The Hong Kong Polytechnic University

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THE EFFECTS OF TREADMILL TRAINING INTENSITIES ON REHABILITATION OUTCOMES IN SUBACUTE STROKE: A FOCAL ISCHEMIC RAT MODEL

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree of diploma, except where due acknowledgement has been made in the text.

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Jing SUN

Jan. 2014

DEDICATION

This thesis is dedicated to my family.

ABSTRACT

Stroke increases risk of mortality and is a major cause of disability. The growing elderly population vulnerable to stroke has substantially increased the burden of medical care in China and other developing countries. Effective rehabilitation is essential to help stroke survivors regain impaired motor function to improve quality of life and to reduce the pressure on current healthcare system. Early intervention of physical training is necessary for effective rehabilitation after stroke. Treadmill is a conventional intervention after stroke in both clinical and animal studies; however, overloaded treadmill training increases stress level during treatment, which may affect the recovery. Effective treadmill training protocols should be further optimized. Animal studies commonly use fixed training intensity throughout rehabilitation period and without adapting the training intensity with their recovered motor ability. This study used a standardized focal ischemic stroke rat model induced by intraluminal suture middle cerebral artery occlusion and reperfusion (MCAo/r) to explore the correlation between training intensity and rehabilitation efficacy in the early stage after stroke.

Forty male Sprague-Dawley (SD) rats (between 2 and 3 months) were used in a pilot study to evaluate neural and motor function assessment tools, and to investigate the effects of treadmill training on motor function recovery, brain infarct, and stress levels. Longa's test and De Ryck's test were selected for post-stroke functional evaluations after comparison. Treadmill training after stroke induced significantly (p<0.05) high stress level than control but still significantly

improved motor function recovery. A separate cohort of 94 male SD rats (between 2 and 3 months) was then used to investigate the effects of differing treadmill training intensities on stroke recovery during 7 early consecutive intervention days. Among the 94 rats, 7 did not meet screening requirements after accommodation, while 10 exhibited no stroke and 17 died within 24 hours after MCAo/r surgery. The rest 60 rats with successful stroke were assigned into four groups: control (CG, n=15), low intensity (LG, n=15), gradually increased intensity (GIG, n=15) and high intensity (HG, n=15). Rats in LG and HG ran at fixed velocities of 5 m/min and 26 m/min, respectively. Rats in GIG ran from 5 m/min on the first treatment day to 26 m/min on the last day, with a gradually increased running speed matching the average recovery rate of the stroke rat. De Ryck's tests were conducted daily to evaluate motor function recovery. Stress level and neural recovery were evaluated via plasma corticosterone and brain-derived neurotrophic factor (BDNF) concentrations in the brain tissues (hippocampus, striatum, and cortex), respectively.

Results showed that GIG rats significantly (p<0.05) recovered motor function and produced higher hippocampal BDNF (112.87±25.18 ng/g). GIG and LG rats exhibited similar stress levels (540.63±117.40 nM/L and 508.07±161.30 nM/L, respectively), which were significantly lower than that (716.90±156.48 nM/L) of HG rats. Training with higher intensity did not result in better motor function recovery and resulted in high stress levels. Training with gradually increased intensity achieved a better recovery outcome with lower stress. These observations indicate that training intensity influences stroke recovery.

This study firstly systematically investigated the relationship of training intensity and rehabilitation after stroke. Gradually increased training intensity was firstly proposed and investigated in this study, suggesting a better improvement of rehabilitation after stroke than fixed training intensities by upregulating cerebral BDNF levels and downregulating stress levels. A training protocol that includes gradually increasing training intensity should be considered in both animal and clinical human studies for better stroke recovery.

PUBLICATIONS ARISING FROM THE THESIS

Journal Papers

1. Sun, J., Ke Z., Yip, Y. P., Hu, X. L., Zheng, X. X., & Tong, K. Y. (2014). Gradually increased training intensity benefits rehabilitation outcome after stroke by BDNF upregulation and stress suppression. *BioMed Research International, 2014*, 925762. http://dx.doi.org/10.1155/2014/925762..

2. Ke, Z., Cui, W., Hu, S. Q., **Sun, J.**, Zhang, S. J., Mak, S. H., … Tong, K. Y. (2013). *Synergistical effects of bis(propyl)-cognitin and treadmill exercises on neuroprotection and motor function rehabilitation after rat brain ischemia*. Manuscript submitted for publication.

Conference Papers

1. Sun, J., Tong, K. Y., Ke, Z., Zhang, S. J., Hu, X. L., & Zheng, X. X. (2012, December). *The effect of treadmill training on motor recovery and neurological outcome in subacute stroke rat*. Paper presented at the 2012 Biomedical Engineering International Conference, Hong Kong, China.

2. Sun, J., Tong, K. Y., Ke, Z., Zhang, S. J., Hu, X. L., & Zheng, X. X. (2013, March). *Treadmill exercise facilitates functional recovery and neuroprotection in subacute stroke rats*. Paper presented at the 5th International Conference on Bioinformatics and Biomedical Technology, Macau, China.

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LIST OF ABBREVIATIONS

ACA	Anterior cerebral artery
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
CCA	Common carotid artery
CORT	Corticorsterone
CVT	Cerebral venous thrombosis
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
H&E	Hematoxylin-eosin
HPA	Hypothalamic-pituitary-adrenal
ICA	Internal carotid artery
ICD	International classification of disease
M1	Primary motor cortex
MCA (O)	Middle cerebral artery (occlusion)
MCAo/r	Middle cerebral artery occlusion / reperfusion surgery
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MWM	Morris water maze
RAM	Radial arm maze
PCA	Posterior cerebral artery
PET	Positron emission tomography
S1FL	Primary somatosensory cortex of left forelimb
S1HL	Primary somatosensory cortex of left hindlimb
SD	Sprague-Dawley
SSS	Superior sagittal sinus
TIA	Transient ischemic attack
TTC	2'3'5' triphenyltetrazolium chloride
VO	Vessel occlusion

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CHAPTER 1 INTRODUCTION

1.1 OVERVIEW OF STROKE

Stroke, a cerebrovascular accident, is caused by a sudden reduction of blood supply to the brain based on the definition in the International Classification of Disease (ICD-10, I64). Stroke can either be acute or chronic, focal or global, and permanent or transient. According to the American Stroke Association (2013), stroke can be classified into three types: ischemic stroke, hemorrhagic stroke, and transient ischemic attack (TIA). Ischemic stroke arises when a cerebral artery is occluded, while hemorrhagic stroke results from the rupture of a brain vessel. TIA occurs when the blood supply to a specific brain area ceases and then returns soon after occlusion; usually no permanent damage occurs. According to the World Stroke Organization (Davis & Norrving, 2013), there are approximately 16 million new stroke cases each year, of which, result in approximately 6 million deaths. In addition, many stroke survivors have a high risk of recurrent stroke, and endure some degree of motor dysfunction on one side of the body (hemiparesis) (Davis & Norrving, 2013).

Among stroke patients, ischemic stroke accounts for more than 80% (Kelly, Pangilinan, & Rodriguez, 2007). According to previous reports, stroke is the third cause of death behind cancer and cardiac diseases (Kelly et al., 2007), and is the leading cause of adult disability in many countries (Pinter & Brainin, 2012), especially in low-income and middle-income populations in countries where case management and treatment are relatively incomplete compared to high-income countries (Strong, Mathers, & Bonita, 2007; Johnston, Mendis, & Mathers, 2009;

Feigin, Lawes, Bennett, Barker-Collo, & Parag, 2009). The overall age-standardized rates of stroke mortality decreased over the last twenty years; however, the absolute number of stroke-related accidents, including new stroke patients, stroke survivors, and deaths, remains high and keeps increasing (Feigin et al., 2013). According to the Hong Kong Authority Statistical Report 2009-2010 (2011), the number of annual stroke admissions to public hospitals has increased from 24,742 cases in 2005 to 25,614 in 2009. In China, healthcare system has improved significantly over recent years with the rapid development of economics; however, stroke is still the leading cause of death and is a tough challenge for the government (Liu, D. Wang, Wong, & Y. Wang, 2011). With the growth of the elderly population who are more vulnerable to stroke (Pinter & Brainin, 2012; Donnan, Marc, Malcolm, & Davis, 2008), it is expected that the burden of post-stroke medical care is likely to increase substantially in the future in all countries, especially in developing countries like China. Stroke care, including diagnosis, management, and prevention, should be more readily available for the general population. Effective rehabilitation is essential to help stroke survivors with disability regain impaired motor functions for improved quality of life, and to relieve the governmental and families' burden on stroke care.

1.2 STROKE REHABILITATION

Since most stroke survivors have some degree of brain damage, and lose the function of the damaged brain area, post-stroke rehabilitation is necessary for them to relearn lost skills, such as motor function, memory, and language production. Over the past two decades, numbers of studies have investigated the

efficacy of post-stroke rehabilitation practices in order to explore better rehabilitation protocols (Richards, Hanson, Wellborn, & Sethi, 2008). Motor rehabilitation is a key area of such research. A few advanced techniques have been developed which demonstrate improved efficacy of rehabilitation after stroke, including robotics-assisted therapy, constraint-induced movement therapy, neuromuscular electrical stimulation, computer-aided instruction, virtual and augmented reality, and biomedical and rehabilitation engineering (Richards et al., 2008; Jones, Mueller, & Morris, 2010; Kalra, 2010). Motor rehabilitation aims to facilitate neural reorganization supporting the relearning of lost motor functions caused by stroke-induced brain damage (Richards et al., 2008); these recovered cerebral systems could also improve motor rehabilitation outcomes.

