Treadmill Training Improves Cognitive Function via Increasing IGF2 Targeted Downregulation of miRNA-483

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Research

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Abstract

Background: Suitable exercise can promote development of cognitive function and improve learning and memory ability of the hippocampus. Nevertheless, mechanisms that elicit these positive effects of exercise are yet needing to be elucidated. IGF2 is known to act as a potent memory and cognitive enhancer, whereas the mechanism by which IGF2 regulates cognitive function related to moderate treadmill exercise remained largely vague.

Methods: In the study, rats were subjected to slight, moderate and high intensity treadmill training for 6 weeks. Then, Morris Water maze test was employed to investigate hippocampus-dependent spatial learning and memory ability in rats subjected to different intensity treadmill exercise. Subsequently, the gene chip and Gene Ontology were used for analysis to explore the expression level of IGF2. Furthermore, The TargetScan_7.1, miRDB, and microRNA.org. databases was used to predict the target gene of IGF2.

Results: After Morris Water maze test, we found that middle intensity treadmill training could obviously enhance learning and memory function of rats. The qRT-PCR and western blot confirmed that the expression of IGF2 was significantly upregulated in hippocampus after moderate treadmill exercise. Through databases, miRNA-483 was screened and predicted as the target gene of IGF2. Moreover, silencing IGF2 inhibited the neurite growth in the hippocampus of rats, while, miRNA-483-inhibitor ameliorated the silencing IGF2 induced hippocampal neurons impairment to promote the neurite outgrowth.

Conclusions: These findings suggested that the treadmill training could enhance the cognitive function, in which the underlying mechanism is involving in elevating the expression level of IGF2 and associated with downregulated miRNA-483. This therefore provide a reliable theoretical explanation on improving cognitive function induced by moderate exercise.

Introduction

Physical activity is beneficial to brain function[1]. While physical exercise strengthens the body, it also enhances the cognitive function of the brain[2]. The suitable intensity exercise can promote the development of cognitive function and personality by enhancing the proliferation, survival and differentiation of neurons and the synapse plasticity, improves the information transmission and learning and memory ability of the hippocampus, and enhances cerebral blood flow function [1, 3, 4], however, the excessive exercise load cause ischemia and hypoxia in the brain and the cells in the hippocampus to be damaged. Therefore, the high intensity exercise could destroy the balance of the body and aggravate cognitive impairment. Comparatively, moderate exercise have been noticed to associate with memory and hippocampal plasticity[5], while the mechanisms for these positive effects of exercise are yet needing to be elucidated.

The insulin like growth factor (IGF) axis plays an essential role in normal growth and development[6]. IGF2 is now known as a multifunctional growth regulator in the insulin-like growth family that promotes

cell differentiation, proliferation and associated with regulation of physical activity[7]. Growing evidence has demonstrated the functional significance of IGF2 in hippocampal-dependent learning and memory of rats[8]. Researchers recently revealed that the increased IGF2 could improve learning memory and activate the neurons in hippocampus[9, 10] suggesting that IGF2 enhance cognitive and memory persistence. Furthermore, the expression of IGF2 was induced to improve cognitive function in mouse models, indicating the IGF2 as represent a target for regulating cognitive functions[11]. The suitable intensity exercise can promote the development of cognitive function. Thus, analysis of IGF2 may broaden our understanding of the molecular mechanisms of learning and memory ability induced by moderate treadmill training.

In this study, we used Morris water maze test to evaluate the learning and memory ability of rats who were subjected to slight, moderate and high intensity treadmill training for 6 weeks. Subsequently, gene chip and Gene Ontology (GO) analyses on hippocampus of rats exerted exercise were performed and exhibited that IGF2 plays a crucial role in regulation of learning and memory ability after treadmill training. Via TargetScan_7.1, miRDB, and microRNA.org. databases and quantitative Polymerase Chain Reaction (qPCR) verification, miRNA-483 was screen out as a target of IGF2. Moreover, to better understand the potential roles of IGF2 and miRNA-483 in the growth of hippocampal neurons, IGF2-siRNA and miRNA-483 (mimic-483, inhibitor-483) were transfected into neuron reflected by immunofluorescence double staining of Tuj1 and IGF2. Taken together, our findings could provide an appropriate motion program and a potential theoretical explanation on improving cognitive function after moderate exercise, which might be associated with the regulation of IGF2 and miRNA-483.

Material And Methods

Animals/Subjects

Thirty-two 2-month-old male (200±20g) and timed pregnant female Sprague-Dawley (SD) rats were purchased from the Center of Experimental Animals, Kunming Medical University. Animal care and all experimental protocols were approved by the guidelines of the Institutional Medical Experimental Animal Care Committee of Kunming Medical University with the approval number: SYXK 2015-0002. Guidelines for laboratory animal care and safety from NIH have been followed. The animals in each group (n=8/group) were kept in a separate container (containing two 57 × 39 × 20 cm^3 laboratory cages with 4 rats per cage)[12] under controlled laboratory conditions, at temperature (22±2 ℃) and humidity (45±10%) room under a 12 h light/dark cycle with food and water available as libitum throughout the study.

Treadmill exercise protocol

Briefly, rats were trained for 2 days by adaptive treadmill training (ran at a speed of 8.2 m/min for 30 min) to adapt the new environment. Then, the normal treadmill training was conducted, and the strength and speed of the treadmill were adjusted to the rats. Rats were randomly divided into 4 groups: Control group,

Slight intensity group (SI group, ran at a speed of 18 m/min for 30 min), Moderate intensity group (MI group, ran at a speed of 24 m/min for 30 min), and High intensity group (HI group, ran at a speed of 30 m/min for 30 min). Additionally, train once-daily for 6 weeks (6 days a week, Sunday off). According to the previous Bedford's methods[13], the treadmill load intensity was made a rough estimate: (1) Less than 200 d: VO2max = 0.19×Weighting (g)+91.16 (male); VO2max = 0.20 ×Weighting (g)+95.58 (female). (2) More than 200 d: VO2max = 0.35 ×Weighting (g).

Morris water maze

Morris water maze was carried out to examine spatial learning and memory at 24 hours (h) after treadmill exercise as previously described[14]. A circular container (50 cm deep, 160 cm wide) was filled with black opaque water and temperature was maintained at 20 ℃-24 ℃. The various prominent visual cues were placed on the walls around the pool. A camera connected to a computer equipped with Tracking System SMART 3.0 (Panlab, Spain) was installed over the pool to automatically record the swimming trace of rats. The rats were put back in cage and placed in standard animal room with food and water available during training sessions. The pool was divided into four equal quadrants (Target quadrant (T), Adjacent right (AR), Adjacent left (AL), and Opposite quadrant (O). An escape platform was submerged 2 cm beneath the water surface in the Target quadrant. The test was conducted including training and probe trial for 4 consecutive days (d). For each training trial, the rats were placed in the water facing the wall at one of the four starting points. Before the training is initiated, rats were allowed to swim freely in the water for 120 s with the platform to adapt the new environment. Each rat received four quadrants trials per day for three consecutive days, with an interval between each the trials of 15-20 min. The rats were given 120 seconds (s) to find the platform and were permitted to stay on the platform for 5 s before being removed, while rats that were unable to find the platform within 120 s were placed on the platform for 10 s before being removed, and the escape latency was recorded by a video camera. On the 4th day, the platform was removed and the number of crossings over the previous platform location was recorded over one 120 s trial. Tracking System SMART 3.0 (Panlab, Spain) automatically recorded the time spent on the target quadrant and the average distance of finding the platform.

