European Review for Medical and Pharmacological Sciences 2016; 20: 2853-2862

Anxiety-like behavior and dysregulation of miR-34a in triple transgenic mice of Alzheimer's disease

Y.-L. ZHANG[1,2], R.-Z. XING[1,2], X.-B. LUO[3,4], H. XU[2], R.C.-C. CHANG[5], L.-Y. ZOU[6], J.-J. LIU[1], X.-F. YANG[1]

[1]Key Laboratory of Modern Toxicology of Shenzhen, Medical Key Laboratory of Guangdong Province, Medical Key Laboratory of Health Toxicology of Shenzhen, Shenzhen Center for Disease Control and Prevention, Shenzhen, China
[2]College of Pharmacy, Jinan University, Guangdong, China
3AND Biotech, Shenzhen, China
[4]Guang Zhou Kai-Tuo Biotech, Guangzhou, China
[5]Laboratory of Neurodegenerative Diseases, Department of Anatomy, The University of Hong Kong, Pokfulam, Hong Kong SAR, China.
[6]Department of Neurology, Shenzhen People's Hospital, Second Clinical College, Jinan University, Shenzhen, China

Yanling Zhang and *Renzhong Xing* contributed equally to this work

Abstract. – OBJECTIVE: MicroRNAs (miRNAs) play an important role in the development of the brain and also implicated in the pathogenesis of neurological diseases such as Alzheimer's disease (AD). Recent studies implied that dysregulation of miRNAs is involved in neuropsychiatric disorders such as anxiety disorder in AD.

MATERIALS AND METHODS: In this study, behavioral experiments such as open field test, elevated plus maze test and light-dark box test were performed to evaluate anxiety-like behaviors in a triple transgenic mouse model of AD (3xTg-AD mice), and Q-PCR was used to measure the change of miR-34a expression.

RESULTS: Behavioral tests revealed anxiety-like behaviors in 3xTg-AD mice. Q-PCR assay showed significantly elevated expression of miR-34a in the hippocampus of 3xTg-AD mice compared with the age- and gender-matched wild-type mice. Western-blot analysis showed that the expression of metabotropic glutamate receptor 7 (GRM7) but not fibroblast growth factor-2 (FGF2), two anxiety disorder-related target genes of miR-34a, was significantly decreased in hippocampus of 3xTg-AD mice compared with the wild-type mice.

CONCLUSIONS: We concluded that anxiety-like behavior occurred in 3xTg-AD mice with an involvement of miR-34a/GRM7.

Key Words:
Alzheimer's disease (AD), miR-34a, Anxiety disorder.

Abbreviations

miRNAs = MicroRNAs; AD = Alzheimer's disease; 3xTg-AD mice = A triple transgenic mouse model of AD; WT = Wild-type; GRM7 = Metabotropic glutamate receptor 7; FGF2 = Fibroblast growth factor-2.

Introduction

Alzheimer's disease (AD), the most common case of dementia, is characterized by a progressive deterioration of cognitive functions[1]. The incidence of AD was estimated to increase double in every five years among the elderly aged more than 65 and that the number of AD cases will reach 115 million by 2050 worldwide[2]. The etiology of AD remains poorly understood. The two pathological hallmarks of AD are the deposition of β-amyloid (aggregates to form plaques) and the progressive formation of neurofibrillary tangles[3]. In addition to progressive cognitive impairment, neuropsychiatric symptoms such as anxiety disorder are most common in AD cases[4-6].

Evidence indicated that the treatment of anxiety could significantly improve their quality of life of the patients with cognitive impairment[7]. A recent report indicated that about two-thirds of AD patients may be undiagnosed[8], and the symptoms such as anxiety in AD are frequently ignored. Enough concerns on neuropsychiatric

Corresponding Author: Xifei Yang, MD; e-mail: xifeiyang@gmail.com

symptoms such as anxiety which often occur at the early stage of AD are helpful for the early diagnosis of AD. However, there is a serious lack of understanding of anxiety in AD and the mechanisms underlying anxiety in AD remain elusive.

