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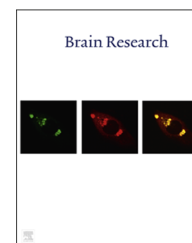

Research Report

Trio gene is required for mouse learning ability

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ABSTRACT

Trio is a guanine nucleotide exchange factor with multiple guanine nucleotide exchange factor domains. Trio regulates cytoskeleton dynamics and actin remodeling and is involved in cell migration and axonal guidance in neuronal development. The null allele of the Trio gene led to embryonic lethality, and Trio null embryos displayed aberrant organization in several regions of the brain at E18.5, including hippocampus. Nestin-Trio−/− mice, in which the Trio gene was deleted specifically in the neuronal system by the Nestin-Cre system, displayed severe phenotypes, including low survival rate, ataxia and multiple developmental defects of the cerebellum. All Nestin-Trio−/− mice died before reaching adulthood, which hinders research on Trio gene function in adult mice. Thus, we generated EMX1-Trio−/− mice by crossing Trio-floxed mice with EMX1-Cre mice in which Cre is expressed in the brain cortex and hippocampus. EMX1-Trio−/− mice can survive to adulthood. Trio gene deletion results in smaller brains, an abnormal hippocampus and disordered granule cells in the dentate gyrus (DG) and cornu ammonis (CA). Behavior tests showed that Trio deletion interfered with the hippocampal-dependent spatial learning in the mice, suggesting that Trio plays critical roles in the learning ability of adult mice. We conclude that the Trio gene regulates the neuronal development of the hippocampus and that it affects the intelligence of adult mice.

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1. Introduction

GTPases act as molecular switches in many cellular processes, and these proteins function in cell growth, differentiation, vesicle-mediated transport, and cytoskeletal organization (Bourne et al., 1991). Rho-family small GTPases, which are activated by guanine nucleotide exchange factors (GEFs), are key regulators of cytoskeleton dynamics (Etienne-Manneville and Hall, 2002). In mammals, the GEF family consists of several subfamilies with different amino acid sequences (Liu et al., 1998). Dbl-homology guanine nucleotide exchange factors (DH-GEFs) can activate Rho GTPases, and this activation regulates cell activities. The Trio gene belongs to a subfamily of DH-GEFs, and it was originally identified in humans in 1996 (Debant et al., 1996; Alam et al., 1997). Since then, Trio orthologs have been identified in the invertebrates *C. elegans* and *Drosophila*, which are called UNC-73 and DTrio, respectively (Steven et al., 1998; Lin and Greenberg, 2000). In mammals, there is a Trio orthlog called kalirin that was originally identified in rats. The structural organization of Trio is evolutionarily very well conserved (O'Brien et al., 2000; Peng et al., 2010).

Trio is a complex protein with multiple domains. Trio consists of 3 domains, including 2 GEF domains and a protein serine/threonine kinase (PSK) domain (Seipel et al., 1999). Each GEF domain has an adjacent pleckstrin homology (PH) domain and a SRC homology 3 (SH3) domain, and the PSK domain has an adjacent immunoglobulin (Ig)-like domain and multiple spectrin-like domains (Seipel et al., 1999). In two GEF domains, the Trio amino-terminal GEF domain (GEF-D1) can catalyze nucleotide exchange for Rac1, activate Jun kinase and produce membrane ruffles (Medley et al., 2000). In addition, Trio GEF-D1 has also been shown to activate RhoG (Blangy et al., 2006). The Trio carboxyl-terminal GEF domain (GEF-D2) acts as an exchange factor for RhoA and induces the synthesis of stress fibers (Seipel et al., 1999). Trio GEF-D2 can promote RhoA binding to the Ig-like domain of Trio, and this process activates RhoA (Medley et al., 2000). In cell cytoskeleton regulation, Trio GEF-D1 activates Rac1 and Rho G, while Trio GEF-D2 modulates Rho A (Bellanger et al., 2000).

GTPase family proteins play important roles in synaptic communication and plasticity in the brain (Ishikawa et al., 2002). As important members of GTPase family, Rac1, Rho G and Rho A can modulate the neuronal cell cytoskeleton. RhoA regulates focal adhesion assembly and actin stress fiber formation; Rac1 induces membrane ruffling and lamellipodia at the plasma membrane; RhoG is involved in nerve growth factor-induced neurite outgrowth and participates in the regulation of neuritogenesis (Ishikawa et al., 2002). In the developing brain, RhoA and Rac1 have been suggested to regulate dendritic growth and remodeling (O'Kane et al., 2003). The Trio protein can affect neuronal cell migration and axon guidance by modulating RhoG, Rac1 and RhoA through its GEF domains (Debant et al., 1996).