Sample collection

Twenty-four hours after Morris Water maze test, animals were equally divided into 2 groups. Animals (First group) were performed for gene analysis and qRT-PCR as well as western blot to predict and verify the increased expression of IGF2 in hippocampus tissue after middle intensity exercise. They were anesthetized with sodium pentobarbitone (10 ml/kg, i.p.) before an incision was made in the middle of the skull to expose the brain. Then the hippocampal part was removed and stored at -80 ℃. For immunofluorescence double staining of NeuN/IGF2 and GFAP/IGF2, samples were obtained after perfusing with 4% paraformaldehyde through the left ventricle. Then samples were kept in 4% paraformaldehyde for 18 h before transferred to 20% sucrose at 4 ℃ until the samples settled in the bottom of the containers. Then, the samples were covered with OCT matrix and left to freeze to -40 ℃.

The frozen tissues were sectioned to 10 μm thick sections. Subsequently, the specimens were stored at -4 ℃ for the following staining.

Differential gene expression profiles

The differentially expressed genes in hippocampus tissue from rats among control, slight intensity, middle intensity and high intensity groups were screened using gene chip. In brief, the RNA was extracted from the frozen hippocampus within a week using the RNesay Mini Kit according to the procedure recommended by the provider company (Qiagen, Hilden, Germany). RNA quality and integrity were measured by 2% Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA). Then, the RNA was sent for sequencing in KangChen Biotech (Shanghai, China). RNA sequences were used to generate heat map and intersection of differential gene expression to detect the upregulated and downregulated genes, which conducted to screen the multiple variational genes. Gene Ontology (GO) analysis is an international standardized gene functional classification system associated with differentially expressed genes. GO analysis comprises three structured networks that describe the properties of gene products (molecular functions, cellular components, and biological processes). P values indicate the significant differences of GO Term enriched in differentially expressed gene lists. Additionally, the smaller the P value, the greater the significance of the gene. The purpose of this experiment is to find the intersection of enriched the highest genes by the four groups' GO-memory, and use TargetScan (http://www.targetscan.org/mamm_31/), miRDB (http://www.mirdb.org/), miRBase (http://www.mirbase.org/) and PubMed literature database (https://www.ncbi.nlm.nih.gov/pubmed/) to predict possible micRNAs regulating IGF2.

Primary culture for hippocampal neuron

Hippocampal neurons were obtained from rat pups according to the method detailed in previous reports[15]. Briefly, animals were culled, brain and meninges were taken, then hippocampus was isolated and cut to the smallest possible pieces and mixed with DMEM and transferred to sterile tubes. The tissue was left to be digested with papain and DNase I at 50 μg/ml. The enzymes were neutralized by adding 10X the original volume of Neurobasal medium with 10% FBS. Afterwards, the cells were collected by centrifugation at 200 g for 5 min. Neurons were incubated in the neurobasal medium at a density of 5×10^5 cells/ml supplemented with 2 mM l-glutamine, 1 μg/ml gentamicin, 2% B27, and 10% FBS in 37 ℃ with 5% CO_2. The medium was changed every third day and neurons were cultured for 10 days.

Screen for the effective fragment of IGF2-siRNA

Three small interfering RNAs (siRNAs) suppressed IGF2 were designed and purchased from RuiBo Company (Guang, Ruibo). The target mRNA sequence of fragment 1 is GTCGCATGCTTGCCAAAGA, fragment 2 is GCAAGTTCTTCAAATTCGA, and fragment 3 is AGAGCTCGAAGC- GTTCAGA. PC12 cell lines were used for screening the effective siRNA. The cell line was transfected with 100 nM siRNA separately. Then, 100 ng/μl siRNA regent was added and the media was replaced after 15 min. Afterwards, qRT-PCR

was used to evaluate the effects of siRNA on the expression of IGF2 mRNA. The most effective one was selected for ideal interfere.

Transfection of si-IGF2 and miRNA-483

To determine the role of IGF2 and miRNA-483 on the cell growth, hippocampal neurons were cultured and randomly divided into the following groups: normal, reagent (Only added transfection reagents), si-negative control (random transfected garbled fragment), si-IGF, miRNA-483-mimic-nc, miRNA-483-inhibitor-nc, miRNA-483-mimic and miRNA-483-inhibitor groups. Subsequently, si-IGF2 was transfected to the cells harboring miRNA-483 and IGF2, we added into the cells with miRNA-483-inhibitor. Then the cell growth, viability and apoptosis were detected at 48 h post transfection using high-content imaging system (Evons, Thermo, USA).

Quantitative Real-Time PCR (qRT-PCR)

RNA from the hippocampus was used as a template synthesise cDNA using SuperScript™ First-Strand Synthesis System (Invitrogen, California, USA). The primers were as follows: IGF2 sense: 5'CAAGTCCGAGAGGGACGTGT3'; antisense: 5'CACGCAGGAGGG- CAGGCAG3' and miRNA-483 forward and reverse primers from GeneCopoeia (Maryland, USA), while β-actin gene was used as housekeeping gene using the specific primers 5' GTAAAGACCTCTA- TGCCAACA3', and 5'GGACTCATCGTACTCCTGCT3'. PCR reaction was prepared by mixing 12.5 μl IQ SYBR Green Supermix (BioRad, California, USA) PCR water nuclear-free 10.5 μl, 0.5 μl of each primer that were stocked at 5 nM and 1 μl cDNA template. PCR reaction set for 35 cycles was as the following: 94 ℃ for 10 sec, 53 ℃（IGF2）/58 ℃ (microRNA） for 10 s, 72 ℃ for 20 s. Relative expressions were calculated with normalization to GAPDH values by using the $2^{-\triangle\triangle Ct}$ method.

