Original Paper

Expression of Amyloid-Associated miRNAs in Both the Forebrain Cortex and Hippocampus of Middle-Aged Rat

Hui Che^a Li-Hua Sun^a Fei Guo^a Hui-Fang Niu^a Xiao-Lin Su^a Ya-Nan Bao^a
Zidong Donna Fu^a Huai-lei Liu^b Xu Hou^b Bao-Feng Yang^a Jing Ai^a

^aDepartment of Pharmacology, Harbin Medical University (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China), ^bDepartment of Neurosurgery, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China

Key Words

Aging • microRNAs • APP • BACE1 • Learning • Memory

Abstract

Background: Aging is associated with the gradual cognitive decline and shows the typical senile plaque formation in the brain, which results from the aggregation of beta amyloid (A β) peptide following the abnormal proteolytic processing of amyloid precursor protein (APP) by β -secretase (BACE1) and γ -secretase. Accumulating evidence indicates that several microRNAs (miRNAs) are involved in the Alzheimer's disease (AD) by regulating the expression of APP and BACE1 proteins. However, the cognitive ability and the expression profile of the APP- and BACE1-associated miRNAs in the middle-aged population are largely unknown.

Methods: The learning and memory ability in rats were determined by Morris Water Maze test. The protein levels of APP and BACE1 were detected by western blotting. The quantitative polymerase chain reaction was used to identify the miRNAs levels in forebrain cortex and the hippocampus. **Results:** Middle-aged rats have declined learning ability without changes in the memory ability, and increased APP and BACE1 protein expression in the forebrain cortex. Computational analysis using Targetscan and Pictar databases reveals that totally 4 predicted miRNAs have conserved binding site with APP, namely *miR-106b*, *-17-5p*, *-153*, *-101*. All of them showed decreased expression in both the forebrain cortex and hippocampus. Among the 10 predicted miRNAs targeting BACE1, different expression profiles were identified in the forebrain cortex (decreased: *miR-9*, *-19a*, *-135a*, *-15b*, *-16*, *-195*, *-29c*, *-214*; increased: *miR-124*; no change: *miR-141*) and the hippocampus (decreased: *miR-9*, *-15b*, *-16*, *-195*, *-29c*, *-124*; increased: *miR-19a*, *-135a*, *-214*, *-141*) in the middle-aged rats compared with the young rats. **Conclusion:** Our results provided the first evidence that middle-aged rats have begun displaying cognitive disability with abnormal expression of APP- and BACE1-related miRNAs in the hippocampus and forebrain cortex.

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Introduction

With the increasing aging population, the incidence of progressive deterioration of cognitive abilities is dramatically increased [1]. Although it is debatable when age-related cognitive decline begins, middle age is the stage when a portion of the population starts to have notable physical decline [2, 3]. The phenomenon suggests that the middle-aged adults may begin to undergo the process of cognitive decline. However, the molecular mechanism is largely unknown.

The decrease of brain volume, loss of synapses, disorder motor, and cognitive impairments are observed in the aging brain, which are risk factors of neurodegenerative diseases such as Alzheimer's disease (AD) [4-6]. Previous data suggested that senile plaque, a major hallmark of both aging and dementia, is triggered by the aggregation of beta amyloid (A β) derived from the endoproteolysis of APP [7-11]. There are two different pathways of proteolytic processing of APP: one is the non-amyloidogenic pathway in which APP is cleaved by α -secretase and γ -secretase, the other is the amyloidogenic pathway processed by β -secretase (BACE1) and γ -secretase [12-15]. BACE1 is the major enzyme of A β formation and has ubiquitously higher expression in neurons of the hippocampus, cortex, and cerebellum, but lower expression in the gliocytes [16]. The activity of BACE1 is increased with aging in human, monkey, and mouse [17]. Furthermore, A β_{42} is found strongly accumulated early in the cortex, and a little later in the hippocampus in aged people between 50 and 79 years [18], and the senile plaques were also found in the cortex of 28-30 month of aged rats [19]. These results suggested that dysfunctions of APP and BACE1 might participate in the progress of normal aging and age-associated disorder. However, whether the changes of APP and BACE1 are present in the cortex and hippocampus of rats at the middle-aged stage are unclear.