Trio can also function in conjunction with other proteins (Astigarraga et al., 2010).Trio interacts with leukocyte common antigen-related protein (LAR), and the Trio-LAR complex coordinates the cell-matrix interactions and cytoskeletal rearrangements (Astigarraga et al., 2010). LAR is a broadly expressed transmembrane protein tyrosine phosphatase (PTPase) with a cell adhesion-like extracellular region that regulates cell-matrix interactions (Serra-Pages et al., 1995). Trio binds to the cytoplasmic region of the LAR, which localizes to the ends of focal adhesions (Ridley and Hall, 1994; Debant et al., 1996). Focal adhesions are large, dynamic protein complexes through which the cytoskeleton of the cell connects to the extracellular matrix (ECM). Focal adhesion dynamics affect the migration of cells by orchestrating cell-matrix and cytoskeletal rearrangements. Through involvement in the regulation of focal adhesion dynamics, the Trio-LAR complex protein can regulate the migration of polarized cells (Bateman and Van Vactor, 2001; Wozniak et al., 2004).

Research on different animals shows the irreplaceable role of the Trio protein in the neuronal development process. UNC-73 and Dtrio, the Trio protein orthologs in *C. elegans* and *Drosophila*, respectively, were reported to be important regulators of axon guidance during nervous system development (Steven et al., 2005). In mammals, Trio is expressed ubiquitously in various tissues, including the central nervous system (Estrach et al., 2002). To determine the role of Trio during mammalian development, a null allele of Trio was generated in mice, and loss of Trio caused embryonic lethality. Sixty percent of Trio null embryos died between E15.5 and E18.5, and the remainder died perinatally. Trio null E18.5 embryos displayed aberrant organization in several regions within the brain, including the hippocampus and olfactory bulb (O'Brien et al., 2000). To address the role of Trio in postnatal brain development, Nestin-Trio−/− mice were generated by crossing Trio-floxed mice with Nestin-Cre mice, in which Cre is mainly expressed in the central and peripheral nervous systems (Dubois et al., 2006). Approximately 90% of Nestin-Trio−/− mice died within 1 day after birth, and the remainder survived for a further 5–22 days. These surviving Nestin-Trio−/− mice showed reduced body weight and smaller brain size. Nestin-Trio−/− mice displayed severe ataxia and defects in the cerebrum and cerebella (Peng et al., 2010). In the cerebellum of Nestin-Trio−/− mice, cerebellar granule neurons were disordered, showing an abnormal pattern of migration. In an in vitro assay, Trio-deficient migrating granule cells showed reduced spreading distance and moved in random directions (Peng et al., 2010). Research using knockout mice models suggests that the Trio gene regulates neural organization and is essential for neural development. However, all Nestin-Trio−/− mice died in infancy, and no adult mice survived (Peng et al., 2010).This phenomenon limited the behavioral studies and Trio functional research in adult mice.

In this study, EMX1-Trio−/− mice were generated by crossing Trio-floxed mice with EMX1-Cre mice in which Cre expression is restricted to the cerebral cortex and hippocampus (Gorski et al., 2002). EMX1-Trio−/− mice can survive to adulthood. The adult mice were useful for Trio gene research and allowed behavioral tests and adult brain analysis of these mice.

2. Results

2.1. Trio gene is deleted in the hippocampus of EMX1-Trio−/− mice

Trio-floxed mice, with exons 22–25 flanked by LoxP sites, were crossed with EMX1-Cre mice to generate EMX1-Trio−/− mice.