Western Blot

To testify the protein expression of IGF2, protein was extracted from hippocampus tissues from each group using RIPA lysis buffer (Beyotime, Jiangsu, China) containing 2 % of cocktail pill. Afterwards, protein concentration was quantitated with BCA protein assay kit (Beyotime Institute). Afterwards, protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis at 60 V for 30 min and then at 120V for 1.2 h. Then, the protein bands were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After being blocked by TBST consisting of 5% evaporated milk at room temperature for 2 h, the membrane was rinsed and incubated with IGF2 antibody (1:500, BIOSS, Massachusetts, USA) and β-actin antibodies (1:1000, Abbkine, Hubei, China) for 18 hours at 4 ℃. Thereafter, the membrane was incubated with secondary antibody (1:5000, goat anti-rabbit IgG and goat anti-mouse IgG; ZSGB-BIO, Beijing, China,) for 2 h. Finally, after being rinsed in TBST, the immunoreactive protein was visualized using Alpha Innotech (Biorad Laboratories, Berkeley, CA, USA) with ECL and quantified by densitometry.

Immunofluorescence staining

To determine the expression of IGF2 in the hippocampus, immunofluorescence analysis was performed. Briefly, brain sections tissues slices were directly washed with PBS and blocked with 5% goat serum for 30 min at 37 ℃. Then the sections were incubated with mouse polyclone antibodies hexaribonucleotide binding protein-3 (NeuN) (1:100, ZSGB-BIO, China), mouse glial fibrillary acidic protein (GFAP) antibodies (1:200, ZSGB-BIO, China), rabbit IGF2 antibodies (1:200, rabbit, BIOSS, China) and 2% goat serum as a negative control at 4 ℃ overnight and rinsed with PBS for 3 times. Subsequently, sections were incubated with species-specific secondary antibody (DyLight 488 labeled Goat-anti-mouse 1:200; DyLight 594 labeled Goat-anti-rabbit, 1:200) for 1 hours at 37 ℃. Finally, sections were observed and obtained using an immunofluorescent microscope (NIKON T1-SM).

To further explore the effect of silencing IGF2 and over-expression and down-expression of miRNA-483 on hippocampal neurons growth *in vitro*, immunocytochemical analysis of Tuj1 and IGF2 was performed and the cells were grown on glass coverslips. The slices were incubated with Rabbit Neuronal Class III β-Tubulin (Tuj1) antibodies (1:200, Abclonal, Boston, USA) and mouse IGF2 antibodies (1:200, Abclonal, Boston, USA) at 4 ℃ overnight. Then the slices were washed with PBS for 3 times, and incubated with species-specific secondary antibodies of 488 (1:200, goat anti-rabbit, Abbkine, Hubei, China) and 594 (1:200, goat anti-mouse, Abbkine, Hubei, China) for 1 hours at 37 ℃. In the end, slices were observed and obtained using High-content cell imaging system (Leica, Wetzlar, Germany). The cells numbers and average length of neuron axon were calculated using Image-Pro Plus 6.0 software (MediaCybernetics, Maryland, USA).

Statistical analysis

All values were expressed as the mean ± standard deviation (SD) and analyzed using SPSS19.0. Statistical significance of the data was calculated by one-way analysis of variance (ANOVA), followed by post-hoc analysis LSD and Tamhane test. In the Morris water maze test, measures of learning during the training trail were averaged within each day, the data were analyzed using repeated measures one-way ANOVA's test, and the LSD was used for post-hoc comparisons. Differences were considered significant at $P < 0.05$.

Results

The middle-intensity treadmill training could enhance cognitive function

The Morris Water maze test was employed to investigate hippocampus-dependent the spatial learning and memory ability of rats at 24 h after treadmill exercise. The training trials for spatial learning was detected on consecutive 3 d. The results showed a significant decrease in latency of target among control, slight intensity and middle intensity groups for days 2 and 3 compared to day 1 **(Fig 1A, $P<0.05$)**, indicating that all animals were capable to learning and finding the platform. Analyses of escape latency for slight intensity training revealed no significant difference compared to control group on day 1, 2 and 3 **(Fig 1A, $P>0.05$)**. Besides, the results revealed a significantly shorter time finding the platform for high intensity training than that of control group on day 3 **(Fig 1A, $P<0.05$)**. The rats in middle intensity group

performed better than the control group in finding the target platform with a better learning performance on day 1, 2 and 3 **(Fig 1A, $P<0.05$)**, indicating that middle treadmill training improved the spatial learning ability of rats. In addition, to assess whether the rats had learned the position of the escape platform, the probe trial for spatial memory was conducted on the 4th d. The time for crossing over the original location of the quadrant in rats receiving middle intensity training were longer compared with opposite quadrant and control rats with no treadmill training **(Fig 1B, $P<0.05$)**. Similarly, the rats undergoing middle intensity training also exhibited more distance in the previous platform, indicating a better spatial memory **(Fig 1C, $P<0.05$)**. Meanwhile, the number for crossing over the target platform location in middle intensity was also remarkably increased compared with opposite quadrant and control group **(Fig 1 D, $P<0.05$)**, indicating that moderate treadmill exercise can improve spatial learning and memory in rats.

IGF2 expression was increased following exercise-induced enhancement of spatial learning and memory

To investigate the underlying molecular mechanism concerning enhancing learning and memory ability after treadmill training, gene chip (Microarray Suite 5.0 [Affymetrix] by GCOS software version 2.0) analysis was carried out among slight, middle and high-intensity groups in hippocampus of rats compared with control group, respectively **(Fig 2A)**. After performing the intersection analysis of these differently expressed genes, we found that 587 genes were up-regulated in hippocampus **(Fig 2B)**. In addition, GO analysis was carried out to identify highly enriched categories of cellular components, molecular functions, and biological processes. The intersection of differently expressed genes in the four groups revealed that there are 431 genes involved in these processes **(Fig 2C)**. In particular, there are 6 differently expressed genes for the critical cellular components involved in learning and memory among control, slight, middle and high-intensity groups **(Fig 2D)**. Finally, combined with gene chip and GO analysis, the six genes are associated with memory function such as IGF2, Ptgs2, Grin2b, Grin2a, Cyp7b1 and Slc24a2 were screened out, and we found that IGF2 has the most powerful function in cognition with the largest fold change **(Fig 2E)**.

The expression of IGF2 mRNA and protein was significantly increased in hippocampus after treadmill training

To confirm the expression of IGF2 after middle intensity treadmill training, qRT-PCR was performed and showed that the expression of the IGF2 was notably elevated in middle intensity trained rats compared with control and slight intensity trained one **(Fig 3A, $P<0.05$)**. Additionally, Western blot results have revealed a similar increase in the expression of IGF2 protein after training in middle intensity compared with control group **(Fig 3B, $P<0.05$)**. Furthermore, immunofluorescence double staining of IGF2/NeuN and IGF2/GFAP were performed to explore the expression and localization of IGF2 in hippocampus from rats following middle intensity treadmill training. Interestingly, the expression of IGF2 in neuron was remarkably higher than its expression in astrocyte **(Fig 3C and 3D)**.

