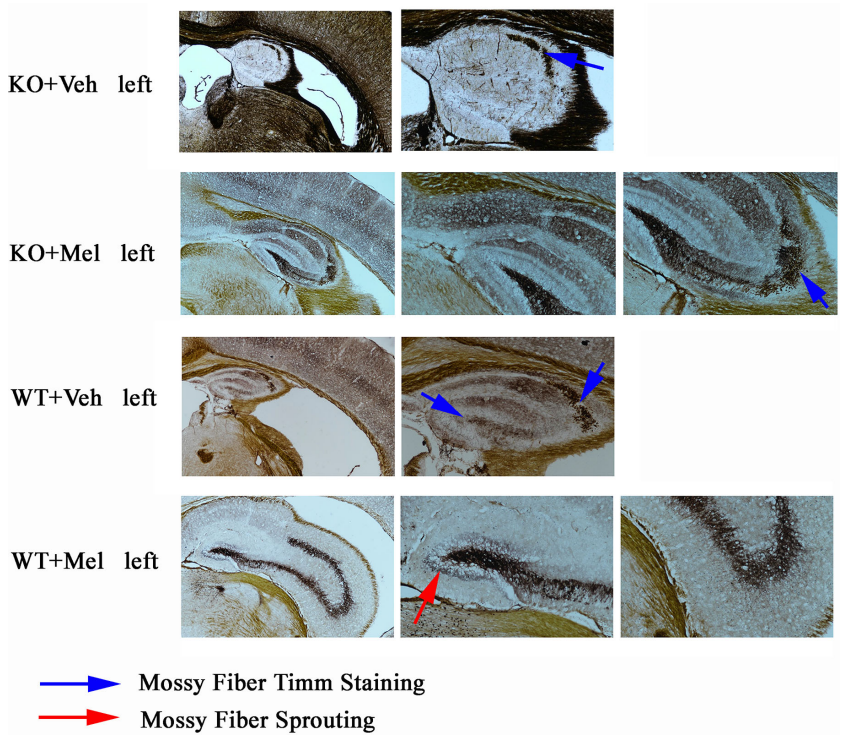

FIGURE 7 | Timm staining results of left brain. Blue arrow means moss fiber staining. Red arrow means moss fire sprouting. Magnification is 40 times for first column (four pictures) and the others are 100 times.

asymmetric limb use, gait changes, etc (49, 54). TTC staining after 24 h also confirmed unilateral carotid ligation causing lateral brain injury, which is consistent with previous studies (38, 39). We found that KO mice are more sensitive to ischemic injury than wild-type mice. Larger ischemic areas in the KO mice suggests that their neurons were more vulnerable to ischemia or abnormalities in blood flow, which needs further research. In general, *plppr5* knockout grow slower than wild-type mice with HI injury. This suggests that *plppr5* contributes to the growth and development of mice.

In most of the tests, there was no interaction between genotype and treatment, but a main effect of genotype and treatment on performance in the tests was found. What deserves our attention the most is that a significant interaction (genotype × treatment) was observed in the novel object recognition test, forelimb suspension test and the surface righting reflex test. Specifically, whether given melatonin or vehicle, mice in the WT group performed better than the KO group. Moreover, melatonin significantly reversed the poor performance in wild-type mice with HI injury but not in *plppr5*–/– mice with HI injury.

Only three of the above behavioral experiments detected interactions between genotype and treatment. The reason for these apparent discrepancies is not known; however, these tests assess different aspects of motor conditions. The novel object recognition test is a commonly used behavioral assay for the investigation of learning and memory, which relies solely on the inherent behavior of rodent searchers. Moreover, it appears to make use of a few brain regions and nervous conduction tracts. In addition, it is also commonly used to evaluate the effects of various drugs on brain damage (44).

The surface righting reflex test, which can be influenced by various factors, is a commonly used behavioral assay for motor function and coordination. As this test involves a reflex, there is no learning component, and it can be repeated throughout the experimental period. It has been reported that ischemia and hypoxia will delay the establishment of reflexes. Our experiment used P10 modeling, and testing was carried out 4 weeks after the modeling when the reflexes should have been established. The forelimb suspensiontest was used to evaluate motor function and deficits following neonatal HI. This suggests that with the current treatment regimen, melatonin improved the cognitive abilities of those mice that had relatively moderate and less severe injury and not those of mice with profound injury.