MicroRNAs (miRNAs) are endogenous, small noncoding RNAs of approximately 21-25 nucleotides that serve as post-transcriptional regulators of gene expression via inhibition of translation initiation or cleavage of mRNA in diverse cellular and developmental processes[9-11]. It is predicted that 1000 miRNAs are expressed in human brain[12]. Up to now, more than 400 miRNAs have been identified in most kinds of cells and tissues such as the brain of humans and chimpanzees[12,13]. MiRNAs have been shown to play important roles in the neurodevelopment and synaptic plasticity[14]. MiRNAs can regulate gene expression more than 30% in the animals[15]. In addition, the regulation of diverse biological processes, such as embryonic development and immune response, also involves miRNAs[11,16]. Aberrant or absent expression of miRNAs is associated with neurological disorders[17,18] such as AD[19-22]. Some miRNAs were found to be differentially expressed in AD patients, and the dysregulation of miRNA target network was implicated in the pathogenesis of AD[23,24].

MiR-34a, whose gene is located at chromosome 1p36, is associated with invasion and metastasis of various cancers. Mature miR-34a is an important component of the p53 tumor suppressor gene regulatory network, which can inhibit tumor growth by regulating the expression of apoptosis-related factors[25]. Wang et al[26] reported that miR-34a caused apoptosis in AD through regulating the expression of bcl2 in a double transgenic mouse model of AD. In mice, miR-34a is ubiquitous with the highest expression in brain, and overexpression of miR-34a in neuroblastoma cell lines modulates the expression of certain neuronal-specific genes[27].

In this study, we are aimed to determine the possible change of neuropsychiatric symptoms such as anxiety behaviors in the 3xTg-AD mice, and the change of miR-34a expression in the hippocampus, a central brain region governing emotion.

Materials and Methods

Experimental Animals

Animals used in this study were 6-month-old triple transgenic mice model of AD (3xTg-AD) harboring $PS1_{M146V}$, APP_{Swe} and Tau_{P301L} transgenes (strain: B6; 129-$Psen1^{tm1Mpm}$ Tg [APPSwe, tauP301L] 1Lfa/Mmjax and wild-type (WT) mice (strain: B6129SF2/J) from the Jackson Laboratory. The mice were group housed of 4 mice per cage (470 × 350 × 200 mm) and kept on a regular 12h light/dark schedule (lights on 6:00 am, lights off 6:00 pm). All the mice were reared under temperature (22-23), and humidity (40%-60%) with food and water available *ad libitum*. All the experiments were conducted in accordance with the approved protocols and animal welfare regulations of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Regulations for Animal Care and Use Committee of Experimental Animal Center at Shenzhen Center for Disease Control and Prevention.

Behavioral Test

All the behavioral tests were performed using groups of 10-15 mice in a quiet room during the dark period (18:00-06:00) and the handlers performing the behavioral tests were blinded to the genotype of the mice. Mice were submitted to the test room for 2 h before the assessment. In this study, the open field test, the light/dark box test and the elevated plus maze test were performed to evaluate anxiety-like behavior.

Open Field Test

The open field test was performed as previously described[28,29]. The open field test is based on an exploration behavior. The more time spent in the central part of the open field, the less anxious it is considered to be. The open field (Shanghai Bio-will Co., Ltd., Shanghai, China) is a square arena consisted of a white Plexiglas box (50 cm x 50 cm x 40 cm, width x length x height), with a lamp providing a dim 120 lux illumination on the floor. The squares along the side are defined as peripheral squares and the others as central squares. The arena was subdivided into 'corner' (12 squares each 12.5 cm x 12.5 cm) and 'center' (4 squares each 12.5 cm x 12.5 cm) zones. Each mouse was placed in the center of the apparatus and allowed to move freely for 5 min. The behavior was recorded using a camera mounted in the apparatus. The time spent in the center of the arena and the total distance traveled were recorded as the indicator of the degree of anxiety behavior.

Elevated Plus Maze Test

The elevated plus maze test was performed as previously described[29-33]. Elevated plus maze

(Shanghai Biowill Co., Ltd, Shanghai, China) is widely used for the assessment of anxiety. The maze was made of black Plexiglas and consisted of two open arms (50 × 10 cm) and two enclosed arm (50 × 10 cm) with walls 60 cm high. The arms extended from a central platform (10 × 10 cm). The maze was elevated 50 cm above the floor. Each mouse was placed onto the center platform facing an open arm and allowed free exploration for 5 min. The behavior of the mice was recorded by a camera mounted in the apparatus. The following parameters were measured during the 5-min test period: (a) the time spent in the open arms, (b) the time spent in the closed arms, (c) the number of entries into the open arms, (d) the number of entries into the closed arms, (e) the number of entries into the open arms/ total entries in arms and (f) the total distance traveled.