Neuroplasticity, the ability of the brain to change its structure and/or function, contributes to motor learning, recovery, and neurorehabilitation after stroke (Hosp & Luft, 2011). Post-stroke motor rehabilitation process takes advantage of neuroplasticity to regain motor skills. Timing during rehabilitation process is an important factor to obtain effective and efficient recovery outcomes (Nudo, Plautz, & Frost, 2001; Barbay et al., 2006). In other words, there is a time window during the first few weeks after stroke (i.e. the subacute stroke period) when the brain is especially sensitive to therapeutic interventions (Hosp & Luft, 2011). Post-stroke behavioural training, with repetitive attempts to move the paretic limbs, can improve functionality of the stroke-damaged efferent pathways more effectively during this time period. Many studies have been dedicated to investigating efficacy, mechanism, and comparison of training methodologies (Hayes et al., 2008; Ke, Yip, Li, Zheng, & Tong, 2011; Wang, Yang, & Yu,

2001). Trainings after stroke show the ability to benefit motor function recovery and promote neurorehabilitation (Wang et al., 2001; Alomari, Khabour, Alzoubi, & Alzubi, 2013).

1.3 TREADMILL TRAINING IN REHABILITATION

Treadmill training, a conventional and easy means, has commonly been used in both human and animal studies for many years (Moseley, Stark, Cameron, & Pollock, 2003; Macko et al., 2005). Treadmill training patterns are different between human and animal studies since they have different motor systems. Stroke induces dysfunction of contralateral forelimb and hindlimb; thus, half of the motor system is impaired for both stroke patients and stroke animal models. Treadmill training forces stroke subjects to repeatedly use the impaired limb(s); thus, the lost motor function can possibly recover. Since animal studies are easier to control the effects of experimental environment and standardize stroke types, animal studies are commonly conducted before clinical studies.

Most of these studies showed that treadmill exercise training can protect neurons from damage (Cechetti et al., 2012; Matsuda, Sakakima, & Yoshida, 2011); for example, Hayes et al. (2008) showed that treadmill exercise induced neuroprotection. Post-stroke treadmill training has been introduced for rehabilitation due to its effectiveness on both functional mobility and cardiovascular fitness in stroke patients in the chronic phase (Macko et al., 2005). Early treadmill training shows significant effects on stroke models in rats by reducing brain infarct size and improving neurologic function (Yang, R. Y. Wang, & P. S. G. Wang, 2003; Yang, R. Y. Wang, P. S. G. Wang, & Yu, 2003).

Moderate treadmill training has been reported to up-regulate brain-derived neurotrophic factor (BDNF) (Ferreira, Real, Rodrigues, Alves, & Britto, 2011).

BDNF is a protein discovered in the early 1980's which is encoded by BDNF gene and expresses broadly in the central and the peripheral nervous systems (Barde, Edgar, & Thoenen, 1982). BDNF is one member of the "neurotrophin" family of growth factors believed to support the neuron survival and encourage new neurons and synapses to grow and differentiate (Weishaupt, Blesch, & Fouad, 2012). BDNF is active in the hippocampus, a region vital to learning, memory and higher thinking (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002). BDNF is related to neuroplasticity contributing to motor learning, recovery and neural rehabilitation after stroke (Hosp & Luft, 2011). Stroke induces the loss of motor function and rehabilitation is the process of relearning; thus, higher BDNF concentration in the brain may imply learning and neural rehabilitation (Soya et al., 2007).

Treadmill training can cause stress, leading to a series of physical changes that inhibit neural recovery during rehabilitation (Leuner & Gould, 2010; McEwen, 1999). Animals suffer from stress and actually develop similar pathology to humans (Moberg & Mench, 2000). In animal studies, stress has been found to affect structural plasticity of the hippocampus in two forms (McEwen, 1999): repeated stress results in dendritic atrophy of neurons in CA3 region, and both acute and chronic stress suppresses neurogenesis of granule cells in the dentate gyrus. Plasma corticosterone (CORT) concentration was widely used as a biomarker of stress in animal models (Ke et al., 2011; R. V. L. Contarteze, De

Barros Manchado, C. A. Gobatto, & De Mello, 2008; R. Contarteze, Manchado-Gobatto, C. Gobatto, & Mello, 2008). CORT could down-regulate BDNF level in the hippocampus (Rothman & Mattson, 2013). Due to the dual effect of treadmill training after stroke, it is important to evaluate its effectiveness in early stroke physical rehabilitation and the relationship between training loads, motor recovery and stress levels. This study, therefore, was designed to investigate these relationships via an ischemic stroke rat model.

Intensity is thought to be a key factor in treadmill training and associated with stress. High-speed treadmill training induces high CORT levels in a stroke rat model (Soya et al., 2007). Stress endurance is also enhanced by exercise (Steiner, Murphy, McClellan, Carmichael, & Davis, 2011). Thus, stress level may not only depend on training intensity, but may be influenced by subjects' conditions. Moreover, adjusted training intensity may be directly correlated to rehabilitation outcomes.

Effects of treadmill training intensity on motor function recovery and neurorehabilitation has not yet been completely elucidated. Both clinical and animal studies have focused on fixed training intensity (Mackay-Lyons, McDonald, Matheson, Eskes, & Klus, 2013; Tian et al., 2013). It remains unclear whether varied training intensity is more effective. This study, thus, employed a focal ischemic stroke rat model to evaluate the effect of differing treadmill training intensities on motor function recovery and neurorehabilitation. This study also analyzed CORT and BDNF levels in early stroke phase. A gradually increased training intensity was designed to investigate the relationship between

intensity, motor recovery, and stress level. This study extends our understanding of treadmill training intensity and influences rehabilitation program design.

1.4 PROJECT OBJECTIVES

This study consisted of two parts in order to investigate the effects of treadmill training on motor function recovery and brain infarct size. In addition, it was also designed to explore the effects of differing treadmill training intensities on motor function recovery and neurorehabilitation. A standardised stroke rat model induced by middle cerebral ischemia was utilized in this study. Study I was a pilot study which evaluated neural and motor function assessments tools and investigated the effects of treadmill training on motor function recovery and brain infarct size. Appropriate assessments for post-stroke functional deficits would be selected after comparison. The hypothesis of the pilot study was that treadmill training after stroke might facilitate motor function recovery and reduce cerebral lesions. Study II was conducted in order to optimize a training protocol with lower stress and better motor function recovery; the effects of different treadmill training intensities on stroke recovery were investigated. The hypothesis of Study II was that post-stroke training intensity might correlate with rehabilitation outcomes.

The objectives in these studies are specified as follows:

Study I (a pilot study):

- To evaluate neural and motor function assessments including Longa's test for neurological deficit, De Ryck's test for locomotor function, and beam

walking test for motor coordination and balance in a focal ischemic rat model;

- To investigate effects of treadmill training on motor function recovery, brain infarct area, and stress levels.

Study II:

- To compare motor function recovery among rats with different treadmill training interventions including control (CG) , low intensity (LG), gradually increased intensity (GIG), and high intensity (HG) by employing De Ryck's locomotor function test over a 7-day intervention;
- To compare BDNF levels in the brain tissues (hippocampus, striatum, and cortex) and plasma CORT levels among the four groups (CG, LG, GIG, and HG) at the end of the 7-day intervention;
- To explore the potential correlations between motor function recovery, stress levels and brain BDNF levels in the hippocampus, striatum, and cortex in stroke rats after the 7-day intervention.

1.5 OUTLINE OF THIS THESIS

Chapter 1 gives an introduction to stroke, the current global concern of stroke, and the importance and advances of post-stroke rehabilitation methods, as well as elaborating the importance of studying the effects of treadmill training intensity on stroke rehabilitation.

Chapter 2 reviews literatures related to this study, including stroke classification, animal models of stroke, post-stroke functional tests, histological evaluations, and chemical evaluations of cerebral BDNF and plasma CORT levels.

Chapter 3 elaborates on the experimental design of the pilot study and Study II, and introduces the methods employed for motor function evaluation, histological evaluation, biochemical detection, and statistical analysis, respectively.

Chapters 4 reports the experimental outcomes in the pilot study and study II; outcomes include body weight changes, motor function recovery, brain infarct volume, cerebral BDNF levels, and plasma CORT levels.

Chapter 5 explains and discusses the potential underlying reasons of these observations including body weight change pattern, post-stroke functional assessments, effects of treadmill training after stroke, and the effects of differing treadmill training intensity on stroke recovery.

Chapter 6 concludes the key findings in the study, and presents several suggestions for future studies.

CHAPTER 2 LITERATURE REVIEW

2.1 STROKE CLASSIFICATION

A stroke occurs when there is a sudden decrease of blood supply to the brain, and can be caused by a blockage of an artery or a rupture of blood vessels (ICD-10, I64). A stroke usually is one of the three types: ischemic stroke, hemorrhagic stroke, and transient ischemic attack (TIA).

2.1.1 ISCHEMIC STROKE

Obstruction of brain blood supply by a blood clot can lead to an ischemic stroke. Figure 2.1 (n.d., 2014a) shows an example of emboli-induced ischemic stroke; a blood clot travels through right common carotid artery and internal carotid artery, and then stops in a small cerebral artery. Blood flow to the downstream tissues decreases.