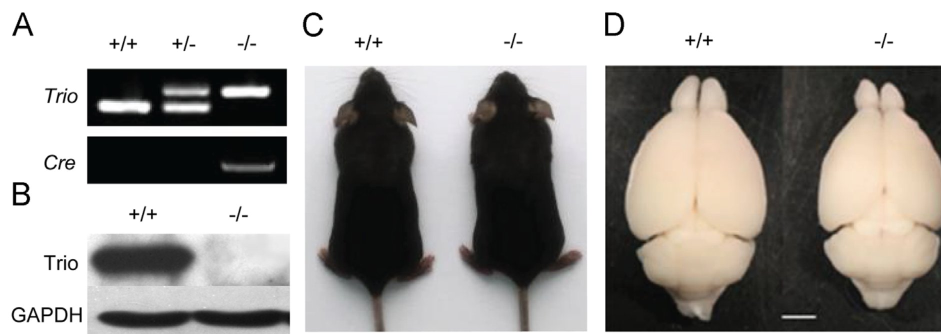

Fig. 1 – Generation of EMX1-Trio−/− mice. (A) Genotyping by PCR analysis. Upper panel: there is a 230 bp band in the wild-type allele (+/+) and a 370 bp band in the mutant allele (−/−). A 230 bp band and a 370 bp band were detected in the heterozygous mice (+/−). Lower panel: a 319 bp band was detected in Cre-positive mice. (B) Western blot analysis. A 358 kD Trio protein was detected in the hippocampus of wild-type (+/+) mice, and no Trio protein was found in Emx1-Trio−/− mice (−/−). GAPDH was used a control. (C) Gross morphology of the mice. There is no significant difference between the wild-type (+/+) and mutant adult mice (−/−). (D) Dorsal view of the whole brain. The brains of mutant mice (−/−) were smaller than those of wild-type mice (+/+). Scale bar=33 mm.

The Trio gene fragment flanked by LoxP sites was removed by Cre upon Cre expression in the early embryo development stage. The genotypes of the pups were determined by PCR analysis (Fig. 1A). The deletion of Trio protein in hippocampus was confirmed by western blot analysis. Western blot was repeated 5 times and it was proved that Trio protein is specifically deleted in the hippocampus (Fig. 1B).

2.2. *Change in gross morphology of EMX1-Trio−/− mice*

EMX1-Trio−/− mice can survive to adulthood. The gross morphology of these adult mice showed no obvious differences compared with wild-type mice (Fig. 1C). The body weight of EMX1-Trio−/− mice was significantly lower than EMX1-Trio+/+ mice before P30 ($P<0.05$, two-way ANOVA with Bonferroni testing, Fig. 2A). Over time, the body weight of EMX1-Trio−/− mice approached that of the wild-type mice, and mice older than one month showed no significant body weight difference (Fig. 2A). However, the mutant adult mice had smaller brains compared with wild-type mice ($P<0.05$, two-way ANOVA with Bonferroni testing, Fig. 1D and Fig. 2B). Although the difference in body weight was reduced($P>0.05$, two-way ANOVA with Bonferroni testing, Fig. 2A), the EMX1-Trio−/− mice brains were still smaller than those of the control even at 2 months postnatal ($P<0.001$, two-way ANOVA with Bonferroni testing, Fig. 2B).

2.3. *Trio deletion resulted in abnormal hippocampus in adult mice*

We examined the hippocampus of the adult mice. Histological results showed that the DG region and CA region in adult wild-type mice had normal shapes, and the granule cells were arranged orderly in both the dorsal and ventral hippocampus (Fig. 3A and B). However, the DG region and CA region in the brain of adult mutant mice had abnormal shapes, and the arrangement of the granule cells were disordered in both the dorsal and ventral hippocampus (Fig. 3C and D). The mutant dorsal hippocampus showed zigzags in the DG region (Fig. 3C) and the shape of the DG region was twisted (Fig. 3C). In the ventral hippocampus, the shape of the DG region twisted slightly, and the granule cells were diffused in both the DG and CA regions (Fig. 3D).

2.4. *Morris water maze*

Animals were subjected to the Morris water maze test, which tests spatial learning and memorizing ability. The Morris water maze task is hippocampus-dependent. In the training stage, mice were trained to find the hidden platform under water in 60 s with four trials per day for five consecutive days. Examining 5 days of escape latency in the water maze training session revealed a significant time effect and genotype effect, but not interaction effect (time, $F_{(4,68)}=24.11$, $P<0.001$; genotype, $F_{(1,17)}=11.92$, $P=0.003$; interaction, $F_{(4,68)}=1.36$, $P=0.26$; two-way repeated measures ANOVA with Student–Newman–Keuls post-hoc testing, Fig. 4A). In the probe test, we removed the platform and tested the time that the mice remained in the quadrant that originally had the platform and the number of times the mice crossed the platform. The EMX1-Trio−/− mice had difficulty locating the quadrant with the hidden platform. The average times that EMX1-Trio+/+ mice crossed the platform region is approximately two times more than the EMX1-Trio−/− mice ($P<0.05$; Student's *t*-test, Fig. 4B). EMX1-Trio−/− mice stayed in the imaginary quadrant with the platform for a much shorter time than EMX1-Trio+/+ mice ($P<0.01$, Student's t-test, Fig. 4C and D).