Light/Dark Box Test

The light/dark box test was performed as previously described[31,33,34]. The apparatus (Shanghai Biowill Co., Ltd, Shanghai, China) was a rectangular box consisted of a plexiglas box divided into two environments by a wall. One compartment (length: 14.5 cm, width: 15.5 cm, height: 15.5 cm) was black and illuminated with an intensity of 70 lux; the other compartment (length: 14.5 cm, width: 15.5 cm, height: 15.5 cm) was white with a lamp providing a dim 400 lux illumination. The compartments were connected by a small central open door (5 cm x 6 cm) located in the middle of the partition. When starting the test, the mice were individually placed in the light box, facing the dark compartment. Then, the mice were allowed to explore freely both compartments for 5 min. The behavior of the mice was recorded by a camera mounted in the apparatus. Behaviors scored were: (a) the number of entries into the white compartment; (b) the time spent in the white compartment; (c) the latency until the first entry into the black compartment; (d) the total distance traveled.

Isolation of Hippocampus, Total RNA Extraction and miRNA Quantitative Analysis

After the behavioral tests, the mice were anesthetized with 10% (200 mg/kg, i.p.) chloral hydrate (Tianjin Kermel Chemical Reagent Co., Ltd, Tianjin, China) and decapitated. The brain was quickly removed from the skull and released into a dissecting tray. The hippocampus is separated from the brain, released into an Eppendorf tube and immediately snap frozen in liquid nitrogen until all the samples were collected.

Total RNA was extracted from whole hippocampi of the mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and miRNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. The final RNA concentration and the purity were determined using a ND-1000 spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE, USA) according to the manufacturer's recommendations. RNA integrity was determined by standard denaturing agarose gel electrophoresis.

Quantitative miRNA expression was analyzed using a ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). For qRT-PCR analysis of miR-34a, The RNA samples were assayed using MMLV reverse transcriptase (Epicentre, Madison, WI, USA) and miRStar™ SYBR® Green Real-Time qPCR Master Mix (Arraystar, Rockville, MD, USA) for each miRNA according to the manufacturer's instructions. The expression of the U6 small nucleolar RNA gene was used as an internal control.

The mixtures including RNase-free water, cDNA and SYBR® Green (Arraystar, Rockville, MD, USA) for each miRNA were loaded into a 384-well plate (Corning, Pittsburgh, PA, USA). Q-PCR amplification included the following thermal cycler conditions: 10 min at 95°C, 40 cycles of 10s at 95°C and 60s at 60°C. PCR was performed in triplicate for each sample. The primers for miR-34a were designed to produce amplicons using Primer Premier v5.0 Software. The expression of U6 snRNA was used to normalize the expression of tested mature miRNAs, and the relative quantification method, $2^{-\Delta\Delta C}T$[35], was used for calculating the relative expression levels of miR-34a compared to the mean of the U6 small nucleolar RNA. All the oligonucleotide primers used in PCR were supplied by Shanghai Brilliance power grid Biotechnology Co. Ltd (Shanghai, China). The reverse transcription primers and the primer sets specific for amplification of miR-34a were shown in Table (for primers, see Supplementary Information, Supplementary Table I and Supplementary Table II).

Western-Blot Analysis

The hippocampi of 3xTg-AD mice and the age- and gender-matched wild-type mice were quickly dissected from the brain tissues and rapidly homogenized in lysis buffer (Beyotime Biotech,

Supplementary Table I. Sequences of the primers used in the RT-PCR validation.

