The Effect of *ARHGEF15* Knockout and Forebrain-Specific Human *ARHGEF15* Knock-In on Seizure Susceptibility in Mice

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Abstract

Background

The *ARHGEF15* gene encodes the Rho guanine nucleotide exchange factor 15. Although multiple evidence indicates that *ARHGEF15* may be related to epilepsy, it is not clear what role it plays.

Methods

Subjects were homozygous *ARHGEF15* knockout ($E5^{-/-}$) mice, wild-type (WT) mice, Cre-positive homozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/CKI}$•Cre+) mice which can overexpress human Ephexin5 in the forebrain, and Cre-positive ($E5^{WT/WT}$•Cre+) mice. Models with epileptic seizures were established by intraperitoneal injection of pentylenetetrazol (PTZ) in 60 mg/kg, and seizures were recorded by video. Then 7 indexes were counted, including “maximum seizure level”, “seizure level classification”, “tonic-clonic seizure or not”, “latency of tonic-clonic seizure”, “number of times of tonic-clonic seizure”, “total duration of tonic-clonic seizure”, and “dead or not”. Western blot was used to detect Ephexin5, RhoA, p-RhoA, ROCK2, and p-ROCK2 in WT mice and $E5^{-/-}$ mice.

Results

Compared with WT mice, $E5^{-/-}$ mice had a shorter “total duration of tonic-clonic seizure”. For levels of RhoA, p-RhoA, ROCK2 and p-ROCK2 in the adult hippocampus, there was no significant difference between WT and $E5^{-/-}$. The level of Ephexin5 was high in the whole brain tissue of 2-day-old WT mice, and an extremely low expression of Ephexin5 was detected in the brain tissue of $E5^{-/-}$ mice of the same age. There was no significant difference between $E5^{CKI/CKI}$•Cre + mice and $E5^{WT/WT}$•Cre + mice in seizures.

Conclusions

ARHGEF15 knockout can decrease the “duration of tonic-clonic seizure” in adult mice with epileptic seizures induced by PTZ. *ARHGEF15* knockout did not affect the expression of the RhoA-ROCK2 pathway in the hippocampus of adult mice, which probably attributed to no expression of Ephexin5 in the hippocampus of adult mice. The overexpression of human Ephexin5 in the forebrain has no significant effect on the behavior of epileptic seizures induced by PTZ in adult mice.

Background

In recent years, the research on the genetic mechanism of epilepsy increased rapidly, exploring new insights for the pathogenesis of epilepsy and providing evidence for identifying potential therapeutic targets [1]. With the application of high throughput sequencing in clinical practice, in addition to epilepsy-

associated genes that have been identified, some genes that probably be related to epilepsy are found [2]. The *ARHGEF15* gene is located on human chromosome 17 and encodes the Rho guanine nucleotide exchange factor 15, also known as Ephexin5, Vsm-RhoGEF, which has guanine nucleotide exchange activity and can regulate the conversion of Rho GTPase from inactive type to active type [3], so that Rho GTPase can play a molecular switch role in cell signal transduction pathways [4].

Previous studies have shown that Ephexin5 plays an important role in the central nervous system. Sahin et al. proved that Ephexin5 is highly expressed in the brain and it has a high homology with the encoding gene of Ephexin1 in mammals [5]. Margolis et al. found that Ephexin5 could limit the formation of excitatory synapses by activating RhoA, a kind of Rho GTPases [6]. Additionally, Hamilton et al. revealed that Ephexin5 plays a dual role in spinogenesis, which is not only a brake on overall spine outgrowth, but also an essential component in the site-specific formation of new spines [7]. Moreover, the study of Ephexin5 in Alzheimer's disease has achieved an initial result that reducing the expression of the synapse inhibitory protein Ephexin5 can ameliorate Alzheimer's-like impairment in mice [8].

