Int J Clin Exp Pathol 2014;7(10):6486-6492
www.ijcep.com /ISSN:1936-2625/IJCEP0001518

Original Article

DSTYK kinase domain ablation impaired the mice capabilities of learning and memory in water maze test

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Received July 23, 2014; Accepted August 23, 2014; Epub September 15, 2014; Published October 1, 2014

Abstract: DSTYK (Dual serine/threonine and tyrosine protein kinase) is a putative dual Ser/Thr and Tyr protein kinase with unique structural features. It is proposed that DSTYK may play important roles in brain because of its high expression in most brain areas. In the present study, a DSTYK knockout (KO) mouse line with the ablation of C-terminal of DSTYK including the kinase domain was generated to study the physiological function of DSTYK. The DSTYK KO mice are fertile and have no significant morphological defects revealed by Nissl staining compared with wildtype mice. Open field test and rotarod test showed there is no obvious difference in basic motor and balance capacity between the DSTYK homozygous KO mice and DSTYK heterozygous KO mice. In water maze test, however, the DSTYK homozygous KO mice show impaired capabilities of learning and memory compared with the DSTYK heterozygous KO mice.

Keywords: DSTYK, knockout mice, brain development, learning and memory

Introduction

DSTYK (dual serine/threonine and tyrosine protein kinase) is originally identified as a novel receptor interacting protein (RIP)-homologous protein designated as receptor interacting protein kinase 5 (RIPK5) [1], because its kinase domain shares homology with kinase domain of RIP protein family. However, evidences from evolution analysis showed DSTYK probably does not belong to RIP protein family, but a divergent protein kinase and is distant from PSK (protein serine/threonine kinase) and PTK (protein tyrosine kinase) [2]. Finally, HUGO (human genome organization) Gene Nomenclature committee (HGNC) changed the official name “RIPK5” to “DSTYK”.

DSTYK widely exists in multicellular organisms. The absence of DSTYK from unicellular organism and some invertebrates and ubiquitous existence in vertebrates suggest that DSTYK is a result of evolution, which may play important roles in higher animals [2]. The kinase domain of DSTYK is strictly conserved, so it has been proposed to have kinase activity to play some potentially important functional roles in the brain and other tissues [2].

Human DSTYK gene is located on chromosome 1, containing 13 exons and encoding a protein with 929 amino acid residues. Northern blot analysis showed that human DSTYK full length (7.9 kb) mRNA was expressed at variable levels in multiple tissues including brain, heart, kidney, lung and muscle. Especially it was expressed highly in adult brain tissues including cerebellum, olfactory, hippocampus, and cerebral cortex [2]. The similar expression pattern was also found in mouse [2]. So we proposed that DSTYK may play some important roles in brain. In the present study we investigated the DSTYK KO mice with DSTYK kinase domain ablation to study the physiological function of DSTYK. The DSTYK KO mice are fertile and have no significant defects in brain gross morphology compared with wildtype mice, and in basic motor and balance capacities com-