Primer name	Primer sequence(5′ → 3′)
U6	5'CGCTTCACGAATTTGCGTGTCAT3'
mmu-miR-34a-5p	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAACCA3'

Shanghai, China) containing protease and phosphatase inhibitor mixture (Thermo Fisher Scientific, Hudson, MA, USA). The lysates of protein were centrifuged at 4°C, 12000 x g for 40 min. The protein concentration was measured with BCA protein assay reagent (Thermo Fisher Scientific, Hudson, MA, USA). The protein samples in the supernatant were denatured with loading buffer at 100°C for 5 min. The proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were incubated overnight with the antibody against mGluR7 (rabbit polyclonal, 1:500) (Santa Cruz Biotech Inc, Santa Cruz, CA, USA) and the anti-FGF2 (mouse monoclonal, 1:500) (Santa Cruz Biotech Inc, Santa Cruz, CA, USA) at 4°C, followed by the anti-rabbit or anti-mouse secondary antibody IgG conjugated to horseradish peroxidase (1:2000) (Santa Cruz Biotech Inc, Santa Cruz, CA, USA) for 1h at room temperature. The membranes were developed with chemiluminescence substrate and an ImageQuant RT ECL System (GE Healthcare, Marlborough, MA, USA). The relative expression levels of the proteins were analyzed using ImageQuant TL-1D analysis tool (GE Healthcare, Marlborough, MA, USA).

Statistical Analysis

The data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). All the tests in this study were analyzed using a two-tailed Student's *t*-test to determine the possible differences between the groups. $p < 0.05$ was considered statistically significant.

Results

3xTg-AD Mice Showed an Anxiety-Like Behavior

To determine the possible neuropsychiatric symptoms in 3xTg-AD mice, we measured the possible change of anxiety-like behaviors using open field test. The data showed that the time spent in the center of the open field was significantly decreased in 3xTg-AD mice compared to the WT mice ($p = 0.000$, Figure 1A). However, no significant difference of the total distance traveled in the open field was observed between 3xTg-AD mice and the WT mice ($p = 0.704$, Figure 1B).

In addition, we also performed elevated plus-maze test. The data showed a significantly decreased number of entries into the open arms ($p = 0.006$, Figure 2A), increased frequency of entries into the closed arms ($p = 0.043$, Figure 2B), and less time spent in the open arms ($p = 0.004$, Figure 2C) in the 3xTg-AD mice compared to the age- and gender-matched wild-type mice. The number of entries into open arms/the number of entries to the open plus closed arms was significantly decreased in the 3xTg-AD mice compared to the wild-type mice ($p = 0.039$, Fig-

Supplementary Table II. Sequences of the primers used in the RT-qPCR validation.

Gene	Annealing temperature (°C)	Product size (bp)	Number gene primer (5′ → 3′)
U6	60	89	F:5'GCTTCGGCAGCACATATACTAAAAT3' R:5'CGCTTCACGAATTTGCGTGTCAT3'
mmu-miR-34a-5p	60	64	GSP:5' GGGGTGGCAGTGTCTTAGC3' R:5'GTGCGTGTCGTGGAGTCG3'

F, forward primer; R, reverse primer; GSP, gene specific primer. MiRNA number and the sequence of a specific miRNA can be obtained from miRBase sequences (http://microrna.sanger.ac.uk.

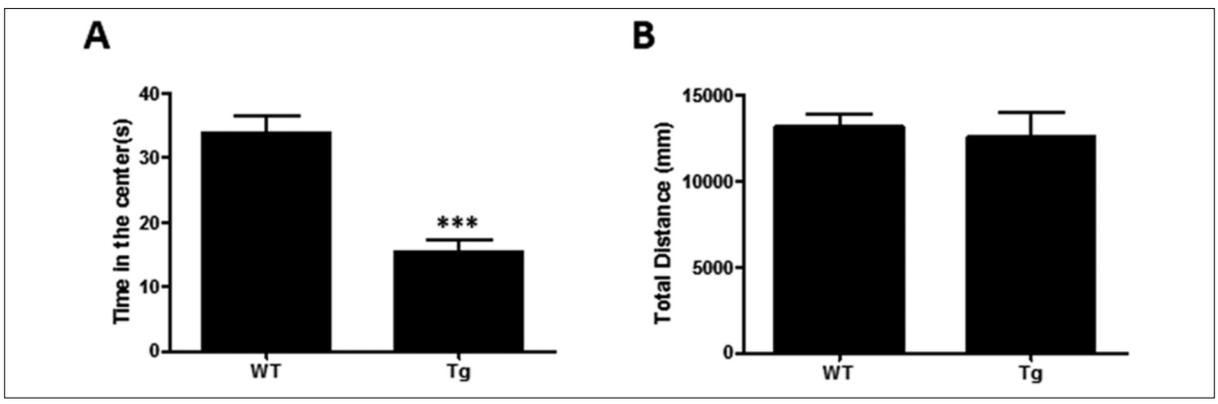

Figure 1. The anxiety-like behavior of 3xTg-AD mice as measured by the open field test. The behavior performance was recorded for 5 min. ***A,*** Time spent in the center of the open field. ***B,*** Total distance traveled in the open field. n = 10-15 for each group. Data were expressed as mean ± SEM ***$p < 0.001$ for the difference between 3xTg-AD mice and the WT mice.