In 2013, a possible link between the *ARHGEF15* gene and epilepsy was reported for the first time [9]. Researchers found a de novo mutation in the *ARHGEF15* gene in a patient with epileptic encephalopathy by the whole-exome sequencing (WES). In vitro analyses using cell-based assays revealed that the mutation in *ARHGEF15* reduced the guanine nucleotide exchange activity of Ephexin5 by about 50% [9]. In a study of copy number variation (CNV) risk factors for rolandic epilepsy, duplicate copies of *ARHGEF15* were found in one patient. Then the researchers performed a network analysis of all the variants detected and found that the variants were significantly enriched in guanine exchange factor activation pathways [10]. Besides, Ephexin5 is ubiquitinated with the assistant of E6AP, the protein product of the *UBE3A* gene, and then degraded by the proteasome. The deficiency of E6AP leads to Angelman syndrome (AS), which is clinically characterized by a lack of speech, abnormal motor development, and epileptic seizures [11]. Although multiple evidence indicates that the function of the *ARHGEF15* gene may be related to epilepsy, it is not clear what role it plays.

To study whether the *ARHGEF15* gene can participate in epilepsy, we generated *ARHGEF15* knockout ($E5^{-/-}$) mice and Cre-positive homozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/CKI}$•Cre+) mice to explore the effect of *ARHGEF15* knockout and forebrain-specific human *ARHGEF15* knock-in on seizure susceptibility in mice. Considering Ephexin5 can activate RhoA specifically [6], and that its downstream signaling molecule ROCK2 [12], was increased in epileptic foci of temporal lobe epilepsy (TLE) patients [13], we also investigated the expression of the RhoA-ROCK2 pathway in the $E5^{-/-}$ mice.

Methods

Animals

Mice were sourced from Cyagen Biosciences Inc. (Guangzhou, China), and housed at the Laboratory Animal Center of Jining Medical University. All animal experiments comply with the REACT guidelines

and ARRIVE guidelines. All experiments are conducted in accordance with the UK Animal (Scientific Procedures) Act (1986) and relevant guidelines. Heterozygous *ARHGEF15* knockout ($E5^{+/-}$) mice, with a C57BL/6 background, were constructed by knocking out the sequence containing exon 2 to exon 8 in the *ARHGEF15* gene causing a frameshift mutation and an early termination, and then bred to obtain homozygous *ARHGEF15* knockout ($E5^{-/-}$) mice and wild-type (WT) mice which were used as the control.

Heterozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/WT}$) mice containing the "CAG promoter-loxP-stop-loxP-kozak-human ARHGEF15 CDS-polyA" in the genome, with a C57BL/6 background, were constructed and bred to obtain homozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/CKI}$) mice. At this time, the human *ARHGEF15* sequence in $E5^{CKI/WT}$ mice and $E5^{CKI/CKI}$ mice can not be transcribed, because the "loxP-stop-loxP" was inserted to separate the CAG promoter and the kozak-human ARHGEF15 CDS. Then $E5^{CKI/CKI}$ mice were bred with Camk2a-cre mice which can drive Cre recombinase expression in the forebrain, including the CA1 pyramidal cell layer of the hippocampus (http://jaxmice.jax.org/strain/005359.html) to obtain Cre-positive heterozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/WT}$•Cre+) mice. Inbreeding of $E5^{CKI/WT}$•Cre + mice produced Cre-positive homozygous human *ARHGEF15* conditional knock-in ($E5^{CKI/CKI}$•Cre+) mice and Cre-positive ($E5^{WT/WT}$•Cre+) mice without human *ARHGEF15* conditional knock-in. $E5^{CKI/WT}$•Cre + mice and $E5^{CKI/CKI}$•Cre + mice expressed the Cre recombinase acting on loxP sites to knock out "loxP-stop-loxP", so the CAG promoter was connected with the kozak-human ARHGEF15 CDS and the mice can express human ARHGEF15 protein (human Ephexin5) in the forebrain specifically.

