creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

의학박사 학위논문

Molecular Mechanism of Exercise Intensity on Neural Plasticity in Rats with Stroke

뇌졸중 백서에서 운동 강도의 신경가소성에 대한 분자생물학적 기전

2013 년 8 월

서울대학교 대학원

의학과 재활의학전공

김 정 윤

의학박사 학위논문

Molecular Mechanism of Exercise Intensity on Neural Plasticity in Rats with Stroke

뇌졸중 백서에서 운동 강도의 신경가소성에 대한 분자생물학적 기전

2013 년 8 월

서울대학교 대학원

의학과 재활의학전공

김 정 윤

Thesis for the Degree of Doctor of Philosophy in Medical Science

Molecular Mechanism of Exercise Intensity on Neural Plasticity in Rats with Stroke

August 2013

Seoul National University College of Medicine

Department of Medicine

Jung Yoon Kim

뇌졸중 백서에서 운동 강도의 신경가소성에 대한 분자생물학적 기전

지도 교수 이 시 욱

이 논문을 의학박사 학위논문으로 제출함
2013 년 4 월

서울대학교 대학원
의학과 재활의학 전공
김 정 윤

김정윤의 의학박사 학위논문을 인준함
2013 년 7 월

위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)
위 원 _____ (인)
위 원 _____ (인)

Molecular Mechanism of Exercise Intensity on Neural Plasticity in Rats with Stroke

by
Jung Yoon Kim
(Directed by Shi-Uk Lee)

A thesis submitted to the Department of Rehabilitation Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Rehabilitation Medicine at Seoul National University College of Medicine

July 2013

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

Professor _____

Professor _____

Abstract

Molecular Mechanism of Exercise Intensity on Neural Plasticity in Rats with Stroke

Jung Yoon Kim

Department of Medicine, Rehabilitation Medicine

The Graduate School

Seoul National University

Introduction: The goal of neuro-rehabilitation is to facilitate neural plasticity. Neural recoveries have been known to increase with exercise training, and effective exercise for recovery has been thought to be early, and mild-to-moderate intensity. However, evidence of mild-to-moderate exercise was mainly focused on hippocampal mRNA expressions previously. Furthermore, relation of exercise intensity to the neurotransmitters and molecules involved in nitric oxide synthase (NOS) pathway were not clearly manifested.

Objectives: Aimed to investigate on the 1) changes in concentration of neurotransmitters during exercise, and 2) changes of neurotrophic, angiogenesis-related factors, and molecules associated with nitric oxide synthase pathway, which affect neural plasticity following different exercise durations and intensities after stroke in rats.

Methods: The middle cerebral artery ischemia was induced in SD rats. One days following ischemia, treadmill training was performed for 30 minutes a

day. In experiment 1, rats were subjected to the control, 3-, 7-, 14-, and 28-day training group. In experiment 2, moderate (60 % of tolerable speed), and high (85% of the tolerable speed) intensity of treadmill trainings were done for 14 days. The Garcia test, and neurotransmitter sampling using the microdialysis were done in the last day of training. Concentrations of acetylcholine, serotonin, GABA, dopamine, and glutamates were quantified in the dialysates. The mRNA expressions of two immediate early genes (arc and c-fos), a neurotrophin (bdnf), and an angiogenesis-related factor (vegf) were measured using the RT-PCR. Proteins concerning NOS pathways (eNOS and nNOS), angiogenesis (VEGF), and long-term potentiation (NR2A/2B) were investigated in the Western blot. Immunofluorescence staining was done with antibodies to DAPI, GFAP, NeuN, nestin, and BrdU, and observed in the peri-infarct borderline zones.

Results: Acetylcholine concentrations were raised in the 3-day exercise group, and then decreased in 14-day group. Glutamate concentrations were elevated in the 3-day training. The expression of bdnf mRNA was raised in day 14 with the increase of c-fos in the ischemic hemisphere. Levels of angiogenesis-related proteins such as eNOS, Akt, and VEGF were raised in day 14 in the contralateral hemisphere (Experiment 1). Following moderate-intensity trainings, NR 2A/2B ratios and VEGF levels increased in day 14. In contrast, high-intensity exercises induced increase of nNOS, in day 3 and 14, accompanied with elevated levels of immediate early genes (Experiment 2). BrdU+, nestin+ cells were observed 3 days following ischemia in the peri-infarct area.

Conclusions: Moderate-intensity exercise for 2 weeks might be associated with neuroprotective mechanism such as neurotrophin, angiogenesis and LTP in rat stroke model. In contrast, high-intensity of training seemed to be associated with nNOS, a neurotoxic factor.

Keywords: exercise intensity, cerebral ischemia, neurotransmitter, neural plasticity

Student Number: 2011-30543

List of tables

Table 1. Garcia Motor Index Scoring System

Table 2. Concentration of the neurotransmitters in each group

Table 3. Concentrations of the neurotransmitters in moderate and high intensity groups in day 14

List if figures

Figure 1. Experimental design (Experiment 1)
Figure 2. Experimental Design (Experiment 2)
Figure 3. Placement of probe using stereotactic frame
Figure 4. Application of microdialysis system during treadmill exercise
Figure 5. Concentrations of the neurotransmitters in each time interval
Figure 6. Garcia test scores in Group 3, 4 and 5
Figure 7. Concentration of dopamine and GABA in the right hemisphere during treadmill training
Figure 8. Concentration of serotonin and acetylcholine in the right hemisphere during treadmill training
Figure 9. Concentration of glutamate in the right hemisphere during treadmill training
Figure 10. c-fos mRNA expression in bilateral hemisphere
Figure 11. bdnf mRNA expression in bilateral hemisphere
Figure 12. nNOS in bilateral hemisphere
Figure 13. eNOS in bilateral hemisphere
Figure 14. Akt in bilateral hemisphere
Figure 15. VEGF in bilateral hemisphere
Figure 16. Ratio of NR2A/2B in bilateral hemisphere
Figure 17. Peri-infarct area of the group 4 (14 days of treadmill training) showed BrdU+, newly-proliferating cells, with highly activated GFAP+ cells.
Figure 18. Peri-infarct area of the group 2 (3 days' of treadmill training) presented BrdU+, Nestin+ cells
Figure 19. Peri-infarct area of the group 4 (14 days' of training) had NeuN+ cells, which seemed to be neurons
Figure 20. arc mRNA in the ischemic hemisphere
Figure 21. c-fos mRNA in the ischemic hemisphere
Figure 22. vegf mRNA in the ischemic hemisphere
Figure 23. nNOS in the ischemic brain
Figure 24. Akt in the ischemic brain
Figure 25. eNOS in the ischemic brain
Figure 26. VEGF in the ischemic brain
Figure 27. NR 2A/2B ratios in the ischemic brain

List of abbreviations and symbols

BDNF	=	brain-derived neurotrophic factor
BrdU	=	5'-bromo-2'-deoxyuridine
BSA	=	bovine serum albumin
CREB	=	cAMP response element binding protein
DLPFC	=	dorsolateral prefrontal cortex
eNOS	=	endothelial nitric oxide synthase
GABA	=	gamma amino-butyric acid
GFAP	=	glial fibrillary acid protein
HPLC	=	high-performance liquid chromatography
MCA	=	middle cerebral artery
NeuN	=	neuronal nuclei
nNOS	=	neuronal nitric oxide synthase
NQ	=	non quantifiable
PAS	=	Periodic acid schiff
PBS	=	phosphate buffer saline
ROS	=	reactive oxygen species
SOD	=	superoxide dismutase
VEGF	=	vascular endothelial growth factor

Contents

Abstract ……i

List of tables ……iv

List of figures……v

List of abbreviations and symbols ……vi

Contents ……vii

Introduction ……1

Materials and Methods ……4

1. Experimental design ……4

2. Experimental Animals ……8

3. Middle Cerebral Artery Ischemia Model in Rats ……8

4. Treadmill Training ……9

5. BrdU injection ……9

6. Outcome Measures ……10

7. Statistical Analysis ……18

Results ……19

1. Preliminary study 1: Establish the microdialysis techniques ……19

2. Preliminary study 2: Determine the optimal time to obtain neurotransmitters……21

3. Experiment 1: Exercise duration ……23

4. Experiment 2: Exercise intensity ……43

Discussion ……55

Conclusion……58

References……59

Abstract in Korean ……61

Introduction

Rapidly aging society has brought about changes in the distributions of diseases. Neurodegenerative diseases, such as dementia and Parkinson disease, and stroke have been the leading cause of disability in elderly population. These trends resulted in the surge of research on the treatment of neurodegenerative diseases. On the other hand, residual disabilities from the diseases increased the demand for neurological rehabilitation (neurorehabilitation).

Neural plasticity is the main topic in recent neurological rehabilitation area. Several studies on the concept of neural plasticity had been conducted concerning changes in the hippocampus and cerebellum. Efforts for its translational research and clinical application have been made. In other words, adopting a strategy to maximize neural plasticity has been a flow of neurological rehabilitation. Exercise, as an integral part of these rehabilitation trainings, has been known to promote the neural plasticity in the brain.

Effects of exercise on neural plasticity

Changes in cortical plasticity following exercise have been investigated in spinal cord injury models as well as brain injury models. (1) In addition, expression of BDNF, VEGF, IGF-1, serotonin in the hippocampus following exercise was reported, which means exercise could facilitate neurogenesis. (2, 3) Exercise was also reported to have protective effects against aging and inflammatory responses by means of mediating BDNF expression.(4) Furthermore, it also showed protective actions to preserve cognitive functions in rats with chronic stroke models. (5) The superoxide dismutase (SOD) activities, BDNF mRNA transcriptions, and cAMP response element binding proteins (CREB) were activated by exercise. These reactions were mediated by the reactive oxygen species (ROS), so exercise might control BDNF

pathways through oxidation-reduction reactions. (6)

Deconditioning and CNS changes

Changes in concentrations of the neurotrophic factors were reported in deconditioning rats without other diseases. Cell proliferation in the subventricular zone and dentate gyrus in rats subjected to hindlimb suspensions, (7) brain-derived neurotrophic factor (BDNF), serum vascular endothelial growth factor (VEGF) concentrations were also raised in hippocampus, suggesting that movement restriction in rats and its correlation with changes in brain neural plasticity. (8, 9) Furthermore, nitric oxides were increased in rat hypothalamus, paraventricular nucleus, when hindlimb suspension was applied. It could be evidences to relation of nitric oxide and cardiovascular deconditioning.

Exercise intensity and neural plasticity

Investigations on the effects of exercise intensities after stroke on motor recovery were also performed previously. Mild to moderate exercise using treadmills were reported to be most effective to recovery in rat stroke models. Rats which had gone through mild to moderate treadmill training showed smaller ischemic lesion areas, less severe neurologic deficits.(10) Furthermore, hippocampal BDNF, VEGF, NADAR1, and Flk-1 mRNA expressions were reported to be higher in rats with mild intensity exercise, compared to those in rats with high intensity training. (11) However, this study did not investigate on the protein synthesis and neurologic deficit, or mRNA from other brain areas rather than hippocampus, and only four mRNAs were analyzed. It has been thought that the neurotransmitter concentrations were closely related to neurologic recovery, but as far as investigators know, real-time extraction and analysis has not been done yet.

Purpose of this study

This study was aimed to investigate on 1) changes in neurotransmitter concentrations during exercise, and 2) evidences of neurogenesis and angiogenesis in peri-infarct borderline zone following exercise of different duration and intensities after cerebral ischemia in rats.