ure 2E). However, there were no significant differences in the time spent in the closed arms (p = 0.390, Figure 2D) and the total distance traveled in the elevated plus maze test (p = 0.120, Figure 2F) between 3xTg-AD mice and the WT mice.

To validate anxiety-like behaviors in the 3xTg-AD mice, we also performed the light/dark exploration test. We found that the time spent in the white box was significantly decreased in 3xTg-AD mice compared to the wild-type mice (p = 0.020, Figure 3A). Both the number of white box entries and the total distance traveled in light for 3xTg-AD mice relative to the wild-type mice were significantly decreased (p = 0.046, Figure 3B; p = 0.048, Figure 3C, respectively). The latency to enter the dark box was significantly decreased in 3xTg-AD mice compared to the wild-type mice (p = 0.004, Figure 3D). Taken together, all these behavioral data indicated that the 3xTg-AD mice developed anxiety-like behaviors.

Up-Regulation of miR-34a in Hippocampus of 3xTg-AD Mice

To determine the possible change of miR-34a expression in the hippocampus in the 3xTg-AD mice, we measured the levels of miR-34a using qRT-PCR. The data showed the levels of miR-34a were significantly up-regulated in the hippocampus of 3xTg-AD mice compared with the matched wild-type mice ($p < 0.001$, Figure 4).

Expression of Potential Anxiety-Related Target Genes of miR-34a

Western-blot analysis showed that the expression of GRM7 (Figure 5) but not FGF2 (data not shown) was significantly down-regulated in hippocampus of 3xTg-AD mice compared to the wild-type mice.

Discussion

AD-like pathologies and spatial learning and memory deficits were well reproduced in transgenic mouse models of AD. However, few studies were focused on neuropsychiatric symptoms in animal models. Anxiety disorder is one of the most common neuropsychiatric symptoms in AD cases, and recognition and treatment of these symptoms can significantly improve the life quality of patients[7]. It is known that anxiety disorder in AD was associated with psychosocial morbidity, disability, and mortality, seriously affecting the quality of life and well-being of the patients. In the elderly patients, anxiety disorder is also associated with both a more rapid increase in the course of AD and reduced survival time of patients[36,37]. In this study, we revealed anxiety-like behavior in 3xTg-AD mice using open field test, elevated plus maze test and light/dark box test.

Some studies suggested that miRNAs play an important role in the molecular control of brain development and neurodegenerative diseases. Scott et al[38] reported that miRNAs were implicated in the development of anxiety disorder and had the potential to serve as biomarkers of disease as well as targets for pharmacological treatment. A recent study[39] showed that miR-183 pathway effectively regulated by environmental enrichment reduced anxiety-like behavior. Although the current models of the hippocampal function empha-