To identify genotypes of offspring mice, the nails were cut at 10 days after birth, and the DNA was extracted, and then PCR and agarose gel electrophoresis were performed (Fig. S1-3).

Testing Seizure Susceptibility

Models with epileptic seizures were established by intraperitoneal injection of pentylenetetrazol (PTZ, Sigma) in 60 mg/kg [14]. Behavior changes in mice of different genotypes were recorded by video within half an hour after drug injection. The seizures of the mice were evaluated according to a revised Racine's scale as follows: stage 0, behavioral arrest; stage 1, mouth clonus; stage 2, head bobbing; stage 3, unilateral forelimb clonus; stage 3.5, alternating forelimb clonus; stage 4, bilateral forelimb clonus with rearing; stage 5, bilateral forelimb clonus with rearing and falling over; stage 6, wild running and jumping with vocalization; stage 7, tonic-clonic seizure [15]. Then we counted 7 indexes according to the video, including "maximum seizure level", "seizure level classification (severe seizure: maximum seizure level ≥ stage 4, mild seizure: maximum seizure level < stage 4)", "tonic-clonic seizure or not", "latency of tonic-clonic seizure", "number of times of tonic-clonic seizure", "total duration of tonic-clonic seizure", and "dead or not". Spontaneous epileptic seizures were not observed in all mice. Mice were euthanized by cervical dislocation.

Western Blot

All the mice applied to detect the expression level of the Ephexin5, RhoA-Rock2 pathway were not injected with PTZ. Mice were anesthetized by intraperitoneal injection of 1.25% tribromoethanol (Sigma) in 0.2ml/10g. Then the brain was taken and the hippocampus was detached. A mixture of 495 μl RIPA lysis buffer and 5 μl PMSF (Beyotime Biotechnology) was added to every 100 mg tissue and then homogenized by an ultrasonic processor. EP tubes with tissue suspension were placed on ice for 30 min, and then centrifuged at 14,000 RPM, 4 ℃ for 20 min. Then the supernatant was transferred into a new EP tube. The protein concentration was measured using BCA Protein Assay Kit (Beyotime Biotechnology). After SDS-PAGE, proteins were transferred to PVDF membranes (Millipore). 5% milk-TBST was used to block the membranes. Then the membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies were as the following: anti-Ephexin5 antibody (Rabbit, Abclonal, raised against a recombinant fragment corresponding to amino acids 140–380 of mouse Ephexin5), anti-human Ephexin5 antibody (Rabbit, Abcam-ab221376), anti-RhoA antibody (Rabbit, Abclonal-A13947), anti-p-RhoA antibody (Rabbit, Abcam-ab41435), anti-ROCK2 antibody (Rabbit, Abclonal-A5698), anti-p-ROCK2 antibody (Rabbit, Abcam-ab228008), anti-ACTB antibody (Rabbit, Abclonal-AC026). HRP Goat Anti-Rabbit IgG (Abclonal-AS014) was used as the secondary antibody.

Statistics

The data of seizure susceptibility were analyzed with IBM SPSS Statistics 25 software. The independent-samples T-test was used to compare the measurement data (“maximum seizure level”, “latency of tonic-clonic seizure”, “number of times of tonic-clonic seizure”, “total duration of tonic-clonic seizure”), and the homogeneity of variance was detected by Levene's tests. Chi-square test was used to compare the counting data (“seizure level classification”, “tonic-clonic seizure or not”, “dead or not”). The level of significance was preset to $P < .05$.

Image J software was used to calculate the results of Western blot, and GraphPad Prism 8 software was used to make statistical graphs. The independent-samples T-test was used to compare the expression of proteins between two groups. The level of significance was preset to $P < .05$.