2.5. *Contextual fear conditioning*

Animals were subjected to the contextual fear conditioning test. In the training stage, mice were placed in the text chamber. After a baseline time (120 s), they received three foot shocks at 1 min intervals. The freezing time after every foot shock at 1 min intervals indicated a significant time effect and genotype effect, but not interaction effect (time, $F_{(2,64)}=93.59$, $P<0.001$; genotype, $F_{(1,32)}=11.42$, $P=0.002$; interaction,

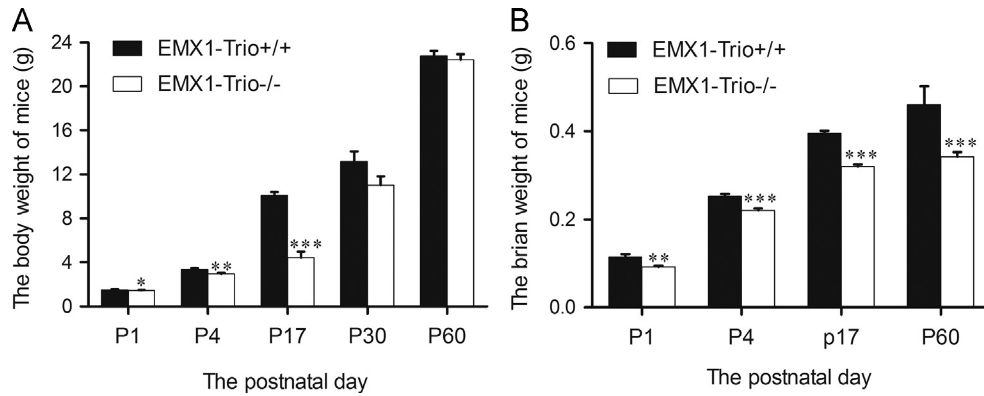

Fig. 2 – The body weight and brain weight of mutant and wild-type mice. (A) The body weight of Trio EMX1-Trio+/+ and Trio EMX1-Trio−/− mice. The body weight of mutant mice before P30 was significantly lower that of the controls ($N=4$–8;$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA with Bonferroni testing). After 1 month, there was no significant difference in the body weight between mutant and wild-type mice. (B) The brain weight of Trio EMX1-Trio+/+ and Trio EMX1-Trio−/− mice. EMX1-Trio−/− mice had smaller brains than EMX1-Trio+/+ mice at every stage. In adults, the mutant brains were still significantly smaller than wild-types brain ($N=5$; $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA with Bonferroni testing).

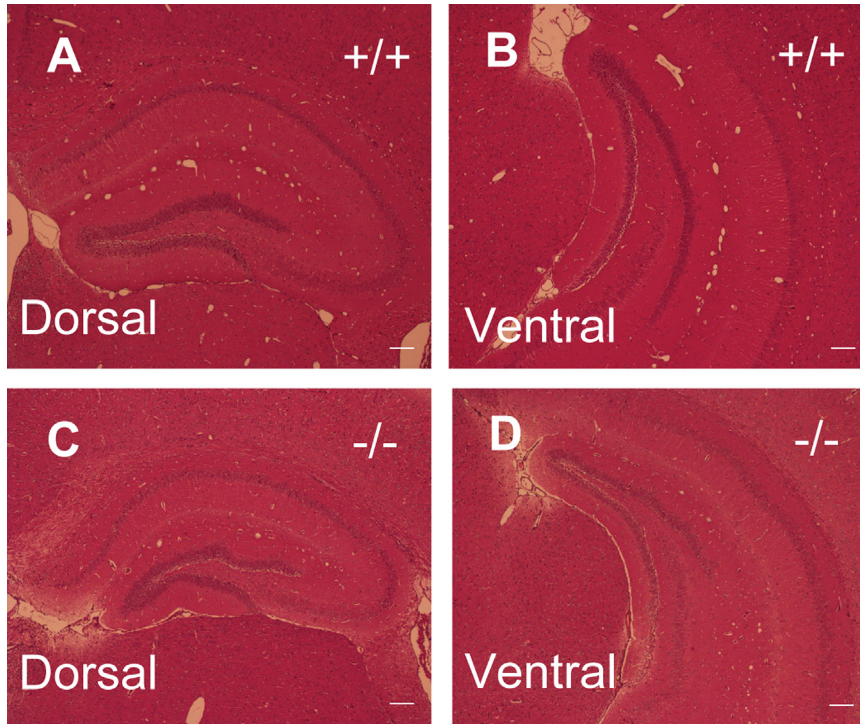

Fig. 3 – The dorsal and ventral hippocampus structure in adult EMX1-Trio+/+ and EMX1-Trio−/−mice. (A and B) In wild-type mice, histological results showed that the DG and CA regions had an ordered shape, and the granule cells were arranged orderly in both the dorsal and ventral hippocampus. (C and D) In mutant mice, histological results showed that the DG and CA regions had a disordered shape, and the arrangement of the granule cells was disorganized. The mutant dorsal hippocampus showed some zigzags in the DG region (arrows in C), which was twisted seriously. In the CA region, the granule cells were in a loose and diffuse arrangement (C). In the mutant ventral hippocampus, there were also some zigzags (arrows in D) in the DG region, and the DG region was twisted slightly (D). Scale bar=50 μm.