Materials and Methods

1. Experimental design

The experiment 1 was performed to know which duration of exercises could result in the maximal effects on neural plasticity, and to determine the optimal time to obtain responses of trainings with different intensities. Furthermore, exercise duration itself could be an important factor in prescribing and applying exercises clinically.

In the experiment 2, duration of the exercise derived from the experiment 1 was applied. Rats had gone through moderate and high intensity exercise to manifest differential effects of exercise intensity.

A. Experiment 1: Exercise duration

Rats were subjected to left middle cerebral artery ischemia. Three days later, exercise using the treadmill was applied with 60% intensity of the maximal speed, which was intended as moderate-intensity exercise. Training durations were as follows; 1) Control group without exercise, 2) 3 days, 3) 14 days, and 4) 28 days of treadmill training for one hour/day (Figure 1).

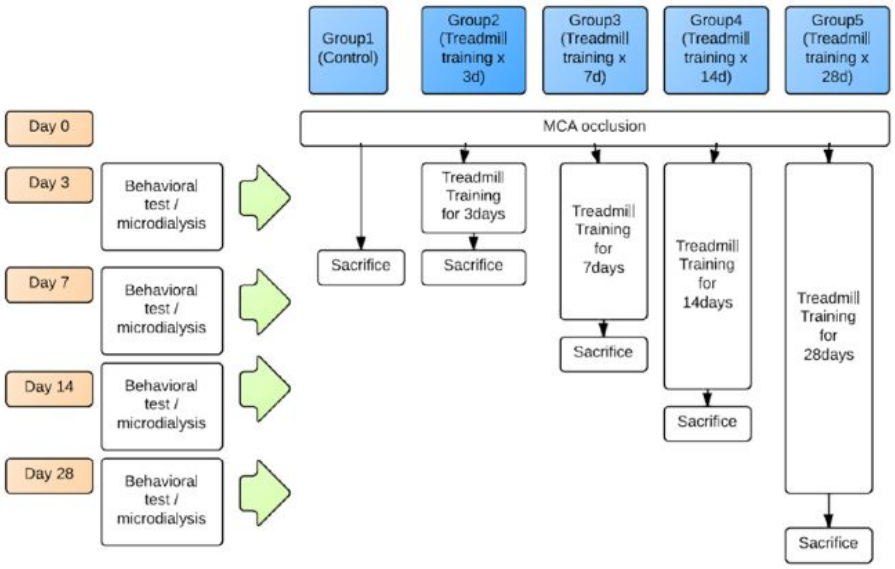
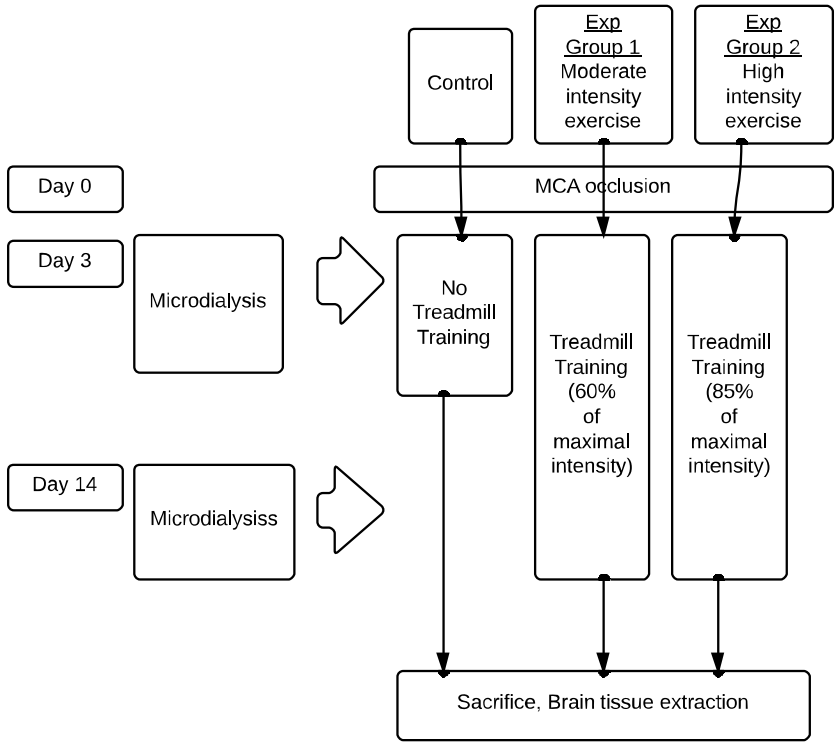


Figure 1. Experimental design (Experiment 1)

B. Experiment 2: Exercise intensity

Middle cerebral artery ischemia was induced, then rats were subjected to the following groups; 1) Control without treadmill training, 2) moderate-intensity training, which was of 60% of the maximum, and 3) high-intensity exercise, which was 85% of maximum (Lee, 2009, Figure 2).



)LJXUH ([SHULPHQWDO HWJQ([SHULPHQW

2. Experimental Animals

Male Sprague-Dawley rats, aged 8-10 weeks, and weighted as 250 – 350 grams, were used. All rats had adaptation period of one week in the laboratory. Temperatures were maintained as 22℃, brightness was controlled in 12-hour cycle. Food and water was supplied *ad libitum*. All experimental procedures were performed between 9:00 A.M and 4:00 P.M. The Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital approved all the procedures described below.

3. Middle Cerebral Artery Ischemia Model in Rats

Occlusion and reperfusion methods to induce MCA ischemia were used as previously reported (Longa et al. 1989). Anesthesia was induced and maintained with 5% and 2% of isoflurane dissolved in 40%/60% and 25%/75% oxygen/nitrogen applied via chamber and nose cone, respectively. After anesthesia with isoflurane inhalation, neck was dissected in the midline. Right common carotid artery was dissected and proximally, external and internal carotid arteries were separated from the surrounding connective tissues. Common and external carotid arteries were clamped, and then small fenestration was made in the bifurcation of the common carotid artery. Through this fenestra, a blunted 4.0 nylon thread (Ethicon, Edinburg, UK) was inserted to the middle cerebral artery. Then, internal carotid artery was clamped. During this operation, continuous monitoring of the rectal temperature was done. Automatic temperature control units (Homothermic Blanket Control Unit, Harvard apparatus Inc., US) were used to monitor and maintain body temperature of the rats.

4. Treadmill Training

After 3 days from the cerebral ischemia, treadmill (Columbus instrument, US) training for 30 minutes a day was applied. The maximal tolerable speeds of treadmill training were measured in advance. The appropriate velocities were determined in each group. Before starting the main training, adaptation period for 5 minutes with the velocity of 5m/min was applied as warm-up exercises. Weak electrical stimulations were applied in the terminal of the treadmill track, to encourage rats to run in appropriate speed. These treadmill training was performed from day 2 to day 28 from the operation according to training protocols in each group.

5. BrdU injection

In day 3 and 7, 5'-bromo-2'-deoxyuridine (BrdU) mixed with phosphate buffer saline (PBS) was injected intra-peritoneally with dose of 100mg/kg to label newly dividing cells.

6. Outcome Measures

A. Behavioral test

Three, seven, and 14 days after the operation, the Garcia test was evaluated. The examiner was blinded to the groups of the experimental animals. To minimize diurnal variations, it was measured in a certain fixed time during the day. The Garcia test has 6 items and each was rated as 0-3 points, resulting in the total score of 3-18 points. Criteria for each point were shown in Table 1.

Table 1. Garcia Motor Index Scoring System

	0	1	2	3
Spontaneous activity	No movement	Barely moves	Moves but does not Approach at least three sides of cage	Moves and approaches at least three sides of cage
Symmetry in four limb movement	Left side: no movement	Left side: slight movement	Left side: moves slowly	Both sides: move symmetrically
Forelimb outstretching	Left side: no movement, no outstretching	Left side: slight movement to outreach	Left side: moves and outreaches less than right ide	Asymmetrical outreach
Climbing		Fail to climb	Weak response on left side	Symmetrical response
Body proprioception		No response on left side	Weak response on left side	Symmetrical response
Response to vibrissae touch		No response on left side	Weak response on left side	Symmetrical response

B. Microdialysis

Microdialysis system is techniques to extract interstitial fluid using the probes, which is a substitute for the vascular system. Brain interstitial fluids are easily extracted using this system. Artificial CSF is continuously perfused during the extraction to maintain equilibrium and to enable the dialysis. Microanalysis CMA Microanalysis system (CMA Microdialysis AB, Solna, Sweden) were used in this study. Experimental animals were positioned supine, and then isoflurane inhalation was performed. Skull was positioned in the stereotaxic frame. Skull bone was exposed, and then intracranial guide cannula (MD-2251, CMA Inc., Sweden) was positioned in the location of interest. Then microdialysis probe (CMA Inc., Sweden) was positioned properly. Microdialysis pump (CMA Inc., Sweden) was used to perfuse artificial CSF (145.0 mM NaCl, 2.7m KCl, 1.0mM MgCl, 1.2 mM CaCl, 0.45 mM NaH2PO4, 2.33 mM Na2HPO4, pH 7.4) with 2μl/min velocity for 60 minutes to obtain equilibrium. After then, microdialysis sample was collected for 30 minutes. Extracted samples were frozen in -80℃.

From extracted specimen, high performance liquid chromatography (HPLC) was used to detect acetylcholine, serotonin, glutamate, dopamine, and gamma amino-butyric acid (GABA) concentrations. Glutamate, GABA, dopamine, serotonin concentrations were analyzed using the high-performance liquid chromatography (HPLC) methods.

In this experiment, samples were extracted from the dorsolateral prefrontal cortex (DLPFC), which were known to be sensitive to the changes in neural plasticity. The locations of the probes were A/P +3.2mm, M/L -3.5mm, depth -1.3mm in stereotactic frame. Guide cannulas were placed in this position (Figure 3). (12, 13)

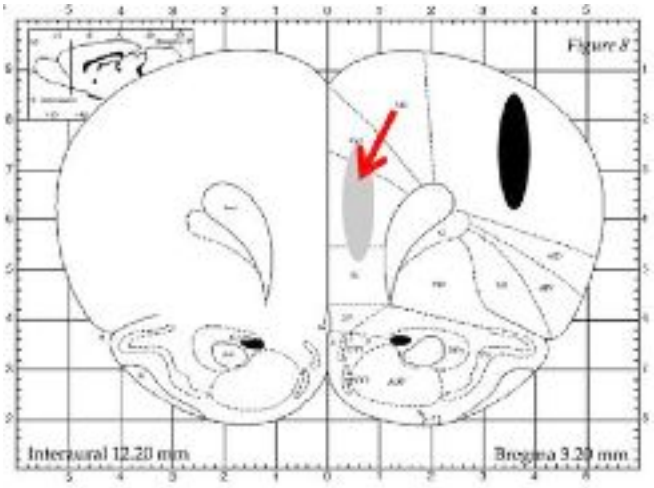


Figure 3. Placement of probe using stereotactic frame (from Beyer et al. 2007 & 2002) (12)

The locations of the probes were A/P +3.2mm, M/L -3.5mm, depth -1.3mm in stereotactic frame. The indicator pointed out the target position of the probe.