MiRNA-483 was screened out and low expressed in moderate treadmill exercise

To further detect the regulatory mechanisms of IGF2, 69 miRNAs were predicted as the targets of IGF2 via three databases, including TargetScan_7.1, miRDB, and microRNA.org. Two miRNAs (miRNA-483 and miRNA665) were found by the intersection of three databases **(Fig 4A)**. However, miRNA-665 did not show the relation with IGF2. Therefore, we selected microRNA-483 as a regulatory mechanism molecule of IGF2. Consequently, the expression of miRNA-483 was further validated by qRT-PCR, and we found that the expression of miRNA-483 mRNA was obviously lower in rats subjected to middle and high intensity training **(Fig 4B, $P<0.05$)** as compared to control group, especially in middle intensity training group **(Fig 4B, $P<0.01$)**.

siRNA-F3 was the most effective production of IGF2-siRNA

In order to further verify the role of IGF2 and miRNA-483 on hippocampal neurons after treadmill training, we estimated the effects of the candidate sequences: F1-F3, on silencing production of IGF2. The results indicated that the neurons were adherent after 1 day and mature after 7 days **(Fig 5A)**. Afterward, the Cy3 labeled siRNA fragments and miRNA-483 (miRNA-483-mimic and miRNA-483-inhibitor) were transfected into neurons for 3 days and found red fluorescence expressed in cells **(Fig 5B)**, indicating that the production of IGF2-siRNA and miRNA-483 was efficiently transfected into the hippocampal neurons. Remarkably, a significant decrease in the expression of IGF2 was observed in siRNA-FI, F2, F3 compared with NC group, especially in the siRNA-F3 by qRT-PCR **(Fig 5C, $P<0.05$)**.

IGF2 could promote hippocampal neurons growth may be involved in miRNA-483 inhibition

Immunofluorescence double staining of Tuj1 and IGF2 was used to determine hippocampal neurons growth after the treatment of NC, miRNA-483-mimic, miRNA-483-inhibitor and IGF2-si **(Fig 6A)**. Additionally, for the rescue experiment, we transfected the miRNA-483-inhibitor-treated cells with IGF2-si **(Fig 6A)** to investigate whether IGF2 regulates cell growth through modulating miRNA-483. As a result, miRNA-483-inhibitor has improved neuron growth. Whereas, miRNA-483-mimic and IGF2-si caused the neuronal damage. Moreover, miRNA-483-inhibitor could markedly counteract with IGF2-si and induce neuron deterioration **(Fig 6A)**. Quantitative analysis revealed that the number and length of cell in miRNA-483-inhibitor was notably increased compared with NC groups **(Fig 6B, C, $P<0.05$)**. Conversely, in the groups of miRNA-483-mimic and IGF2-si as well as IGF2-si+inhibitor exhibited cell numbers lower than NC **(Fig 6B, $P<0.05$)**. Furthermore, we also found that the damaged neurons induced by IGF2-si were obviously rescued with miRNA-483-inhibitor **(Fig 6C, $P<0.05$)**.

Discussion

In this study, we found that middle intensity treadmill training could obviously enhance learning and memory function as it is shown by Morris water maze test. Subsequently, we have revealed that the expression of IGF2 was remarkably upregulated in hippocampus after middle intensity training. Through TargetScan_7.1, miRDB, and microRNA.org. databases, miRNA-483 was screened as the target gene of IGF2, and low expressed in moderate treadmill exercise compared with control groups. Furthermore, *in vitro* studies indicated that the damaged hippocampal neurons induced by silencing of IGF2 were

obviously rescued with miRNA-483-inhibitor, and promote the neurite outgrowth. These results revealed that treadmill training elevated the learning and memory ability in rats may be associated with increasing IGF2 and downregulation of miRNA-483.

The treadmill training enhances cognitive function by upregulating IGF2.

Our data revealed that the rats following the middle intensity treadmill training performed remarkably better in learning and memory ability. Previous studies suggested that exercise induces the expression of enhancing neurogenesis and neurotrophic factors, and ameliorating hippocampal neuronal activities[16-18]. Additionally, moderate exercise could regulate the brain functions for learning and memory ability through modulating central and peripheral factors[19]. It is reported that moderate aerobic exercise could promote the vitality of hippocampal neurons[20, 21] and attenuate the increase of nitric oxide (NO) concentration. It has been proved that NO is involved in many stress responses in hippocampus, such as neuronal injury in hippocampal CA1 region [22]. Whereas, the over-load exercise can lead to cells apoptosis and even necrosis, resulting in toxicity. Besides, improper load management is an important risk factor for acute illness and overtraining syndrome[23]. Therefore, in present study, the middle intensity training (24 m/min for 30 min) was applied to explore the neuroprotective effects of treadmill exercise on hippocampus in rats. Moreover, through conjoint analysis of gene chip and GO prediction and qRT-PCR verification, we have found that IGF2 was evidently upregulated after middle intensity exercise. Likewise, immunofluorescence double staining of IGF2/NeuN and IGF2/GFAP results demonstrated that the IGF2 was abundantly expressed in the hippocampal neurons, which is consistent with studies indicating that IGF2 levels are extensively elevated in the central nervous system, and are particularly concentrated in hippocampus [9, 24]. Moreover, it was proved that IGF2 improved the cognitive by inducing synapse formation and maturation, and improving hippocampal dependent memory ability in mice[25, 26]. Although animal disease models were not applied in the study, our data pointed out that treadmill training has significantly increased learning and memory abilities via IGF2 upregulation.

IGF2 enhancement for the cognitive function after middle intensity treadmill training is associated with inhibiting miRNA-483.

To investigate the interaction between brain function, exercise and IGF2 upregulation, TargetScan_7.1, miRDB, and microRNA. org databases and qRT-PCR verification were performed. We found that miRNA-483 was a target of IGF2 in hippocampus and low expressed in middle intensity training rats. Accumulating evidence showed that microRNAs could dynamically regulate synaptic plasticity, function and morphology[27] and resulting in lower learning and memory abilities in aging process, while aerobic exercise could reverse micro-RNA-induced cognitive impairment[28, 29]. It is known that in the second intron of the IGF2 gene on chromosome 11p15, human miRNA-483 is considered as co-expression with its IGF2 host gene [30]. miRNA-483 plays different roles in a variety of cancer types. Recent studies have shown that microRNA-483 is involved in the development of proliferation of tumor by upregulating the expression of IGF2 in nephroblastoma, colon cance[31, 32]. Whereas, miRNA-483 was not reported in the IGF2-related cognitive function. Our data revealed that silencing IGF2 inhibited the neurite growth in the

hippocampus of rats. In contrast, miRNA-483-inhibitor ameliorated the silencing IGF2 induced hippocampal neurons impairment, promote the neurite outgrowth.