Atherosclerosis is the underlying reason for ischemic stroke, which consists of two kinds of obstruction: cerebral thrombosis and cerebral embolism. The main difference between cerebral thrombosis and cerebral embolism is the site at which the blood clot develops. Cerebral thrombosis means that a blood clot develops right at the blocked site and is a part of the targeted vessel, while embolism means that a clot forms at other sites of the circulatory system, such as the heart and other large arteries, where it then dislodge and travels to the brain through the bloodstream. Ischemic stroke is a common type of clinical episodes, and accounts for more than 80% of all cases (Kelly et al., 2007).

2.1.2 HEMORRHAGIC STROKE

Hemorrhagic stroke occurs when a weakened brain vessel bursts and bleeds into surrounding tissues. It consists of two types: intracerebral hemorrhage and subarachnoid hemorrhage, according to the sites of burst brain vessel. Intracerebral hemorrhage occurs in the deep brain, while subarachnoid hemorrhage occurs under the arachnoid (Figure 2.2 (n.d., 2014b)). Hemorrhagic stroke is not as common as ischemic stroke, accounting for around 15% of all stroke cases, according to epidemiologic studies (Feigin, Lawes, Bennett, & Anderson, 2003).

2.1.3 TRANSIENT ISCHEMIC ATTACK

TIA is defined as a short-term episode of neurologic dysfunction caused by a sudden reduction of cerebral blood supply (Easton et al., 2009). The blood clot in TIA only temporarily works and disappears within a few minutes (Figure 2.3 (n.d., 2014c)). TIA is also called as a mini-stroke. Its symptoms are the same as ischemic stroke. Different from ischemic stroke, symptoms of TIA patients persist for less than 24 hours based on the definition of World Health Organization (Truelsen, Begg, & Mathers, 2000). No permanent damage is caused by TIA. However, it is difficult to tell if a patient has experienced a TIA or ischemic stroke shortly after onset. Those who exhibit stroke symptoms should be hospitalized to reduce the risk of recurrent stroke (Easton et al., 2009). TIA is considered a warning of future strokes.

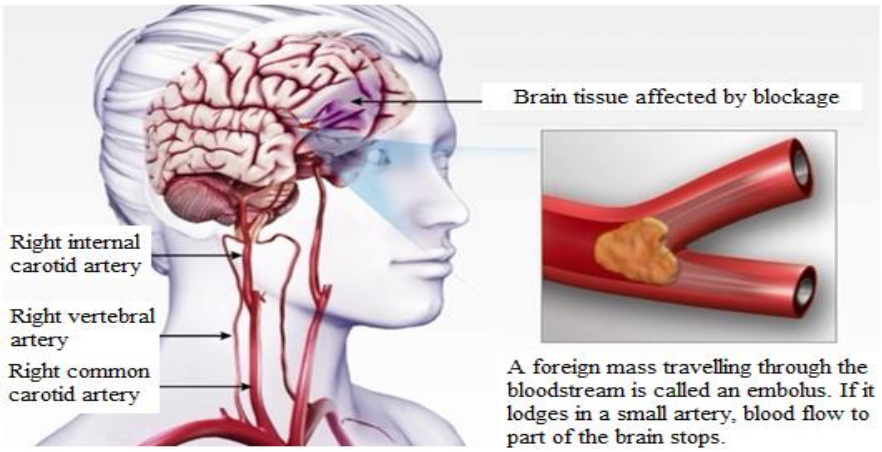

Figure 2.1 A schematic diagram of emboli-induced ischemic stroke (Picture adapted from Ischemic Stroke, In *American Heart Association: Watch, Learn and Live*, n.d., Retrieved 6 Jan 2014, from http://watchlearnlive.heart.org/CVML_Player.php?moduleSelect=iscstr. Copyright 2014 by American Heart Association, Inc.)

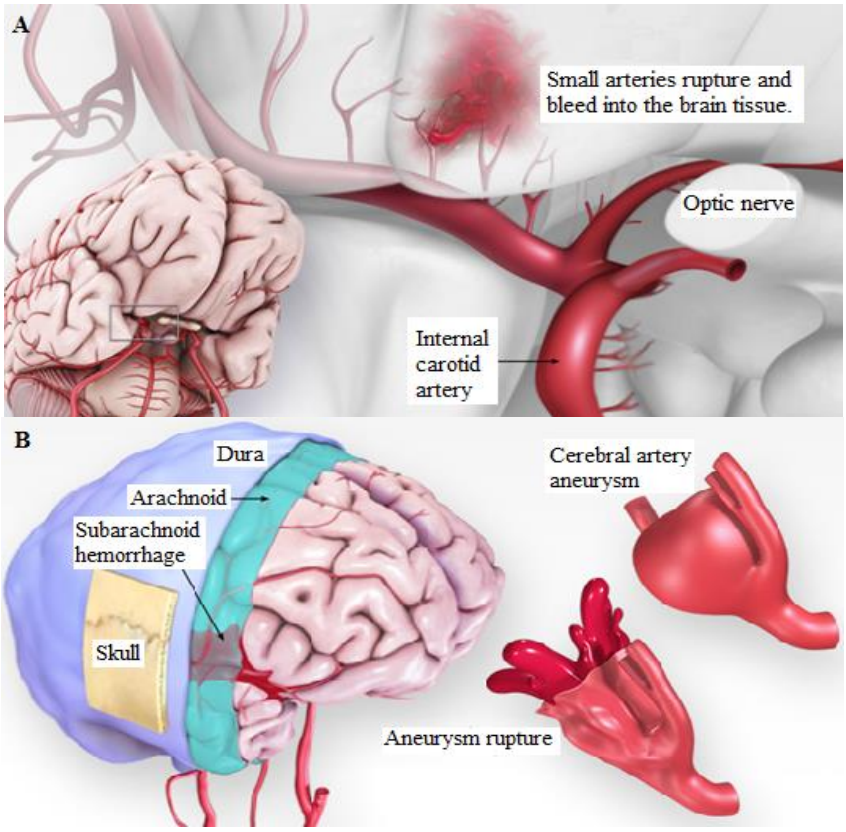

Figure 2.2 A schematic diagram of hemorrhagic strokes. A) Intracerebral haemorrhage; B) Subarachnoid hemorrahage (Picture adapted from Hemorrhagic Stroke, In *American Heart Association: Watch, Learn and Live*, n.d., Retrieved 6 Jan 2014, from http://watchlearnlive.heart.org/CVML_Player.php?moduleSelect=hemstr. Copyright 2014 by American Heart Association, Inc.).

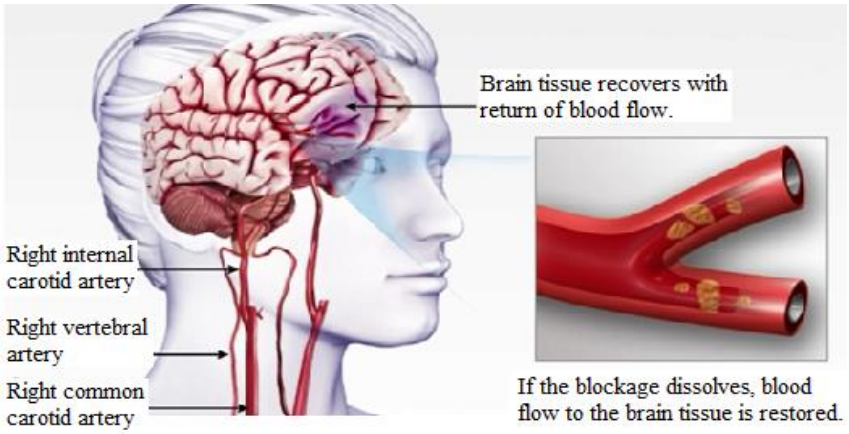

Figure 2.3 A schematic diagram of transient ischemic attack (Picture adapted from Transient Ischemic Attack, In *American Heart Association: Watch, Learn and Live,* n.d., Retrieved 6 Jan 2014, from http://watchlearnlive.heart.org/CVML_Player.php?moduleSelect=tisatk. Copyright 2014 by American Heart Association, Inc.).

2.1.4 SUMMARY OF STROKE TYPES

Since ischemic stroke is the most common clinical type (80%) over hemorrhagic stroke and TIA, it is the most studied. Almost 90 thousand articles related to brain ischemia have been published since 1945. There is still lack of understanding of underlying mechanisms of ischemic stroke on prevention, treatment and rehabilitation; thus, ischemic stroke should be further studied.

2.2 ANIMAL MODELS: CEREBRAL ISCHEMIC STROKE

Studies on the mechanisms underlying stroke and its rehabilitation can help promote rehabilitation after stroke. Animal stroke models must be used, especially when experiments cannot be done on human beings. Several types of ischemic and hemorrhagic stroke models have been developed to mimic human stroke, extending our understanding of stroke physiopathology and providing evidence for better stroke treatments (Carmichael, 2005). Animal species such as rabbits, pigs, dogs, primates and rodents have been used to develop stroke

models (Bailey, McCulloch, Sudlow, & Wardlaw, 2009). The brain volume and grey-white ratio (Figure 2.4 (Krafft et al., 2012)) of rodent brains are not the most similar to human brains; however, rodent stroke models are the most frequently utilized in both pathophysiological and preclinical investigations due to the considerations on cost, ethics, availability of standardized neural and behavioural assessments, easily physiological monitoring, and similar cerebrovascular anatomy and pathophysiology (Casals et al., 2011; Krafft et al., 2012).