$F_{(2,64)}=0.67$, $P=0.52$; two-way repeated measures ANOVA with Student–Newman–Keuls post-hoc testing, Fig. 5A). After 24 h, the mice were placed into the same text chamber and were not given a foot shock. EMX1-Trio−/− mice had shorter freezing time than EMX1-Trio+/+ mice ($P<0.01$, Student's t-test, Fig. 5B).

3. Discussion

Trio is a guanine nucleotide exchange factor with multiple guanine nucleotide exchange factor domains. There have been studies demonstrating the role for Trio in regulating

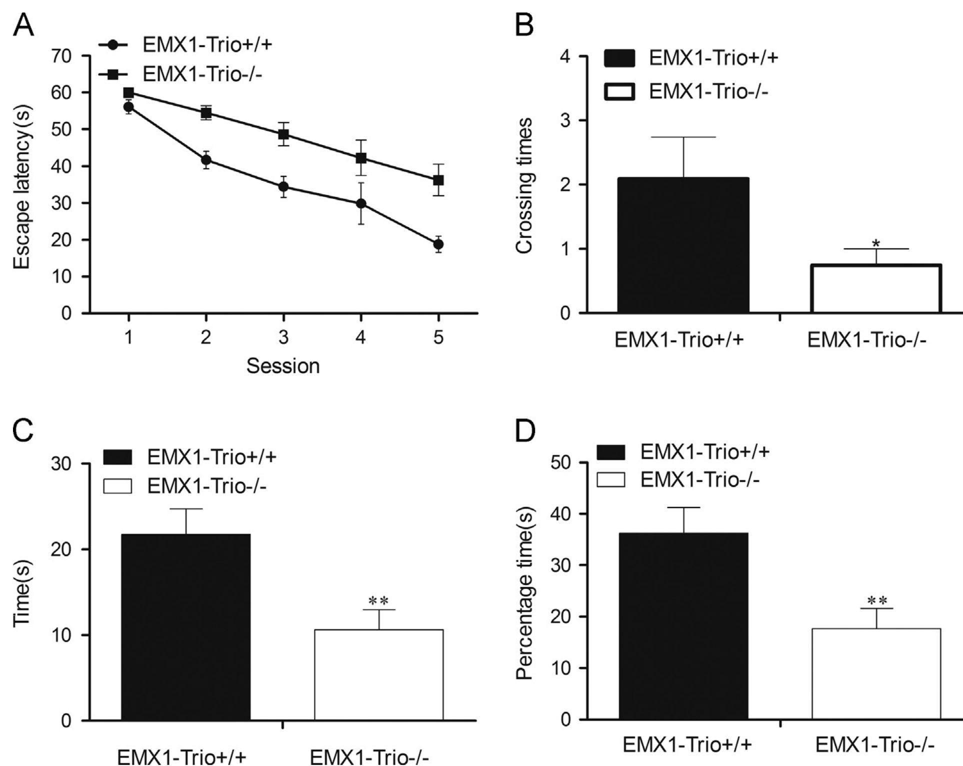

Fig. 4 – Morris water maze test in adult wild-type and mutant mice. (A) Average daily escape latency to reach the platform for each genotypic group in the Morris water maze training test. EMX1-Trio−/− mice displayed a significantly longer latency to reach the platform compared to EMX1-Trio+/+ mice. ($N=8$–11, time effect, $F_{4,68}=24.11$, $P<0.001$, genotype effect, $F_{1,17}=11.92$, $P=0.003$, interaction effect, $F_{4,68}=1.36$, $P=0.26$, two-way repeated measures ANOVA with Student–Newman–Keuls post-hoc testing). (B) Number of platform crosses in the probe trial. In the Morris water maze probe test, we removed the platform, and trained mice were tested after 1day. The number of crosses in the target platform location by WT mice is approximately 2 times more than that of the mutant mice ($N=8$–11, $^{*}P<0.05$, Student's *t*-test). (C and D) Remaining time and remaining time proportion of mice staying in target quadrant. The EMX1-Trio−/− mice had problems locating the quadrant with the platform, and the time they stayed in this quadrant was much shorter than that of the EMX1-Trio+/+ mice. ($N=8$–11, $^{**}P<0.01$, Student's *t*-test).

neuronal cells growth and migration and axon guidance using *Caenorhabditis elegans* and *Drosophila* animal models (Hu et al., 2011; Shivalkar and Giniger, 2012). Knocking out genes is an effective method of studying the function of genes in mice. Two types of Trio knock-out mouse lines were generated, and the role of Trio during mammalian development was studied. However, these mice did not survive to adulthood (O'Brien et al., 2000; Peng et al., 2010).