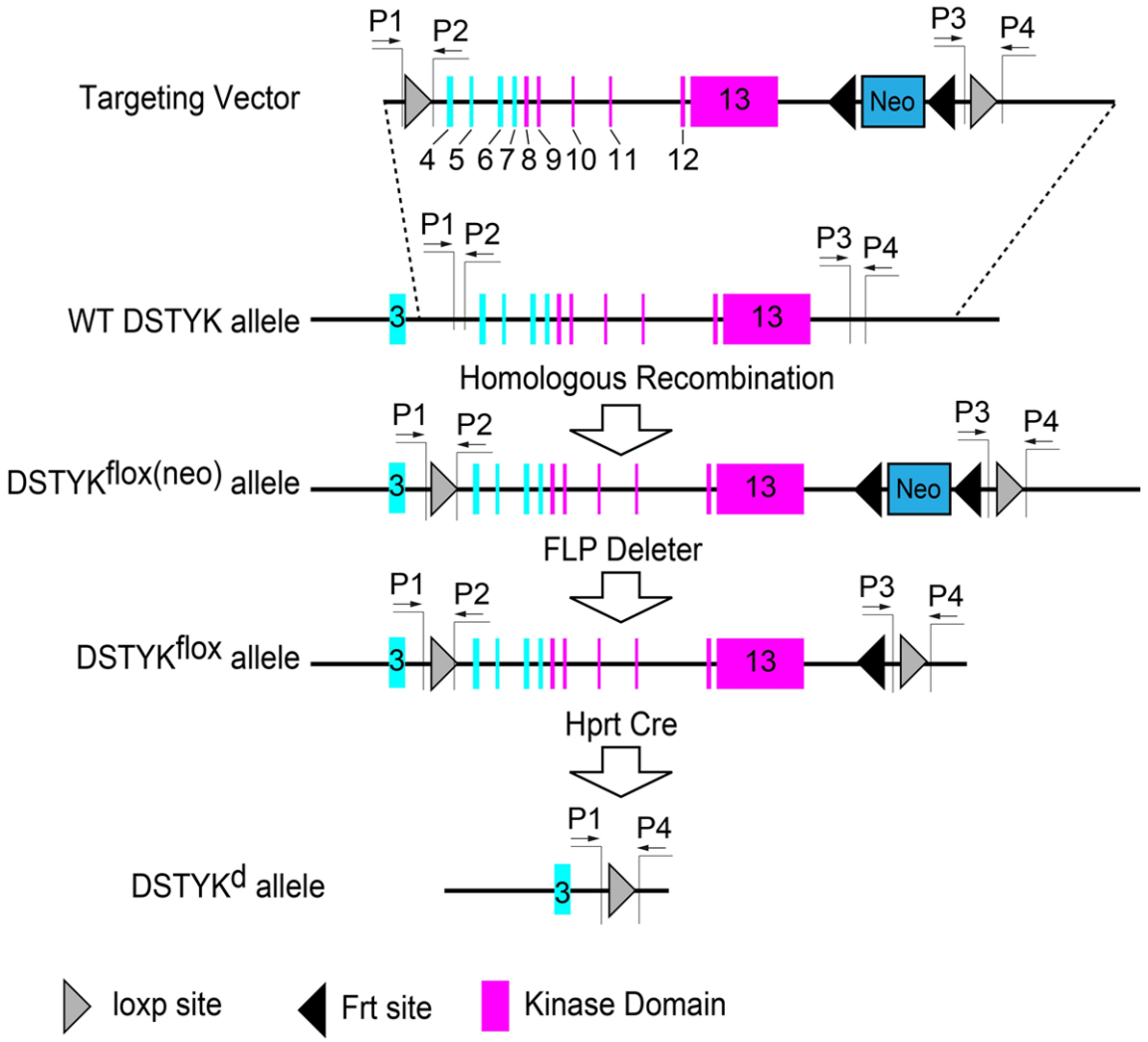

Figure 1. Schematic diagram showing the knockout strategy of DSTYK in mice. Exons 4-13 was removed from mouse genomic DNA leading to the kinase domain ablation of DSTYK protein. The sites for binding of genotyping Primers (P1-P4) were also showed.

pared with DSTYK heterozygous KO mice. But the homozygous KO mouse showed impaired capabilities of learning and memory in water maze test, which suggested DSTYK may play roles in the process of learning and memory.