MicroRNAs (miRNAs) are conserved ~22 nucleotides non-coding RNAs that regulate gene expression by translation inhibition or mRNA degradation [20]. Accumulating studies suggest that miRNAs can affect the aging-related pathways. There is evidence that approximately one third of miRNAs in *C. elegans* is altered in the expression level across the lifespan [21]. In addition to *C. elegans*, the expression levels of miRNAs were changed in aged mice and the elderly [22, 23]. It is noteworthy that increasing evidence indicated that BACE1 expression is regulated by *miR-328*, *-298*, *-29c* and *miR-107* in AD patients and mice [24-26]. Meanwhile, the expressions of *miR-17*, *-101* and *miR-16* decreased, which was accompanied with significantly high level of APP [27, 28]. These data indicated that miRNAs might be involved in the age-related pathologies.

In the present study, we reported for the first time that rats at ages of 18 months showed cognitive deficit with changes in APP- and BACE1-related miRNAs expression.

Materials and Methods

Animal

Male Wistar young (6 months old) and middle-aged (18 months old) rats (obtained from the Animal Center of the Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China) were housed in the temperature-control room (23 $\pm$ 1°C) with humidity of 50 $\pm$ 10% and allowed free access to food and water. All animals and experimental procedures were approved and carried out in accordance with the Experimental Animal Ethic Committee of Harbin Medical University, China (No.HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01). All procedures were conformed to the Directive 2010/63/EU of the European Parliament.

Morris water maze (MWM)

MWM hidden platform trail was performed using a previous method with some modifications [29]. Briefly, the maze was filled with opaque water (23 $\pm$ 1°C) via addition of black food nontoxic pigment and divided into four quadrants. A submerged escape platform (20 cm diameter platform, top surface 2.0 cm below water level) was located in the center of the first quadrant. Before training, the pupillary light reflex

was tested through all rats, and rats with disabled pupillary light reflex were excluded from the experiment to avoid the effect of the animals' vision on the test. On the first day, rats were released into the water facing the pool wall and allowed to swim to search the hidden platform. If they failed to find the target within 120 s, they were guided to the platform and allowed to rest for at least 20 s. In the following 5 consecutive days, rats were trained to find a hidden platform. The length of the swim path (distance in centimeters) was recorded using a video camera-system. MWM probe trial session was conducted on the 6th day. In the probe trial, the platform was removed from the pool and each rat received one 120 s swim. The times of crossing platform (X) and percentage time spent in target quadrant (Q) were monitored by an online DigBehav-Morris water maze Video Analysis System (Mobile Datum Software Technology Co. Ltd., Shanghai, China).

Western blot analysis

Proteins were extracted from the hippocampus and forebrain cortex of young and middle-aged rats for immunoblotting analysis. Briefly, tissue were lysed with RIPA buffer (50mM Tris-HCl, pH7.4; 150mM NaCl; 0.1% Tryton X-100; 0.25% Na-deoxycholate; 0.1M EDTA and 1% SDS) containing protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) on ice for 30 min. The supernatant were collected after centrifuging 13500rpm at 4°C for 15 min. The concentration of protein samples were measured spectrophotometrically using a BCA kit (Universal Microplate Spectrophotometer; Bio-Tek Instruments, Winooski, VT, USA). The proteins were loaded onto a 10% SDS-PAGE gel, and then transferred onto nitrocellulose membranes, which were incubated in 5% non-fat dry milk in TBST for 2 h at room temperature. Primary antibodies used were the following: anti-APP (Cat no. MAB348; 1:1000; Millipore, United States, US), anti-BACE1 (Cat no. ab2077; 1:1000; Abcam, Cambridge, MA) and GAPDH (Cat no. sc-137179; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was incubated in room temperature for 1 h. The bands were captured on Odyssey Infrared Imaging System (LI-COR) and quantified with Odyssey v3.0 software by measuring the band intensity (area×OD) in each group and normalizing to GAPDH.