Stroke animal models could be separated into two categories: artificial means induced models with neurological deficits caused by acute vessel injury, and models with spontaneous stroke (Krafft et al., 2012). Artificially induced models include ischemic, intracerebral hemorrhagic and subarachnoid hemorrhagic stroke models. Since over 80% stroke accidents are caused by ischemia (Kelly et al., 2007), in this thesis, the ischemic stroke was utilized.

Generally, ischemic stroke models can be assorted into two categories: global and focal cerebral ischemic models. Global ischemic stroke models imitate the clinical conditions of brain ischemia following profound systemic hypotension or cardiac arrest, whereas focal ischemic models mimic the most common clinical ischemic stroke subtype (Liu, Zhen, Meloni, Campbell, & Winn 2010). Figure 2.5 showed the pathophysiological mechanisms involved in global and focal cerebral ischemia in the brain (Traystman, 2003).

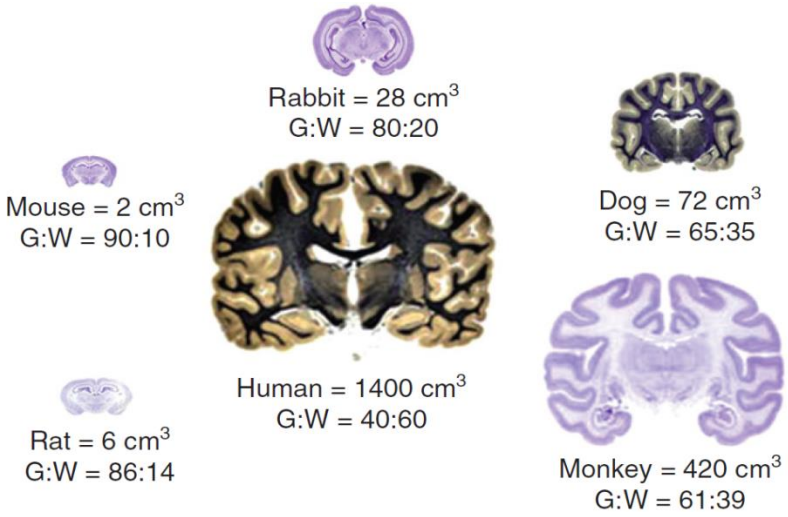

Figure 2.4 Approximate brain volumes (cm^3) and the grey-white ratio (G : W) of commonly utilized animal species (healthy and young) compared to the human brain (Reprinted from P. R. Krafft et al., 2012, *International Journal of Stroke, 7*, pp. 403. Copyright 2012 by World Stroke Organization).

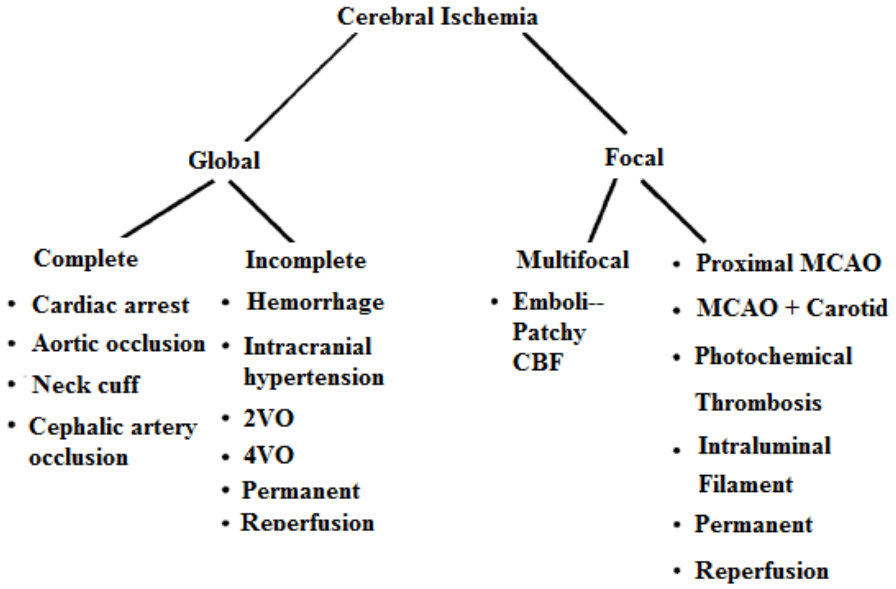

Figure 2.5 Pathophysiological mechanisms of global and focal cerebral ischemia in the brain. VO: vessel occlusion; CBF: cerebral blood flow; MCAO: middle cerebral artery occlusion (Reprinted and modified from R. J. Traystman, 2003, *ILAR Journal*, 44(2), pp. 87. Copyright 2003 by Institure for Laboratory Animal Research).

2.2.1 MORPHOLOGY OF CEREBRAL CIRCULATION

To facilitate understanding of the cerebral ischemia and ischemic stroke models, a discussion of the encephalic vascular structure is necessary. Vessels that supply blood to the brain form into the Willis circle (Figure 2.6 (n.d., 2014d)) which consists of several main cerebral arteries, such as the middle cerebral artery (MCA), anterior cerebral artery (ACA), posterior cerebral artery, and other communicating arteries.

The Willis circle can be divided into two main parts: the anterior and the posterior. In the anterior part, blood from the internal carotid artery (ICA) flows through two different paths when the ICA enters the cranial cavity; one portion flows into the ACA and the rest advances along the MCA. The encephalon is symmetrical, and the left and right ACAs are united by the anterior communicating artery. In the posterior part, the basilar artery which is formed by the bilateral vertebral arteries, divides into left and right posterior cerebral arteries (PCAs). The PCAs join the circle of Willis via the posterior communicating artery.

Vessels forming the Willis circles differ in human and rats (Table 2.1 (Casals et al., 2011)); however, both species have a similar ICA-MCA route, a structure which plays a significant role in developing stroke animal models (Casals et al., 2011). The MCA is the most commonly affected vessel in stroke (Durukan & Tatlisumak, 2007), and has been used to induce cerebral ischemia via occlusion. The MCA is composed of three segments: the horizontal, the sylvian and the cortical (Figure 2.7 (Figliolini & Piazze, 2011)). The brain regions supplied by

the MCA include internal capsule, part of the caudate putamen, the globus pallidus, an most of the temporal, frontal, parietal lobes. Symptoms caused by the right MCA occlusion include contralesional hemiplegia and ipsilesional head/eye deviation.

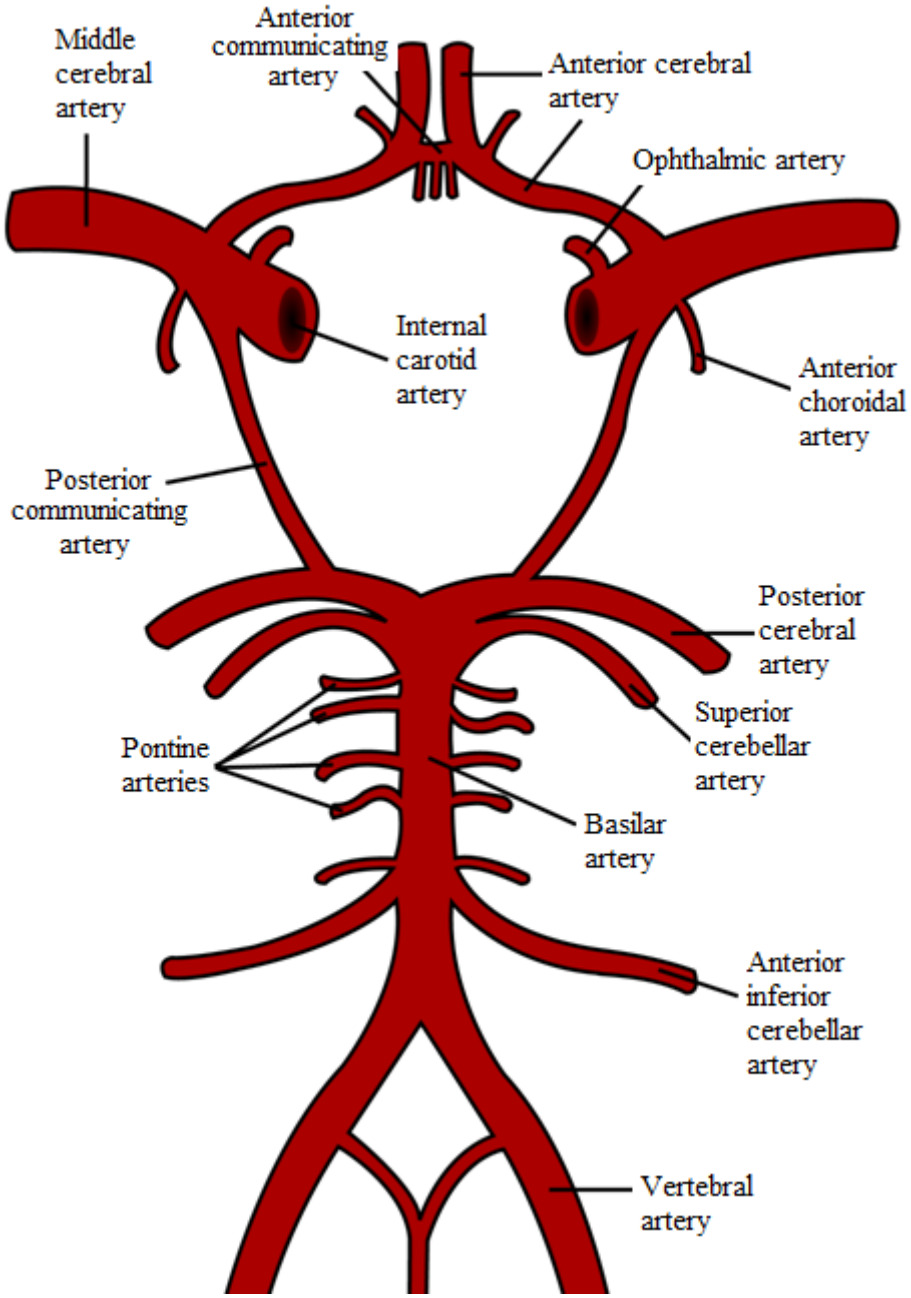

Figure 2.6 Morphology of the Willis circle. (Reprinted and modified from File: Circle of Willis en.svg, In *Wikipedia*, n.d., Retrieved 19 Jan 2014, from http://en.wikipedia.org/wiki/File:Circle_of_Willis_en.svg.).