Materials and methods

Animals

The DSTYK heterozygous knockout mice was generated in Baylor College of Medicine using a strategy similar as previously reported [3]. Briefly, a DSTYK-targeting vector containing two loxp sites flanking the exons 4-13 of DSTYK gene was constructed, with an Frt-Neo-Frt cassette for selecting (**Figure 1**). The targeting vector was linearized, purified, and electroporated into murine embryonic stem cells to obtain high quality male chimeras. Then the chimeras were crossed with C57BL/6 to generate $DSTYK^{flox(neo)}$ mice. $DSTYK^{flox(neo)}$ mice were crossed with Frt deleter mice to generate $DSTYK^{flox}$ mice. To obtain a DSTYK knockout mouse line, the Hprt Cre mice were crossed with $DSTYK^{flox}$ mice to generate DSTYK null allele ($DSTYK^{d}$) as the activation of Cre protein at fertilization. The exons 4-13 of DSTYK gene were deleted in this mouse line, which means that the kinase domain of DSTYK protein was removed (**Figure 1**). Animals were kept in a room with 12 light/12 dark cycle. Mice were allowed *ad libitum* access to the food and water. Male DSTYK homozygous knockout mouse were mated with female

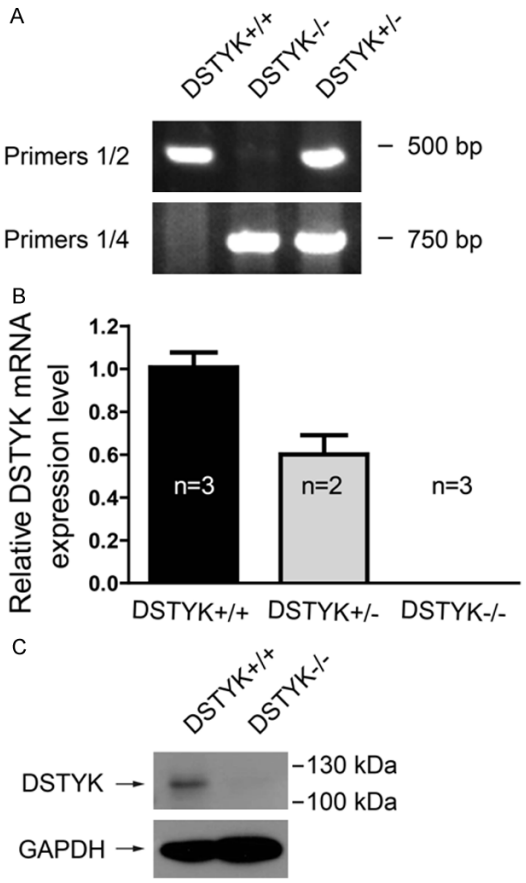

Figure 2. Genotyping, mRNA expression and protein expression of mice with kinase domain ablation of DSTYK. A. Representative Genotyping PCR results using different primer sets. B. The mRNA level of DSTYK in DSTYK$^{+/+}$, DSTYK$^{+/-}$ and DSTYK$^{-/-}$ mice analyzed by quantitative PCR. Sample size was marked in the columns. C. DSTYK protein expression in DSTYK$^{+/+}$ and DSTYK$^{-/-}$ mice assessed by western blot.

DSTYK heterozygous mouse to generate offsprings to perform behavioral test.

Genotyping

Polymerase chain reaction (PCR) was used to confirm the genotype of the offsprings. PCR Primer sequences are: P1, 5'-GATGCAATGTTTGGCTTAAATAGATA-3'; P2, 5'-GCATAAAAAGTTTTGACATGTAACTCC-3'; P3, 5'-CAGCACTGTCTTCCTGTTCCTAC-3'; P4, 5'-CTGCCTCTGAGACTGTGAGAAAC-3'. Since the binding sites of P2 and P3 were deleted in DSTYK knockout mouse, using primer sets P1/P2 or P3/P4 can't get specific products in DSTYK knockout mouse. While it can get specific products in wildtype mouse using these primer sets. As there is more than 16 kb distance between primer binding sites of P1 and P4 in wildtype mouse genome, no specific product can be amplified in wildtype mouse with limited extension time. While in DSTYK knockout mouse, it can generate an about 700 bp product with primer set P1/P4 since the DSTYK kinase domain ablation shortened the distance between P1 and P4. Thus P1/P2 or P3/P4 can be used to identify wildtype DSTYK genome, and P1/P4 can be used to identify DSTYK mutant genome. An example of genotyping was provided (**Figure 2A**).