Numerous studies on the role of melatonin in various brain regions and signaling systems provide strong support for a neuroprotective role of melatonin. It was previously reported that melatonin activates protein kinase C and Rho-related kinases and induces neuritogenesis at early stages in N1E-115 cells and has the ability to regulate neuroplastic processes (56). Acute melatonin treatment increases apical dendritic length and dendritic complexity in the CA1 region (57). Bharati Sinha et al. (27) found that the expression of melatonin receptor 1 (MT1) in the brain of mice after HI injury was reduced. FJB staining showed that the neuron damage was significantly reduced, and the brain atrophy

and hippocampal atrophy area were generally reduced. The OGD model further confirmed the role of the MT1 receptor. Silvia Carloni et al. (58) injected 15 mg/kg of melatonin 5 min after HI to study the effect of melatonin on cell necrosis and autophagy in the short term. Their experimental results showed that melatonin can block the downregulation of SIRT1 after HI. There was significantly reduced cell death at 1 h after HI, revealing the connection between SIRT1 and melatonin neuroprotection.

Function of melatonin is often triggered through interactions with its membrane receptors (MT1 and MT2) which are G protein-coupled receptors. MT1 and MT2 have been localized to discrete brain areas of the rodent nervous system, including the suprachiasmatic nucleus (SCN), cerebellum, thalamus, hippocampus, and peripheral tissues (59), for example, MT1 is expressed in the dentate gyrus, CA3, CA1 regions and the subiculum of the hippocampus (one of the major areas affected in newborn H-I injury) (27).

The hippocampus is an important part of the central nervous system (CNS) and is especially vulnerable to deleterious conditions, such as ischemia, epilepsy. Previous studies have elucidated the cognitive dysfunction-attenuating capabilities of exogenous melatonin administration which is consistent with our experimental results.

It is known that the cerebellum is critically involved in learning, balance, impulse control, memory and the formation of synapses. Luciana Pinato's study showed that cerebellar can synthesize melatonin as a response to inflammation (23). Guissoni Campos, L. M. et al. reported that the effects of melatonin on cerebellum may be related to sensorimotor and neuroprotection (60). It should be mentioned that melatonin is a neuroprotective drug when cerebellar cells are attacked. However, if the normal balance of the NF-κB pathway is disturbed, melatonin can also cause cell death (61). LPS treatment resulted in the death of hippocampal and cortical neurons, but did not lead to the death of cerebellar neurons, indicating that the local production of melatonin can protect cerebellar neurons from LPS toxicity (23). In addition to its pivotal role in improving neurobehavioral deficits, Mel is able to scavenge free radicals, stimulate antioxidant defenses of the cells, and decrease the activity of pro-oxidant enzymes (62, 63).

Currently, most research indicates that *plppr5* is involved in the development and stabilization of dendritic spines (10). The body is a unified whole. Although some of our performance statistics indicated that there was no interaction between genotype and treatment, this does not mean that there was no interaction in molecular biology. Additional molecular mechanisms need further study. The *plppr5* gene is a member of the plasticity-related protein family and is strongly expressed during mouse brain development beginning on the 14th day of the embryonic period (E14), peaks at birth, and remains stable at least into early adulthood, but after brain injury, changes may occur. It affects synaptic morphology and changes the number of dendritic spines (16). *Plppr5* is involved in dendritic spine formation and stabilization. Dendritic spines are the postsynaptic components of most excitatory synapses. They are highly plastic during development, but are generally stable in adulthood. Studies have reported that their morphological or functional changes are closely related to the pathological state of the body, such as neurodegenerative diseases (10).

However, most dendritic spines in the adult cortex persist throughout life, the loss of dendritic spines after can detrimental to the functions of synaptic networks (64). That may explain that wild-type mice performed better than *plppr5* knockout mice in learning and memory.

In our experiment, melatonin was injected intraperitoneally 5 min after HI injury at 10 mg/kg and continued to be injected every 24 h for 4 weeks in order to observe the long-term effects of melatonin on cognition, learning and memory, motor ability, and neurodevelopmental reflexes. At present, the most commonly used method of drug dose conversion is body surface area-based (BSA-based) dose calculation and body weight based dose calculation. Food and Drug Administration has suggested that BSA-based dose calculation is the most appropriate method. However, when designing clinical trials, especially phase I and phase II, the dose, administration route, and timing are critical points to identify effective results. The safety, pharmacokinetics (PK), dosing, and effectiveness of melatonin in infants with HIE undergoing hypothermia is still unclear, which need further research.