Table 2.1 Major Brain Arteries and their Branches and Supplied Brain Areas in Human and Rats (Casals et al., 2011).

Species	Major Artery	Branches of major artery	Region vascularized
Human			
	Internal carotid artery	Middle cerebral artery (MCA)	Frontal lobe to the parieto-occipital sulcus (ACA)
			Superolateral face of hemispheres (MCA)
		Anterior cerebral artery (ACA)	Midbrain, occipital and temporal lobe (PCA)
	Basilar artery	Posterior cerebral artery (PCA)	
	Posterior cerebral artery	Posterior communicating artery	
	Anterior cerebral artery	Anterior communicating artery	
Rat			
	Internal carotid artery	Posterior communicating artery	Lateral surface of the olfactory tract and cerebral cortex (MCA)
		Hypothalamic artery	
		Anterior choroidal artery	
		Midderl cerebral artery	
		Anterior cerebral artery	
	Basilar artery	Posterior cerebral artery	Posterior portion of the arterial circle of Willis (posterior communicating artery)
		Vertebral artery	
	Posterior cerebral artery	Posterior communicating artery	

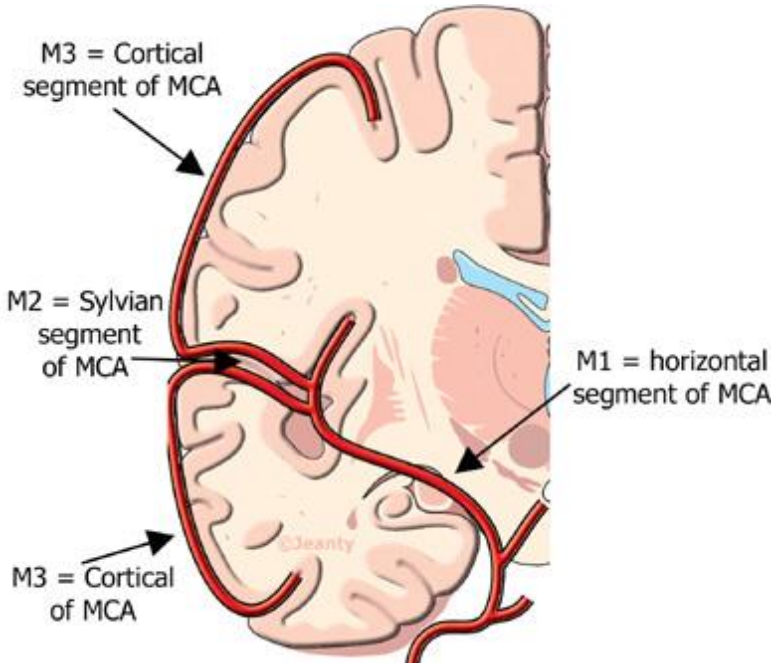

Figure 2.7 A schematic diagram of the MCA in the left hemisphere (Reprinted from C. Figliolini & J. Piazze, 2011, Middle Cerebral Artery, Bilateral Early Bifurcation, In *TheFetus.net*, Retrieved 28 Nov 2013, from http://www.sonoworld.com/fetus/page.aspx?id=3015. Copyright 2011 by TheFetus.net.).

2.2.2 GLOBAL CEREBRAL ISCHEMIC MODELS

Global cerebral ischemia occurs when most or all of the brain lacks blood supply, leading to neurologic impairment in vulnerable brain regions like the hippocampus by the reduction of oxygen and glucose supply (Traystman, 2003; Nikonenko, Radenovic, Andjus, & Skibo, 2009). Acute global ischemia is usually transient and caused by cardiac arrest, resulting in approximating 50% survivors who acquire moderate-to-severe, persistent cognitive deficits (Roine, Kajaste, & Kaste, 1993), while long-term hypoperfusion induced chronic global ischemia in humans may result from small vessel diseases in the brain (Krafft et al., 2012). Both acute and chronic global cerebral ischemic models have been developed in order to mimic these neurophysiological deficits.

One of the earliest acute ischemic models without any reperfusion used decapitation of a mouse to investigate the biochemical changes after global ischemia (Lowry, Passonneau, Hasselberger, & Schulz, 1964); however, no other manipulations are possible in this model. A neck tourniquet used in rats (Siemkowicz & Gjedde, 1980) and neck cuff inflation in big animals like dogs (Symon, 1993) have also been used to induce ischemia in the entire brain. The disadvantages of the neck tourniquet method include vagal nerve compression and venous congestion, while the neck cuff inflation method does not block the vertebral arteries, thus, requiring separate occlusion. Ventricular fibrillation has also been used to mimic global ischemia resulting from cardiac arrest in clinic (Eleff et al., 1991); however, large animals are generally used in this method, leading to huge expense and extensive labor. A four-vessel occlusion model is another technique in which both vertebral arteries are permanently occluded and two common carotid arteries are temporarily ligated (Sugio, Horigome, & Goto, 1988; Pulsinelli & Brierley, 1979; Chen, Z. C. Xu, X. M. Xu, & Zhang, 2009; Schmidt-Kastner, Paschen, Ophoff, & Hossmann, 1989). A two-vessel occlusion model involves temporarily occluding the common carotid arteries in combination with induced systemic hypotension (Smith et al., 1984; Gionet, Warner, Verhaegen, Thomas, & Todd, 1992). Compared to the four-vessel occlusion-induced global ischemia with variable effect of ischemia and partial reversibility via a two-stage surgery, the two-vessel occlusion model is an easier procedure and less invasive with full reversibility (McBean & Kelly, 1998). A three-vessel occlusion rodent model has also been developed to produce bilateral hemispheric ischemia via occlusion of the common carotid arteries and the basilar artery (Kameyama, Suzuki, Shirane, & Ogawa, 1985).

Chronic global hypoperfusion models with white matter lesions are produced by using either external microcoil-induced bilateral common carotid artery stenosis (Shibata, Ohtani, Ihara, & Tomimoto, 2004) or permanent ligation of both common carotid arteries (Ueno, Tomimoto, Akiguchi, Wakita, & Sakamoto, 2002).

2.2.3 FOCAL CEREBRAL ISCHEMIC MODELS

The focal cerebral ischemic models present the most common clinical subtype of stroke resulting from major cerebral vessel occlusion (typically, the middle cerebral artery (MCA) or its branches) by either thrombosis or embolism (Durukan & Tatlisumak, 2007; Del Zoppo et al., 1992). Several MCA occlusion (MCAO)-induced focal ischemic stroke models have been developed. These MCAO models can be divided into several categories based on different grouping considerations; for example, they can be categorized into permanent and transient ischemic models based on occlusion duration and severity or proximal and distal ischemic models based on occlusion sites. These models can be further specified with or without craniectomy based on the procedures used.

There are approximately 10 rodent stroke models routinely used in current experimental paradigms (Carmichael, 2005), including the intraluminal suture, thromboembolic, photothrombosis, and endothelin-1 induced models (Howells et al., 2010). These models vary widely in their ability to recapitulate the symptoms of clinical stroke, and in their application of the study of cell death and neural repair. The choice of appropriate focal ischemic models to use in aparticular study should be made based on specific research question being addressed, as

each model differs in pathophysiology. Major focal ischemic stroke models were reviewed and described in Table 2.2.

Table 2.2 Major Focal Ischemic Stroke Models.

Focal Ischemic Models	Features		
	Craniectomy	Permanent (P) or Transient (T)	Proximal (P) or Distal (D)
Intraluminal suture MCAO model	No	P/T	P
Embolic model	Mostly No	P/T	P/D
Photothrombosis model	No	P	D
Endothelin-1 induced model	Mostly No	T	P
Posterior cerebral circulation models	Yes	P/T	P/D
Cerebral venous thrombosis models	Yes	P/T	P/D

2.2.3.1 INTRALUMINAL SUTURE MCAO MODEL

The intraluminal suture MCAO rodent model has been most frequently used for many years because it is less invasive, and it is procedurally easier to induce both transient and permanent focal ischemia (Liu et al., 2010). In this model, the MCA is occluded by using a monofilament which is inserted into the internal cerebral artery and is advanced until the tip of the monofilament reaches the origin of the MCA. This model causes reproducible infarction in the MCA reachable brain territories, including the lateral caudoputamen and frontoparietal cortex (M1, M2, and M3, in Figure 2.7 (Figliolini & Piazze, 2011)); in addition, this model can readily induce reperfusion by withdrawing the monofilament.