Quantitative PCR

Adult male mice were decapitated, and brain tissues were rapidly dissected to homogenate in Trizol (Invitrogen, Carlsbad, USA) reagent. Total RNA was extracted, reverse transcribed (RT M-MLV, Promega, Beijing, China) and analyzed by quantitative real-time PCR. Quantitative PCR kit SYBR Premix Ex Taq (Takara, Dalian, China) was used following the instructions of the manufacturer. The primer sets for quantitative PCR were: DSTYK, 5'-TGCCGCTTACCATGTTGAGG-3', 5'-CACCCATGTGATACGCTGGAT-3'; β-Actin, 5'-GGCTGTATTCCCCTCCATCG-3', 5'-CCAGTTGGTAACAATGCCATGT-3'.

Western blot

Mice were decapitated, and the brain cerebral cortex tissues were rapidly dissected. Tissues were then immersed in liquid nitrogen until homogenization using Dounce homogenizer. Tissues were lysed in RIPA lysis buffer (Beyotime, Nantong, China) supplemented with 1 x PMSF (Beyotime, Nantong, China) and 1 x protease inhibitor cocktail (Sangon, Shanghai, China). 8% SDS-PAGE was used to separate the proteins. We used antibodies specific for DSTYK (Santa Cruz: sc-162109, Shanghai, China), horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz: sc-2020, Shanghai, China) and GAPDH (Kangchen, Shanghai, China). The antibody dilution rates were: anti-DSTYK, 1:200, donkey anti-goat secondary antibody, 1:4000, anti-GAPDH, 1:8000.

Nissl staining

Adult male mice were anesthetized and perfused with saline and 4% paraformaldehyde, and their brains were removed for cryosections at 40 μm per slice for Nissl staining. Tissue sections were mounted on gelatin-coated slides

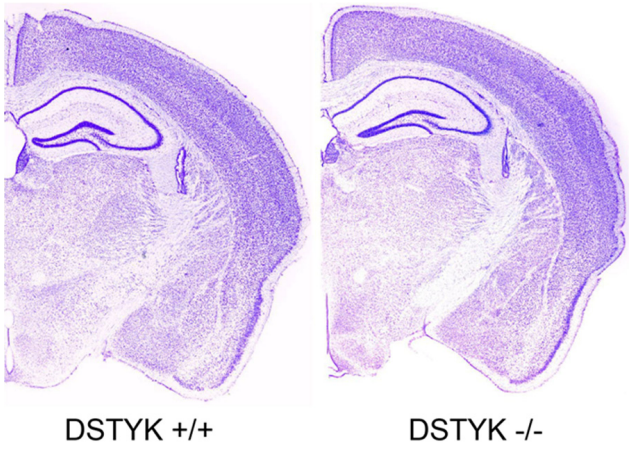

Figure 3. Representative Nissl staining of brain sections showed no significant morphological defects of mouse brain.

and baked for more than two hours in 50°C oven. The sections were defatted in a solution containing 50% chloroform and 50% alcohol for 30 min. Then the sections were hydrated in 100% alcohol (for 5 min), 95% alcohol (for 5 min), 70% alcohol (for 5 min), 50% alcohol (for 5 min) and distilled water (for 5 min). The hydrated sections were stained in Nissl staining solution for 30 min. To make Nissl staining solution, 60 ml 0.24% (w/v) cresyl violet in distilled water, 30 ml 1.36% (w/v) sodium acetate in distilled water and 470 ml 6 mM acetic acid were mixed and the pH was adjusted to 3.5. Then the sections were rinsed with distilled water and dehydrated in 50% alcohol (for 5 min), 70% alcohol (for 5 min), 95% alcohol (for 5 min) and 100% alcohol (for 5 min). Finally, slides were cleared in xylene for 2 × 5 min and covered with glass coverslips. Images were captured using microscope Nikon Ti-s.

