Effects of a Long Term Physical Exercise on the mRNA Level of BDNF in Mice after Middle Cerebral Artery Occlusion

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Abstract

Background: BDNF plays an important role in maintaining survival and function of neurons. Exercise may be increasing the mRNA and protein level of BDNF in hippocampus and cortex.

Objective: To investigate the impact of a long-term physical training on the mRNA and protein level of BDNF after Middle Cerebral Artery Occlusion (MCAO) in mice.

Design, Time and Setting: A randomized, controlled, animal experiment was performed at the department of rehabilitation medicine, Chinese PLA general hospital.

Materials: Twenty-six male C57 BL/6J mice (20–30g, 2-3 months old) were provided by Experimental Animal Center of Institute of Medicine, Chinese PLA general hospital.

Methods: Twenty-six male C57 BL/6J mice underwent MCAO (described by Bederson JB et al) and then were divided into training group I (n=9), training group II (n=9) and control group (n=8). The mice of training group I were trained with voluntary running wheel for one hour daily (6 days per week) for 90 days; The mice of training group II were trained for 15 days, stopped for 30 days and retrained for 45 days after MCAO. The running wheel of control group was fixed. The mRNA level of BDNF mice brain was tested by RT-PCR method 90 days after MCAO. The design of study is completely randomized among three groups. Wilcoxon rank sum test and Kruskal-Wallis test were performed.

Main Measures: Quantitation of the expression of BDNF genes is determined by RT-PCR for mRNA level techniques and Western blotting for protein level.

Results: Both the mRNA and protein levels of BDNF mice brain are significantly higher in training group I and training group II than that in control group in 90 days after MCAO (T values respectively are 5.1 and 3.9, P<0.01). There was no marked difference in the levels of BDNF mRNA or BDNF expression between training group I and in training group II, respectively (T value is 1.2,or 1.3 P>0.05).

Conclusion: The long term physical training can elevate the mRNA and protein levels of BDNF in mice brain after Middle Cerebral Artery Occlusion.

Keywords: Physical Training; MCAO; BDNF; Mouse

Introduction

Stroke rehabilitation is based on neuron plasticity and cerebral activity reorganization. In the past few years with the development of new technologies, the researchers have employed the "functional imaging" (fMRI, PET) technologies, intracortical micro- stimulation (ICMS) ICMS, magnetic stimulation, and gene technologies that allow scientists to observe neural activity on macro and micro level. The capacity of the brain to reorganize itself is perhaps the most interesting phenomenon [1-4].

It is found that enriched environment can functionally improve synapse plasticity in stroke mice. Some scientists analyzed enriched environment components and suggested that physical activity is the most important factor. Many studies have substantiated the beneficial effects of exercise on stroke recovery in animal models. Exercise increases brain-derived neurotrophic factor (BDNF), which has been proved to involve in neuroplastic processes contributing to recovery from ischemia [5-7].

Previously research data report that the behavioral training and complex environmental experience contribute to the two major categories of structure alteration: changes in pre-existing synapses and the number of synapses. In the animal's brain, after behavioral trainings and complex environmental experience, the synaptic interface structure in pre-existing synapses changed. It is found that learning and memory was not determined by alterations of the size of...
synapses or the number of synaptic vesicles, but significantly induced by an increase in BDNF.

Brain derived neurotrophic factor (BDNF), a member of neurotrophic factor family, is widely distributed in the brain, including Cerebral cortex, hippocampus, basal forebrain. There are some evidences proving that the BDNF plays a critical role in the activity-dependent processes, including the synapse development and plasticity. The activity of BDNF through the tyrosine kinase receptor (TrkB) regulates neuronal development and functions. BDNF is also involved in memory formation, including behavior and learning, synaptic efficacy and neuronal connectivity, synaptic plasticity. BDNF also promotes the development of immature neurons and improves the survival of adult neurons. The receptor of BDNF has been discovered in cholinergic neurons and it’s ending of basal forebrain [8-14]. In this study, the data demonstrated that exercises exerted beneficial effect on the pullulation and regeneration of peripheral neurons after injury. It was reasonable to hypothesize that these pullulation and regeneration must have its own neural biochemistry foundation, in which the increment of BDNF may play an important role. Previous studies have suggested that exercise increases BDNF mRNA levels in the normal brain. But we do not know physical activity whether elevate the BDNF protein and mRNA levels after brains were insulted. Therefore the present study try to further investigated whether exercise training can change the expression of BDNF protein and mRNA after Middle cerebral Artery Occlusion (MCAO) in mice.