C. RT-PCR

mRNA extraction

Rat brain tissues were obtained from each hemisphere. Tissues of the peri-infarct area, weighted 30-40mg, were extracted from the ischemic hemisphere, and corresponding areas were obtained from the contralateral hemisphere. RNA extraction was performed using RNeasy Lipid Tissue Mini Kit (QIAGEN, Germany)

Tissues were disrupted and sonicated in 1ml QIAzol, then incubated at room temperature for 5min. The 200㎕ of chloroform was added and shaken for 15s. Samples were incubated at room temperature for 2-3min, and centrifuged at 12,000 rpm for 15min at 4℃. The upper, aqueous phases were transferred to a new tube, 700㎕ of 70% ethanol was added, and were vortexed. About 700㎕ of the samples were transferred to RNeasy Mini spin column in the 2ml collection tube. These were centrifuged at room temperature for 15s at 10,000rpm, and the flow-through was discarded. Using the same collection tube, transfer to RNeasy Mini spin column and centrifuge were repeated. The 700㎕ of Buffer RW1 was added to the RNeasy column, and centrifuged for 15s at 10,000rpm. Then the 500㎕ of Buffer RPE was added to RNeasy column, and centrifuged for 15s at 10,000rpm. The same amount of Buffer RPE was added again, then centrifuged for 2min at 10,000rpm. The RNeasy column was placed in a new 2ml tube, centrifuged at full speed for 1min, and then located in a new 1.5ml tube. The 30-50㎕ of RNase-free water was added, and centrifuged for 1min at 10,000rpm.

cDNA synthesis

The 1 μg of total RNA, 1 μl of dNTPs, and 1 μl of oligo (dT) were incubated at 65 °C for 5 min followed by cooling on ice for 1 min. The 2 μl of 10X RT buffer, 4 μl of 20mN $MgCl_2$, 2 μl of 0.1 M DTT, SuperScript III Reverse

Transcriptase (Invitrogen, CA, US) and 1 µl RNaseOUT (Invitrogen, CA, US) were mixed at 50°C for 50 min, 85°C for 5 min followed by cooling on ice for 1 min. The RNA strands in the RNA–NA hybrid were removed by adding 1 µl RNaseH (Invitrogen, CA, US) and incubating for 20 min at 37°C.

RT-PCR

Polymerase chain reaction (PCR) was performed in a total volume of 25 µl containing 1 µl cDNA, 1.5 µl dNTPs, 0.5 µl of each primer pair, and 0.5 µl Taq DNA polymerase (Bioneer, Daejon, Korea) in Taq DNA polymerase reaction buffer as provided by the supplier. For the PCR, a total of 35 cycles were run under the following schemes; a denaturing at 94°C for 30 s, a primer annealing at 52°C (c-fos), 57°C (vegf), 51°C (BDNF), 55°C (arc), 57°C (glyceraldehyde-3-phosphate dehydrogenase (gapdh) for 30s, and an extension step at 72°C for 30 s in the T100 Thermal Cycler (BIO-RAD). The following sets of primers were used in the PCR amplification: c-fos (forward 5'AGA CAG ATC AAC TTG AAG A-3', reverse 5'AAA CAA GAA GTC ATC AAA-3'); vegf (forward 5'GAT CAT GCG GAT CAA ACC TCA CC-3' reverse 5'CCT CCG GAC CCA AAG TGC TC-3'; bdnf (forward 5'CAT CCT TTT CCT TAC TAT GGT T-3' reverse 5'TTC CAG TGC CTT TTG TCT ATG-3'; and arc (forward 5'GAG GTT CCT CCA TCT GCC TG-3' reverse 5'GGC TTG GTG AAA ATA GGG GA-3'). Each sample was tested in duplicate. Optical densities of the bands were quantified with Image J analyzer (NIH, US, http://rsb.info.nih.gov/ij/).

D. Western Blot

Protein extraction

The RRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Inc., Seongnam-si, Korea) was used for the Western blot.

Brain tissue about 30-40mg were added to the 400㎕ PRO-PREP™ solution, sonicated, incubated for 20-30min on ice at 20℃, and then centrifuged at 13,000rpm at 4℃ for 5min.The supernatants were transferred to fresh 1.5ml tubes. The protein concentrations were measured by the Bradford method.

Western blot

The protein samples of 30ug were loaded for 90min (with 80V and 120V for 10 and 80 min respectively), transferred for 60min with 100V on ice. After 5% skim milk blocking buffer was applied, samples were incubated at room temperature for one

Used first Antibody were as follows; rabbit anti-eNOS (Cell Signaling Technology #9572), diluted 1:1,000; rabbit anti-nNOS (Abcam #ab90443), diluted 1:1,000; mouse anti-Akt (Cell Signaling Technology #9272), diluted 1:1,000; mouse anti-VEGF (Abcam #ab1316), diluted 1:500; rabbit anti-NR2a (Abcam #ab77980), diluted 1:1,000; mouse anti-NR2b (Abcam #ab78383), diluted 1:1,000; and rabbit anti-GAPDH (Cell Signaling Technology #2118). Samples were incubated at room temperature for 2 hours, washed with TBS/T buffer for 10 min, repeated 3 times. The 2nd Antibodies were applied and incubated for 30 min at room temperature, then washed with the TBS/T buffer for10min, repeated 4 times. The Lumi-plus ECL detection (Tech & Innovation, Chuncheon-si, Korea) was applied. The optical densities of the protein bands were quantified with Image J analyzer (NIH, US, http://rsb.info.nih.gov/ij/). The ratios of each protein to those of the GAPDH were calculated.

E. Immunofluorescence staining

Primary antibodies to the glial fibrillary acid protein (GFAP), neuronal nuclei (NeuN), nestin, and BruU were used to detect glial cell activations, neuronal cells, and newly produced cells. Peri-infarct borderline zones and hippocampus were investigated in each group.

Preparation of slides and samples: Frozen sections

Before extracting brain tissues for staining, 50ml of heparinized phosphate buffer saline (PBS) and the same amount of 4% paraformaldehyde (PFA) were perfused transcardially twice and once respectively.

Tissues were fixed in 4% PFA for 24 hours, then immerged in 10%, 20% and 30% sucrose solution for 24 hours each, sequentially. After sucrose was removed, tissues were embedded in OCT compound, then stored in liquid nitrogen tank at -70°C. Blocks were cut into 2 μm-thick cryostat sections and mounted on superfrost slides.

Double Immunofluorescence staining

Sections were washed with in PBS for 5 min, then were immerged in blocking solution (1% BSA, 0.3% Triton X-100 in PBS) for 1 hour with gentle agitation at room temperature, and rinsed in PBS. Slides were then incubated at room temperature for 1 hour, in the primary antibodies as follows; FITC-conjugated anti-BrdU (Abcam, #ab74545), diluted 1:200; anti-NeuN (Millipore, #MAB377B), diluted 1:200; anti-GFAP (Abcam, #ab7260), diluted 1:1000; and anti-Nestin (Abcam, #ab6142), diluted 1:50. Slides were rinsed for 5 min in PBS/0.3% Triton X-100, and incubated at room temperature for 2 hour with secondary antibodies as follows; Texas Red ® -conjugated goat anti-mouse IgG (Invitrogen, #T862) diluted 1:200 for anti-

NeuN and anti-Nestin; Texas Red ® sheep anti-rabbit IgG (Abcam #Ab6793) diluted 1:200 for anti-GFAP.

Following rinsing the sections in PBS/0.3% Triton X-100 and in 0.05M PBS, VECTASHIELD ® Mounting Medium with DAPI (Vector Laboratories, US) was put on the slides. After 1 minute, coverslips were mounted then sealed with nail polisher to prevent drying and movement under microscope.

7. Statistical Analysis

All values were written in mean ± standard error of means. Statistical differences were analyzed using the SPSS for Windows 19.0 version (IBM corporation, US). Concentrations of neurotransmitters measured using the microdialysis were compared using the one-way ANOVA, and non-parametric values such as Garcia scales, and fold changed measured in the PCR and Western blot were compared with the Kruskal-Wallis test. A p-value below 0.05 was considered to be significant.

Graphs were made using the GraphPad Prism for Mac 6.0 c version (GraphPad Software, Inc., US).

Results

1. Preliminary study 1: Establish the Microdialysis Techniques

Pilot study was performed in 3 healthy rats. As formerly described, rats were dissected in the scalp to expose the skull, then were trephined in the frontal area. A microdialysis guide cannula was inserted and fixed with bone cements. An anchor was fixated to prevent movement of the cannula during the movements. Then scalp was sutured. After 24 hours from the operation, researchers made sure that the anchor and probes were fixated without any movement in the skull. The microdialysis probe was positioned through the cannula, and 10 minutes of adaptation period was applied to the rats to minimize effects of probe placement. During this adaptation period, rats were let to ambulate in the cylindrical cage while artificial CSF was perfused through the probe. After then, treadmill adaptation was applied with a velocity of 5m/min. Velocities of the treadmill was 12m/min for 10 minutes, then increased to 18m/min. Specimens were extracted continuously during the treadmill training (Figure 4). Perfusing velocity was 5ul/min. Extracted specimens were analyzed using the high performance liquid chromatography (HPLC).

Quantifiable limits of the HPLC were 1ng/ml in dopamine and GABA, 0.1ng/ml in serotonin and acetylcholine, 2ng/ml in glutamate.

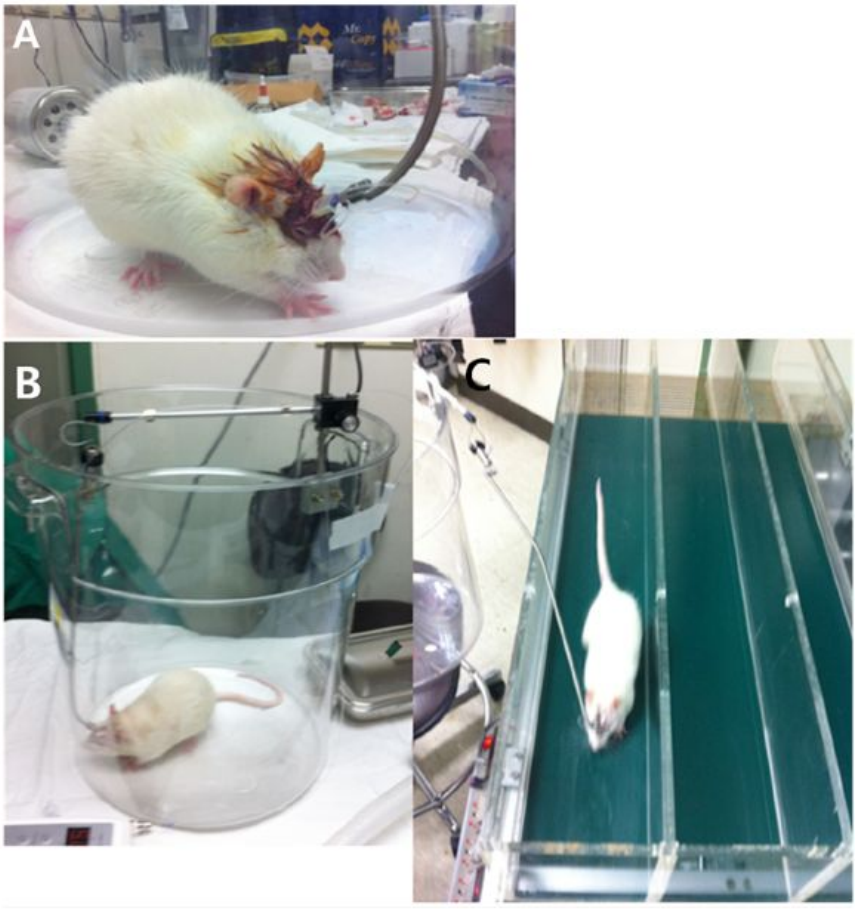


Figure 4. Application of microdialysis system during treadmill exercise

(A) A guide cannula and an anchor were fixed to the skull. (B) Adaptation was allowed in a cylindrical plastic cage for 10 minutes. (C) Collecting the specimen for the microdialysis could be performed during treadmill training without complications such as disconnection or bleeding.