Conclusion

The middle intensity treadmill training could enhance learning and memory ability. The underlying mechanism might be associated with the upregulation of IGF2 and downregulating miRNA-483. These results develop the understanding of exercise-induced regulatory factors and their effect on neuron activity and provide a reliable theoretical basis on improving cognitive function after moderate exercise.

Declarations

Ethics approval and consent to participate

All experimental protocols and animal handling procedures were approved by the Animal Care and Use committee of Kunming Medical University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

All authors agreed to be published.

Availability of data and materials

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

Competing interests

No competing financial interests exist. There is no conflict of interest for each contributing author in the manuscript.

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Authors' contributions

All authors are responsible for the accuracy of data in this study and approved the final version of the manuscript. LLX and XJD designed and supervised the whole study; MAH and LLX performed all the experiments; JJC and ZQZ contributed the data collection and analysis; THW participated in the part of experiments guidance and data verification.

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List Of Abbreviations

Full name	Abbreviations
Insulin-like growth factor	IGF
Insulin-like growth factor 2	IGF2
Gene Ontology	GO
quantitative Polymerase Chain Reaction	qPCR
Sprague-Dawley	SD
small interfering RNAs	siRNAs

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Figures

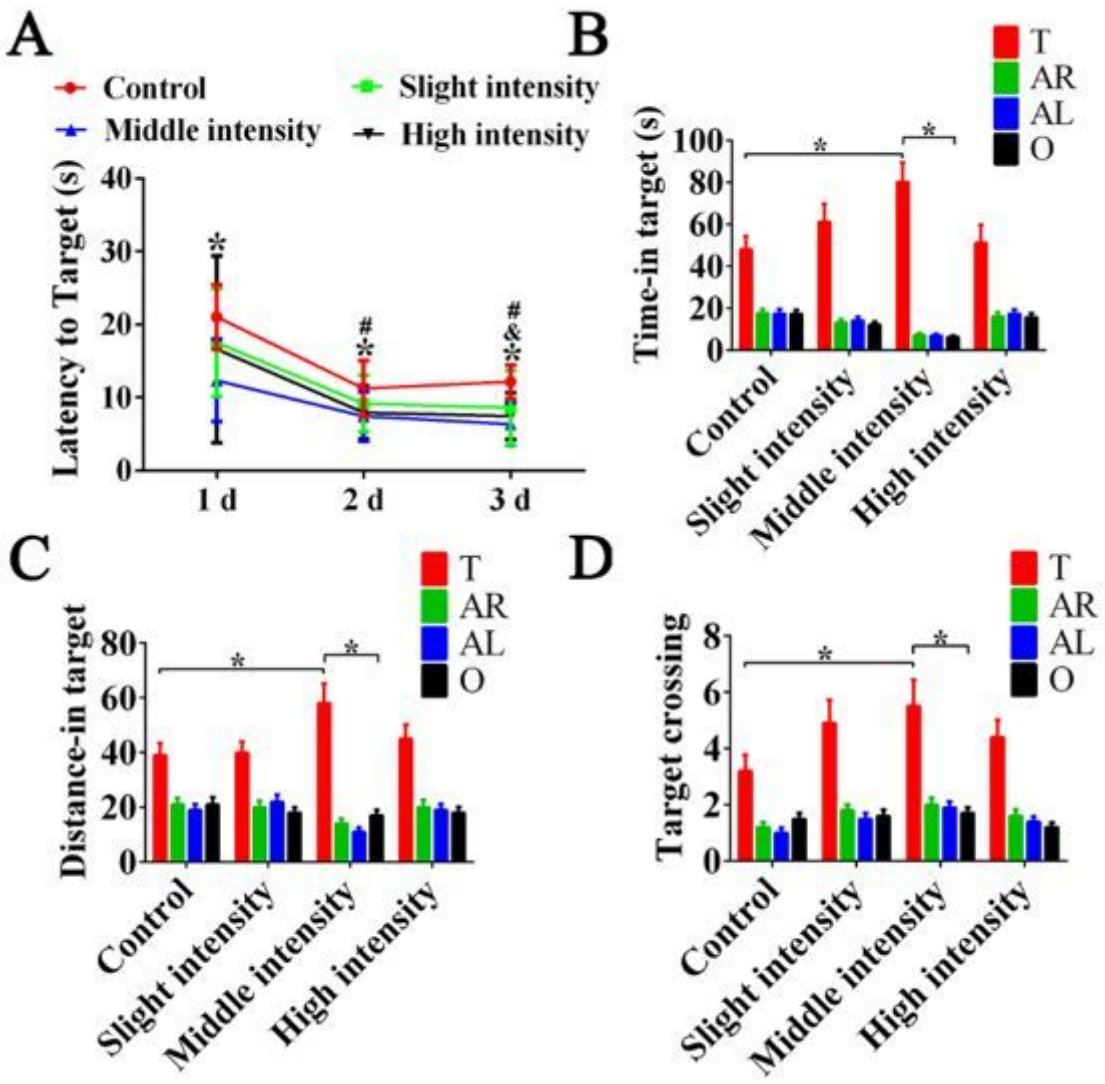

Figure 1

Behavioral performance by Morris water maze test (A) The time of latency to target in rats with control and training intensity among slight, middle and high groups for 3 days in the Morris water maze test. *P<0.05: control vs. middle intensity. &P<0.05: control vs. high intensity. #P<0.05: day 1 vs. day 2; day 1 vs. day 3 in control, slight intensity and middle intensity groups, respectively. (B, C) The time and distance for crossing the quadrants in these groups at the 4th day of the test. (D) The number of targets crossing on the 4th of Morris water maze test among the groups of control, slight, middle and high intensity. Pool quadrants: target quadrant (T), adjacent right (AR), adjacent left (AL), opposite quadrant (O). All data were presented as mean ± SD, *P<0.05, n=8/group.