This model was initially developed by Koizumi et al. (1986) and subsequently optimized to reduce the incidence of subarachnoid hemorrhage and premature reperfusion (Hossmann, 1998; Longa, Weinstein, Carlson, & Cummins, 1989;

Schmid-Elsaesser, Zausinger, Hungerhuber, Baethmann, & Reulen, 1998). Characteristics of the suture, such as diameter, coating, and insertion length, highly correlate with brain infarct volume. Sutures can be classified into coated sutures (silicone or poly-1-lysine) (Koizumi, Yoshida, Nakazawa, & Ooneda, 1986) and traditionally uncoated nylon sutures (Longa et al., 1989). Figure 2.8 shows the two typical sutures applied in focal ischemic stroke models (Durukan & Tatlisumak, 2007). Previous studies have compared the uncoated and coated sutures, showing that coated sutures demonstrate better performance in MCAO with deeper and more consistent ischemia, with less perforation incidence of the intracranial internal carotid (Shimamura, Matchett, Tsubokawa, Ohkuma, & Zhang, 2006; Laing, Jakubowski, & Laing, 1993).

Disadvantages of this model include vessel rupture, subarachnoid haemorrhage, inadequate MCAO, and hyperthermia. Experimental success when using this model may be increased by combing the use of silicone coated 4-0 filament with laser-Doppler flowmetry to monitor sufficient MCAO (Schmid-Elsaesser et al., 1998).

2.2.3.2 EMBOLIC MODEL

The embolic model closely mimics thromboembolic stroke, which accounts for a high rate of clinical stroke. This model has shortcomings, including varied infarct size and unpredicted and inconsistent embolic location; however, it has also been widely used for testing thrombosis agents and neuroprotective drugs before clinical trials (Lapergue et al., 2010; Wilhelm-Schwenkmezger et al., 2007) and for investigating the mechanisms like inflammation of embolic stroke (Abulafia

et al., 2009). The types of emboli used in previous studies involves autologous blood clots (Kudo, Aoyama, Ichimori, & Fukunaga, 1982; Kaneko, Nakamura, & Ogawa, 1985) and artificially emboli including microspheres (Takeo et al., 1992; Miyake, Tanonaka, Minematsu, Inoue, & Takeo, 1989), plastic beads (Roos, Sperber, Johansson, & Bill, 1996), and preformed clots (Wang, Todd, Yang, Gordon, & Shuaib, 2001). Direct injection of different emboli into the external carotid artery (ECA) (Z. Zhang, Chopp, R. L. Zhang, & Goussev, 1997; Omae, Mayzel-Oreg, Li, Sotak, & Fisher, 2000) or internal carotid artery (ICA) (Atochin et al., 2004) is widely used and can induce reproducible brain injury. Figure 2.9 gives an example of emboli injection through the ECA and ICA in a mouse model (Zhang et al., 1997). After a catheter is inserted into the ECA and ICA (the tip arrives at the origin of the MCA), a clot is injected and enters the distal MCA (Zhang et al., 1997). The degree of neurologic deficits caused by microemboli has been found to be dose-dependent (Atochin et al., 2004). Embolic models are commonly developed without the use of craniectomy; however, a thromboembolic stroke can be induced by *in situ* microinjection of a purified murine thrombin to produce a local clot in a mouse, resulting in reproducible brain impairment (Figure 2.10) (Orset et al., 2007).

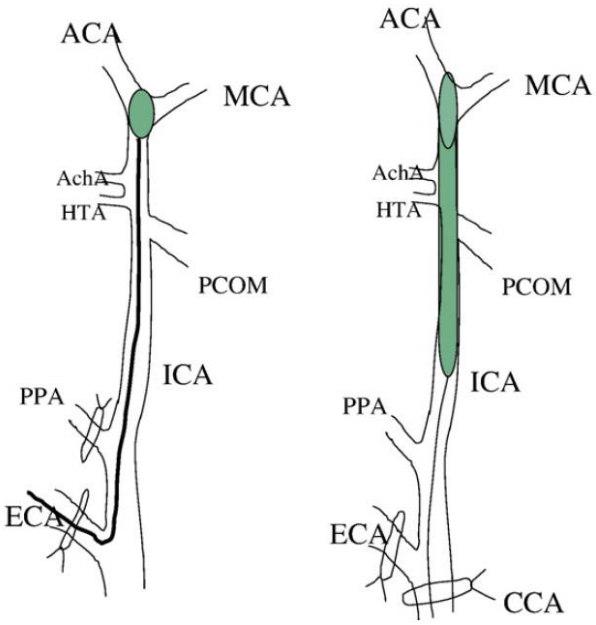

Figure 2.8 Two typical intraluminal sutures MCA occlusion in the application of focal ischemic stroke models. The left: traditionally uncoated nylon suture used by Longa et al. (1989); the right: coated filament introduced by Koizumi et al. (1986). ACA: anterior carotid artery; AchA: anterior choroidal artery; HTA: hypothalamic artery; PCOM: posterior communicating artery; ICA: internal carotid artery; PPA: pterygopalatine artery; ECA: external carotid artery; CCA: common carotid artery (Reprinted from A. Durukan & T. Tatlisumak, 2007, *Pharmacology, Biochemistry, and Behavior, 87*, pp. 181. Copyright 2007 by Elsvier, Inc.).

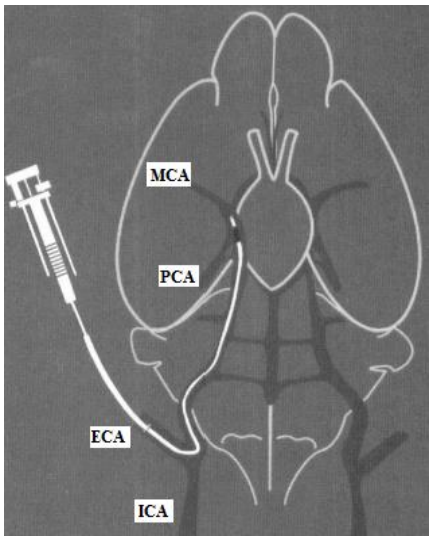

Figure 2.9 Example of an emboli injection method in a mouse model. PCA: posterior cerebral artery; ECA: external carotid artery; ICA: internal carotid artery (Reprinted and modified from Z. Zhang et al., 1997, *Journal of Cerebral Blood Flow and Metabolism, 17*, pp. 1082. Copyright 1997 by the International Society of Cerebral Bllod Flow and Metabolism).

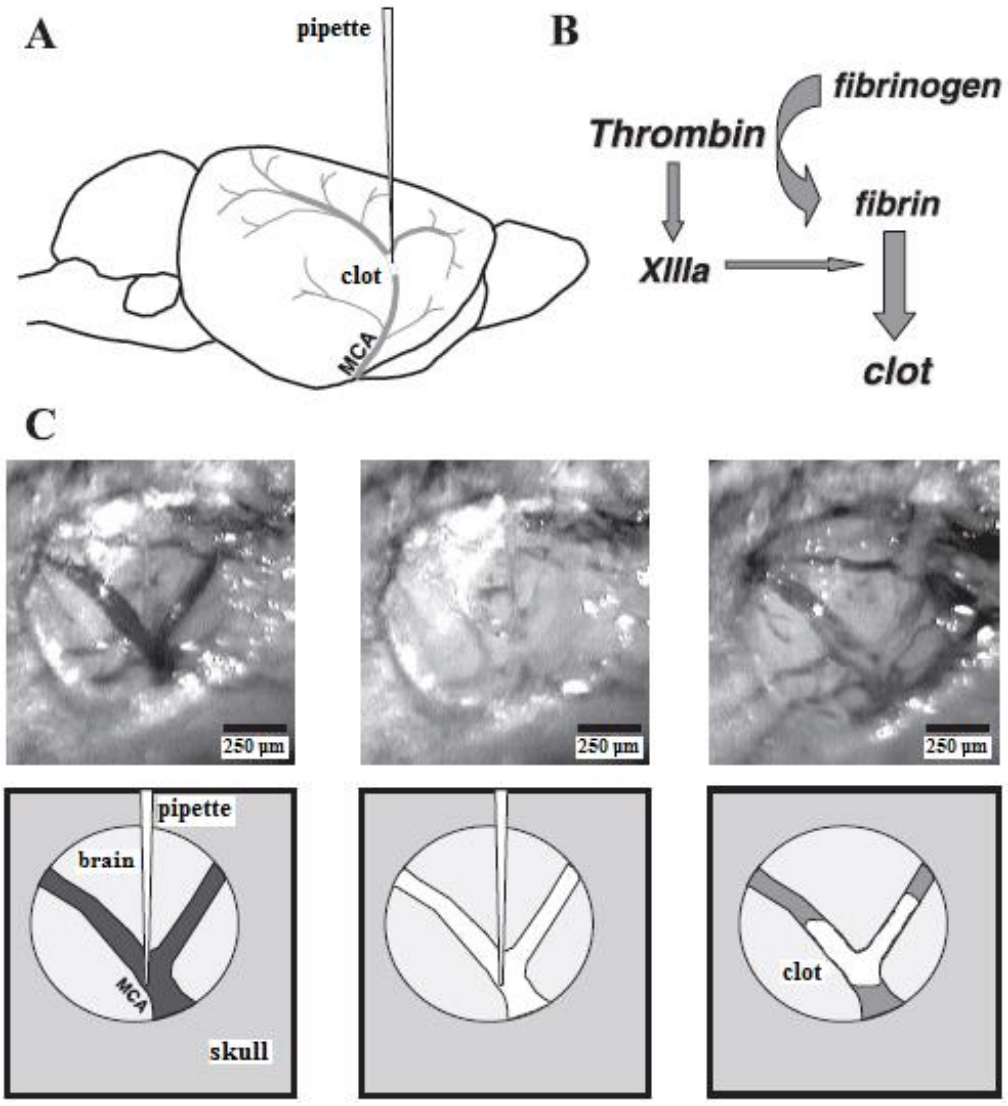

Figure 2.10 *In situ* thromboembolic mouse stroke model using craniotomies. A) Schematic diagram of this method; B) The fibrin clot formation process; C) Photomicrograph and schematic diagram of the different steps of the surgical procedure from injection of thrombin into the MCA to the formation of a clot (Reprinted and modified from C. Orset et al., 2007, *Stroke*, *38*, pp. 2772. Copyright 2007 American Heart Association, Inc.).