What's more, melatonin can regulate autophagy. A recent study provides evidence that autophagy can regulate synaptic plasticity at CA1 synapses and serves as a significant regulator of structural plasticity, synaptic strength, and memory consolidation (65). Autophagy activation was found only in the ischemic side of the brain after HI injury (66). Consistent with our experimental results, *plppr5* knockout weakens melatonin's protection against learning and cognition. Thus, we suspect that melatonin might regulate autophagy through the plasticity-related molecules represented by *plppr5*, thereby achieving neuroprotective effects, which needs our further research to confirm.

To our knowledge, this is the first study designed to examine the interaction between *plppr5* and melatonin. A limitation of this study is that this is an one dose study (0.1 mg/10 g body weight per day, at 5 min after the surgery and once every 24 h for 4 weeks). In addition, *plppr5* knockout may have potential confounding effects on other tissues. (Neuron-specific promoters can be used to eliminate potential confusion, but our experiments did not.) Ma and colleagues' research (67) showed that melatonin was detected in the urine of mice within 24 h after oral administration of melatonin, while the littermates may consume other mice's urine while playing, which may affect their melatonin supplementation. However, due to the need to consider the sex, genotype and different treatment groups in the same litter, the influence of this factor cannot be excluded before weaning, and dividing the cage immediately after weaning can minimize its impact. This is indeed a question worth exploring, and relevant experiments can be specially designed to explore.

To summarize, melatonin was administered at a certain dose immediately after HI, which had a protective effect on the mice and could reduce later injury; at the same time, the HI injury was aggravated when *plppr5* was knocked out. These data suggest that the *plppr5* gene plays a protective role in HI injury, and it is related to the protective role of melatonin. Our results provide new insights into reducing the delayed neurological damage caused by hypoxia ischemia. Further research is needed in

regard to the forelimb suspension reflex, surface righting reflex and novel object recognition test, especially the underlying molecular mechanisms of these abnormalities.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Soochow University.