2. Preliminary study 2: Determine the optimal time to obtain neurotransmitters

To know when is the appropriate time interval to obtain stable neurotransmitter levels, extracellular fluids were extracted every 15 minutes from 20 minutes to 140 minutes after probe placement. Artificial CSF was perfused for initial 20 minutes to reach the equilibrium. Healthy SD rats (N=4) were used.

Concentrations of dopamine, GABA, acetylcholine, serotonin, and glutamate were not eligible for statistical analysis due to small number of quantifiable samples. However, 60 minutes following probe placement seem to be an appropriate time to get stable levels of neurotransmitters (Figure 5).

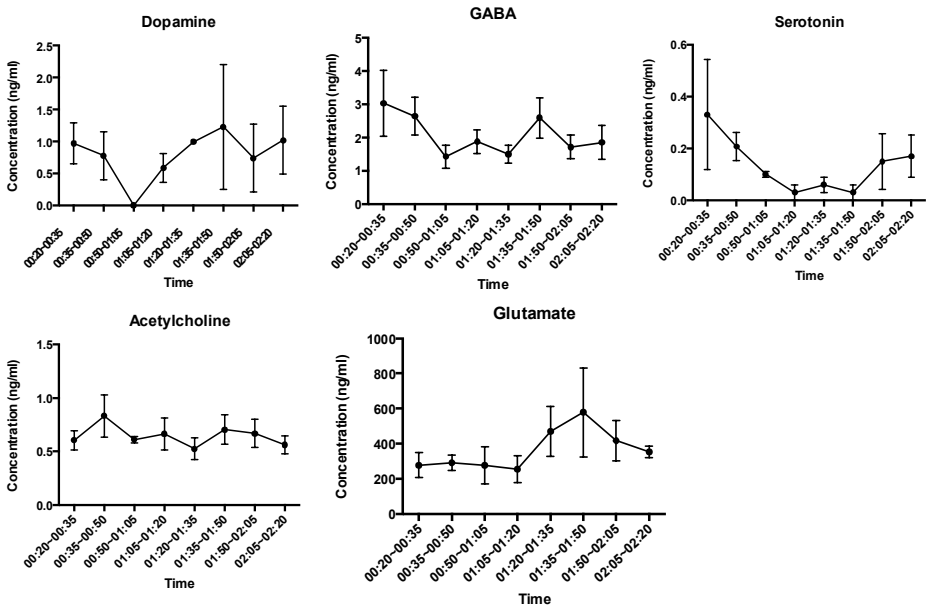


Figure 5. Concentrations of the neurotransmitters in each time interval

Samples were extracted every 15 minutes from 20 to 140 minutes following the probe placement and the beginning of artificial CSF perfusion. Although statistical analysis was not available owing to a couple of points with non-quantifiable level of neurotransmitters, each concentration was assumed to be stabilized after 50 to 65 minutes

3. Experiment 1: Exercise duration

Moralities following left MCA occlusion were 28.6% (12 rats out of 42). All mortal cases were within 3 days of the operation, which might be related to the direct complication from the surgery. No significant differences between pre-operative and 3-days after the operations were found in all experimental groups.

A. Behavioral tests

Score of the Garcia test did not show any difference after 3 days of the operation. Treadmill training for 7 days also resulted in no definite differences between group 3, 4, and 5. However, 14-days of treadmill training seem to elevate the scores in the 14-day training group, which were 15.5±0.5 in group 4, and 13.0±0.8 in group 5 (p=. 029). No significant differences were detected in each group according to the measurement time (Figure 6).

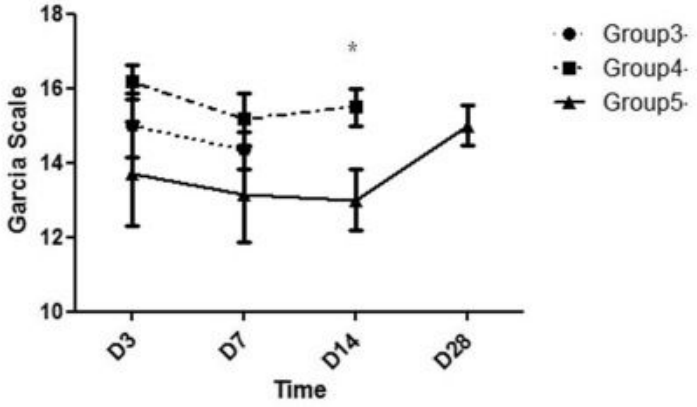


Figure 6. Garcia test scores in Group 3, 4 and 5 (* $p<.05$)

Except for the Garcia test score between group 4 and 5 in day 14, no significant differences were found among every time points and groups.

B. Concentrations of the neurotransmitters

Neurotransmitter concentrations were analyzed in the specimens extracted from the interstitial tissues during exercise training. Some of the samples, dopamine in all of the groups, serotonin and glutamate in 28-day training group, had non-quantifiable levels of neurotransmitters (Table 2). Dopamine and GABA, acetylcholine and serotonin showed similar trends of changes between each other (Figure 7, 8 and 9). Dopamine and GABA seemed to be lower in the 3-, and 14-day group when compared to the control group, but GABA did not show statistically significant differences. Dopamine concentrations were not suitable for statistical analysis because each time point had only one quantifiable sample. Acetylcholine level rose in the 3-day exercise group, and then decreased in 14-day group (p=.015, Figure 8). Glutamate concentrations were higher in the 3-day training group than those of control and the 14-day group (p=.038 and .023 respectively, Figure 9).

Table 2. Concentration of the neurotransmitters in each group (ng/ml, Mean ± SEM)

	Acetylcholine	Serotonin	Dopamine	GABA	Glutamate
Control	0.78±0.36	0.67±0.60	5.60	16.83±13.94	207.03±60.22
Day 3	1.59±0.25	2.44±1.94	2.02	6.50±2.39	892.67±563.02
Day 14	0.38±0.07	0.11±0.05	1.10	0.99±0.34	60.60±21.57
Day28	0.55	0.05±0.01	1.26	5.19±4.32	218.0

* Values without SEM had only one available level.

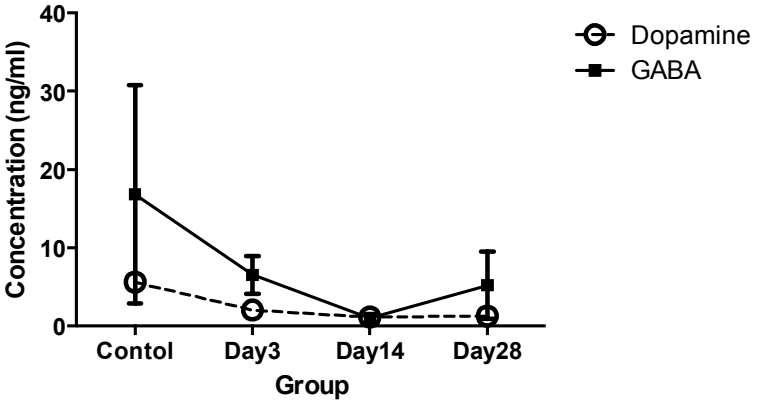


Figure 7. Concentration of dopamine and GABA in the right hemisphere during treadmill training

Dopamine concentrations were not suitable for statistical analysis due to small number of quantifiable samples. GABA concentrations did not show significant differences between each group.

*GABA: Gamma amino-butyric acid

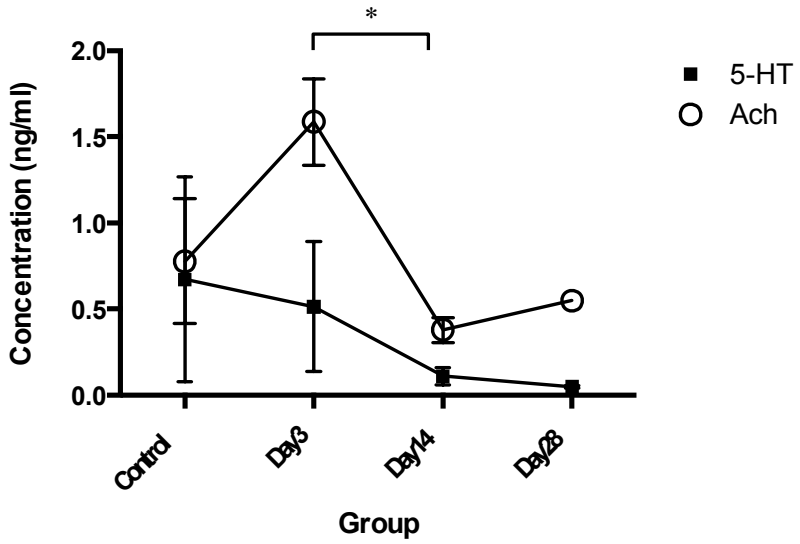


Figure 8. Concentration of serotonin and acetylcholine in the right hemisphere during treadmill training

Acetylcholine levels were higher in the 3-day exercise group, than those in the 14-day group (p=.015). Serotonin concentrations did not differ between each group.

*p<.05

** Ach: Acetylcholine, 5-HT: Serotonin

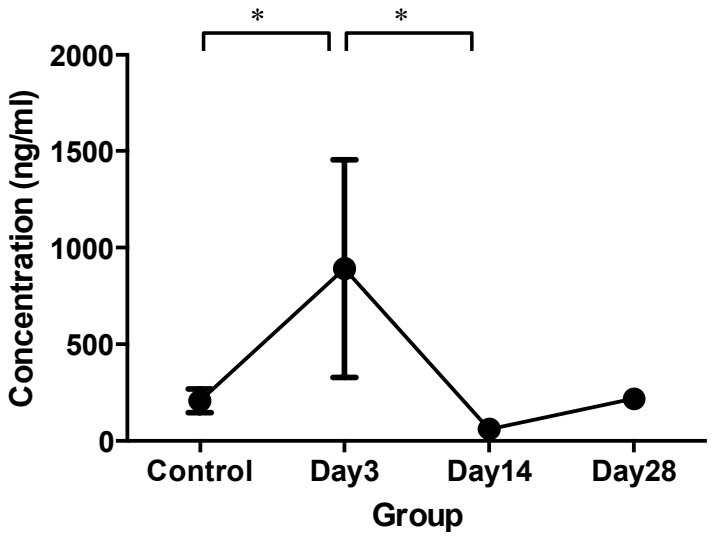


Figure 9. Concentration of glutamate in the right hemisphere during treadmill training

Glutamate levels were higher in the 3-day training group than those of control and the 14-day group (p=.038 and .023 respectively, Figure 9).

*p<.05

C. RT-PCR

The levels of c-fos mRNA in the right hemisphere of 14-day group was higher than those of 3-day group (Figure 10). Also, bdnf levels from the right hemisphere were significantly higher than those from the left hemisphere (Figure 11). Another mRNAs measured in this experiment, which were arc, akt, and vegf, did not show differential expression in bilateral hemisphere following the duration of training.