In this study, EMX1-Trio−/− mice were generated to address the role of the Trio gene in postnatal hippocampal development and learning ability. Emx1-Cre mice expresses the Cre recombinase in radial glia, Cajal–Retzius cells, glutamatergic neurons, astrocytes, and oligodendrocytes of most pallial structures but not in GABAergic neurons at E12.5 (Gorski et al., 2002). EMX1-Trio−/− mice can live to adulthood, which allows investigation of adult mice. Trio gene deletion resulted in smaller brains, abnormal adult hippocampal structure and disordered granule cells in the DG and CA regions. In the Morris water maze test, EMX1-Trio−/− mice showed impaired learning ability, while there was no evidence that the memory was affected in the mutant mice. In contextual fear conditioning test, EMX1-Trio−/− mice showed impaired learning ability. Our data demonstrated that the Trio gene can regulate brain development in young and adult mice, and deletion of Trio severely affects the intelligence of the mice.

3.1. *Trio plays an irreplaceable role in brain development*

The deletion of Trio in EMX1-Trio−/− mice affected brain development, and mutant mice had smaller brains and abnormal hippocampal structure. The brain phenotype of EMX1-Trio−/− mice is consistent with previous research. Both Trio null mice and Nestin-Trio−/− mice showed abnormal hippocampal structure and disorderly arrangement of granule cells in the DG and CA region (O'Brien et al., 2000; Peng et al., 2010). In Nestin-Trio−/− mice the abnormal structure of hippocampus is the result of disordered migration of granule cells in brain development (Peng et al., 2010). It is reasonable to infer that the impaired structure of the hippocampus EMX1-Trio−/− mice was caused by abnormal migration of granule cells.

EMX1-Trio−/− mice also showed reduced body weight in the developmental stage. Over time, the body weight of EMX1-Trio−/− mice increased to match the body weight of

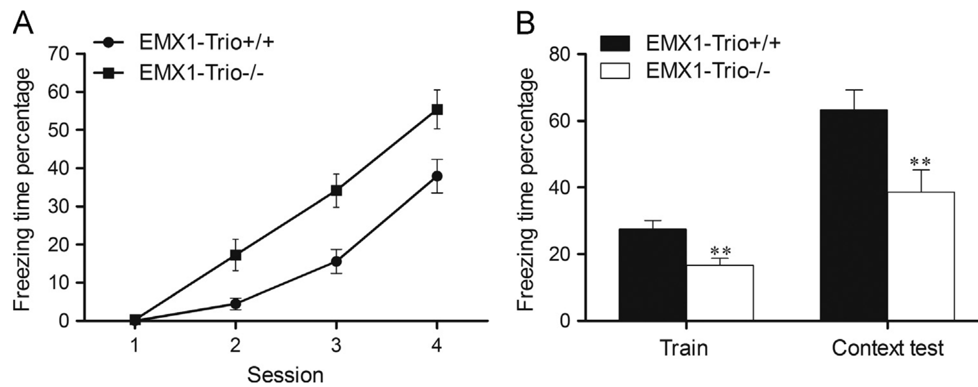

Fig. 5 – Contextual fear conditioning test in adult wild-type and mutant mice. (A) Freezing time percentage curve of each genotype in the training stage. The results of the fear conditioning training test shows that EMX1-Trio−/− mice had shorter freezing time than EMX1-Trio+/+ mice ($N=16$–18, time effect, $F_{2,64}=93.59$, $P<0.001$, genotype effect, $F_{1,32}=11.42$, $P=0.002$, interaction effect, $F_{2,64}=0.67$, $P=0.52$, two-way repeated measures ANOVA with Student–Newman–Keuls post-hoc testing). (B) Average freezing time percentage of the mice in the training and context stage. EMX1-Trio−/− mice had smaller training freezing time percentage and context freezing time percentage ($N=16$–18, $^{**}P<0.01$, Student's *t*-test).

EMX1-Trio+/+ mice, and adult mice showed no significant difference in body weight. The reduction of body weight in early developmental stages may be a secondary effect of the brain defects. The mutant mice had impaired learning and memory ability, which affected the competition for suckling milk. Failure to compete with other pups for suckling milk reduced the body weight of the mutant mice. After weaning, there was no more competition for food, so the body weight of the mutants increased to that of wild-type mice.