AUTHOR CONTRIBUTIONS

HN designed the study. YS and HN analyzed the data and wrote the manuscript. YS, LM, YZ, MJ, and DW were the operators of the experiment and were responsible for the statistical analysis of data. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Marret S, Vanhulle C, Laquerriere A. Pathophysiology of cerebral palsy. *Handb Clin Neurol* (2013) 111:169–76. doi: 10.1016/B978-0-444-52891-9.00016-6
2. Colver A, Fairhurst C, Pharoah PO. Cerebral palsy. *Lancet* (2014) 383:1240–9. doi: 10.1016/S0140-6736(13)61835-8
3. Coq JO, Delcour M, Massicotte VS, Baud O, Barbe MF. Prenatal ischemia deteriorates white matter, brain organization, and function: implications for prematurity and cerebral palsy. *Dev Med Child Neurol* (2016) 58 Suppl 4:7–11. doi: 10.1111/dmcn.13040
4. Kirby RS, Wingate MS, Van Naarden Braun K, Doernberg NS, Arneson CL, Benedict RE, et al. Prevalence and functioning of children with cerebral palsy in four areas of the United States in 2006: a report from the Autism and Developmental Disabilities Monitoring Network. *Res Dev Disabil* (2011) 32:462–9. doi: 10.1016/j.ridd.2010.12.042
5. Pin TW, Elmasry J, Lewis J. Efficacy of botulinum toxin a in children with cerebral palsy in gross motor function classification system levels IV and V: a systematic review. *Dev Med Child Neurol* (2013) 55:304–13. doi: 10.1111/j.1469-8749.2012.04438.x
6. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res* (2016) 61:253–78. doi: 10.1111/jpi.12360
7. Ng KY, Leong MK, Liang H, Paxinos G. Melatonin receptors: distribution in mammalian brain and their respective putative functions. *Brain Struct Funct* (2017) 222:2921–39. doi: 10.1007/s00429-017-1439-6
8. Daher I, Le Dieu-Lugon B, Lecointre M, Dupré N, Voisin C, Leroux P, et al. Time- and sex-dependent efficacy of magnesium sulfate to prevent behavioral impairments and cerebral damage in a mouse model of cerebral palsy. *Neurobiol Dis* (2018) 120:151–64. doi: 10.1016/j.nbd.2018.08.020
9. Bräuer AU, Nitsch R. Plasticity-related genes (PRGs/LRPs): a brain-specific class of lysophospholipid-modifying proteins. *Biochim Biophys Acta* (2008) 1781:595–600. doi: 10.1016/j.bbalip.2008.04.004
10. Coiro P, Stoenica L, Strauss U, Bräuer AU. Plasticity-related gene 5 promotes spine formation in murine hippocampal neurons. *J Biol Chem* (2014) 289:24956–70. doi: 10.1074/jbc.M114.597880
11. Schneider P, Petzold S, Sommer A, Nitsch R, Schwegler H, Vogt J, et al. Altered synaptic phospholipid signaling in PRG-1 deficient mice induces exploratory behavior and motor hyperactivity resembling psychiatric disorders. *Behav Brain Res* (2018) 336:1–7. doi: 10.1016/j.bbr.2017.08.032
12. Liu X, Huai J, Endle H, Schlüter L, Fan W, Li Y, et al. PRG-1 regulates synaptic plasticity via intracellular PP2A/β1-integrin signaling. *Dev Cell* (2016) 38:275–90. doi: 10.1016/j.devcel.2016.06.019
13. Ni H, Jiang YW, Xiao ZJ, Tao LY, Jin MF, Wu XR. Dynamic pattern of gene expression of ZnT-1, ZnT-3 and PRG-1 in rat brain following flurothyl-induced recurrent neonatal seizures. *Toxicol Lett* (2010) 194:86–93. doi: 10.1016/j.toxlet.2010.02.008
14. Brosig A, Fuchs J, Ipek F, Kroon C, Schrötter S, Vadhvani M, et al. The axonal membrane protein PRG2 inhibits PTEN and directs growth to branches. *Cell Rep* (2019) 29:2028–40.e8. doi: 10.1016/j.celrep.2019.10.039
15. Broggini T, Schnell L, Ghoochani A, Mateos JM, Buchfelder M, Wiendieck K, et al. Plasticity related gene 3 (PRG3) overcomes myelin-associated growth inhibition and promotes functional recovery after spinal cord injury. *Aging (Albany NY)* (2016) 8:2463–87. doi: 10.18632/aging.101066
16. Broggini T, Nitsch R, Savaskan NE. Plasticity-related gene 5 (PRG5) induces filopodia and neurite growth and impedes lysophosphatidic acid- and nogo-A-mediated axonal retraction. *Mol Biol Cell* (2010) 21:521–37. doi: 10.1091/mbc.e09-06-0506
17. Ni H, Ren SY, Zhang LL, Sun Q, Tian T, Feng X. Expression profiles of hippocampal regenerative sprouting-related genes and their regulation by E-64d in a developmental rat model of penicillin-induced recurrent epilepticus. *Toxicol Lett* (2013) 217:162–9. doi: 10.1016/j.toxlet.2012.12.010
18. Tan F, Wang J, Liu JX, Wang C, Li M, Gu Y. Electroacupuncture stimulates the proliferation and differentiation of endogenous neural stem cells in a rat model of ischemic stroke. *Exp Ther Med* (2018) 16:4943–50. doi: 10.3892/etm.2018.6848
19. Chollat C, Marret S. Magnesium sulfate and fetal neuroprotection: overview of clinical evidence. *Neural Regener Res* (2018) 13:2044–9. doi: 10.4103/1673-5374.241441
20. Sargin D, Friedrichs H, El-Kordi A, Ehrenreich H. Erythropoietin as neuroprotective and neuroregenerative treatment strategy: comprehensive overview of 12 years of preclinical and clinical research. *Best Pract Res Clin Anaesthesiol* (2010) 24:573–94. doi: 10.1016/j.bpa.2010.10.005
21. Xiong T, Qu Y, Mu D, Ferriero D. Erythropoietin for neonatal brain injury: opportunity and challenge. *Int J Dev Neurosci* (2011) 29:583–91. doi: 10.1016/j.ijdevneu.2010.12.007
22. Markus RP, Cecon E, Pires-Lapa MA. Immune-pineal axis: nuclear factor κB (NF-kB) mediates the shift in the melatonin source from pinealocytes to immune competent cells. *Int J Mol Sci* (2013) 14:10979–97. doi: 10.3390/ijms140610979
23. Pinato L, da Silveira Cruz-Machado S, Franco DG, Campos LM, Cecon E, Fernandes PA, et al. Selective protection of the cerebellum against intracerebroventricular LPS is mediated by local melatonin synthesis. *Brain Struct Funct* (2015) 220:827–40. doi: 10.1007/s00429-013-0686-4
24. Mukherjee S, Maitra SK. Gut Melatonin in Vertebrates: Chronobiology and Physiology. *Front Endocrinol (Lausanne)* (2015) 6:112. doi: 10.3389/fendo.2015.00112
25. Hayter CL, Bishop GM, Robinson SR. Pharmacological but not physiological concentrations of melatonin reduce iron-induced neuronal death in rat cerebral cortex. *Neurosci Lett* (2004) 362:182–4. doi: 10.1016/j.neulet.2004.02.024