Materials and Methods Design

Time and setting

The experiment was performed at the department of rehabilitation medicine, Chinese PLA general hospital from 2010 to 2012.

Materials

Twenty-six male C57 BL/6J mice (20–30g, 2-3-months old) were provided by Experimental Animal Center of Institute of Medicine, Chinese PLA general hospital. On arrival, the animals were housed six per cage and acclimatized to a colony room with controlled ambient temperature (22±1), humidity (50±10%) and a 12 h light/dark cycle. They were fed with standard diet and water ad libitum and were allowed to acclimate for 7 days before they were used. All experiments were conducted in accordance with the Chinese Community guidelines for the use of experimental animals and approved by the Chinese PLA general hospital Committee on Animal Care and Use.

Methods

Model of MCAO

Twenty-six male C57 BL/6J mice (20–30g, 2-3-months old) were allowed free access to food and water before and after all procedures. Mice were weighed and placed in an ether jar until they were immobilized, and anesthetized with 3.5% chloral hydrate in normal saline (35 mg/100 gm, intraperitoneally). Body temperature was monitored and maintained within normal limits with a heating pad. Under the operating microscope the left MCA was exposed transcranially 20 without damage to the zygomatic bone. Transection of the facial nerve was avoided during exposure of the temporalis muscle, which was divided caudally and retracted inferiorly to avoid compression of the orbital contents. The circle of Willis and the origin of the MCA were exposed in all mice by gently retracting the brain with a spatula on a flexible arm. The MCA was occluded with microbipolar coagulation using a low power setting and continuous saline irrigation, and then transected to avoid recanalization. Temporalis muscle and skin were closed in layers, and mice were allowed to recover from anesthesia on the heating pad. They were returned to their cages for the remainder of the 24-hour period.

Grouping and training

Mice that underwent MCAO (described by Bederson JB et al) [15] were housed and randomly divided into three experimental groups: training group I (n=9), training group II (n=9) and control group (n=8). Mice began to be trained three days after MCAO. Mice in training group I were trained with voluntary running wheel for one hour daily (6 days per week) for 90 days; Mice in training group II were trained for 15 days in the beginning, then they had an interval of 30 days and retrained for the last 45 days; The running wheel in control group was fixed for the same period of time. After 90 days, decapitation was used when mice’s brain tissue of the ischemic side were removed. For the running wheel test, the equipment was programmed to present an initial speed of 1 rpm and a final speed of 40 rpm, 300 seconds later.

Quantitation of the expression of genes in BDNF mRNA using RT-PCR techniques [16]