Results

The seizure susceptibility of the ARHGEF15 knockout mice

The experimental group was $E5^{-/-}$ mice, and the control group was WT mice. There were 6 males and 6 females in each group, and all were 8–10 weeks old. Compared with WT mice, $E5^{-/-}$ mice had a shorter “total duration of tonic-clonic seizure” ($P < .05$). There were no significant differences in “maximum seizure level”, “latency of tonic-clonic seizure”, “number of times of tonic-clonic seizure”, “seizure level classification”, “tonic-clonic seizure or not”, and “dead or not” between the two groups (Table 1, Table 2).

Table 1
The seizure susceptibility of the *ARHGEF15* knockout mice (measurement data, $\bar{x}\pm S$).

	WT mice (n = 12)	$E5^{-/-}$ mice (n = 12)	P value
Maximum seizure level	6.50 ± 1.24	6.13 ± 1.65	0.536
Latency of tonic-clonic seizure (s)	230.60 ± 141.78	210.75 ± 88.56	0.735
Number of times of tonic-clonic seizure	0.83 ± 0.39	0.67 ± 0.50	0.368
Total duration of tonic-clonic seizure (s)	18.33 ± 12.00	8.25 ± 6.73	0.019*

*P < .05

Table 2
The seizure susceptibility of the *ARHGEF15* knockout mice (counting data, rate).

	WT mice (n = 12)	$E5^{-/-}$ mice (n = 12)	P value
Seizure level classification (Severe seizure rate)	91.67%	83.33%	0.537
Tonic-clonic seizure or not (Tonic-clonic seizure rate)	83.33%	66.67%	0.346
Dead or not (Death rate)	25.00%	8.33%	0.273

The seizure susceptibility of the forebrain-specific human ARHGEF15 **knock-in mice**

The experimental group was $E5^{CKI/CKI}$•Cre + mice, and the control group was $E5^{WT/WT}$•Cre + mice. There were 6 males and 6 females in each group, and all were 8–10 weeks old. There were no significant differences between the two groups in all the 7 indexes including “maximum seizure level”, “latency of tonic-clonic seizure”, “number of times of tonic-clonic seizure”, “total duration of tonic-clonic seizure”, “seizure level classification”, “tonic-clonic seizure or not”, and “dead or not” (Table 3, Table 4).

Table 3
The seizure susceptibility of the forebrain-specific human *ARHGEF15* knock-in mice (measurement data, $\bar{x}\pm S$).

	$E5^{WT/WT}$•Cre + mice (n = 12)	$E5^{CKI/CKI}$•Cre + mice (n = 12)	P value
Maximum seizure level	6.67 ± 0.78	6.75 ± 0.87	0.807
Latency of tonic-clonic seizure (s)	304.20 ± 357.30	179.55 ± 179.05	0.338
Number of times of tonic-clonic seizure	1.08 ± 0.67	1.33 ± 0.78	0.408
Total duration of tonic-clonic seizure (s)	34.08 ± 29.66	66.17 ± 58.74	0.110

Table 4
The seizure susceptibility of the forebrain-specific human *ARHGEF15* knock-in mice (counting data, rate).

	$E5^{WT/WT}$•Cre + mice (n = 12)	$E5^{CKI/CKI}$•Cre + mice (n = 12)	P value
Seizure level classification (Severe seizure rate)	100.00%	100.00%	—
Tonic-clonic seizure or not (Tonic-clonic seizure rate)	83.33%	91.67%	0.537
Dead or not (Death rate)	33.33%	58.33%	0.219

The expression of Ephexin5 in the ARHGEF15 **knockout mice and the forebrain-specific human** ARHGEF15 **knock-in mice**