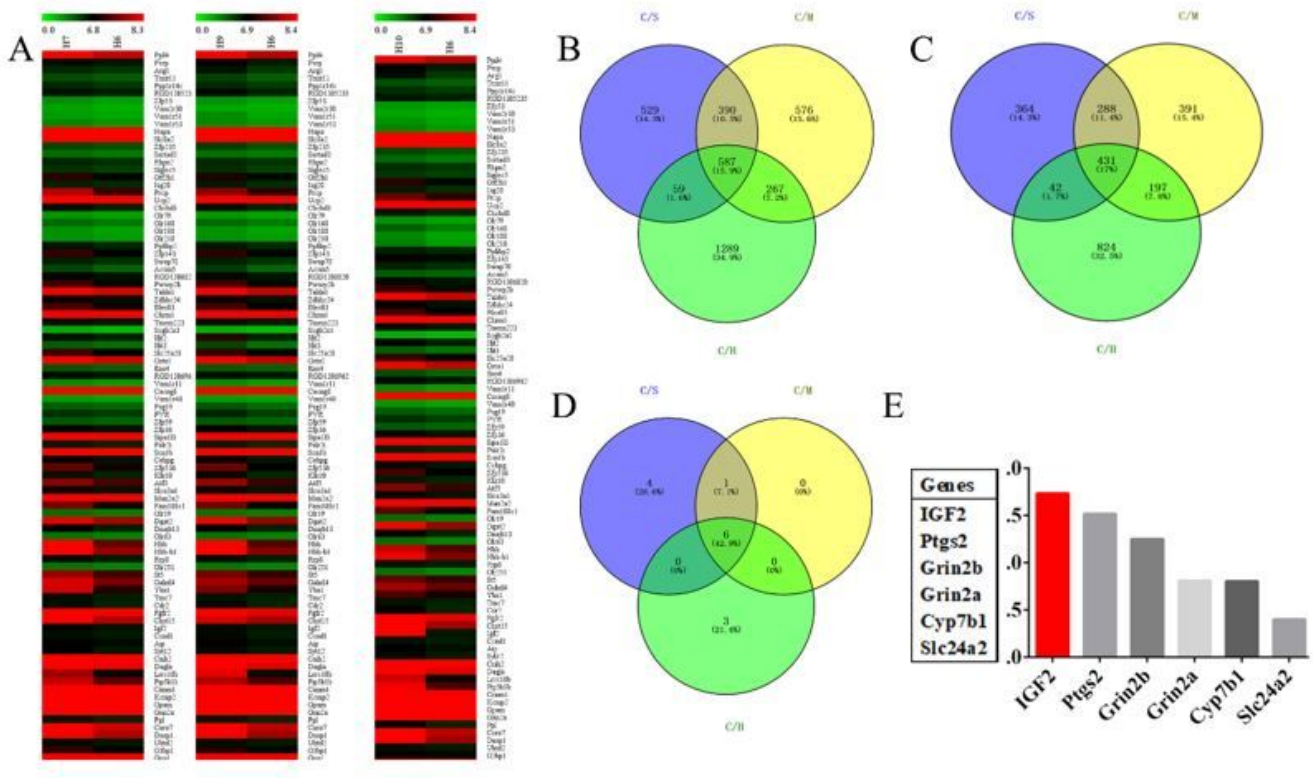

Figure 2

Bioinformatics analysis of IGF2 (A) The differentially expressed genes among the groups of control (H6), slight (H7), middle (H9) and high (H10) intensity were performed using VAM software and visualized with robust multiarray averaging. Gene symbols are labeled on the right. A color tag represents gene expression level, red spectrum denotes up-regulated genes, green for down-regulated ones and the middle was black. (B) Intersection of differentially expressed genes in these groups. (C, D) Intersection of these genes through Gene Ontology (GO) analysis for selecting the target gene are associated with memory in the venny 2.1 software. (E) The fold change of six gene including IGF2, Ptgs2, Grin2b, Grin2a, Cyp7b1 and Slc24a2. Fold change: the maximum value difference was expressed in red. C/S: control / slight intensity; C/M: control / middle intensity; C/H: control / high intensity, n=8/group.