2.2.3.3 PHOTOTHROMBOTIC MODEL

The photothrombosis-induced stroke model can be developed by intravenously injecting a photosensitizing dye and using a specific laser beam to target a selected region of the brain (Carmichael, 2005). The photoactive dyes commonly used include Rose Bengal and Photofrin II (Omae et al., 2000), with Rose Bengal

being the most frequently utilized (Futrell & Riddle, 1993; Schmidt et al., 2012). During the laser process, neurological deficits will form within minutes in the illuminated brain regions through the action of singlet oxygen, a substance generated during illumination which is able to induce focal endothelial damage, platelet activation, and aggregation (Carmichael, 2005). Figure 2.11 shows an example of surgical procedure to develop a photothrombotic model by using a rat (the left) and the induced brain lesion (the right) (Schmidt et al., 2012).

The advantages of this model include controlled infarct size, lesion location, and minimal trauma caused by surgical procedure. A disadvantage of this model is that the penumbra is small; this technique results in micro-vascular insults and brain lesion is developed within minutes (Durukan & Tatlisumak, 2007), such that this model is limited in the application of penumbra-targeted studies. However, applications can be used in preclinical studies on restorative drugs tests and evaluation of neural repair (Brunkhorst et al., 2013; Song et al., 2012).

2.2.3.4 ENDOTHELIN-1 (ET-1) MODEL

ET-1 is a strong vasoconstrictor with the application in the development of stroke animal models. Focal ischemia within the MCA supported brain area can be induced by microinjecting ET-1 into the MCA via a cannula, leading to dose-dependent neurological deficits and functional impairment (Sharkey, Ritchie, & Kelly, 1993; Callaway, Knight, Watkins, Beart, & Jarrott, 1999). ET-1 was found to have a more potent effect in rats than mice in a study where different types of mouse strains received the same ET-1 injection, but inducing small and strain-dependent infarct sizes (Horie et al., 2008). In an ET-1 induced MCAO model,

axons and oligodendrocytes injury was investigated; a few injured axons were found to have kept their structural integrity, indicating the possibility of delayed therapy (Gresle, Jarrott, Jones, & Callaway, 2006). Most of ET-1 induced stroke models do not require craniotomy; however, ET-1 can be injected through the sensorimotor cortex into the subcortical white matter to mimic human subcortical stroke (Sozmen, Kolekar, Havton, & Carmichael, 2009). The ET-1 stroke model has often been used to study therapeutic interventions and the mechanisms underlying subcortical stroke (Frost, Barbay, Mumert, Stowe, & Nudo, 2006; Hughes et al., 2003).

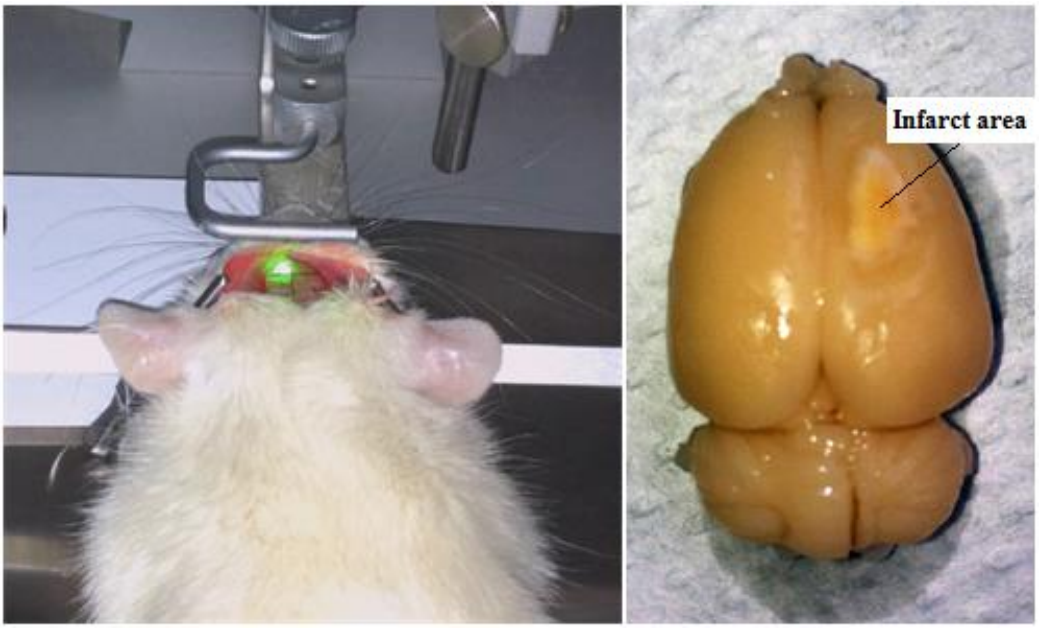

Figure 2.11 An example of a rat photothrombotic model. The left shows a laser beam illuminating on a rat's skull after injection of rose bengal; the right shows the size and location of photochemically induced lesion on the sensorimotor cortex (Reprinted and modified from A. Schmidt et al., 2012, *Experimental & Translational Stroke Medicine*, *4*(13), pp. 3-4. Copyright 2012 Schmidt ed al.; licensee BioMed Central Ltd.).

2.2.3.5 POSTERIOR CEREBRAL CIRCULATION MODEL

The posterior cerebral circulation stroke is a common subtype in clinical stroke and accounts for approximately 20% of ischemic stroke (Gulli, Khan, & Markus, 2009). Vertebrobasilar is one of the key segments in the posterior circulation. Patients with vertebrobasilar stenosis have a high risk of perioperative stroke (Blacker, Flemming, & Wijdicks, 2003) and early recurrent stroke (Gulli et al., 2009). The mortality of vertebrobasilar occlusion-induced stroke is approximately 27% within one week after stroke onset, based on an analysis of 37 patients; this rate is almost 2.5 times higher than that of other carotid system diseases (Jones, Millikan, & Sandok, 1980). Intraarterial thrombolysis for vertebrobasilar recanalization is necessary for these patients; however, this procedure is not enough to reduce the high incidence of mortality caused by rethrombosis and hemorrhage after successful thrombolysis (unless thrombolysis is used in combination with other treatment) (Becker et al., 1996; Schulte-Altedorneburg et al., 2006; Hacke, Zeumer, Ferbert, Bruckmann, & Del Zoppo, 1988).

Animal models are lacking to mimic the symptoms of posterior cerebral circulation and thus, there are not enough investigations on the prognosis of vertebrobasilar occlusion and on effective interventions for clinical treatment. A vertebrobasilar occlusion stroke model has been successfully developed with neurological deficits via the injection of preformed autologous blood clots directly into the vertebral artery after thoracotomy (rat-based model) (Henninger et al., 2006). Rabbits have also been utilized to produce posterior circulation stroke by either injecting autologous clots into distal vertebral artery (Amiridze,

Gullapalli, Hoffman, & Darwish, 2009) or by occluding the unilateral vertebral artery (Cai et al., 2012). Other studies (Guo, Liao, Preston, & Batjer, 1995; Kuwabara, Uno, & Ishikawa, 1988) have used dogs to develop posterior circulation stroke models by occluding the perforating arteries, resulting in brain stem ischemia and consistent neurological deficits. The surgical procedures in these models are relatively invasive and more complicated compared to other focal ischemic stroke models.

2.2.3.6 CEREBRAL VENOUS THROMBOSIS (CVT) MODEL

CVT stroke is such a cerebrovascular disease with a low-incidence (approximately 0.5% of all stroke types), wide age distribution (from newborns to the elderly), various causes (often transtentorial herniation (Canhão et al., 2005)), and low mortality rate (Bousser & Ferro, 2007). Inherited or acquired hypercoagulable states provide potential conditions for thrombotic formation, such as in the case of pregnancy, malignancy, and the use of oral contraceptives (Di Paola, 2002). CVT stroke patients are usually prescribed anticoagulant agents for post-stroke treatment, even though there is evidence lacking to demonstrate a correlation between sinus recanalization and clinical outcomes (Schultz, Davis, Tress, Kilpatrick, & King, 1996). Cytoprotective drugs have been suggested to use in thrombolytic treatment because both vasogenic and early cytotoxic edema were found in a study using CVT stroke rat models induced by injecting a thrombogenic cephalin suspension after ligating the rostral and caudal sites of the superior sagittal sinus (SSS) (Röther, Waggie, Van Bruggen, De Crespigny, & Moseley, 1996). SSS occlusion has often been employed for the development of CVT models in various animals, such as the mouse (Röttger et al., 2005), rat

(Srivastava, Kalita, Dohare, Ray, & Misra, 2009), gerbil (Miyamoto, Heimann, & Kempski, 2001), and cat (Schaller, Graf, Wienhard, & Heiss, 2003). Magnetic resonance imaging (MRI) is frequently used to diagnose CVT for patients (Schultz et al., 1996; Bousser & Ferro, 2007) or to confirm the success of CVT modeling in animal models (Röttger et al., 2005; Srivastava, Kalita, Haris, Gupta, & Misra, 2009), while positron emission tomography (PET) is also used to monitor the cerebral blood flow after CVT (Figure 2.12) (Schaller et al., 2003).