We used western blot to detect the expression level of Ephexin5 in $E5^{-/-}$ mice and WT mice. At 2 days after birth, the expression of Ephexin5 was detected in the whole brain tissue of WT mice, but almost no Ephexin5 was expressed in the brain of $E5^{-/-}$ mice at the same age. In adult mice aged 8–10 weeks, Ephexin5 was not expressed in the hippocampus of WT mice and $E5^{-/-}$ mice (Fig. 1a). Western blot was also used to detect the expression of human Ephexin5 in the hippocampus of $E5^{WT/WT}$•Cre + mice, $E5^{CKI/WT}$•Cre + mice, $E5^{CKI/CKI}$•Cre + mice, and $E5^{CKI/CKI}$ mice aged 8–10 weeks. As a result, $E5^{CKI/WT}$•Cre + mice and $E5^{CKI/CKI}$•Cre + mice carrying both the human *ARHGEF15* gene and the *Cre* gene expressed human Ephexin5 in the hippocampus, while $E5^{WT/WT}$•Cre + mice with the *Cre* gene only and $E5^{CKI/CKI}$ mice with the human *ARHGEF15* gene only expressed no human Ephexin5 in the hippocampus (Fig. 1b).

3.4 The expression of the RhoA-ROCK2 pathway in the *ARHGEF15* knockout mice

The expression of the RhoA-ROCK2 pathway in the hippocampus of 8–10 weeks mice was detected by western blot. As a result, the levels of RhoA, p-RhoA, ROCK2, and p-ROCK2 were not significantly different between WT mice and $E5^{-/-}$ mice (Fig. 2).

Discussion

In this study, to explore whether the *ARHGEF15* gene can participate in epilepsy, the *ARHGEF15* knockout mice were constructed. In the seizure susceptibility experiment, because none of the mice had spontaneous epileptic seizures, the PTZ-induced epileptic animal model was selected, which is used widely in investigating the pathophysiology of epilepsy and related genes [16]. Compared to WT mice, $E5^{-/-}$ mice had no other significant difference in seizures induced by PTZ, except a shorter “total duration of tonic-clonic seizure”, indicating that *ARHGEF15* knockout can decrease “duration of tonic-clonic seizure” in adult mice with epileptic seizures induced by PTZ, but it cannot influence other indexes including “maximum seizure level”, “latency of tonic-clonic seizure”, “number of times of tonic-clonic seizure”, “seizure level classification”, “tonic-clonic seizure or not”, and “dead or not”.

The level of Ephexin5 was high in the whole brain tissue of 2-day-old WT mice, and an extremely low expression of Ephexin5 was detected in the brain tissue of $E5^{-/-}$ mice of the same age, but no expression of Ephexin5 was found in the adult hippocampus of the two genotypes, illuminating that the level of Ephexin5 in the brain tissue of mice is age-dependent, which was consistent with the study of Margolis et al. [6]. In the brain of WT mice, Ephexin5 was expressed during 12 days of the embryonic period to 21 days after birth [6].

In previous studies, Ephexin5 can activate RhoA specifically [6], a kind of Rho GTPases, and the expression of ROCK2, a downstream signaling molecule of RhoA [12], was increased in epileptic foci of temporal lobe epilepsy (TLE) patients [13]. In this study, we investigated the expression of the RhoA-ROCK2 pathway in the hippocampus of WT mice and $E5^{-/-}$ mice. However, there was no significant difference in levels of RhoA, p-RhoA, ROCK2, and p-ROCK2 in the adult hippocampus between WT and $E5^{-/-}$, which probably attributes to no expression of Ephexin5 in the hippocampus of adult mice. Considering *ARHGEF15* knockout did not affect the expression of the RhoA-ROCK2 pathway in the hippocampus of adult mice, although Ephexin5 was demonstrated to be a RhoA guanine nucleotide exchange factor [6], we could not conclude that the effect of *ARHGEF15* knockout on “duration of tonic-clonic seizure” in adult mice was mediated by the RhoA-ROCK2 pathway. Accordingly, we speculated that during the embryonic period and early childhood when Ephexin5 can express in the brain, *ARHGEF15* knockout may cause some changes in the brain of mice, thus affecting the seizure susceptibility in adulthood. In addition to Ephexin5, other members of the Ephexin family such as Ephexin1 can also activate RhoA in neurons [5]. Perhaps after the *ARHGEF15* gene knockout, the body's negative feedback regulatory function could maintain the RhoA activation at a normal level by enhancing the expression of other Ephexins.