Although abundant food after weaning can help the mutant mice reach normal body weight, the brain of the mutant mice was still smaller than that of with adult wild-type mice. Neurogenesis and therefore major brain development activity in mice stop soon after birth. Therefore, this growth retardation cannot be compensated by sufficient nutrition because the brain structure and size became fixed and unchangeable at birth. As a result, the mutant brains remained smaller even as the mutant mice grew to adulthood. These data prove that Trio plays an irreplaceable role in brain development, and Trio deficiency in early developmental stages will lead to irreversible defects.

3.2. *Impairment of learning ability was caused by hippocampus lesions in EMX1-Trio−/− mice*

In the behavior tests, EMX1-Trio−/− mice showed impaired learning ability, which may be caused by hippocampus lesions. The hippocampus, which consists of DG, CA1 and CA3 regions, is the organ in the brain related to learning and memory functions. The structure of the hippocampus can influence the intelligence of the mice. In the early development stage of the hippocampus, the migration of the granule cells and the growth of the neural cells are important for the structure of the hippocampus and thus affect the learning and memory ability of the adult mice (Bruel-Jungerman et al., 2007). The hippocampus plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. The hippocampus is the core center for learning and memory, and adult hippocampal neurogenesis may participate in hippocampal function related to learning and memory (Lee and Son, 2009). The specific knockdown of adult neurogenesis impaired spatial learning and memory functions in the hippocampus. Trio is an important factor regulating neurogenesis and participates in hippocampus development. The knockout of Trio gene by EMX1-Cre affected the neurogenesis in the hippocampus and produced messy granule cell order and abnormal structure of dorsal hippocampus and ventral hippocampus. Due to the relationship between hippocampus and spatial learning ability, it can be inferred that the deletion of Trio gene in brain caused the mess in granule cell migration, influenced the hippocampus neurogenesis in and resulted in lesion to hippocampus structure, leading to the impairment of spatial learning function in the hippocampus. We conclude that the Trio gene is involved in hippocampal development and plays critical roles in the learning behavior of mice.

3.3. *Some Rho GTPases and its regulator Trio are involved in learning ability in mice*

Trio is a key GEF regulator of Rho GTPases and is noted for its role in axon guidance and axonagenesis in *Drosophila* and *C. elegans* (Haditsch et al., 2009; Hu et al., 2011). Trio consists of two GEF domains, which can activate Rho GTPases RhoA and Rac1 (Seipel et al., 1999; Bellanger et al., 2000; Medley et al., 2000). The activation levels of RhoA and Rac1 were significantly reduced in the brain of Nestin-Trio−/− mice (Peng et al., 2010). In our study, EMX1-Trio−/− mice showed impaired learning ability. So far, no report has been made on RhoA knockout mouse model. Selective deletion of Rac1 in excitatory neurons in the forebrain of mice resulted in disorganization of cortical lamination, impairment of neuronal migration and increased complexity of neurite (Haditsch et al., 2009). Rac1-deficient animals have impaired learning and working memory (Haditsch et al., 2009). In the knockout mice of another Rho GTPase Cdc42, the remote memory was injured (Kim et al., 2014). Both our results and previous

reports by other researchers indicated that some Rho GTPases and its regulator Trio are closely related to the learning functions of mice.

3.4. EMX1-Trio−/− mice may be used as an animal model for human diseases

Trio is involved in human diseases. Trio was reported to be highly expressed in glioblastoma, breast tumors, soft tissue sarcomas and urinary bladder tumors (van Rijssel and van Buul, 2012). There is an alternative splice variant called Tgat that encodes the DH domain of the Trio GEF-D2. Tgat was identified in patients with adult T-cell leukemia and was demonstrated to induce cell transformation and tumour formation, indicating that Trio might potentially regulate cancer progression (van Rijssel and van Buul, 2012). In Huntington's disease, Huntingtin-associated protein 1(HAP1) is a protein closely related to cerebellar degeneration, and there is a protein called Duo that interacts specifically with HAP1. Duo is involved in the pathogenesis of HD, and human Duo is highly similar to human Trio, especially in the GEF domain (Colomer et al., 1997; Peng et al., 2010). Trio may be relevant to Huntington's disease through involvement in the Duo signal pathway because the Trio protein has similar structural features to Duo protein. Due to the lack of availability of human material, animal models are especially important in the study of human disease. The mutant mice generated in this study are the first Trio-deficient animal model that can survive to adulthood. EMX1-Trio−/− mice may potentially serve as an animal model in cancer research or in the study of the pathological mechanism and clinical diagnosis of Huntington's disease.