(1) Extract the RNA from mouse brain tissue of the ischemic side. BNDNF gene primer design: Primers were designed according to the complete sequence [17] of BNDNF mRNA (Gen Bank Accession no. X55573). The forward primer was a 21mer with sequence 5’-GGA TGA GGA CCA GAA GGT TCG –3’ (NT 296-316) and the reverse primer was a 22mer, 5’-ACC CTC ATA GAC ATG TTT GCG G–3’ (NT 460-439). Amplification product size was 164bp and was synthesized by Sheng Gong Biological Engineering Incorporation in Shanghai, 10mmol/ml for use. Preparation for total RNA from brain: Operating according to the instruction of TRIzol Reagent kit, intact 5S 18S and 28S bands were observed through gel electrophoresis (Figure 1) Ultraviolet spectrophotometer was used to detect the OD values at A260 and A280. (1OD=40μg/ml RNA), then the amount of total RNA abstracted could be calculated. The value of A260/A280 has to be more than 1.8, the RNA was stored at -70 and the whole process should be finished within a week. (2) Synthesis of RT-cDNA. 2μg RNA from sample was added in each of samples and transcripted to cDNA. Reaction volumes (20μl) comprised 5×RT Reaction Buffer (2μl), 10mM dNTP 1μl, forward primer and reverse primer (0.5μl, 12polM, respectively), reverse transcriptase (10μl, made by Sheng Gong Biological Engineering Incorporation in Shanghai), RNA (2μg), RNase a 10u and water homogenized by DEPC. Conditions of the reaction: After heated for 60min at 37°C and 5min at 95°C in water bath ,the template cDNA was finished and should be preserved in -20. (3) The reaction of RT-PCR. We make PCR with the above-mentioned coda –the product of reverse transcription10ml and the primer BDNF. Reaction volumes (30μl) comprised cDNA 10μl (reverse transcript as previously described), BDNF (primer), Taq DNA polymerase 1μl, 5×RT Reaction Buffer 4μl, 10mM dNTP 2μl,forward primer 0.5μl, reverse primer 1μl and water. Proper amount of paraffin oil was needed. Centrifugation for a few seconds then the amplification reaction could be carried on as follow: denaturation 3min at 93, followed by 35 cycles including 30s at 93, 30s at 57 and 45s at 72, then
an extension for 5 min at 72. 5μl RT-PCR reaction products were pipetted out and mixed with 1μl bromphenol blue thoroughly, followed by an electrophoresis on 2% agarose gels (10v/cm) for 10~30 minutes. The result could be observed and analysis on gelatin imaging analysis system. (4) Quantitative real-time PCR. RNA was purified using the Qiaex II Gel, Extraction Kit (Qiaigen) by following the manufacturer’s protocol. After that, the OD value at A260 of the product was detected using ultraviolet spectrophotometer. (1OD at A260 was equal to 50μg/ml cDNA). The density of cDNA was 200μg/ml. 5μl cDNA (1μg) were used as determine standard curves. cDNA reactions were then diluted tenfold in nuclease-free water (10-3-10-11μcDNA) and 5μl per sample were used in PCR. Reaction volumes (20μl) comprised 5μl products of RT-PCR, 5μl standard substance, 1.5μl Taq DNA polymerase, 6μl 5×RT Reaction Buffer, 2μl 10mM dNTP, 1μl forward primer, 1 μl reverse primer and water. Centrifugation for a few minutes after proper amount of paraffin oil was added in. The amplification reaction could be carried on. Standard cycling parameters were as follows: 3min at 93, followed by 30 cycles comprising 45s at 94, 45s at 57 and 60s at 72, with a final extension for 5 min at 72. 5μl product of RT-PCR in the underlayer of tube were mixed with 1μl bromphenol blue and the mixture were analyzed on agarose gels (10v/cm) for 20-30min (Figure 2,3).

Figure 1: Total RNA from brain of SS 18S and TRIzol prepared 28S bands through gel electrophoresis in nerve cells

Figure 2: The RT-PCR amplification products of standard samples of BDNF gene in nerve cells.

Each value of sample peak area was in the range of standard samples. Take pictures of the standard concentration and peak area values using the gel imaging and analysis device. Line scan analysis of electrophoresis strap in film was done using Gel Base / Gel Blot Support software.

Figure 3: The RT-PCR amplification products of BDNF samples in nerve cells.

Quantitation of the expression of BDNF protein using Western blot

The fresh mouse brain tissues of ischemic side (1.0g) were homogenized in RIPA DOC lysis buffer (1% Triton X100, 1% sodium deoxycholate, 4% SDS, 0.15M NaCl, 0.05M Tris-HCl, pH 7.2) supplemented with protease inhibitors (Roch, USA) and sonicated. Supernatant were separated after 12,000g for 15min at 4°C. The concentration of protein was detected by Bradford assay. 12% Tris-glycine gels applied brain tissue sample lysates. Immuno-blotting was performed as described [18]. After that, the proteins were transferred to PVDF membranes. BDNF protein was detected by monoclonal BDNF antibody (35928.11, Abcam). The membranes were blocked in 5% milk and 3%BSA at room temperature for 1 h. Then the membranes incubated with primary antibodies. Antibodies was diluted in 5% (w/v) nonfat dry milk and incubated with the PDVF blot overnight at 4°C. Incubation with secondary peroxidase-conjugated anti-mouse antibodies was performed at room temperature for 1 h. Blot was developed by using ECL system (Amersham Pharmacia Biotech). [Figure 4]

Figure 4: Blot was developed by using ECL system

Statistical analysis

Calculation was done with the SPLM software. Standard curve was made with the concentrations of standard sample and peak area. Then we made curve fitting and selected the best curve. The value of sample peak's area was dragged in the curve to calculate the concentrations of cDNA. That was the initial expression quantity of mRNA. The nonparametric multisamples analysis was carried out using the Kruskal-wallis test to determine the difference between the three groups and Wilcoxon test to contrast with each other.
Results

Quantitative analysis of experimental animals

There were no operative deaths and seizures observed in rats after surgery. The incidence of infarction after occlusion of the MCA was calculated as the percentage of rats with identifiable areas of infarction through examination by TTC-stained brain slices.