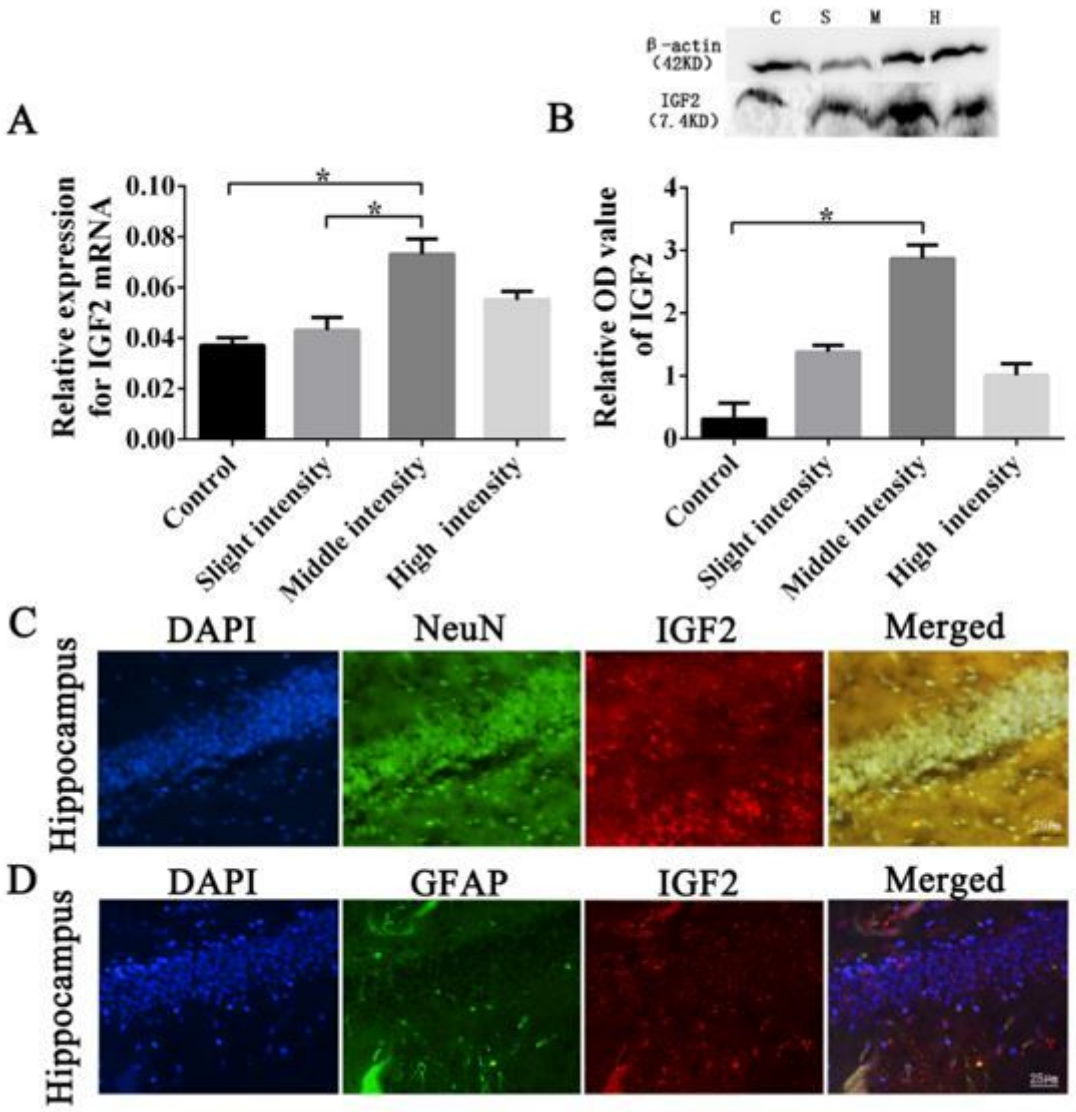

Figure 3

The change of expression of IGF2 in hippocampal tissues (A) The expression of IGF2 mRNA in the rats with control, slight, middle and high intensity training, respectively. (B) The western blot stripes and relative OD value of IGF2 in these groups. β-actin is treated as an internal control. (C, D) Immunofluorescence double staining of IGF2 and NeuN as well as IGF2 and GFAP in the hippocampal tissues following middle intensity training. IGF2 was stained by red colors, the neurons are stained by green colors, which are NeuN positive, the GFAP positive areas represent the astrocytes, which are stained by green, and the co-location of IGF2 with NeuN and GFAP represent yellow colors (merged). NueN: hexaribonucleotide binding protein-3. GFAP: glial fibrillary acidic protein. DAPI: 4', 6-diamidino-2-phenylindole. C: control. S: slight intensity. M: middle intensity. H: high intensity. β-actin is treated as an internal control. OD: optical density. Data are presented as the means ± SD.*P<0.05. Scale bar = 25 μm, n=8/group.

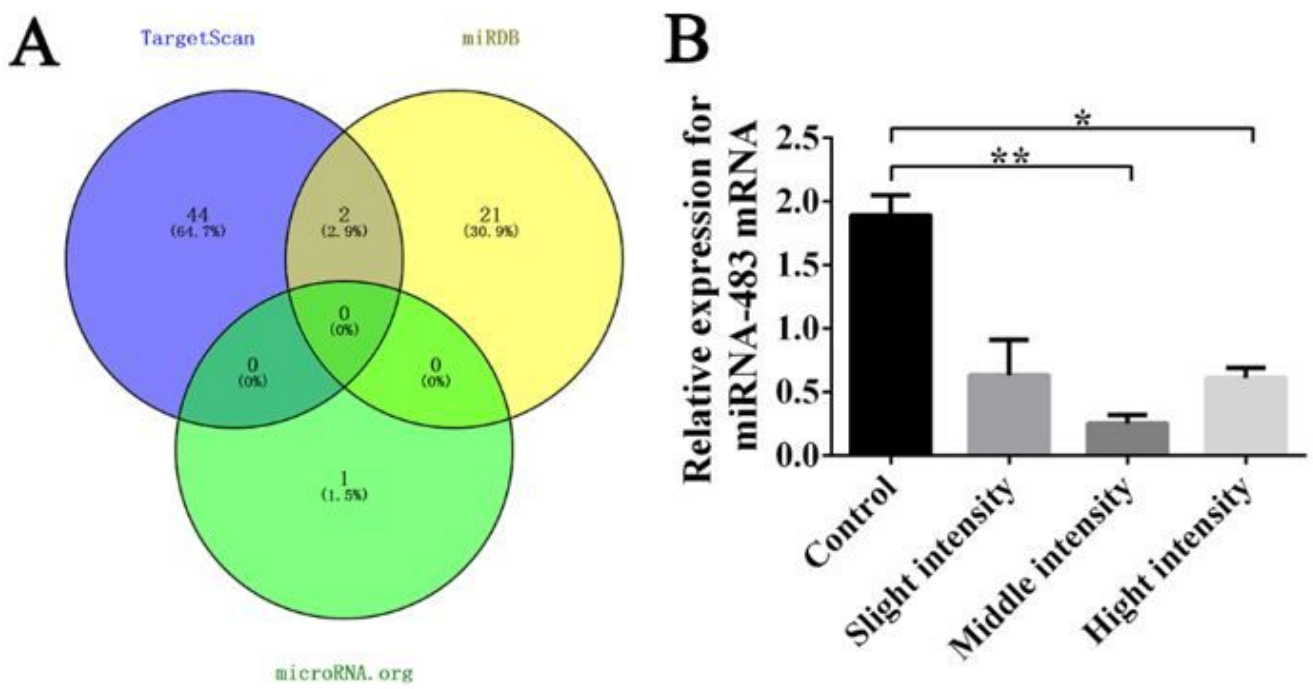

Figure 4

Changes of expression level of miRNA-483 mRNA in hippocampus after treadmill training (A) The intersection of TargetScan_7.1, miRDB, and microRNA.org. for selecting the target gene to IGF2 in the venny 2.1 software. (B) The relative expression of miRNA-483 mRNA in control, slight, middle and high groups, respectively in hippocampus. Data are presented as the means ± SD.*P<0.05. **P<0.01, n=8/group.