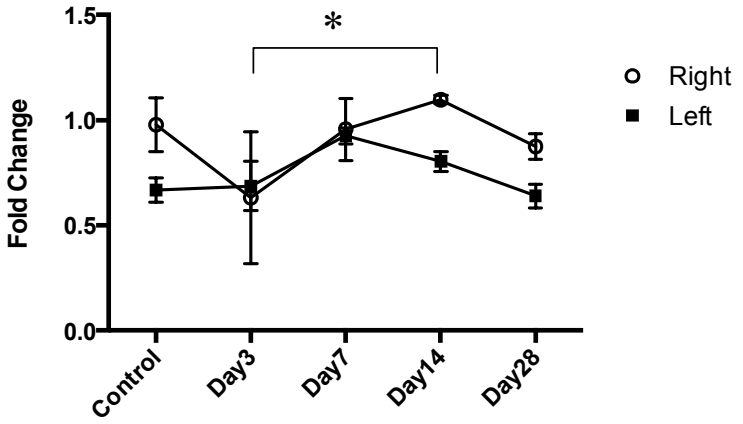


Figure 10. c-fos mRNA expression in bilateral hemisphere

The mRNAs of the c-fos were significantly higher following 14-days of exercise than those following 3-days of training in the right ischemic hemisphere.

***p*<.05**

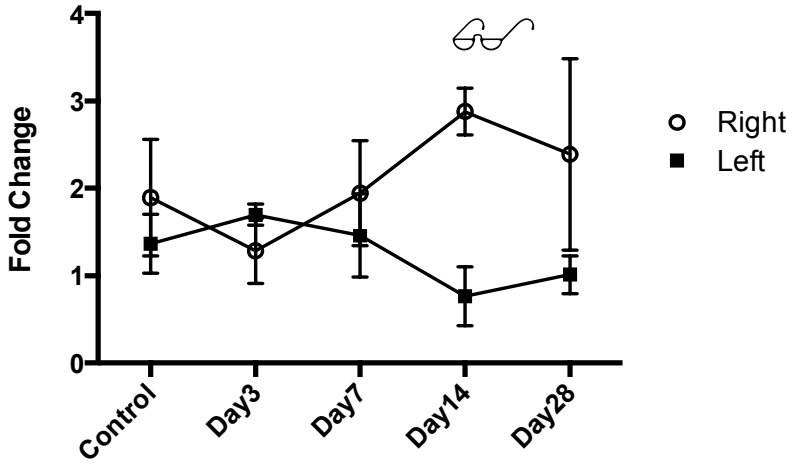


Figure 11. bdnf mRNA levels in bilateral hemisphere

Levels of bdnf mRNA was higher in the ischemic hemisphere than the contralateral one in 14-dy training group. No significant differences were found between each group and hemisphere.

***p*<.05, difference between right and left hemisphere**

D. Western Blot

In ischemic brain (right hemisphere), tissues from the peri-infarct zone were analyzed. Levels of nNOS were significantly higher in the 3-days of treadmill training group when compared to those of the control group, and to that of the contralateral side (Figure 12). The eNOS level increased in day 28 in the ischemic hemisphere. Meanwhile, it reached the highest level in day 14 then decreased in the contralateral hemisphere (Figure 13). In contrast, Akt level reached the highest value in day 14 in the contralateral hemisphere, and these were significantly higher than those of the ischemic side of the brain (Figure 14). VEGF showed the same tendency of changes with those of Akt. Expression levels were the highest in the day 14, and then decreased in day 28 (Figure 15).

In the contralateral left hemisphere, NR2A/2B ratio was significantly higher in day 14 (Figure 16).

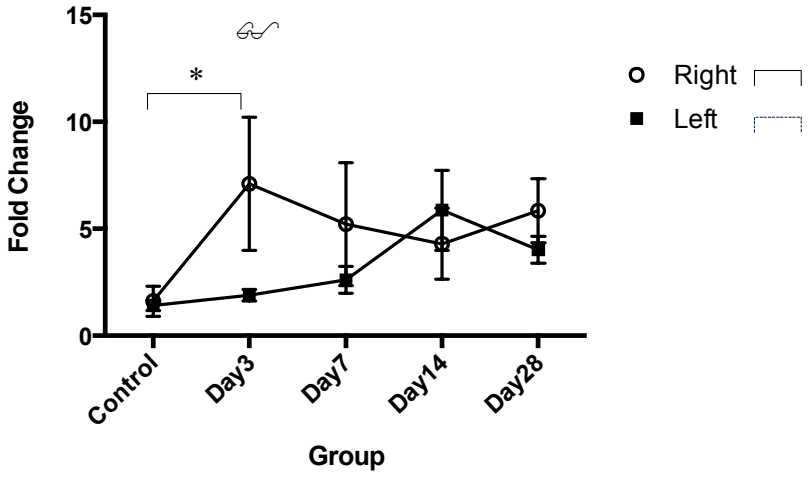


Figure 12. nNOS in bilateral hemisphere

The nNOS expression levels from the ischemic hemisphere were significantly higher in the 3-day training group than those of the control group. Differences between ischemic and contralateral hemispheres were only found in the 3-day group.

***p<.05, between each group**

◆ p<.05, between each hemisphere

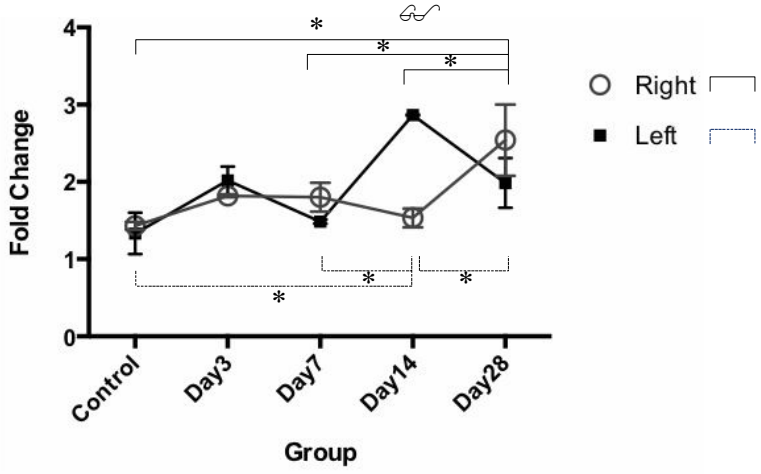


Figure 13. eNOS in bilateral hemisphere

In the ischemic right hemisphere, eNOS level was the highest in day 28 and was significantly higher than those of the control, day 7, and day 14. In the contralateral, left hemisphere, the eNOS level reached maximal value in day 14, then decreased in Day 28.

*p<.05

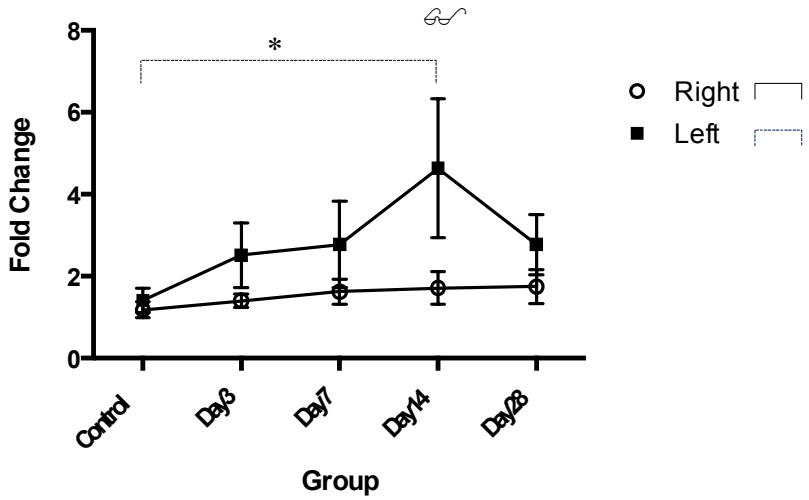


Figure 14. Akt in bilateral hemisphere

Akt level showed increasing tendency from control to day 7, and reached the highest value in day 14, then decreased in day 28 in the contralateral hemisphere. Values in day 14, in the contralateral hemisphere, were significantly higher than those of ischemic hemisphere.

***p*<.05**

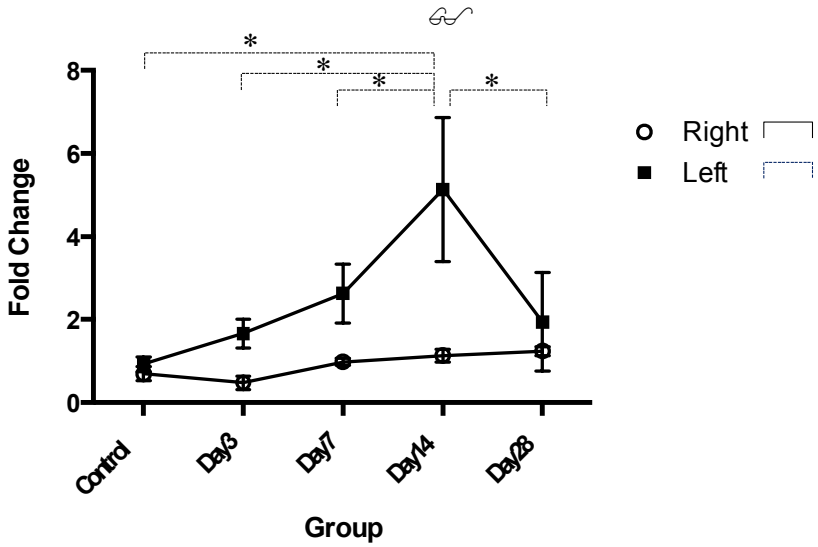


Figure 15. VEGF in bilateral hemisphere

VEGF levels of the contralateral hemisphere increased from control to day 14, and attained the maximal values in day 14, then decreased in day 28. Significant differences were found between the levels of day 14 and those from the rest control, day3, day7 and day 28.

***p*<.05, between each group**

◆*p*<.05, between each hemisphere

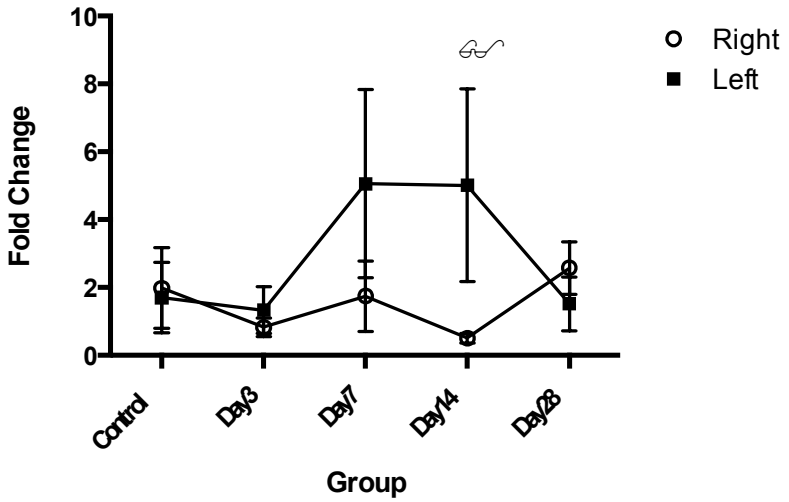


Figure 16. Ratio of NR2A/2B in bilateral hemisphere

Ratio of NR2A/2B was higher in the contralateral side than that of the ischemic hemisphere in day 14. No significant differences were found between each group.