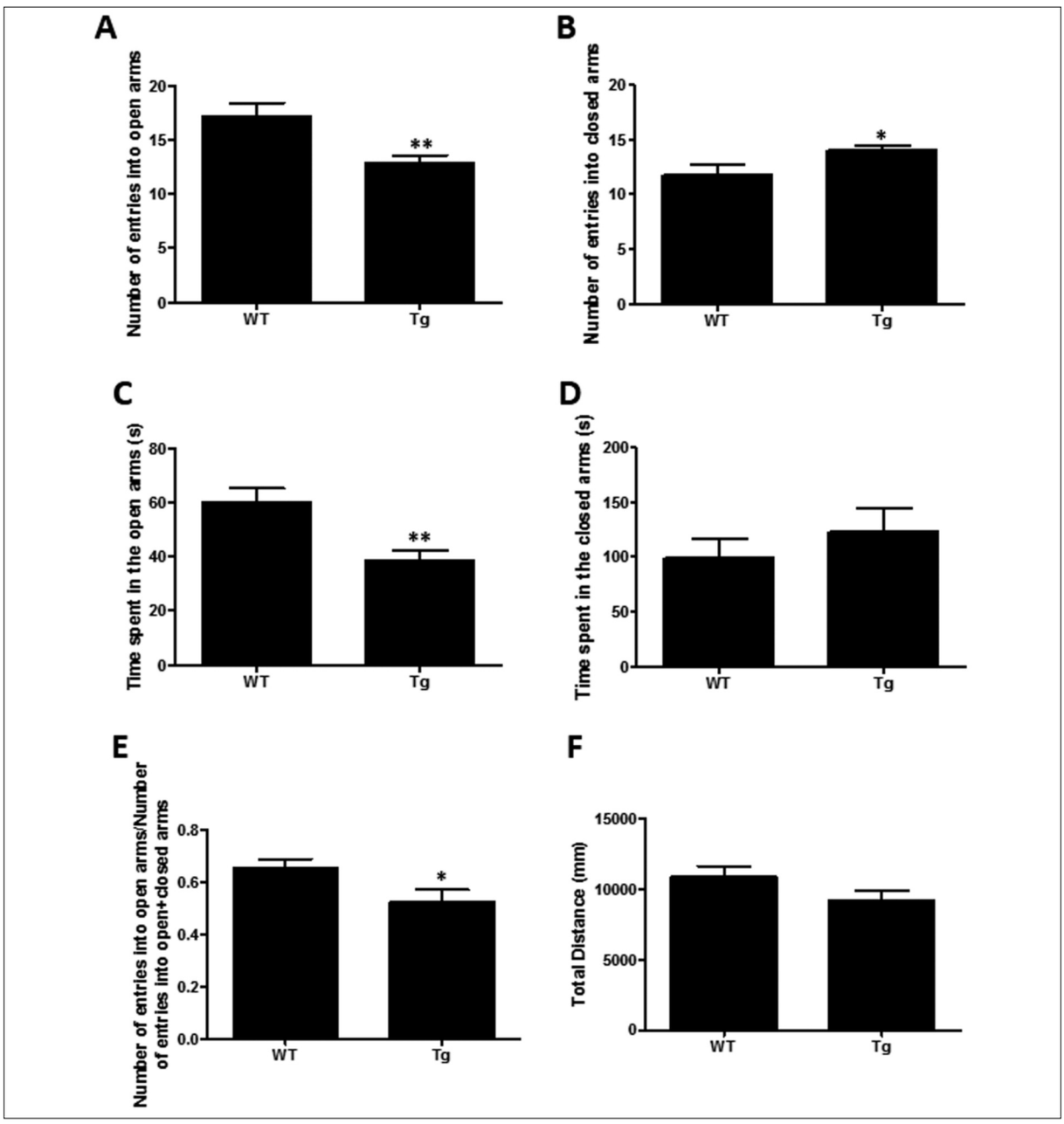

Figure 2. The anxiety-like behavior of 3xTg-AD mice as measured by the elevated plus maze test. ***A,*** The number of entries into open arms. ***B,*** The number of entries into the closed arms. ***C,*** The time spent in the open arms. ***D,*** The time spent in the closed arms. ***E,*** The number of entries into open arms/number of entries to the open plus closed arms and ***(F)*** The total distance traveled during 5 min exploration in the elevated plus maze. n = 10-15 for each group. All the values represent mean ± SEM. *$p < 0.05$ and **$p < 0.01$ for the differences between 3xTg-AD mice and the WT mice.

size the role of its well-known cognitive function, historically it is also regarded as a neural medium of emotion. The recent data[40] clearly suggested that hippocampus is importantly and directly involved in the mediation of untrained anxiety reactions in animals. Mòdol et al[41] demonstrated that the dorsal hippocampus had an important effect on several neurosteroids behavior, for instance, anxiety, learning and memory. A most recent work[42] revealed that increasing adult hippocampal neurogenesis is sufficient to reduce anxiety and depression-related behaviors in mice treated chronically with corticosterone.