Moreover, to explore the direct effect of *ARHGEF15* gene expression product, Ephexin5, on seizure susceptibility in mice, we constructed $E5^{CKI/CKI}$•Cre + mice which can significantly produce human Ephexin5 in the forebrain and found that there was no significant difference between $E5^{CKI/CKI}$•Cre + mice and $E5^{WT/WT}$•Cre + mice in seizures induced by PTZ, indicating that the overexpression of human Ephexin5 in the forebrain has no significant effect on the behavior of epileptic seizures induced by PTZ in adult mice. However, no significant difference in behavior does not mean no difference in electrophysiological activity.

Conclusions

In conclusion, the effect of the *ARHGEF15* knockout and the effect of the forebrain-specific human *ARHGEF15* knock-in on the seizure susceptibility in mice still need to be verified by electrophysiological activity tests, such as the video electroencephalography. The mechanism for the differences in “duration of tonic-clonic seizure” induced by PTZ between WT mice and $E5^{-/-}$ mice also needs to be further explored.

Abbreviations

WES, whole-exome sequencing; CNV, copy number variation; AS, Angelman syndrome; E5-/-, homozygous ARHGEF15 knockout; E5CKI/CKI•Cre+, Cre-positive homozygous human ARHGEF15 conditional knock-in; TLE, temporal lobe epilepsy; E5+/-, heterozygous ARHGEF15 knockout; WT, wild-type; E5CKI/WT, heterozygous human ARHGEF15 conditional knock-in; E5CKI/CKI, homozygous human ARHGEF15 conditional knock-in; E5CKI/WT•Cre+, Cre-positive heterozygous human ARHGEF15 conditional knock-in; E5WT/WT•Cre+, Cre-positive; PTZ, pentylenetetrazol.

Declarations

Ethics approval and consent to participate

All experiments were conducted with approval from the Ethics Committee of Jining Medical University (Project identification code: 2019-FY-021).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YDL, MJD, QXK, YLW, and YLH designed the present study. YLH, YY, and YKZ guided the research methods. YDL, MYM, and JHF completed the experiments. YDL, ZCL, MYM, and YLW conducted the data analysis. YDL and MYM wrote the main manuscript text. QXK and YLW revised the manuscript. All authors read and approved the final manuscript.