4. Experimental procedures

4.1. Animals

The EMX1-Cre and Trio-floxed mouse lines were used and genotyped as described previously (Iwasato et al., 2000; Peng et al., 2010). We crossed Trio-floxed mice with EMX1-Cre mice. All animals used in this study were C57BL/6 background and they were by backcrossing breeding over 10 generations. The mice housed in an animal room at 22±2 °C with a 12-h light/dark cycle (light on 07:00–19:00 h). The day of birth was defined as postnatal day 0 (P0). Body weight and brain weight were determined at various ages. All of the mice used for other experiments were 8–10 weeks old. The experiments were conducted in accordance with the standards of the Shandong University Ethics Committee.

4.2. Genotyping

The following PCR primers were used to determine genotypes: Trio LoxP/LoxP left primer 5′-TTGTTCCATTTACGTCACCG-3′; Trio LoxP/LoxP right primer 5′-CCTCCTCAGGGAAGAGACTA-3′. The PCR cycling conditions were as follows: 30 cycles of 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. The EMX1-Cre left primer is 5′-TGCAACGAGTGATGAGGTTC-3′; the EMX1-Cre right primer is 5′-GCTTGCATGATCTCCGGTAT-3′. The PCR cycling conditions were 30 cycles of 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min.

4.3. Histological analyses

To generate paraffin sections, the mice were perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. The brains were removed and fixed in 4% paraformaldehyde at 4 °C overnight and dehydrated via an ethanol series. The tissues were embedded with the proper orientation in paraffin, sectioned at 4 μm thickness and stained using haematoxylin and eosin (H&E) for microscopic analysis.

4.4. Western blot analysis

The hippocampus was dissected, and the proteins were extracted in cell lysis buffer (150 mmol NaCl, 1% Triton X-100, 10 mmol Tris, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 1 mmol ethylenediamine tetraacetic acid). The proteins from the samples (50 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The primary antibodies used were anti-Trio (Santa Cruz: 1:200) and anti-GAPDH mouse monoclonal antibodies (Millipore: 1:3000). Subsequently, the membrane was incubated for 1 h at room temperature with the appropriate secondary antibodies coupled with horseradish peroxidase. Finally, immunoreactive bands were visualized using electrochemiluminescence detection reagents.

4.5. Morris water maze test

The Morris water maze method was performed as described previously (Yu et al., 2012). EMX1-Trio+/+ mice (WT, $N=8$) and EMX1-Trio−/− mice (KO, $N=11$) were trained to swim to a visible platform and then swim to a hidden platform. The water maze consisted of a round tank 170 cm in diameter and 70 cm deep filled with water. The water temperature was maintained at 25 °C. A platform ($11\times14\ cm^2$) submerged 2 cm under the water surface was placed in the center of one of the four imaginary quadrants of the tank and was maintained in the same position during all trials. Several distal visual cues were placed on the walls of the water maze room. The training session consisted of four consecutive trials during which the animals were placed in the tank facing the wall and allowed to swim freely to the platform. If the animal did not find the platform in 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 30 s after reaching it removed from the tank. Afterwards, the mouse was placed at the next starting point in the tank. This procedure was repeated 4 times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. In the training stage, escape latency, which was the time mice took to find the platform was recorded. The test session was performed 24 h later and was similar to the training session. After 5 days training, we removed the platform and put the mice in the opposite imaginary quadrant of the platform. The mice were allowed to swim for 60 s. We recorded the times

that the mice crossed the platform region and the time that the mice remained in the imaginary quadrant with the platform.

4.6. *Contextual fear conditioning test*

In the training test, EMX1-Trio+/+ mice (WT, $N=18$) and EMX1-Trio−/− mice (KO, $N=16$) were put into the conditioning chamber ($25 \times 25 \times 25\ cm^3$). These mice were allowed to habituate for 120 s without any stimulation (habituation). They then received three consecutive foot shocks (0.7 mA, 2 s duration each) through a stainless steel grid floor (Panlab, Barcelona, Spain). Each foot shock was separated by a 60 s time period. The freezing time at every session test was recorded. Freezing time percentages (freezing time/60 s) were used to create curves. After an additional 60 s following the last shock, the mice were placed back in their home cage. Context test was conducted 24 h after training. The mice were returned to the previous chamber in which the training occurred and kept there for 5 min without foot shock. The time of freezing behavior was recorded (Huff et al., 2006).

4.7. *Statistical analysis*

All data are presented as means ± SEM and data analyses were performed using the SPSS statistical program, version 17.0. The escape latencies in the water maze and the freezing time of training test in the contextual fear conditioning were analyzed by repeated measures ANOVA with Student–Newman–Keuls post-hoc testing. Student's *t*-test was used to analyze the differences between two groups (EMX1-Trio+/+ mice and EMX1-Trio−/− mice). The body weight and brain weight were analyzed by two-way ANOVA with Bonferroni testing.

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