26. Ni H, Sun Q, Tian T, Feng X, Sun BL. Long-term expression of metabolism-associated genes in the rat hippocampus following recurrent neonatal seizures and its regulation by melatonin. *Mol Med Rep* (2015) 12:2727–34. doi: 10.3892/mmr.2015.3691
27. Sinha B, Wu Q, Li W, Tu Y, Sirianni AC, Chen Y, et al. Protection of melatonin in experimental models of newborn hypoxic-ischemic brain injury through MT1 receptor. *J Pineal Res* (2018) 64:e12443. doi: 10.1111/jpi.12443
28. Naskar A, Manivasagam T, Chakraborty J, Singh R, Thomas B, Dhanasekaran M, et al. Melatonin synergizes with low doses of L-DOPA to improve dendritic spine density in the mouse striatum in experimental Parkinsonism. *J Pineal Res* (2013) 55:304–12. doi: 10.1111/jpi.12076
29. Chakraborty J, Nthenge-Ngumbau DN, Rajamma U, Mohanakumar KP. Melatonin protects against behavioural dysfunctions and dendritic spine damage in 3-nitropropionic acid-induced rat model of Huntington's disease. *Behav Brain Res* (2014) 264:91–104. doi: 10.1016/j.bbr.2014.01.048
30. Nobili A, Krashia P, Cordella A, La Barbera L, Dell'Acqua MC, Caruso A, et al. Ambra1 Shapes Hippocampal Inhibition/Excitation Balance: Role in Neurodevelopmental Disorders. *Mol Neurobiol* (2018) 55:7921–40. doi: 10.1007/s12035-018-0911-5
31. Companys-Alemany J, Turcu AL, Bellver-Sanchis A, Loza MI, Brea JM, Canudas AM, et al. A Novel NMDA Receptor Antagonist Protects against Cognitive Decline Presented by Senescent Mice. *Pharmaceutics* (2020) 12:284. doi: 10.3390/pharmaceutics12030284
32. Cikla U, Chanana V, Kintner DB, Udho E, Eickhoff J, Sun W, et al. ERα Signaling Is Required for TrkB-Mediated Hippocampal Neuroprotection in Female Neonatal Mice after Hypoxic Ischemic Encephalopathy(1,2,3). *eNeuro* (2016) 3:ENEURO.0025-15.2015. doi: 10.1523/ENEURO.0025-15.2015
33. Hishiyama S, Kotoda M, Ishiyama T, Mitsui K, Matsukawa T. Neuroprotective effects of neurotropin in a mouse model of hypoxic-ischemic brain injury. *J Anesth* (2019) 33:495–500. doi: 10.1007/s00540-019-02655-z
34. Rice JE3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* (1981) 9:131–41. doi: 10.1002/ana.410090206
35. Rodríguez-Fanjul J, Durán Fernández-Feijóo C, Lopez-Abad M, Lopez Ramos MG, Balada Caballé R, Alcántara-Horillo S, et al. Neuroprotection with hypothermia and allopurinol in an animal model of hypoxic-ischemic injury: Is it a gender question. *PLoS One* (2017) 12:e0184643. doi: 10.1371/journal.pone.0184643
36. Sharma HS, Muresanu DF, Nozari A, Castellani RJ, Dey PK, Wiklund L, et al. Anesthetics influence concussive head injury induced blood-brain barrier breakdown, brain edema formation, cerebral blood flow, serotonin levels, brain pathology and functional outcome. *Int Rev Neurobiol* (2019) 146:45–81. doi: 10.1016/bs.irn.2019.06.006
37. Omur AD, Yildirim B, Saglam YS, Comakli S, Ozkaraca M. Activity of resveratrol on the influence of aflatoxin B1 on the testes of Sprague dawley rats. *Pol J Vet Sci* (2019) 22:313–20. doi: 10.24425/pjvs.2019.129222
38. Wong R, Abussaud A, Leung JW, Xu BF, Li FY, Huang S, et al. Blockade of the swelling-induced chloride current attenuates the mouse neonatal hypoxic-ischemic brain injury in vivo. *Acta Pharmacol Sin* (2018) 39:858–65. doi: 10.1038/aps.2018.1
39. Tu L, Wang Y, Chen D, Xiang P, Shen J, Li Y, et al. Protective Effects of Notoginsenoside R1 via Regulation of the PI3K-Akt-mTOR/JNK Pathway in Neonatal Cerebral Hypoxic-Ischemic Brain Injury. *Neurochem Res* (2018) 43:1210–26. doi: 10.1007/s11064-018-2538-3
40. Xie D, Ge X, Ma Y, Tang J, Wang Y, Zhu Y, et al. Clemastine improves hypomyelination in rats with hypoxic-ischemic brain injury by reducing microglia-derived IL-1β via P38 signaling pathway. *J Neuroinflamm* (2020) 17:57. doi: 10.1186/s12974-019-1662-6
41. Naik AA, Patro IK, Patro N. Slow Physical Growth, Delayed Reflex Ontogeny, and Permanent Behavioral as Well as Cognitive Impairments in Rats Following Intra-generational Protein Malnutrition. *Front Neurosci* (2015) 9:446. doi: 10.3389/fnins.2015.00446
42. Ch S, Reddy PS. Prenatal exposure to aflatoxin B1: developmental, behavioral, and reproductive alterations in male rats. *Naturwissenschaften* (2015) 102:26. doi: 10.1007/s00114-015-1274-7
43. Chen NN, Zhao DJ, Sun YX, Wang DD, Ni H. Long-Term Effects of Zinc Deficiency and Zinc Supplementation on Developmental Seizure-Induced Brain Damage and the Underlying GPR39/ZnT-3 and MBP Expression in the Hippocampus. *Front Neurosci* (2019) 13:920. doi: 10.3389/fnins.2019.00920