Curve fitting

After the standard curve had been made, we did curve fitting. The most fitting curve was the one, which contained all the value of standard product. That is \( \ln Y = a + b \ln (1000-X) \), \( a = 14.442, b =-2.667, r =-0.8332, p = 0.0199 \). [Figure 5]

The result of immunohistochemical staining

We calculated the initial expression of mRNA in three groups respectively according to the peak area of each sample. [Table 1]

| Group            | Samples | Median | Range  |
|------------------|---------|--------|--------|
| Training Group I | 9       | 1833   | 3583   |
| Training Group II| 8       | 1139   | 5650   |
| Control Group    | 8       | 233    | 305    |

Table 1: The median and range of mRNA level of BDNF in MCAO mice's metencephalon tissue. (PgmRNA/gm of total RNA)

According to the Kruskal-wallis test (\( H = 13.8, P <0.01 \)), differences among the three groups were significant.

The comparison of the two samples in the threes. [Table 2]

| Training Group I and II | Samples | Na | Nb | R\(^{-}\)R\(^{b}\) | T  | P   |
|-------------------------|---------|----|----|-----------------|----|-----|
| Training Group II and control | 9       | 8  | 13.3| 5.1             | <0.01|     |
| Training Group II and control | 9       | 8  | 10.9| 3.9             | <0.01|     |

Table 2: The result of wilcoxon test Groups the sample is different between the sums of ranks T value P

Discussion

Our findings indicated that 90 days after MCAO in mice, properly exercise training could increase the mRNA level of BDNF. There are Significant differences between training groups and control group, however, no marked difference was observed, in the different training groups. Data indicated that after MCAO, exercise retraining following an interval was still in effect, because level of BDNF mRNA was still increased. This result indicated that there is a second window of treatment opportunity during the recovery phase of MCAO --may be true.

Previous studies have found that early rehabilitation could improve the outcome of the stroke patients. The delayed rehabilitation (on rehabilitant after three months to one year onset of stroke) could not get good result, therefore; there exists the first window of recovery of stroke. The former studies show that the mice of MCAO were early trained, stopped on period of time and retrained, could still improve their impair behavior. The second therapeutic window exists in history of recovery of stroke. The study indicates one of the mechanisms responsible for this training improved the functional outcomes, is the elevate of expression level of BDNF mRNA.

BDNF is a powerful differentiation factor distributed widely in the central nervous system (CNS). Studies have suggested [17, 18, and 19] that BDNF plays an important role in maintaining survival and function of neurons. Endogenous BDNF, an important member of neurotrophic factor, laid the foundation for the function of cortex. As we known, BDNF is widely expressed in the cortex and could be regulated by neuronal activity. Neeper SA [20] work described that exercise can increase the transcription of BDNF gene in hippocampus and cortex. The activation of Voltage-sensitive calcium channel (VSCC) can promote the expression of BDNF, which exerts positive effect on survival of neurons in cortex. These also have been demonstrated by Ghosh A [21-25] et al. using embryonic neurons from mice. That is to say, the survival of neurons in cortex is activity-dependent. External stimulation and exercise training are associated with increased neuronal activity and metabolism, and have positive effects on the survival and plasticity of neurons. In Recent years, we have got a further understanding of the relationship between neurotrophic factors and neuronal plasticity. Studies indicated that BDNF could be regulated by physiological stimulation such as visual impulse. The block of impulses transmission to visual cortex by toxin and darkness can result in a quick down regulation of mRNA level of BDNF. Similarly, the use of physiological stimulation such as changes of osmotic pressure in lateral ventricle or stimulation of LTP can up-regulate the expression of BDNF mRNA. The LTP in BDNF knockout mice is decreased significantly and can be reversed by transferring the adenovirus-mediated BDNF gene into its hippocampal CA1 area.