MiR-34a is a member of the miR-34a family,

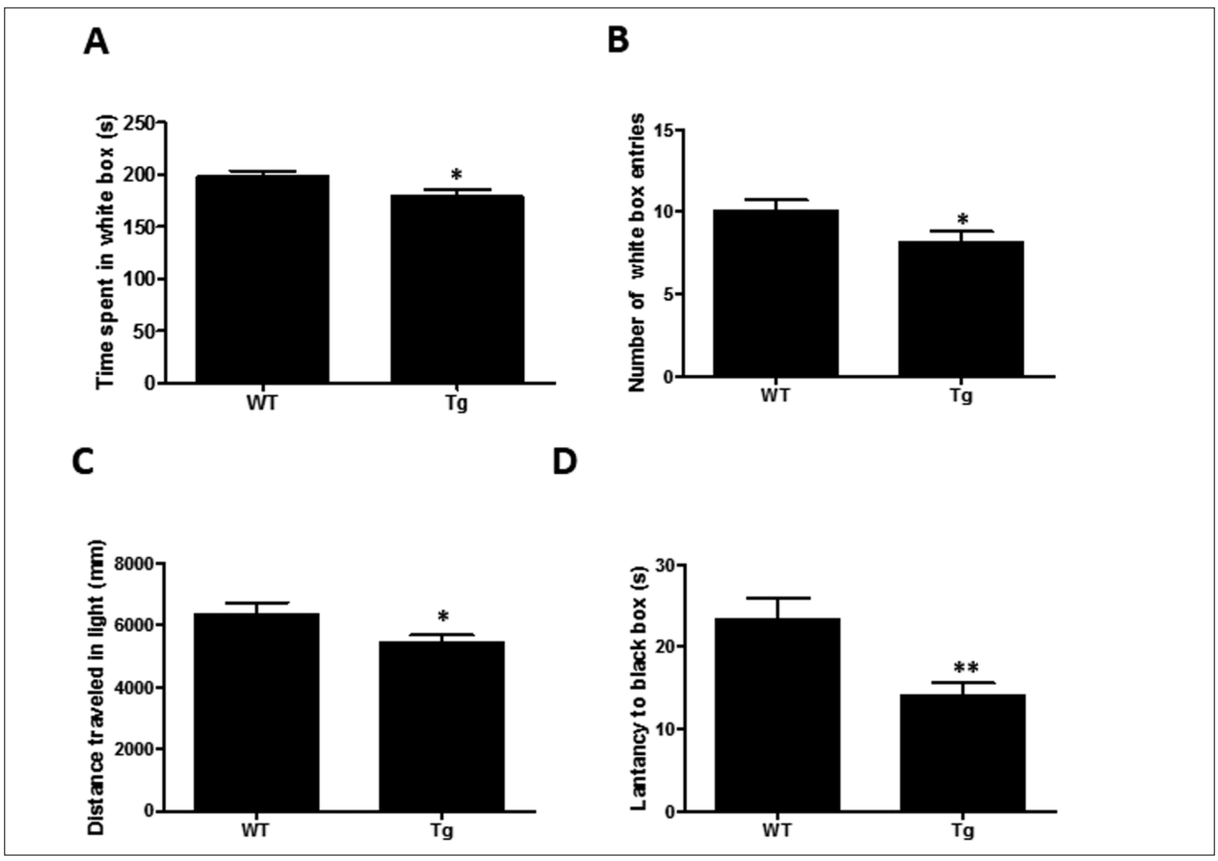

Figure 3. Anxiety-like behavior as measured by the light/dark box test. ***A,*** The time spent in the white box. ***B,*** The number of white box entries. ***C,*** The total distance traveled in the white box and ***(D),*** The latency of mice went into the black box in the light/dark exploration test. The mice were allowed to explore the apparatus for 5 min. The data values were expressed as mean ± SEM (n = 10-15 mice/group). *$p < 0.05$ and **$p < 0.01$ for the differences between 3xTg-AD mice and the WT mice.