44. Lueptow LM. Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. *J Vis Exp* (2017) 126:55718. doi: 10.3791/55718
45. Kuniishi H, Ichisaka S, Yamamoto M, Ikubo N, Matsuda S, Futora E, et al. Early deprivation increases high-leaning behavior, a novel anxiety-like behavior, in the open field test in rats. *Neurosci Res* (2017) 123:27–35. doi: 10.1016/j.neures.2017.04.012
46. Wang GW, Cai JX. Reversible disconnection of the hippocampal-prelimbic cortical circuit impairs spatial learning but not passive avoidance learning in rats. *Neurobiol Learn Mem* (2008) 90:365–73. doi: 10.1016/j.nlm.2008.05.009
47. Karimipour M, Rahbarghazi R, Tayefi H, Shimia M, Ghanadian M, Mahmoudi J, et al. Quercetin promotes learning and memory performance concomitantly with neural stem/progenitor cell proliferation and neurogenesis in the adult rat dentate gyrus. *Int J Dev Neurosci* (2019) 74:18–26. doi: 10.1016/j.ijdevneu.2019.02.005
48. Zhang Y, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A, et al. Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg* (2015) 122:856–67. doi: 10.3171/2014.11.JNS14770
49. Ohshima M, Taguchi A, Sato Y, Ogawa Y, Saito S, Yamahara K, et al. Evaluations of Intravenous Administration of CD34+ Human Umbilical Cord Blood Cells in a Mouse Model of Neonatal Hypoxic-Ischemic Encephalopathy. *Dev Neurosci* (2016) 38:331–41. doi: 10.1159/000454830
50. Li LL, Li YC, Zhao DJ, Jin MF, Ni H. Leptin-regulated autophagy plays a role in long-term neurobehavioral injury after neonatal seizures and the regulation of zinc/cPLA2 and CaMK II signaling in cerebral cortex. *Epilepsy Res* (2018) 146:103–11. doi: 10.1016/j.eplepsyres.2018.07.023
51. Hsu M, Buzsáki G. Vulnerability of mossy fiber targets in the rat hippocampus to forebrain ischemia. *J Neurosci* (1993) 13:3964–79. doi: 10.1523/JNEUROSCI.13-09-03964.1993
52. Ni H, Jiang YW, Tao LY, Jin MF, Wu XR. ZnT-1, ZnT-3, CaMK II, PRG-1 expressions in hippocampus following neonatal seizure-induced cognitive deficit in rats. *Toxicol Lett* (2009) 184:145–50. doi: 10.1016/j.toxlet.2008.11.003
53. Yang Y, Jing XP, Zhang SP, Gu RX, Tang FX, Wang XL, et al. High dose zinc supplementation induces hippocampal zinc deficiency and memory impairment with inhibition of BDNF signaling. *PLoS One* (2013) 8:e55384. doi: 10.1371/journal.pone.0055384
54. Crowther CA, Middleton PF, Voysey M, Askie L, Duley L, Pryde PG, et al. Assessing the neuroprotective benefits for babies of antenatal magnesium sulphate: An individual participant data meta-analysis. *PLoS Med* (2017) 14:e1002398. doi: 10.1371/journal.pmed.1002398
55. Douglas-Escobar M, Weiss MD. Hypoxic-ischemic encephalopathy: a review for the clinician. *JAMA Pediatr* (2015) 169:397–403. doi: 10.1001/jamapediatrics.2014.3269
56. Bellon A, Ortíz-López L, Ramírez-Rodríguez G, Antón-Tay F, Benítez-King G. Melatonin induces neuritogenesis at early stages in N1E-115 cells through actin rearrangements via activation of protein kinase C and Rho-associated kinase. *J Pineal Res* (2007) 42:214–21. doi: 10.1111/j.1600-079X.2006.00408.x
57. Ikeno T, Nelson RJ. Acute melatonin treatment alters dendritic morphology and circadian clock gene expression in the hippocampus of Siberian hamsters. *Hippocampus* (2015) 25:142–8. doi: 10.1002/hipo.22358
58. Carloni S, Riparini G, Buonocore G, Balduini W. Rapid modulation of the silent information regulator 1 by melatonin after hypoxia-ischemia in the neonatal rat brain. *J Pineal Res* (2017) 63:e12434. doi: 10.1111/jpi.12434
59. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. MT1 and MT2 Melatonin Receptors: A Therapeutic Perspective. *Annu Rev Pharmacol Toxicol* (2016) 56:361–83. doi: 10.1146/annurev-pharmtox-010814-124742
60. Guissoni Campos LM, Hataka A, Vieira IZ, Buchaim RL, Robalinho IF, Arantes G, et al. Circadian Clock Proteins and Melatonin Receptors in Neurons and Glia of the Sapajus apella Cerebellum. *Front Physiol* (2018) 9:5. doi: 10.3389/fphys.2018.00005
61. Franco DG, Markus RP. The cellular state determines the effect of melatonin on the survival of mixed cerebellar cell culture. *PLoS One* (2014) 9:e106332. doi: 10.1371/journal.pone.0106332
62. O'Neill LA, Kaltschmidt C. NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci* (1997) 20:252–8. doi: 10.1016/s0166-2236(96)01035-1