Our study results showed that voluntary wheel running can up-regulate brain neurotrophic factor (BDNF) after brain ischemia. BDNF has been widely implicated in neuroplasticity and to enable essential functions such as learning and memory [26-30]. Recent studies suggested that exercise-induced enhancement in learning and memory is dependent on an increased BDNF level in hippocampus and the behavioral improvement was also correlated with a cell
and receptor changes. In adult rodents, BDNF also showed neuroprotective properties: promoting survival of hippocampal, striatal, and septal neurons in vivo and in vitro by preventing the brain against such insults as focal brain ischemia [21-28]. Our work supported the possibility that exercise-induced increase of neurotrophins may be the result of elevated neuronal activity after brain insult. And all results shown above indicate that BDNF is of great importance to the plasticity of brain.

When cerebral ischemia happens, BDNF can up-regulate TrkB protein to protect the neurons from death. BDNF may also act directly on neurons to prevent ischemia-induced neuron death [11, 12]. Previous studies indicate that immediately after cutting off the cortical spinal nerve axons, BDNF can protect red nucleus neurons against atrophy, stimulate the expression of GAP43 and promote axonal regeneration. In this study, exercise training was exerted after MCAO. The level of BDNF mRNA was up regulated, and similar results had been observed by the study. Significant differences existing between training group and control group, rather than training group I indicated that retraining after an interval could still effectively promote the transcription of BDNF gene.

According to the current understanding, functional recovery of cerebral ischemia includes two processes [26-29]. Early recovery is mainly the disappearance of edema and the protection of "Penumbra Area". More recently, hyperacute imaging studies have shown that early rapid recovery of function corresponds with successful reperfusion of the ischemic penumbra, suggesting that successful thrombolysis, optimization of collateral flow and even angiogenesis may be the first step for successful rehabilitation. Our data also demonstrated that the diversity and complexity of reorganization patterns make it dynamic and dependent on the nature of injury, substrates involved and the duration since the initial insult. The late functional recovery is mainly the sprouting of axon and the plasticity of synapse and so on. Examples of neural plasticity are: Neuronal reorganization, collateral sprouting, unmasking. Cortical Reorganization and Chemical Communication changes, such as peptides, hormones, amino acids, and various "factors" (especially, NGF, BDNF) have been shown regulate neuronal activity; transmitter and receptor changes. [30-32]

It has been studied that Environmental enrichment (EE), as a stimulation paradigm, involves a combination of increased social interaction, physical exercise and continuous exposure to learning tasks and produces interesting effects. It has been reported that abundant experience in the complex environment following ischemic injuries promotes neural plasticity, including enhanced neurogenesis, dendritic reconstruction and reactive synaptogenesis. [33-38].

The physical exercise is the most important factor among EE components and voluntary wheel running is the commonly adopted exercise model. Apart from its physical benefits, the exercise has been demonstrated to improve cognitive function and facilitate neural rehabilitation after brain damage. Moreover, other studies found that voluntary exercise showed superior effects in terms of plastic changes in the dentate gyrus. In accordance with this, Huang and his colleagues showed that up-regulation of BDNF lasted seven and two days in the wheel group and the treadmill group respectively [39-40].

Stroke rehabilitation with different exercise paradigms of continuous training has been investigated, but whether retraining after an interval could still up-regulate brain neurotrophic factor (BDNF) after brain ischemia would be interesting in clinics. Our results have demonstrated retraining after a one-month pause can also enhance the level of BDNF mRNA, suggesting that there appears to be a second therapeutic window in the process of stroke recovery. We infer from the experiments that stroke rehabilitation not only benefits linearly intervention but also improves a lot in late recovery time. [41-43]

Experimental methods in this study needed to discuss as well. C57 BL/6J mice were used because they have better athletic ability than the counterparts and are more suitable for long-term exercise experiments. Running in a cage was a proactive way of movement and the volume of physical activity was easy to calculate. Mice exercise 1 hour per day with reference to the former anti-aging experiment so that the physical exercise won't be too limited to make a difference. But it should not be too much because it was more in line with physiological characteristics other than depletion of movement. Clinically, stroke patients would suffer functional decline if they stopped training during recovery, Moreover, Function collapse always happens in patients with stroke during the recovery period after they stop training, but once again training was carried on, it is still effective.

Training group II was designed to study the recovery period after stroke and to determine whether there was a "second therapeutic window". For time choice, such as having a rest for 45 days was primarily to eliminate the impact of pre-training. It was not economic to have an interval for a long time.

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