Quantitative RT-PCR analysis

The analysis of mRNA expression was performed as described. Total RNA was isolated from the forebrain cortex and hippocampus of young or middle-aged rats using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions [30]. 500ng total RNA was reverse-transcribed by the High Capacity cDNA Reverse Transcription kit (Cat no. 4368814, Applied Biosystems, Foster City, CA) with random primer following the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was done using the TaqMan® Gene Expression Master Mix (Cat no. 4369016, Applied Biosystems, Foster City, CA) with probes for APP (ID: Rn00570673_m1), BACE 1 (ID: Rn00569988_m1) and GAPDH (ID: Rn01462662_g1) purchased from Applied Biosystems. The PCR procedure was as follows: 95°C for 10 min, followed by 40 cycles with 95 °C for 15 sec and 60 °C for 1min. GAPDH was used as internal control. $\Delta\Delta C_t$ was calculated for each sample, and the expression levels of mRNA were calculated using the $2^{-\Delta\Delta C_t}$ method.

The detection of mature miRNAs was performed by qRT-PCR. First, 0.5µg total RNA was reverse-transcribed by the High Capacity cDNA Reverse Transcription kit (Cat no. 4368814, Applied Biosystems, Foster City, CA) with miR-specific stem-loop reverse-transcription (RT) primer (Table 1). The qPCR was performed by the SYBR Green PCR Master mix (Cat no. 4309115, Applied Biosystems, Foster City, CA). The reactions containing miR-specific forward and reverse primer for each mature miRNA (Table 2) were done in thermocycler ABI Prism® 7500 fast detection system (Applied Biosystems, Foster City, CA). The PCR procedure was as follows: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. U6 was used as internal control. $\Delta\Delta C_t$ was calculated for each sample, and expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Bioinformatics analysis

TargetScan 5.2 (http://www.targetscan.org/vert_50/) and Pictar (version: 2010) were used to identify the miRNAs that target APP and BACE1.

Data analysis

All values were described as mean ± SEM. Day-by-day comparisons between two groups were performed using one-way repeated measure ANOVA. The two-tail Student's t-test was used for comparisons between two groups with SPSS19.0. $p < 0.05$ was considered as statistically significant.

Table 1. MiR-specific reverse-transcription primers

miRNA	primer sequence (5'-3')
miR-106b	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACATCTGC
miR-17-5p	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACCTACCT
miR-153	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACGATCAC
miR-101	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTTCAG
miR-9	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCATAAC
miR-19a	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAGTTT
miR-135a	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCACATA
miR-15b	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGTAAAC
miR-16	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGCCAAT
miR-195	GTCGATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCCAAT
miR-29c	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACTAACCG
miR-124	GTCGATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGCAT
miR-214	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACCTGCCTG
miR-141	GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCATCTT
u6	CGCTTCACGAATTTGCGTGTCTAT