Open field test

The open field test box was a plastic box with 49 cm long, 49 cm wide and 49 cm high. Its walls and floor were all white. Two 8W fluorescent tube were placed on two top sides of the box. Mice were allowed to explore for 30 min. Their movements were automatically recorded and analyzed by AniLab Software (Ningbo, China).

Rotarod test

Rota rod test was performed as previously reported [4]. The rota rod test device and associated control software was bought from Taimeng Software (Chengdu, China). When training, the speed was fixed at 24 rpm. Mice were trained 4 trials per day, less than 1 minute in a trial. Intervals between two training trails were about 10 minutes. After 3 consecutive days of training, the test trials were performed on the fourth day. When testing, the speed of the rod accelerated from 0 to 35 rpm in 180 seconds. Mice were tested for 3 trials per mouse and the average latency to fall from the rod was calculated.

Morris water maze test

The diameter of tank for morris water maze test is 120 cm. The tank is divided into virtual 4 quadrants, with a virtual round position for placing platform in the central location of each quadrant. Both visible platform water maze test and hidden platform water maze test were performed. In visible platform water maze test, the platform was set just above the water with a black triangle paper over it. Every mouse was tested for four trials per day. The location of the platform and the drop location of mice always changed but kept a constant distance between them in the four trials. In hidden platform water maze test, the platform was put just under the water and its position was fixed at northwest. Every mouse was also trained for four trials per day. The order of drop location (east, south, west and north) changed among 7 training days. After 7 days of training, the hidden platform was removed and mice were giving one probe trial test. AniLab Software (Ningbo, China) was used to record and analyze the data.

Data analysis

Data were presented as means ± standard error of mean (SEM). Data were analyzed by t test using Graphpad Prism 5 software. The levels of significance were measured based upon respective graph values. Symbols * and *** represent significant values $P < 0.05$ and $P < 0.001$, respectively.

Results

The expression of DSTYK mRNA and protein is deleted in DSTYK homozygous KO mice

To compare DSTYK mRNA expression level in $DSTYK^{+/+}$, $DSTYK^{+/-}$ and $DSTYK^{-/-}$ mouse, 2-3

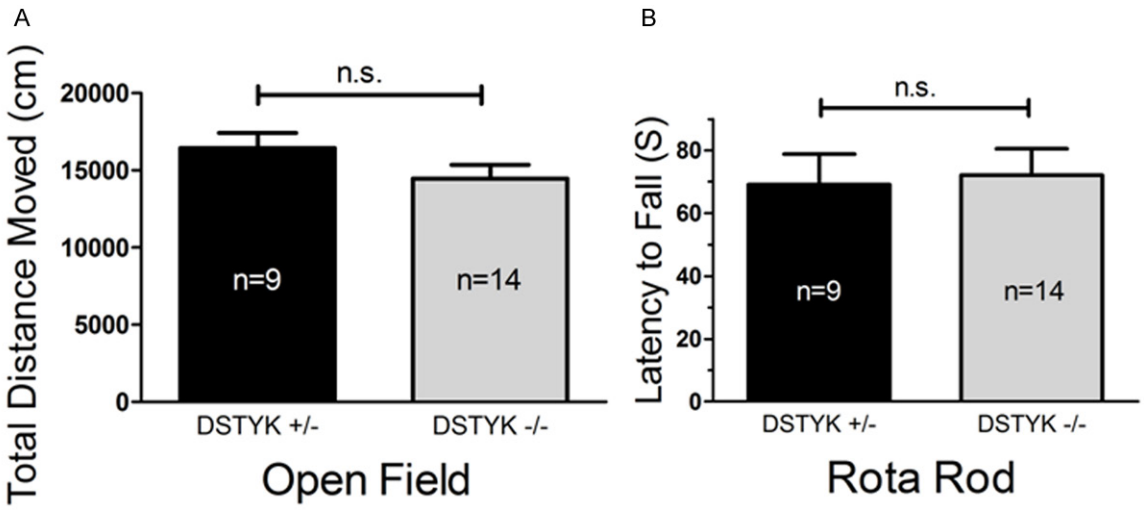

Figure 4. DSTYK knockout mice showed no significant defects in basic motor and balance capacity. A. Total distance moved in open field test. B. Rota rod test showed no significant difference between DSTYK heterozygous and homozygous knockout mice. N.S. No Significance.