63. Lamtai M, Azirar S, Zghari O, Ouakki S, El Hessni A, Mesfioui A, et al. Melatonin Ameliorates Cadmium-Induced Affective and Cognitive Impairments and Hippocampal Oxidative Stress in Rat. *Biol Trace Elem Res* (2020). doi: 10.1007/s12011-020-02247-z
64. Yang G, Pan F, Gan WB. Stably maintained dendritic spines are associated with lifelong memories. *Nature* (2009) 462:920–4. doi: 10.1038/nature08577
65. Nikoletopoulou V, Tavernarakis N. Regulation and Roles of Autophagy at Synapses. *Trends Cell Biol* (2018) 28:646–61. doi: 10.1016/j.tcb.2018.03.006
66. Sadigh-Eteghad S, Geranmayeh MH, Majdi A, Salehpour F, Mahmoudi J, Farhoudi M. Intranasal cerebrolysin improves cognitive function and structural synaptic plasticity in photothrombotic mouse model of medial prefrontal cortex ischemia. *Neuropeptides* (2018) 71:61–9. doi: 10.1016/j.npep.2018.07.002
67. Ma X, Idle JR, Krausz KW, Tan DX, Ceraulo L, Gonzalez FJ. Urinary metabolites and antioxidant products of exogenous melatonin in the mouse. *J Pineal Res* (2006) 40:343–9. doi: 10.1111/j.1600-079X.2006.00321.x

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