Table 2. MiR-specific forward and Reverse primers for PCR

miRNA	primer sequence (5'-3')	
miR-106b	Forward: GGGGTAAAGTGCTGACAG	Reverse: TATCCAGTGCCTGTCGTG
miR-17-5p	Forward: GGGCAAAGTGCTTACAGT	Reverse: TGTCGTGGAGTCGGCAATT
miR-153	Forward: GGGGTTCATAGTCACAA	Reverse: CAGTGCCTGTCGTGGAGT
miR-101	Forward: GGGGTACAGTACTGTGATAA	Reverse: CAATTGCACTGGATACGA
miR-9	Forward: GGGTCTTTGGTTATCTAGCT	Reverse: ATCCAGTGCCTGTCGTGGA
miR-19a	Forward: GGGTGTGCAATCTATGC	Reverse: GCAATTGCACTGGATACG
miR-135a	Forward: GCGGTATGGCTTTTATTCC	Reverse: TGTCGTGGAGTCGGCAATT
miR-15b	Forward: GGGTAGCAGCACATCATG	Reverse: TATCCAGTGCCTGTCGTG
miR-16	Forward: GGGGTAGCAGCACGTAAAT	Reverse: TGTCGTGGAGTCGGCAATTG
miR-195	Forward: GGGGTAGCAGCACAGAAAT	Reverse: TCCAGTGCCTGTCGTGGA
miR-29c	Forward: GGGGTAGCACCATTGAAATC	Reverse: TGTCGTGGAGTCGGCAATT
miR-124	Forward: TAAGGCACGCGGTGAAT	Reverse: CAGTGCCTGTCGTGGAGTC
miR-214	Forward: GACAGCAGGCACAGACA	Reverse: GGCAATTGCACTGGATAC
miR-141	Forward: GGGGTAACTGTCTGGTAA	Reverse: GCAATTGCACTGGATACG
U6	Forward: GCTTCGGCAGCACATAIACTAAAT	Reverse: CGCTTCACGAATTTGCGTGTCTAT

Results

Cognitive deficit in middle-aged rats

Using the MWM test, we found the ability of spatial learning was significantly impaired in middle-aged rats compared with young rats. First, after guiding a direct path to the hidden platform located in the first quadrant from the second quadrant of the tank, the middle-aged rats had longer swim distance to navigate to the hidden platform than young rats after starting from the third and fourth quadrant on day one (Fig. 1A. for quadrant: $F_{(2,28)}=9.19$, $p=0.001$; for group: $F_{(1,14)}=6.214$, $p=0.026$), indicating that the learning abilities of using

Fig. 1. Impairment of cognition in middle-aged rats in the Morris Water Maze test (A) Average distance to platform from other three nontarget quadrants (Q) at training day 1 were compared (ANOVA: for quadrant: $F(2,28)=9.19$, $p=0.001$; for group: $F(1,14)=6.214$, $p=0.026$. $n=8$ for each group). (B-D) Mean daily distance to swim from the start point to the hidden platform in Q2, 3, and 4 (ANOVA: Q2: For groups: $F(1,14)=19.38$, $p=0.001$; for days $F(4,56)=20.108$, $p<0.0001$; for groups $\times$ training days: $F(4,56)=1.37$, $p=0.256$; Q3: For groups: $F(1,14)=11.278$, $p=0.005$; for days $F(4,56)=6.189$, $p<0.0001$; for groups $\times$ training days: $F(4,56)=0.723$, $p=0.58$; Q4: For groups: $F(1,14)=12.789$, $p=0.003$; for days $F(4,56)=2.731$, $p=0.038$; for groups $\times$ training days: $F(4,56)=0.165$, $p=0.955$. $n=8$ for each group). (E) Percentage time spent in target quadrant relative to the total time in the pool during probe trial. (F) Number of times crossed the previous target platform in probe trial.

cues as navigational guides in middle-aged rats are declined. Second, in learning trial, the middle-aged rats also took more swim distance to find the hidden platform after they had been released into the tank in all of the three non-target quadrants day-by-day (Fig. 1B-D, Q2: For groups: $F_{(1,14)}=19.38$, $p=0.001$; for days $F_{(4,56)}=20.108$, $p<0.0001$; for groups $\times$ training days: $F_{(4,56)}=1.37$, $p=0.256$; Q3: For groups: $F_{(1,14)}=11.278$, $p=0.005$; for days $F_{(4,56)}=6.189$, $p<0.0001$; for groups $\times$ training days: $F_{(4,56)}=0.723$, $p=0.58$; Q4: For groups: $F_{(1,14)}=12.789$, $p=0.003$; for days $F_{(4,56)}=2.731$, $p=0.038$; for groups $\times$ training days: $F_{(4,56)}=0.165$, $p=0.955$). The results suggested that middle-aged rats have the decreased ability to combine cues with memory to reach the target. In addition, in the probe trial, both platform crossings and spending time in the target quadrant of middle-aged rats were similar with those in young rats ($p>0.05$). These results indicated that middle-aged rats might not display declined memory ability (Fig. 1E, F).