◆ ***p*<.05, between each hemisphere**

E. Immunofluorescence staining

GFAP-positive cells seemed to be increased in the group 4 (14 days of treadmill training after stroke) in conjugation with BrdU-positive cells in the peri-infarct zones of the group 4 (Figure 17).

Nestin-positive cells were observed in the group 2, which could be evidence of neurogenesis in the peri-infarct area (Figure 18).

NeuN-positive neurons were shown in the group 4 (Figure 19).

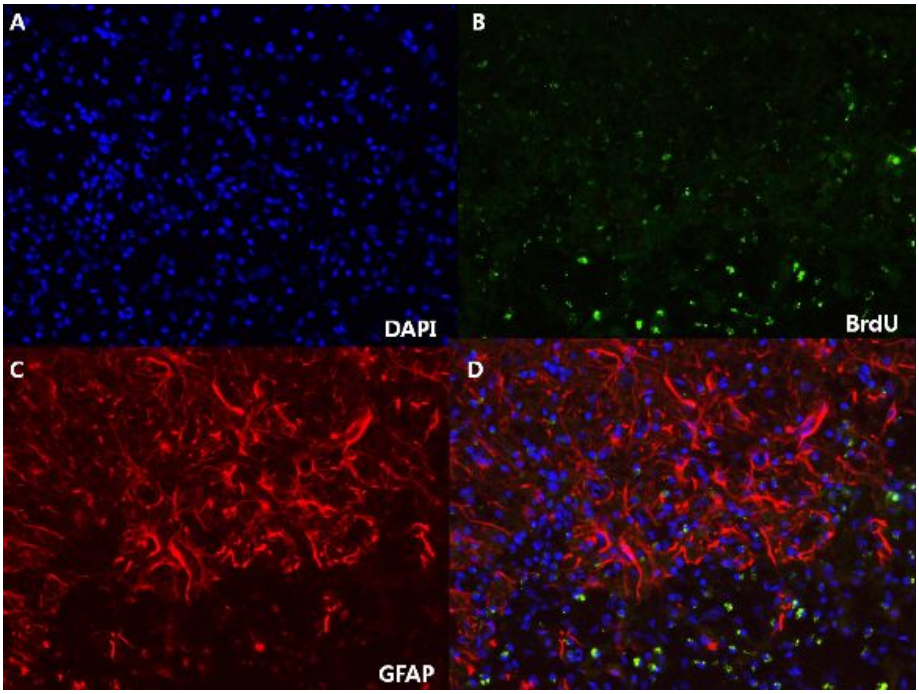


Figure 17. Peri-infarct area of the group 4 (14 days of treadmill training) showed BrdU+, newly-proliferating cells, with highly activated GFAP+ cells. A merged view (D) demonstrated GFAP+ astrocytes adjacent to BrdU+ cells in the peri-infarct areas.

(A) DAPI +, (B) BrdU +, (C) GFAP +, and (D) merged image

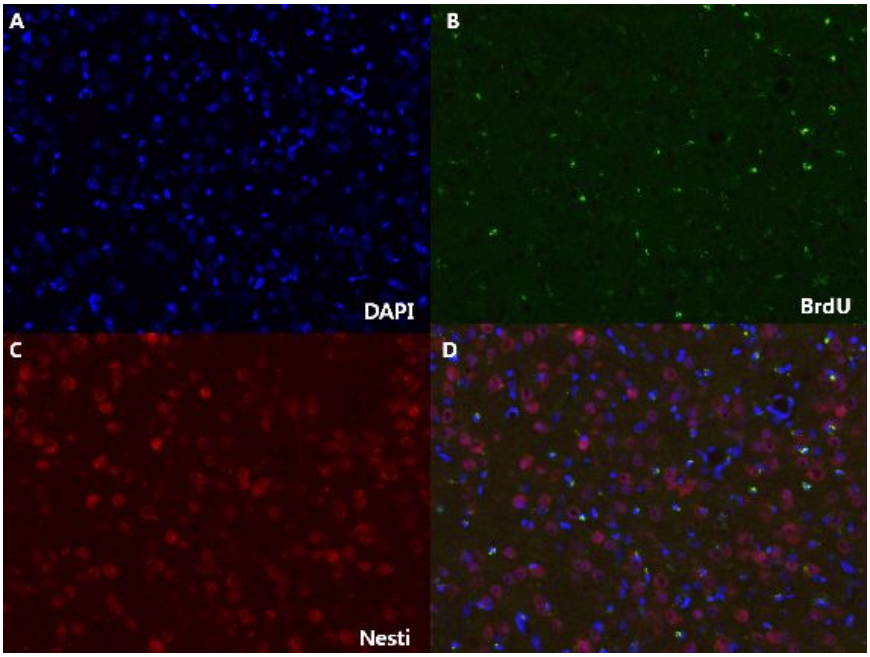


Figure 18. Peri-infarct area of the group 2 (3 days' of treadmill training) presented BrdU+, Nestin+ cells, which might represent neurogenesis following cerebral ischemia.

(A) DAPI +, (B) BrdU +, (C) Nestin +, and (D) merged image

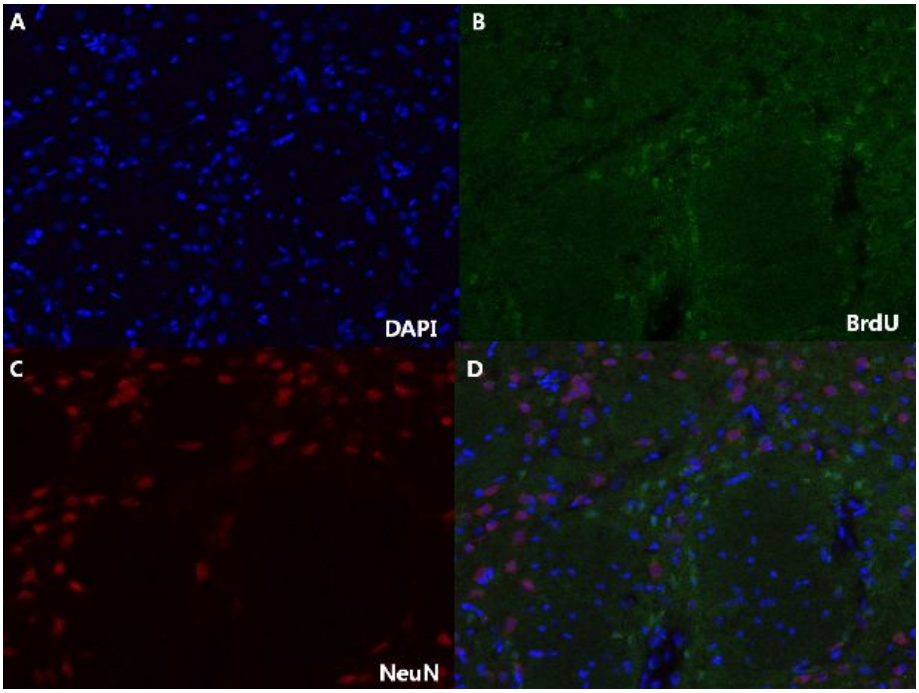


Figure 19. Peri-infarct area of the group 4 (14 days' of training) had NeuN+ cells, which seemed to be neurons.

(A) DAPI +, (B) BrdU +, (C) NeuN +, and (D) merged image.

4. Experiment 2: Exercise intensity

As shown in Figure 2, rats were subjected to the following groups; 1) Control without treadmill training, 2) moderate-intensity training of 60%, 3) high-intensity exercise, which is 85% of maximum.

A. Garcia test

Scores of the Garcia test did not show significant differences between moderate and high intensity group.

B. Neurotransmitter

Neurotransmitter concentrations were compared in day 14 between two groups with moderate and high intensity exercise. No significant changes were found because each group had only 2 to 3 quantifiable values. Means with standard error of means were presented in Table 3.

Table 3. Concentration of the neurotransmitters in moderate and high intensity groups in day 14 (ng/ml, Mean ± SEM)

	Acetylcholine	Serotonin	Dopamine	GABA	Glutamate
Moderate intensity exercise	3.58±2.89	0.22	1.26±0.33	37.38±11.11	2265±1865
High intensity exercise	1.79±1.06	4.68±3.74	5.59±4.01	219.23±225.37	2516±1805

* Values without SEM had only one available level.

C. RT-PCR

Two immediate early gene mRNAs showed similar changes with high-intensity exercise. The arc mRNAs were higher in day 14 than those of the control (Figure 20). The c-fos mRNAs were also elevated with high intensity exercise in day 14; however, these differences were significant between day 3 and day 14 (Figure 21).

The bdnf level did not show differences between each group.

Levels of the vegf increased in day 14 with high intensity of exercise (Figure 22).

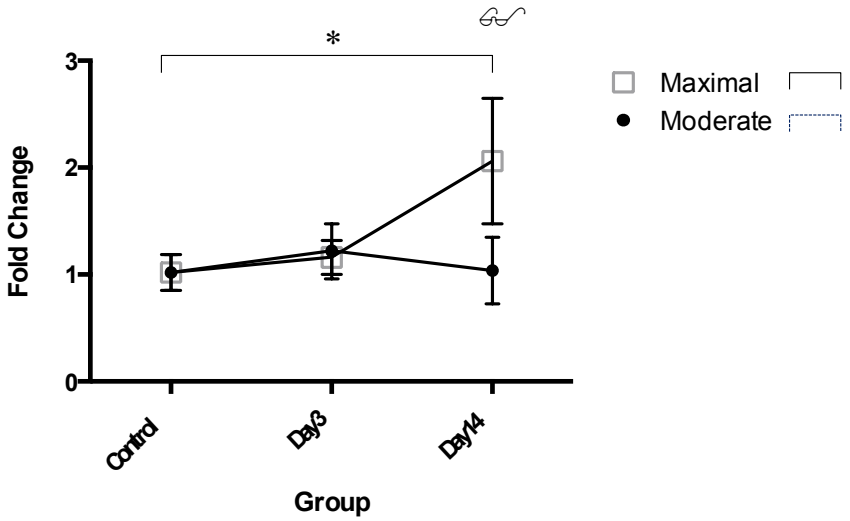


Figure 20. arc mRNA in the ischemic hemisphere

Levels of arc mRNAs were the highest in day 14. Significant differences were detected between control and day 14. Values in day 14 with high intensity exercise were also higher than those with moderate intensity training.

***p*<.05, between each group**

◆ *p*<.05, between each hemisphere

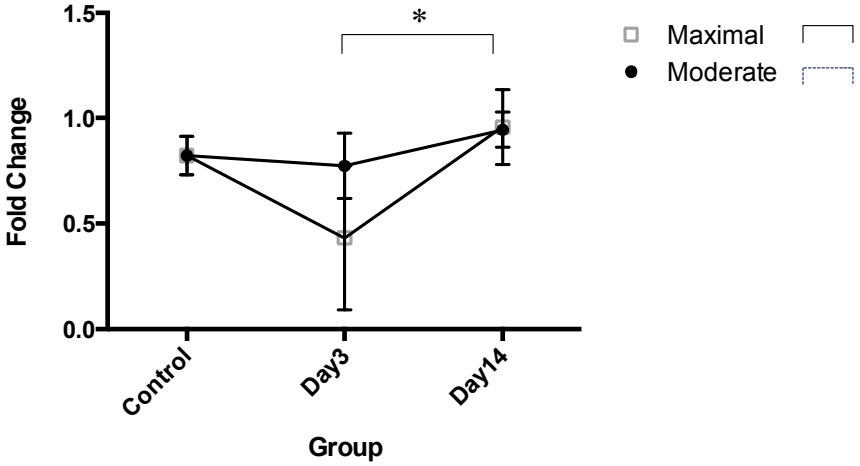


Figure 21. c-fos mRNA in the ischemic hemisphere

Levels of c-fos mRNAs were the highest in day 14 with high intensity training. Significant differences were detected between day 7 and day 14.