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Figures

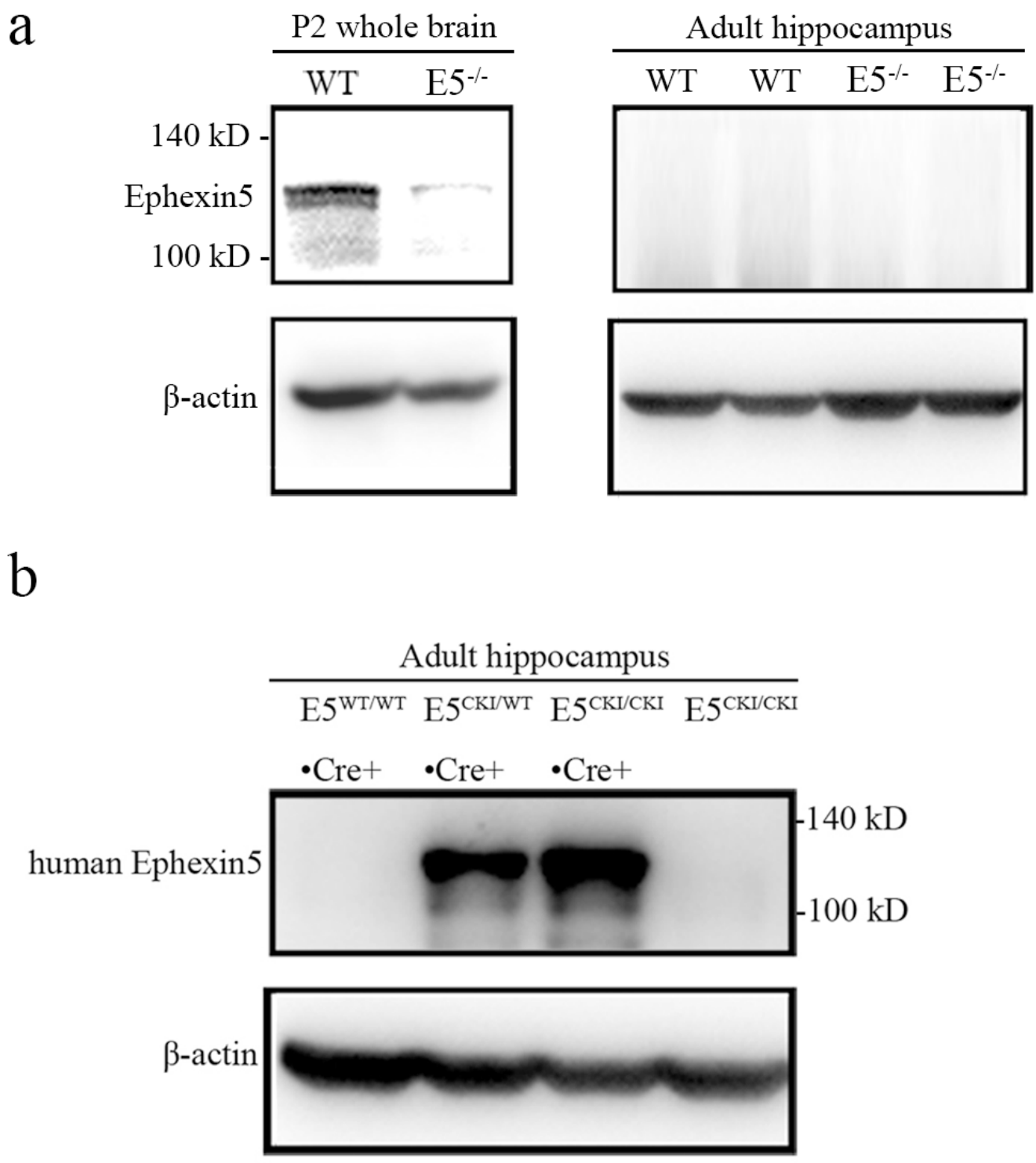

Figure 1

The expression of Ephexin5 in the ARHGEF15 knockout mice and forebrain-specific human ARHGEF15 knock-in mice. a: The level of Ephexin5 was high in the whole brain tissue of 2-day-old WT mice, and an extremely low expression of Ephexin5 was detected in the brain tissue of E5-/- mice of the same age. No expression of Ephexin5 was found in the hippocampus of the two genotypes aged 8-10 weeks. b: The 8-10 weeks E5CKI/CKI•Cre+ mice and E5CKI/WT•Cre+ mice expressed human Ephexin5 in the hippocampus, and E5WT/WT•Cre+ mice and E5CKI/CKI mice expressed no human Ephexin5 in the hippocampus.