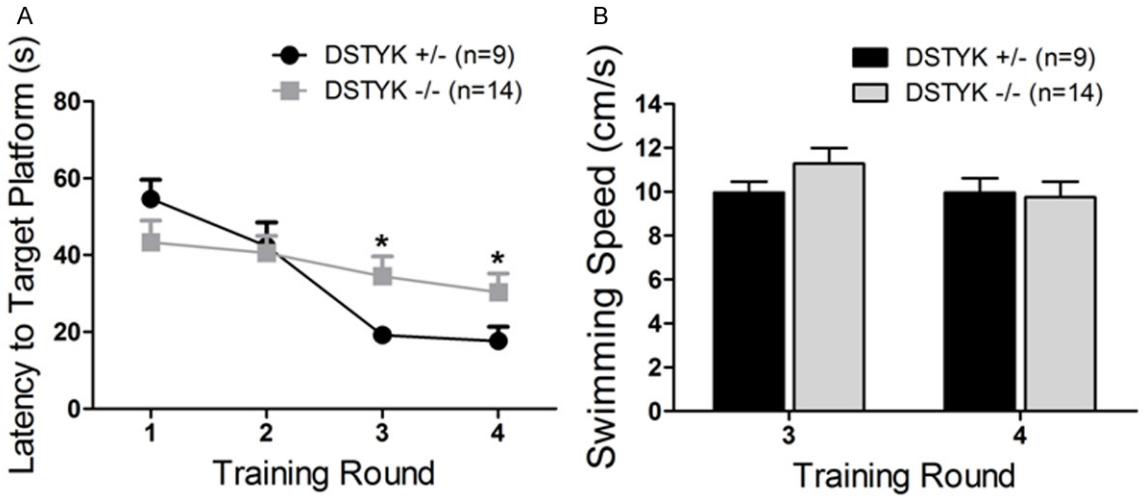

Figure 5. Visible task results of water maze test. A. DSTYK$^{-/-}$ mice showed a longer Latency to target platform than DSTYK$^{+/-}$ mice in training round 3 and 4. B. There is no significant difference in average speed between DSTYK$^{+/-}$ mice and DSTYK$^{-/-}$ mice. "*", $P < 0.05$.

mice for each genotype were sacrificed to obtain mRNA from cerebral cortex. Quantitative PCR analysis showed that the DSTYK mRNA level of DSTYK$^{+/-}$ mouse were reduced to about 60% of DSTYK$^{+/+}$ mouse and no DSTYK mRNA was detected in DSTYK$^{-/-}$ mice (**Figure 2B**). To confirm the deletion of full-length DSTYK protein in DSTYK$^{-/-}$ mice, adult male mice were sacrificed to obtain protein from cerebral cortex. Results from western blot showed that a band about 119 kDa disappeared in DSTYK$^{-/-}$ mice, which confirmed the knockout of DSTYK. The observed protein size of DSTYK was larger than the predicted size 105 kDa, which may be due to unknown posttranslational modification. The antibody manufacturer also observes the change in molecular weight of DSTYK protein in mice.