Expression of APP and BACE1 in both the forebrain cortex and hippocampus of middle-aged rats

Overexpression of APP and BACE1 contribute to the formation of A β and participate in the aging process [17, 18, 31-33]. In this study, we found that the protein level of APP was elevated in both the forebrain cortex and hippocampus of the middle-aged rats compared to young rats (Fig. 2A, B, $*p<0.05$, $n=6$ for each group). Interestingly, the mRNA level of APP was not changed in either the forebrain cortex or hippocampus of middle-aged group compared to young rats (Fig. 2C, D, $p>0.05$, $n=6$ for each group).

Fig. 2. The protein and mRNA level of APP in both the forebrain cortex and hippocampus. Western blot analysis with forebrain cortex (A) and hippocampus (B) tissues in young and middle-aged rats. Top, representative immunoblotting image of APP was showed. Bottom, the relative quantification of image analysis. The density of each band was evaluated and normalized to internal control, followed by further normalization to the values from young brain tissues (* $p < 0.05$ vs young group, $n=6$ for each group). The relative quantification of APP mRNA level in the forebrain cortex (C) and hippocampus (D) tissues were detected by qRT-PCR and normalized to GAPDH ($n=6$ for each group).

Fig. 3. The mRNA and protein level of BACE1 in both the forebrain cortex and hippocampus. The relative quantification of BACE1 protein levels from forebrain cortex (A) and hippocampus (B) tissues were attained by normalizing to the values from young group after correction for protein loading with GAPDH (* $p < 0.05$ vs young group, $n=6$ for each group). The relative quantification of BACE1 mRNA level in the forebrain cortex (C) and hippocampus (D) tissues were expressed as fold decrease from young brain tissues by using GAPDH as internal control (* $p < 0.05$ vs young group, $n=6$ for each group).

Strikingly, as illustrated in Fig.3A and 3B, the protein level of BACE1 significantly increased in the forebrain cortex, however, it was lower in the hippocampus in rats at middle-age compared to young rats (* $p < 0.05$, $n=6$ for each group). The mRNA level of BACE1 decreased in the forebrain cortex (Fig. 3C, * $p < 0.05$, $n=6$ for each group). However, it showed

Fig. 4. APP-associated miRNAs expression in both the forebrain cortex and hippocampus. (A) APP-related miRNAs in Targetscan database, among 23 miRNAs, 4 miRNAs had conserved sites with 3'UTR of APP. The overlapping indicated 4 potential miRNAs were sifted by Pictar database. The relative quantification of APP-associated miRNAs level in forebrain cortex (B) and hippocampus (C) tissues were analysed by normalizing to U6, followed by calculating the fold change relative to the values from young brain tissues (* $p < 0.05$, ** $p < 0.01$ vs young group, $n=6$ for each group).

no significant change in the hippocampus of middle-aged rats compared to young rats (Fig. 3D, $p > 0.05$, $n=6$ for each group).

These data implicated that the post-transcriptional regulation of APP and BACE1 plays an important role in aging process.

Different expression patterns of APP-associated miRNAs between the young and middle-aged rats