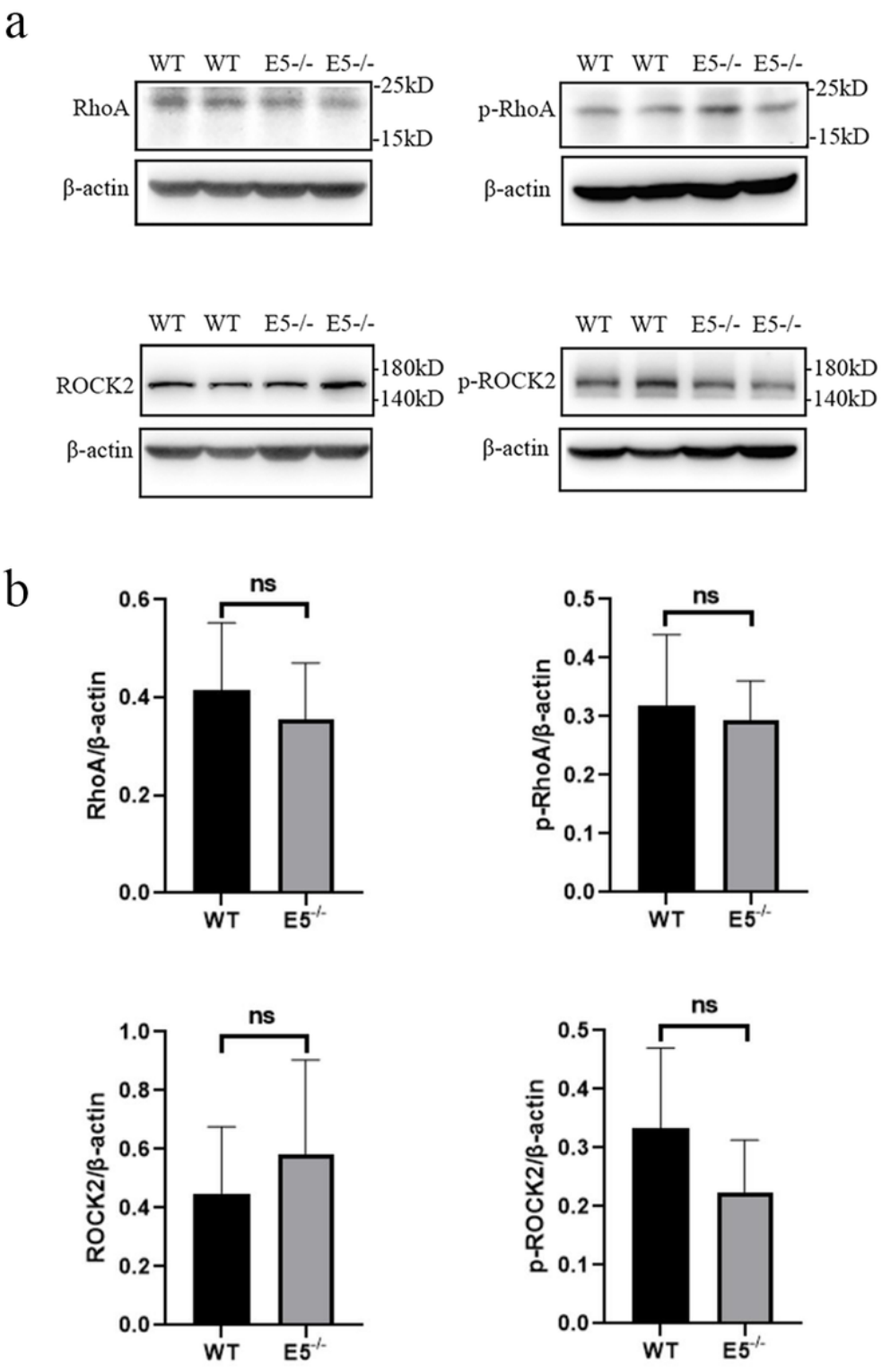

Figure 2

The expression of the RhoA-ROCK2 pathway in the ARHGEF15 knockout mice. a: The expression of RhoA, p-RhoA, ROCK2, and p-ROCK2 in the hippocampus of WT mice and E5-/- mice aged 8-10 weeks detected by western blot. b: There was no significant difference between WT mice (n=6) and E5-/- mice (n=6) in the levels of RhoA, p-RhoA, ROCK2, and p-ROCK2 by the independent-samples T-test (P > .05).

Supplementary Files

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