DSTYK$^{-/-}$ mice showed no detectable developmental defects in brain revealed by Nissl staining

The knockout of DSTYK is not lethal to mouse. The DSTYK$^{-/-}$ mice can normally develop and is fertile. To check the effect of DSTYK knockout on the brain development, adult DSTYK$^{+/+}$ and DSTYK$^{-/-}$ mice were sacrificed to conduct Nissl

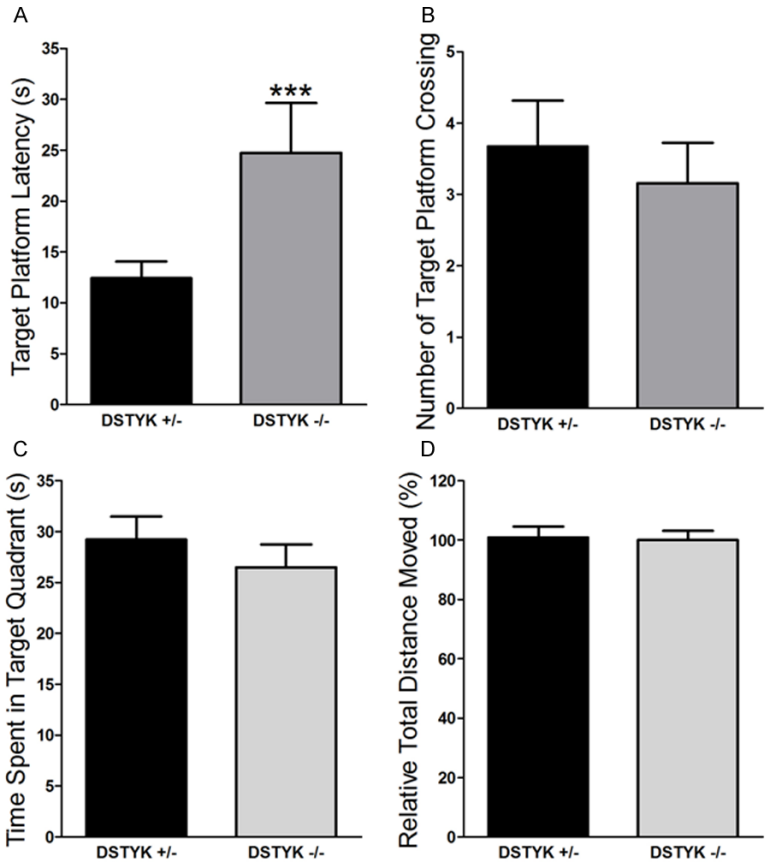

Figure 6. Probe trial results of water maze test. A. Target platform latency. B. Number of target platform crossing. C. Time spent in target quadrant. D. Relative total distance moved. DSTYK$^{+/-}$, n=9; DSTYK$^{-/-}$, n=14. "***", $P < 0.001$.

staining. The results showed there are no detectable defects both in DSTYK$^{+/+}$ and DSTYK$^{-/-}$ mice (**Figure 3**).

DSTYK$^{-/-}$ mice showed no basic locomotion defects

To identify the effect of DSTYK knockout on the basic locomotion activity of mouse, open field test and rotarod test were performed. The total distance moved in open field test (**Figure 4A**) and the latency to fall from rod in rota rod test (**Figure 4B**) were not significantly different between DSTYK$^{+/-}$ and DSTYK$^{-/-}$ mice. These results indicated that DSTYK knockout did not cause severe defects in basic movement and balance capacity.