Accumulating evidence suggested that miRNAs regulated the expression of APP both *in vitro* and *in vivo* [27, 28]. Thus, we hypothesized that the disturbed level of APP during middle-aged stage was in part due to post-transcriptional regulation of miRNAs. In order to select APP-associated miRNAs, the Targetscan database was used. Typically, miRNAs have both conserved and poorly conserved binding sites in the 3' untranslated region (3'UTR) of target genes. Friedman *et al.* reported that the miRNAs with evolutionarily conserved target sites cause stronger effects than non-conserved sites [34]. Therefore, we selected miRNAs that have conserved binding sites in the 3'UTR of target genes by Targetscan database, and then sifted by the Pictar database. In the present study, 23 miRNAs were found in Targetscan database and 26 miRNAs were found in the Pictar database. Four miRNAs, namely *miR-106b*, *-17-5p*, *-101* and *-153*, were found in both two databases (Fig.4A) and identified to be important to the regulation of APP expression by previous studies[28, 35, 36]. We then performed qRT-PCR to quantify the levels of these four miRNAs in both the forebrain cortex and hippocampus tissues of young and middle-aged rats. All of these miRNAs were significantly decreased in both the forebrain cortex and hippocampus of middle-aged rats compared to young rats (Fig.4B, C, * $p < 0.05$, ** $p < 0.01$, $n=6$ for each group).

Fig. 5. The expression of BACE1-related miRNAs in both the forebrain cortex and hippocampus. (A) BACE1-associated miRNAs in Targetscan database, among 58 miRNAs, in total 10 miRNAs had conserved sites with 3'UTR of BACE1. The overlapping indicated 10 potential miRNAs were sifted by Pictar database. The relative quantification of BACE1-related miRNAs level in forebrain cortex (B) and hippocampus tissues (C) were detected (* $p < 0.05$ vs young group, $n=6$ for each group).

Different expression profile of BACE1-associated miRNAs between young and middle-aged rats

Using the same strategy with APP, 58 miRNAs were found in Targetscan database, and 23 miRNAs were found in the Pictar database. After taking the intersection, ten miRNAs were sifted (Fig.5A). As shown in Fig.5B, in the forebrain cortex, *miR-9*, *-19a*, *-135a*, *-15b*, *-16*, *-195*, *-29c* and *miR-214* were lower in middle-aged rats. However, the *miR-124* was higher, but *miR-141* showed no significant change (* $p < 0.05$, $n=6$ for each group). In the hippocampus, *miR-9*, *-15b*, *-16*, *-195*, *-29c* and *miR-124* were lower, but *miR-19a*, *-135a*, *-214* and *miR-141* were higher in the middle-aged rats (Fig. 5C, * $p < 0.05$, $n=6$ for each group).

Discussion

In the present study, we found that 18-month-old Wistar rats displayed cognitive impairment with changes in APP and BACE1 expression in both the hippocampus and forebrain cortex. In addition, we reported for the first time that different expression profile of amyloid-related miRNAs in the hippocampus and forebrain cortex between the young and middle-aged rats. This suggests that imbalanced expression of amyloid-associated miRNAs might be involved in the declined cognitive ability with abnormal expression of APP and BACE1 proteins in the middle-aged rats.

Aging is characterized by the gradual decline in learning and memory ability [37, 38]. Clinical study displayed that the age-related cognitive deficit could start from midlife of human, but it is relatively greater in person at the age 70 years old or more [39]. Disclosing the early risk factors for cognitive decline prior to aging is very important to prevent the onset and deterioration of aging-induced cognitive decline. Although some studies have reported that aging rats displayed significant cognitive deficit, they all used rats at the

ages of over 20 months old [40-42]. In the present study, we selected rats at the age of 18 months as the middle-aged stage because it matches with the age of 45 years old in human, which is the beginning stage of the cognitive decline reported previously [43-45]. Here, we found that 18-month-old rats displayed impaired cognitive ability by MWM technique. The main characteristic of impaired cognitive ability in middle-aged rats is the decreased learning ability without marked memory decline as evaluated by MWM. This result is similar with previous study, which demonstrated that middle-aged rats (18 months old) showed significantly slower escape latency and longer swimming distance in cued trial than those in young rats, without significant difference in probe trial between two groups [46].

Over the last decades, emerging evidence supported that a variety of neurobiological variables contributed to the age-related cognitive decline in normal aging brain. APP and BACE1 play critical roles in brain atrophy, cortical thickness and neuron loss via the proteolytic product A β during normal aging [47-49]. In our present study, loss of cognition was found in the middle-aged rats. Therefore, we focused on the APP and BACE1, and determined whether dysfunction of APP and BACE1 are associated with cognitive decline in middle-aged rats.