***p*<.05**

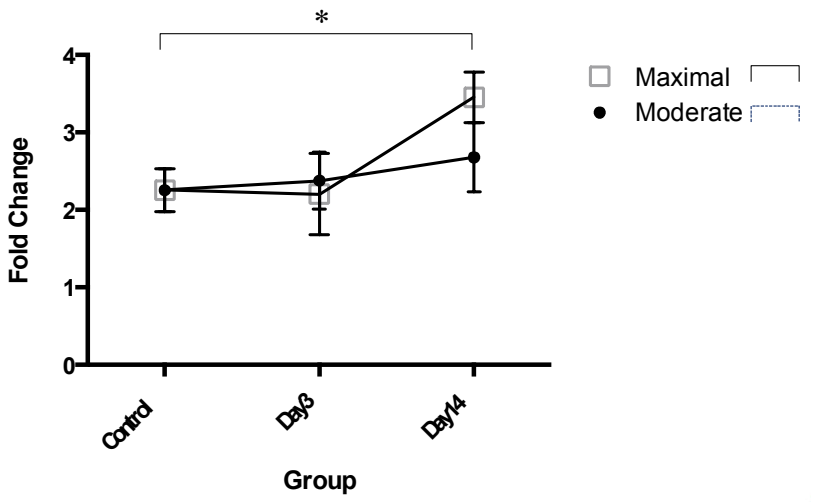


Figure 22. vegf mRNA in the ischemic hemisphere

The vegf mRNAs had the highest levels in day 14 with high intensity training. Significant differences were detected between control and day 14.

***p*<.05**

D. Western Blot

In rats that went through high intensity of treadmill training, nNOS was raised in day 3 and 14 (Figure 23). Akt levels were also the highest in day 14 with high intensity exercise (Figure 24).

In contrast, VEGF levels and NR2A/2B ratios reached the highest value in day 14 with moderate intensity exercise (Figure 26 and 27). However, nNOS, Akt did not show differences. Levels of eNOS did not differ between each time points and exercise intensity (Figure 25).

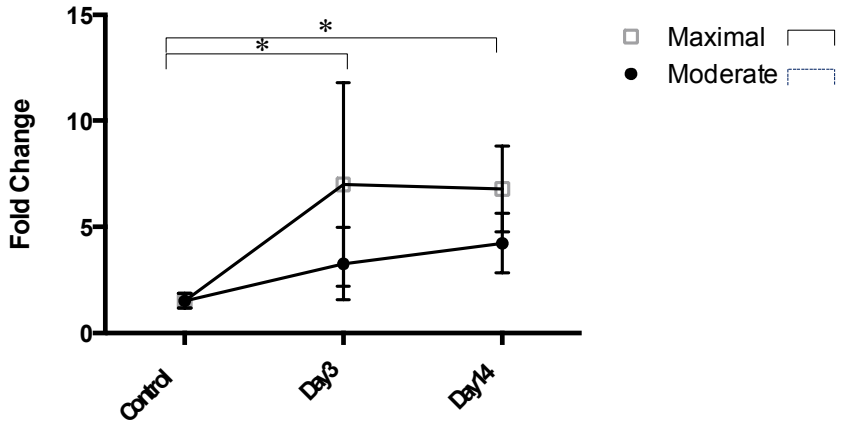


Figure 23. nNOS in the ischemic brain

Levels of nNOS was raised in day 3 and sustained until day 14 with high intensity exercise. Meanwhile, no significant increases were detected with moderate intensity training.

***p*<.05**

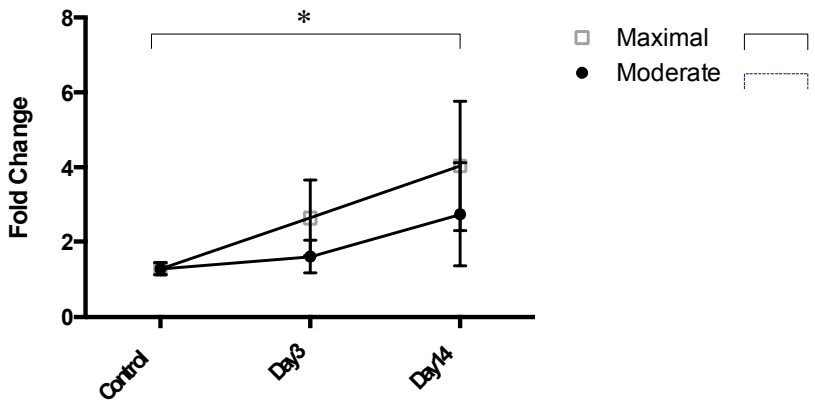


Figure 24. Akt in the ischemic brain

The Akt levels were the highest in day 14 with high intensity exercise, when compared to those of the control group. No definite increases were detected with moderate intensity training.

***p*<.05**

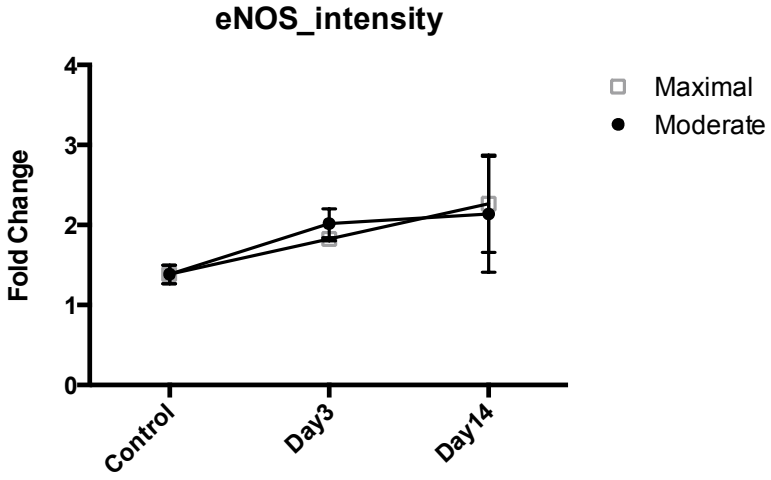


Figure 25. eNOS in the ischemic brain

Levels of eNOS did not differ between each time points (control, day 3 and day 14) and exercise intensity.

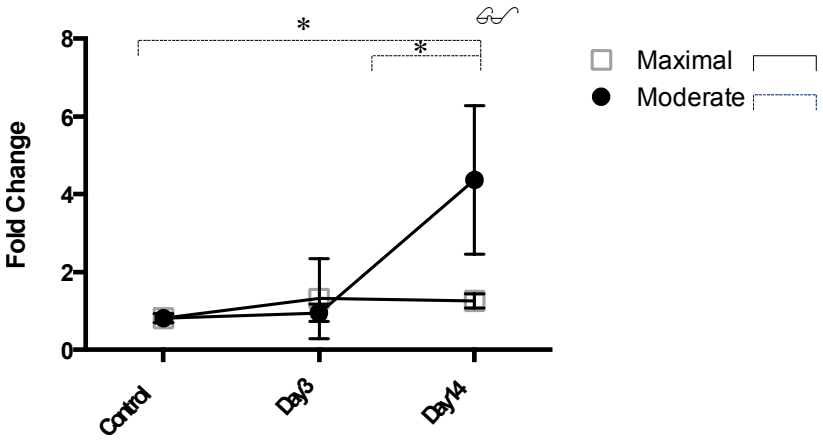


Figure 26. VEGF in the ischemic brain

VEGF levels reached the highest value in day 14 with moderate intensity exercise. These values were higher compared to those with high intensity exercise. No increases in VEGF levels were shown with high intensity training.

***p<.05**

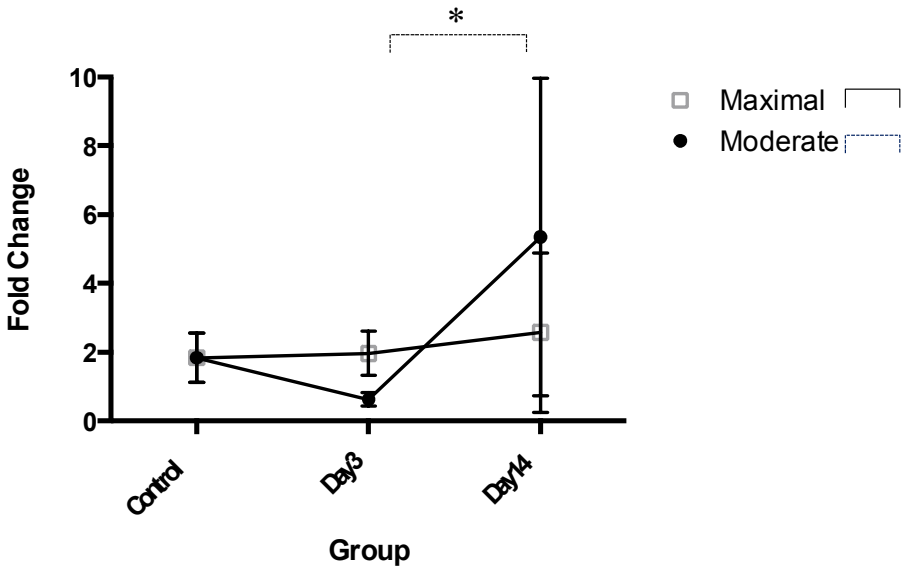


Figure 27. NR 2A/2B ratios in the ischemic brain

The NR2A/2B ratios increased in day 14 with moderate intensity training. A significant difference was between day 3 and day 14.

***p*<.05**

Discussion

This study showed increased neurotoxic factors including glutamate(14, 15) and nNOS(16-19) in day 3 in the ischemic hemisphere even with exercise training following cerebral ischemia. In contrast, bdnf, which is known to be a neurotrophic factor, was raised in day 14 with the increase of c-fos. Levels of angiogenesis-related proteins such as eNOS, Akt, and VEGF were elevated in day 14 in the contralateral hemisphere (Experiment 1).

Concerning exercise intensities, moderate-intensity trainings seem to raise NR 2A/2B ratio and VEGF in day 14, and eNOS and Akt in day 28. However, high-intensity exercises induced increase of nNOS, a neurotoxic factor, in day 3 and 14, accompanied with elevated levels of immediate early genes (Experiment 2).

1. Concentration of the neurotransmitters during exercise

Neurotransmitters from the interstitial tissue of the dorsolateral prefrontal cortex were analyzed on the final day of treadmill training in each group. Although statistical analysis was not applicable to some of the samples, owing to small sample size, certain tendency was observed throughout the groups. The glutamate concentration, which is known to be an excitatory neurotransmitter, was higher in 3 days after the cerebral ischemia. This could reflect the excito-toxicities following stroke.

Changes following cerebral ischemia have been not well known, which makes it difficult to compare results from this study. However, complications following stroke, such as cognitive impairment, inattention, and post-stroke depressions have been closely related to the changes in neurotransmitters.