DSTYK$^{-/-}$ mice showed poor performance in morris water maze test

To test if DSTYK knockout has any effects on spacial learning and memory, 9 DSTYK$^{+/-}$ mice and 14 DSTYK$^{-/-}$ mice were given morris water maze test. In trial 1-2 of visible platform water maze test, DSTYK$^{-/-}$ mice did not show great difference compared with DSTYK$^{+/-}$ mice (**Figure 5A**). But In trial 3-4 of visible platform water maze test, DSTYK$^{-/-}$ mice cost significantly more time to swim to the target platform than DSTYK$^{+/-}$ mice (**Figure 5A**). The poor performance of DSTYK$^{-/-}$ mice may not be caused by the motor defects but by the defects in learning and memory because there was no significant difference in the average swimming speed of trial 3-4 between DSTYK$^{+/-}$ and DSTYK$^{-/-}$ mice (**Figure 5B**). In the probe trial of hidden platform water maze test, the latency to target platform of DSTYK$^{-/-}$ mice were significantly higher than that of DSTYK$^{+/-}$ mice (**Figure 6A**). But the other index in probe trial such as number of target platform crossing (**Figure 6B**), time spent in target quadrant (**Figure 6C**) and relative total distance moved (**Figure 6D**) showed no significant difference between DSTYK$^{+/-}$ and DSTYK$^{-/-}$ mice. Taken together, our findings suggest that DSTYK may be involved in the process of spacial learning and memory.

Discussion

DSTYK is reported to be widely distributed in multiple tissues of vertebrates from the developmental stage to the adult [2]. Human DSTYK full-length (7.9 kb) mRNA is presented in many organs and tissues such as heart, brain, placenta, skeletal muscle, kidney, lung and pancreas assessed by northern blot analysis. Another short DSTYK mRNA (about 3.6 kb) was detected in testes [2]. Like human DSTYK, mouse DSTYK also located on Chromosome 1, containing 13 exons and encoding a protein with 927 amino acid residues. Northern Blot experiments found that full length mRNA (7.6 kb) of DSTYK existed in E9.5 mouse embryo and in adult mouse brain, heart, lungs, kidneys, and skin [2]. A smaller mRNA fragment (3.6 kb)

was also detected in mouse testes [2]. Results from fluorescence *in situ* hybridization showed that DSTYK mRNA exists in E14.5 mouse embryo and in adult mouse brain, heart, kidney and other organs, which is consistent with results of northern blot [2]. Western Blot test showed that DSTYK protein exists in the mouse brain, heart, kidney, lung and skeletal muscle [2]. Northern Blot has also detected the expression of DSTYK in multiple tissues of rats and chickens [2]. However, few studies are focusing on the physiological function of DSTYK until now.

In vitro study revealed that DSTYK could induce cell death in a dose dependent manner and also cause DNA fragmentation [1]. Pan-caspase inhibitors did not rescue the cell death induced by DSTYK, suggesting that DSTYK induce cell death through caspase-dependent and caspase-independent pathways [1]. DSTYK knockdown in zebrafish caused severe developmental defects. Many organs such as heart, fins, tail, and cloaca were not normally developed. Cloaca of zebra fish is considered as the homologous organ of the urogenital system of higher animals [5]. Genome analysis of a family suffering from a congenital kidney disease pointed out a linkage between DSTYK and kidney development [5]. In this family, a mutation in DSTYK genomic DNA was detected between exon 2 and exon 3, which was believed to cause mRNA splicing errors and the formation of mRNA missing 27 bp in exon 2. The protein product of this abnormal mRNA lacked amino acid residues 210-218 (VTMHHALLQ), which was considered as the cause of congenital kidney disease in this family. DSTYK mutations were also detected in 2.3% of 311 other unrelated patients suffering from congenital kidney disease. Thus mutations in DSTYK were considered as one of the causes of congenital kidney disease [5].

In summary, previous studies showed that DSTYK plays multiple roles in cell death and the development of many organs. To our surprise, our results showed that kinase domain ablation of DSTYK did not cause severe developmental defects especially in brain development. It is noted that the identified DSTYK mutant in the family suffering from congenital kidney disease occurs at exon 2-3. But exon 1-3 is not ablated in the knockout mice we used. The truncated DSTYK protein may be translated from exons 1-3 in this DSTYK knockout mice line, which may still play some roles as wildtype DSTYK in development. In addition, we can't exclude the possibility that other genes may compensate the function of DSTYK. Thus the ablation of full-length DSTYK in mouse is needed to further clarify the role of DSTYK in the development of mouse.

Disclosure of conflict of interest

None.

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