Interestingly, in this study, the protein level of APP is higher in both the forebrain cortex and hippocampus of middle-aged rat than young rats without changes of APP mRNA level. The mismatch in APP mRNA level from its protein level in middle-aged rats suggested a possible post-transcriptional depression effect. Emerging evidence showed that post-transcriptional repression mediated by miRNA occurred through translation repression and degradation of target mRNA. MiRNA-mediated translational repression could result in the inhibition of ribosome assembly onto the target mRNA, leading to the reduction in protein synthesis [50-52]. Moreover, miRNAs could also degrade target mRNAs [53]. Mehmet Somel et al. [54] reported that aged-related miRNAs expression profiles show a negative correlation with their targets' expression profiles at mRNA and protein level. It is shown that miRNAs played a critical role in regulating the expression of target gene in post-transcriptional level during normal aging. We hence hypothesized that miRNAs might participate in the declined cognitive function, and the abnormal expression of APP and BACE1 proteins in rats at ages of 18 months old. As expected, we found that all of the four APP-associated miRNAs, compared with young rats, decreased in both the cortex and hippocampus of middle-aged rats. That could explain why the protein level of APP increased in both the cortex and hippocampus of middle-aged rats. It is noteworthy that *miR-17-5p*, *miR-106b*, *miR-153* and *miR-101* were all identified to regulate APP expression in neuronal cell lines [28, 35, 36, 55]. The results suggested that changes of *miR-17-5p*, *miR-106b*, *miR-153* and *miR-101* may be important risk factors of cognitive decline in the middle-aged rats.

In addition, we found there are ten miRNAs that have conserved binding site with 3'UTR of BACE1 based on the two databases. Among these miRNAs, *miR-195*, *-29c* and *-124* were identified to regulate the expression of BACE1 [24, 56, 57], and *miR-9*, *-19a*, *-135a*, *-15b*, *-16*, *-214* and *-141* were reported to be associated with aging [58-60]. Interestingly, we found different expression profile between the forebrain cortex and hippocampus in the middle-aged rats. There are eight of them that are decreased in the forebrain cortex, while only six miRNAs are decreased in the hippocampus. On the other hand, there is one miRNA in the forebrain cortex, but there are four miRNAs in the hippocampus that are increased in the middle-aged rats. Considering the inconsistent expression of BACE1 between the forebrain cortex and hippocampus of the middle-aged rats, we hypothesized that it might be associated with the different expression of miRNAs in these two regions. For detail, in the forebrain cortex, the expression of BACE1 is increased. There are eight miRNAs that are decreased, whereas only one miRNA was increased. In the hippocampus, the expression of BACE1 is decreased. Coincidentally, there were four miRNAs that are increased, and six that are decreased. However, whether the discrepancy expression of BACE1 in the forebrain cortex and hippocampus results from the combination effect of the changes of all these miRNAs still need to be investigated in the future. In addition, for the decreased expression of BACE1 in the hippocampus, we could not exclude the following reasons: (1)

other upregulated miRNAs may not be found based on the limited predictions by present database. (2) regulatory mechanisms of non-miRNAs may be involved in the process as well.

In the present study, we reported that middle-aged rats have begun to display the cognitive decline accompanied with changes of APP and BACE1 expression. In addition, we here for the first time reported that abnormal expression of amyloid-associated miRNAs has begun in the middle-aged rats, suggesting that miRNAs participate in the process of aging associated with cognitive decline. However, whether these changed miRNAs participate in the regulatory process of APP and BACE expression in middle-aged rats and whether these changed miRNAs combination could be biomarkers to predict the early cognitive decline, still need to be studied further.

Acknowledgements

This work was supported by Natural Science Foundation of China (81271207, 81070882, 81000499), Creative Research Groups of The National Natural Science Foundation of China (81121003), The postgraduate innovation scientific research project of Heilongjiang Province (YJSCX2012-244HLJ).

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