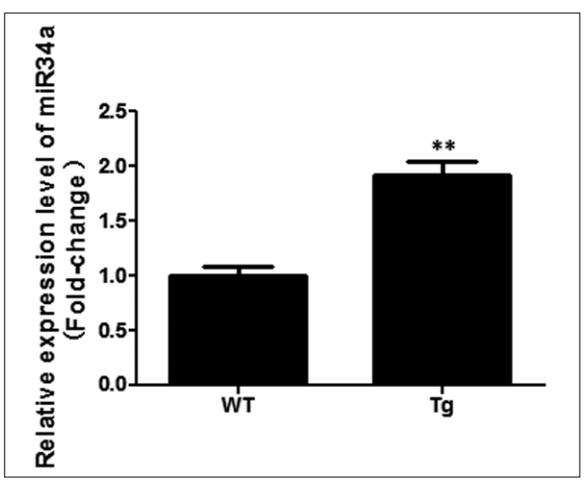

Figure 4. The expression of miR-34a in the hippocampus of 3xTg-AD mice. The relative expression of miR-34a was normalized to the expression of the internal control (U6 small nucleolar RNA). The p values were calculated by 2-tailed Student's *t*-test. The values were expressed as mean ± SEM (n = 4 mice/group). **$p < 0.01$ for the difference between 3xTg-AD mice and the WT mice.

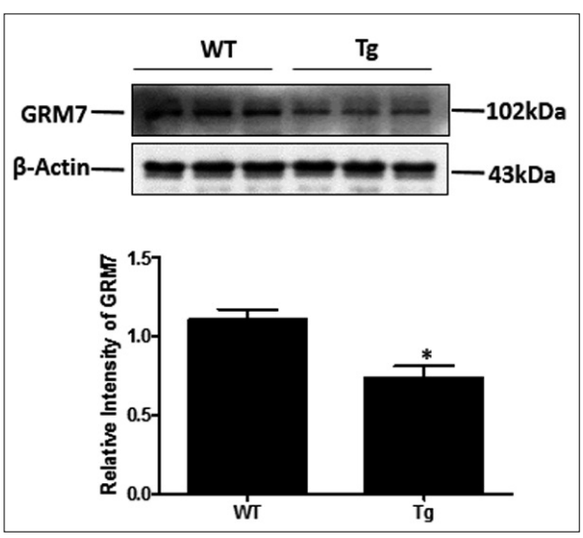

Figure 5. The expression of GRM7 in the hippocampus by Western-blot analysis. Western-blot analysis showed the change of hippocampus GRM7 in 3xTg-AD mice compared to the WT mice. The data were presented as mean ± SEM (n = 3 mice/group). *$p < 0.05$ compared with WT mice.

which also includes miR-34b, and miR-34c in mammals[43]. Previous studies[26,44] demonstrated the up-regulation of miR-34a in AD patient brains and double transgenic mice model of AD. Here it has been demonstrated a significant elevated expression of miR-34a in the hippocampus of 3xTg-AD mice compared with the control mice. Zhou et al[45] showed that anxiety-like behavior occurred in GRM7 knockout mice and that the treatment of the mice with lithium and VPA could attenuate anxiety-like behaviors through modulating the expression of anxiety-related genes such as GRM7 (an identified target gene of miR-34a) by miR-34a. The GRM7 is a kind of excitatory neurotransmitter in the central nervous system of mammals, which is closely related to the fast excitatory synaptic transmission, rapid development and death of neurons, synaptic plasticity, and certain neurological diseases[46]. GRM7 has been associated with autism, drug abuse, anxiety, and depression. O'Connor et al[47] suggested targeting GRM7 receptors with selective antagonist drugs may provide a safe and effective strategy for the treatment of anxiety disorder. In this study, we observed a significant decreased expression of GRM7 in the hippocampus of 3xTg-AD mice, while no significant change of anxiety disorder-related gene FGF2, a potential target gene of miR-34a (data not shown). We, therefore, speculated that miR-34a/GRM7 could be involved in anxiety-like behavior in 3xTg-AD mice as observed.

Conclusions

We revealed anxiety-like behaviors in 3xTg-AD mice at a relatively early stage, up-regulated expression of miR-34a and down-regulation of anxiety-related target gene GRM7 of miR-34a, in the hippocampus. We observed that anxiety-like behavior in 3xTg-AD mice could be attributed to dysregulation of miR-34a/GRM7 in the hippocampus. We concluded that anxiety-like behaviors occurred in 3xTg-AD mice with an involvement of miR-34a/GRM7.

Acknowledgements

This work was supported by NSFC (the National Natural Science Foundation of China) (81171191; 81501213), Guangdong Provincial Natural Science Foundation (2014A030313715) and Shenzhen Special Fund Project on Strategic Emerging Industry Development (JCYJ20130329103949650, JCYJ20120616144140857, JCYJ20140416122812017, JSGG20140703163838793).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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