Medications including dopamine agonists, selective serotonin reuptake inhibitors, and acetylcholine esterase inhibitors have been used clinically to modify the concentrations of neurotransmitters. These medications have also been administered to facilitate motor recoveries following stroke. Therefore, further studies on the neurotransmitter concentrations during exercise could establish neurophysiologic basis on these medications and their effects.

2. Changes in mRNA transcription and protein expression following differential duration of treadmill training

Experiment 1 was performed first to investigate on the effects of exercise duration on the neural plasticity. As previously reported in the articles (10) and as clinically relevant, exercise intensities in stroke rehabilitation are most commonly moderate-intensity trainings. These were applied to each group with differential durations.

Although no consistent differences in mRNA were shown, bdnf expression in day 14 in the ischemic hemisphere with increase of c-fos could be an evidence of enhanced neurogenesis in the peri-infarction area. The elevated expression of the nNOS, which had been known to exert neurotoxicity, was observed in day 3 in the ischemic hemisphere. It could reflect neurotoxicity early after cerebral ischemia, with increased glutamate concentrations. In addition, VEGF and Akt, which were thought to be a surrogate marker of angiogenesis, were raised in the contralateral hemisphere in day 14. LTP-related NR2A/2B ratio(20, 21) was also increased at the same time. Plastic changes of the contralateral hemisphere might have pathways through angiogenesis.

3. Changes in mRNA transcription and protein expression following moderate and high intensity of treadmill training

Moderate-intensity exercise induced increases in NR2A/2B and VEGF in day 14, while high-intensity training resulted in nNOS, Akt, and immediate early gene expressions in the same time period. Moderate intensity of training seemed to induce neural plasticity through the angiogenesis and long-term potentiation. Meanwhile, high intensity of exercise increased nNOS, a neurotoxic factor from day 3 to day 14.

However, short-term exercise did not seem to be beneficial enough to reduce neurotoxic effects following cerebral ischemia, because neurotoxic factor remained elevated in day 3 even with moderate-intensity exercise.

Experiment 2 revealed differential effects of moderate and high intensity exercise, and could enhance evidence on mechanisms of benefits of moderate exercises rather than high intensity.

4. Limitations

Techniques of microdialysis have been utilized in animal research, especially in the pharmaceutical area, and preliminary study did not reveal troubles in extracting specimen during exercise. However, analyzing the neurotransmitter concentration using the HPLC techniques might have high quantifiable limits, which makes it difficult to clearly differentiate unquantifiable values and meaningful concentrations. Especially when each group and specimen had limited amounts, HPLC might show certain limitations in sensitivity.

Because each group has 6 or 7 rats, variability between objects could have influenced reliable measurement. Analysis on the neurotransmitters using the

microdialysis should have enough number of the rats in each group. Furthermore, controls need to be differentiated from natural growth changes and effects of the exercises. In other words, 7, 14, and 28 days of training needs their own controls groups without exercise to known changes from growth and maturation.

Furthermore, tissues analyzed in this study were lesion areas could be seen from the light microscope. Neuronal cell sorting using the FACS cell sorting technique could give more specified analysis for changes in the neurons.

Conclusions

Moderate-intensity exercise for 2 weeks might be associated with neuroprotective mechanism such as neurotrophin, angiogenesis and LTP in rat stroke model. In contrast, high-intensity of training seemed to be associated with nNOS, a neurotoxic factor.

References

1. Kao T, Shumsky JS, Murray M, Moxon KA. Exercise induces cortical plasticity after neonatal spinal cord injury in the rat. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(23):7549-57.
2. Fabel K, Kempermann G. Physical activity and the regulation of neurogenesis in the adult and aging brain. Neuromolecular medicine. 2008;10(2):59-66.
3. Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, et al. Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. The Journal of comparative neurology. 2003;467(4):455-63.
4. Barrientos RM, Frank MG, Crysdale NY, Chapman TR, Ahrendsen JT, Day HE, et al. Little exercise, big effects: reversing aging and infection-induced memory deficits, and underlying processes. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(32):11578-86.
5. Cechetti F, Worm PV, Elsner VR, Bertoldi K, Sanches E, Ben J, et al. Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. Neurobiology of learning and memory. 2012;97(1):90-6.
6. Radak Z, Kumagai S, Taylor AW, Naito H, Goto S. Effects of exercise on brain function: role of free radicals. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2007;32(5):942-6.
7. Yasuhara T, Hara K, Maki M, Matsukawa N, Fujino H, Date I, et al. Lack of exercise, via hindlimb suspension, impedes endogenous neurogenesis. Neuroscience. 2007;149(1):182-91.
8. Holmes MM, Galea LA, Mistlberger RE, Kempermann G. Adult hippocampal neurogenesis and voluntary running activity: circadian and dose-dependent effects. Journal of neuroscience research. 2004;76(2):216-22.
9. Mueller PJ, Cunningham JT, Patel KP, Hasser EM. Proposed role of the paraventricular nucleus in cardiovascular deconditioning. Acta physiologica Scandinavica. 2003;177(1):27-35.
10. Lee SU, Kim DY, Park SH, Choi DH, Park HW, Han TR. Mild to moderate early exercise promotes recovery from cerebral ischemia in rats. The Canadian journal of neurological sciences Le journal canadien des sciences neurologiques. 2009;36(4):443-9.

11. Lou SJ, Liu JY, Chang H, Chen PJ. Hippocampal neurogenesis and gene expression depend on exercise intensity in juvenile rats. Brain research. 2008;1210:48-55.
12. Beyer CE, Cremers TI. Do selective serotonin reuptake inhibitors acutely increase frontal cortex levels of serotonin? European journal of pharmacology. 2008;580(3):350-4.
13. Beyer CE, Boikess S, Luo B, Dawson LA. Comparison of the effects of antidepressants on norepinephrine and serotonin concentrations in the rat frontal cortex: an in-vivo microdialysis study. Journal of psychopharmacology. 2002;16(4):297-304.
14. Hossmann KA. [Glutamate hypothesis of stroke]. Fortschr Neurol Psychiatr. 2003;71 Suppl 1:S10-5. Epub 2003/08/30. Die Glutamathypothese des Schlaganfalls.
15. Bashkatova VG, Rayevsky KS. Nitric oxide in mechanisms of brain damage induced by neurotoxic effect of glutamate. Biochemistry (Mosc). 1998;63(7):866-73. Epub 1998/08/29.
16. Samdani AF, Dawson TM, Dawson VL. Nitric oxide synthase in models of focal ischemia. Stroke. 1997;28(6):1283-8. Epub 1997/06/01.
17. Mahairaki V, Xu L, Farah MH, Hatfield G, Kizana E, Marban E, et al. Targeted knock-down of neuronal nitric oxide synthase expression in basal forebrain with RNA interference. J Neurosci Methods. 2009;179(2):292-9. Epub 2009/05/12.
18. Gulturk S, Kozan R, Bostanci MO, Sefil F, Bagirici F. Inhibition of neuronal nitric oxide synthase prevents iron-induced cerebellar Purkinje cell loss in the rat. Acta Neurobiol Exp (Wars). 2008;68(1):26-31. Epub 2008/04/05.
19. Bizzoco E, Vannucchi MG, Faussone-Pellegrini MS. Transient ischemia increases neuronal nitric oxide synthase, argininosuccinate synthetase and argininosuccinate lyase co-expression in rat striatal neurons. Exp Neurol. 2007;204(1):252-9. Epub 2007/01/03.
20. Gardoni F, Mauceri D, Malinverno M, Polli F, Costa C, Tozzi A, et al. Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. J Neurosci. 2009;29(3):669-77. Epub 2009/01/23.
21. Muller L, Tokay T, Porath K, Kohling R, Kirschstein T. Enhanced NMDA receptor-dependent LTP in the epileptic CA1 area via upregulation of NR2B. Neurobiol Dis. 2013;54:183-93. Epub 2013/01/15.

국문 초록

서론: 신경재활의 목표는 신경 가소성을 촉진하는 것이며, 이러한 신경 회복은 운동에 의해 증대된다고 알려져 있다. 효과적인 운동은 손상 후 조기의 저-중 강도 운동으로 생각되고 있다. 그러나 과거 저-중 강도의 운동이 회복을 촉진시키는데 대한 근거는 대부분 해마의 관련 mRNA 발현에 초점을 맞추어 연구 되었다. 또한 운동시 변화하며, 신경가소성과도 관련 있다고 알려진 신경전달물질, 산화질소 합성 효소 경로의 인자들이 운동 강도와 어떤 관계를 가지고 있는지는 명확히 밝혀진 바가 없었다. 이에 본 연구는 뇌졸중 유발 랫드에서 1) 운동 중의 신경전달 물질 농도, 2) 신경생성과 혈관생성 관련 인자들, 산화질소 합성 효소 경로의 물질들이 각각 서로 다른 운동 기간과 운동 강도에 따라 차이를 보이는 지를 알아보고자 하였다.

방법: SD 랫드에서 중대뇌동맥 경색을 유발하였고, 하루 후부터 트레드밀을 사용한 운동을 하루 30 분간 적용하였다. 실험 1 에서는 각 군이 3 일, 7 일, 14 일, 28 일로 운동 지속 기간을 달리하였다. 실험 2 에서는 운동강도를 중등도(최대 가능 속도의 60%), 고강도(최대 가능 속도의 85%)로 14 일 간 적용하였다. 신경행동학적 검사인 Garcia 검사와 미세 투석을 이용한 뇌 신경전달물질 추출이 각 군에서 운동 마지막 날 이루어졌다. 투석액에서 신경전달물질 중 아세틸콜린, 세로토닌, GABA, 도파민, 글루탐산 농도를 정량화 하였다. 전초기 유전자인 arc, c-fos, 신경생성 관련된 bdnf, 혈관생성 관련인자인 vegf 의 mRNA 발현을 RT-PCR 을 사용해 측정하였다. 산화질소 합성 효소 경로의 단백인 eNOS 와 nNOS, 혈관생성 인자 VEGF, 장기강화와 관련된다고 알려진 NR2A/2B 비를 Western blot 을 이용하여 측정하였다. 형광염색은 DAPI, GFAP, NeuN, nestin, BrdU 에 대하여 염색하였고, 허혈 주변 경계부위에서 관찰하였다.

결과: 뇌의 신경전달물질 농도는 허혈 후 3 일 운동 시 아세틸콜린 농도가 증가되었다가 14 일 째는 감소하였으며, 글루탐산 농도 또한 3 일째에 최대로 상승하였다. 허혈 측 대뇌 반구 피질에서 bdnf 와 c-fos mRNA 가 14 일 째 증가하였고, 혈관생성과 관련된 Akt, VEGF, eNOS 단백은 14 일 째 반대측 대뇌 반구에서 증가되었다(실험 1). 중등도의 운동 후 NR2A/2B 비와 VEGF 생성이 14 일 째 상승된 반면, 고강도 운동은 3 일과 14 일에 nNOS 상승과 전초기 유전자 발현 증가를 유발하였다. 형광 염색에서 BrdU+ , nestin+ 세포들은 뇌 경색 이후 3 일 째부터 허혈주변부에서 관찰되었다.

결론: 중등도의 운동을 2 주간 적용하였을 때 신경성장인자, 혈관생성, 장기강화 등의 신경보호 기전과 연관이 있었으며, 반면 고강도의 운동은 신경독성 인자인 nNOS 유발과 관련되어 있었다.

Keywords: 운동 강도, 뇌 허혈, 신경전달물질, 신경 가소성
학 번: 2011-30543