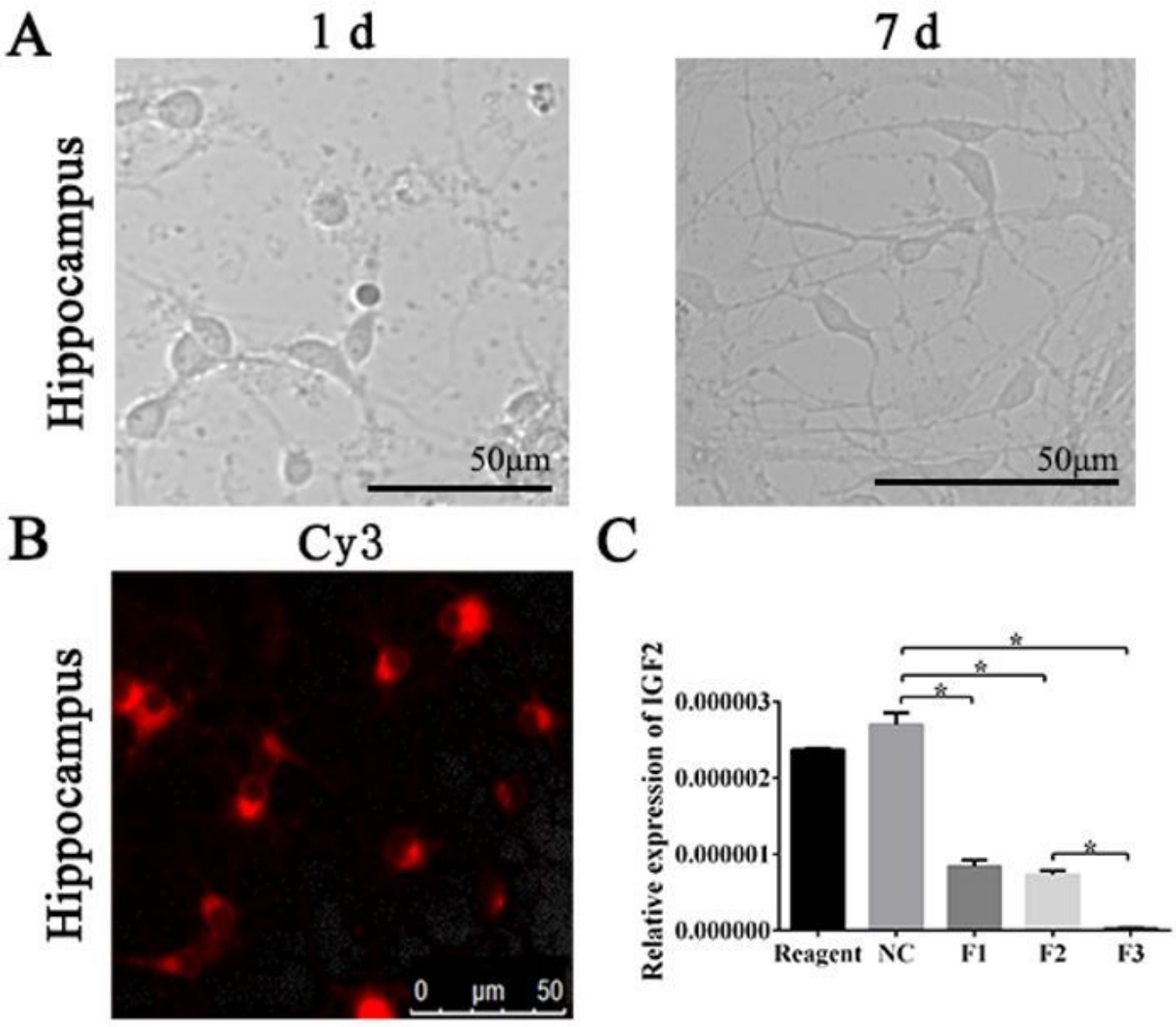

Figure 5

Successfully transfecting and screening effective IGF2-siRNA fragment (A) The morphology of hippocampal neurons at 1 d and 7 d. (B) The hippocampal neurons were transfected by IGF2-siRNA and miRNA-483 (mimic-483, inhibitor-483), which carries the red Cy3 were then visualized by fluorescent microscope. (C) Quantitative histograms of the relative expression of IGF2 in the groups of Reagent, NC, F1, F2 and F3. siRNA: silencing RNA. F1: treatment with No.1 siRNA fragment. F2: treatment with No.2 siRNA fragment. F3: treatment with No.3 siRNA fragment. NC: negative control group. d: day. Data are presented as the means ± SD.*P<0.05. Scale bar = 50 μm, n=6/group.

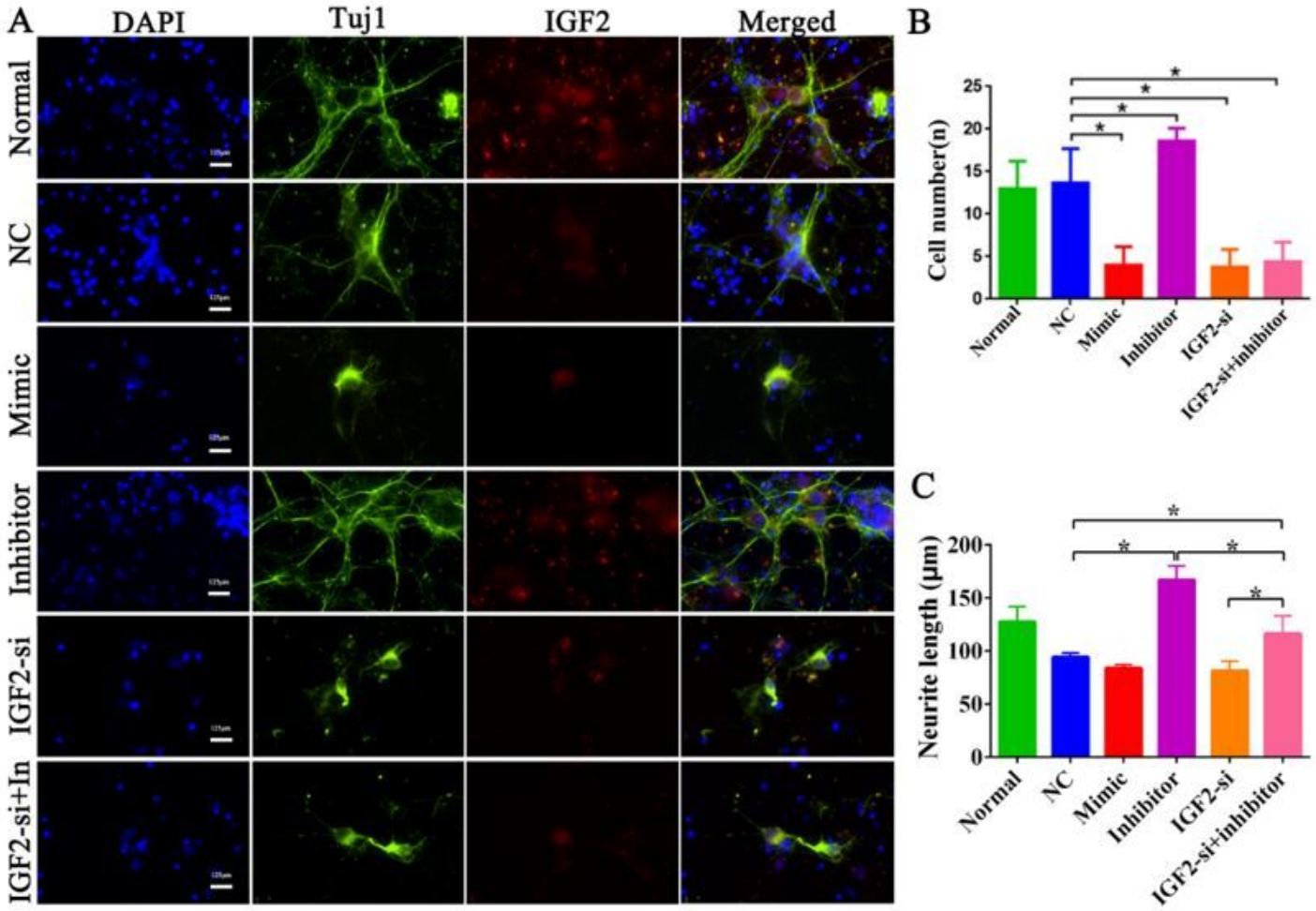

Figure 6

IGF2 and miRNA-483 regulation of the hippocampal neuron's growth (A) Immunofluorescence double staining of Tuj1 and IGF2 in the groups of normal, NC, miRNA-483-mimic, miRNA-483-inhibitor, IGF2-si and IGF2-si+inhibitor in hippocampal neurons. Green staining represents the Tuj1 positive cells, and the nucleus is stained by blue. IGF2 was stained by red colors. Scale bar= 50 μm. (B, C) The bar charts for quantitative analysis of the number and length of cells in these groups. Tuj1: Neuronal Class III β-Tubulin. IGF2-si: silencing of IGF2. Data are presented as the means ± SD.*P<0.05. Scale bar = 125 μm, n=6/group.