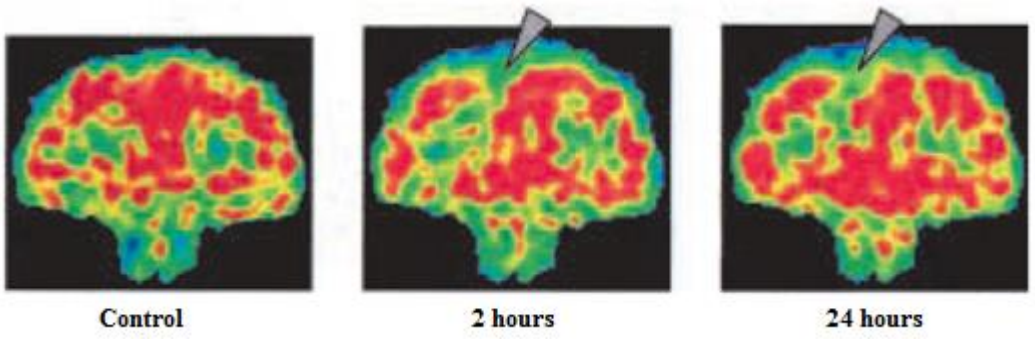

Figure 2.12 Example of positron emission tomography (PET) of cerebral blood flow monitoring in a healthy cat (the left), 2 hours (the middle), and 24 hours (the right) after superior sagittal sinus occlusion. The arrows point to the main ischemic brain regions (Reprinted from B. Schaller et al., 2003, *Swiss Medical Weekly, 133*, pp. 414. Copyright 2003 by Swiss Medical Weekly).

2.2.3.7 SUMMARY OF FOCAL CEREBRAL ISCHEMIC MODELS

Each focal cerebral ischemic animal model has its own specific strengths or weaknesses. In this study, we need a focal ischemic animal model which should have favorable reproducibility and produce consistent infarcts in brain tissues, especially in the sensorimotor cortex. After reviewing the current models commonly used in studies (Table 2.3), intraluminal suture MCAO stroke rat model is the most appropriate model for this study. This rat stroke model allows the use of a relatively large quantity of rats for a relatively low cost in order to

obtain statistical significance of rehabilitation outcomes. Moreover, it is also less invasive and is less complicated to conduct the surgical procedures.

Table 2.3 Summary of Characteristics of Major Focal Ischemic Stroke Models.

Focal Ischemic Models	Characteristics
Intraluminal suture MCAO model	Favorable reproducibility; consistent infarct in brain tissues; readily realize reperfusion; uncomplicated surgical procedures; less invasive.
Embolic model	Varied infarct size; unpredicted and inconsistent embolic location.
Photothrombosis model	Controlled infarct size and lesion location; minimal trauma; small penumbra (not suitable for rehabilitation study)
Endothelin-1 induced model	Dose-dependent neurological deficits and functional impairment; strain-dependent infarct sizes.
Posterior cerebral circulation models	Lack of animal models; relatively invasive and complicated surgical procedures.
Cerebral venous thrombosis models	Relatively invasive and complicated surgical procedures.

2.3 FUNCTIONAL EVALUATIONS

A successful stroke model will exhibit neurological deficits, including motor dysfunctions and impaired cognition. Several specific tasks have been developed and are commonly used to assess the degree of motor function (sensorimotor integration, motor coordination and balance, and limb use) and cognition improvement following intervention (Brooks & Dunnett, 2009; Hunter et al., 2000; DeVries, Nelson, Traystman, & Hurn, 2001); involve such tasks include neurological assessments (Bederson's (Bederson et al., 1986b) and Longa's (Longa et al., 1989) tests), sensorimotor tasks (the rotarod (Hamm, Pike, O'Dell, Lyeth, & Jenkins, 1994), corner (Schallert et al., 1982), and staircase (Bouët et al., 2007) tests), and cognitive tasks (the water maze (Schallert, 2006) and cylinder (Li et al., 2004) tests).

2.3.1 NEUROLOGICAL DEFICITS ASSESSMENT

Neurological deficits were assessed in order to determine the success of the stroke model. Two commonly used assessment systems are Bederson's (Bederson et al., 1986b) and Longa's (Longa et al., 1989) tests. The scoring scales and related rules of Bederson's test and Longa's test are described in Table 2.4 and Table 2.5. Bederson's grading system has 4 levels with scores from 0 to 3, where 0 points indicates no observable neurological deficits, while 3 points indicates severe neurological deficits. Longa's grading system has similar criteria except that it has an additional level to describe unconsciousness in stroke rat models. Modified and improved assessments with wider grading scales have also been developed which are time-based (Zausinger, Hungerhuber, Baethmann, Reulen, & Schmid-Elsaesser, 2000; Ding et al., 2001).

Table 2.4 Bederson's Grading System for Neurological Evaluation (Bederson et al., 1986b).

Grade	Neurological deficits
0	No neurologic deficit
1	Flexion of the affected forelimb
2	Decreased resistance to lateral push (and forelimb flexion) without circling
3	Same behaviour as grade 2, with circling

Table 2.5 Longa's Grading System for Neurological Evaluation (Longa et al., 1989).

Grade	Neurological deficits
0	No neurologic deficit
1	Failure to fully extend the impaired forepaw
2	Circling to the impaired side
3	Falling to the impaired side
4	Walk non-spontaneously and have a decreased consciousness level

2.3.2 SENSORIMOTOR FUNCTION ASSESSMENT

Many tasks have been developed to assess sensorimotor performance, such as locomotor activity, motor coordination and balance, and limb use in rodents after MCAO stroke. The most commonly used tasks include the rotarod, corner, staircase, foot fault, cylinder, pole, rope and ladder climbing, parallel bar crossing, and adhesive removal tasks (Modo et al., 2000; Ding et al., 2001; Li et al., 2004).

2.3.2.1 THE ROTAROD TEST

The rotarod test is used to evaluate motor coordination and balance after traumatic (Hamm et al., 1994) or ischemic (Bouët et al., 2007; Zausinger et al., 2000) brain injury in rodents. The basic apparatus is shown in Figure 2.13 (Carroll & Wild, 2012). All the experimental rodents are trained across several days by walking on the central rods while they rotate. The time that the rodents stay on the rods is a key parameter for comparing their motor coordination and balance before and after stroke, or between strokes.

Figure 2.13 Eample of the rotarod apparatus (Reprinted from J. Carroll & E. Wild, 2012, Special 'brain fat' Injection Helps HD Mice, In *HDBuzz*, Retrieved 28 Nov 2013, from http://en.hdbuzz.net/072. Copyright 2011-2014 by HDBuzz).

2.3.2.2 THE BEAM BALANCE AND WALKING TEST

The beam balance and walking test is reliably used to measure rodents' balance abilities and complex neuromotor function (Schallert & Woodlee, 2005; Petullo et al., 1999; De Ryck, Van Reempts, Borgers, Wauquier, & Janssen, 1989). The apparatus size varies in different studies; for example, Schallert et al. (2002) used a 2 cm wide × 130 cm long wooden beam apparatus which was elevated 110 cm away from the ground with the home cage at one end. Jolkkonen et al. (2000) adopted a 2.5 cm wide × 122 cm long square beam which was positioned 42 cm above the ground and connected to a black box (20.5 × 25 × 25cm) at one end. In Petullo et al. (1999)'s study, a narrow beam (30 cm × 1.5cm) was used for a balance test and another elevated beam (90 × 4 × 1.5cm) with a box at one end was used to assess beam walking ability. De Ryck et al. (1989) used two elevated wooden beams (30 cm above the table surface) 4 cm and 2.5 cm wide and 2 m long.

The rating scales also differ across these studies, but they all indicate the degree of impairment. Goldstein et al. (1990) rated motor performance on a 7-point scale, where a score of '1' indicated that the rat failed to lay the affected hindpaw on the beam surface and a score of '7' indicated that the rat could traverse the beam with less than 2 foot slips. In Petullo et al. (1999)'s study, noise was used to trigger the rat to go into the box, and its scoring system (Table 2.6) was based on the time that rats spent crossing the beam, with a higher score indicating greater limb function impairment. Jolkkonen et al. (2000) employed a modified rating scale developed by Feeney et al. (1982), ranging from 0 to 6 (Table 2.7), with a higher score representing greater function.

Table 2.6 Scoring System used in Petullo et al. (1999)'s Study for Beam Balance and Walking Test.

Score	Description	
	Beam Balance	Beam Walking
0	Balances with 4 paws on the beam top	≤ 4 s
1	Put paws on side of beam or wavers	5 – 7s
2	1 or 2 limbs slip off beam	8 – 10s
3	3 limbs slip off beam	11 -15s
4	Attempts to balance but falls off	> 15s
5	Animal drapes on beam then falls	Not able to run
6	Fall without attempting to balance	-

Table 2.7 Scoring System in Jolkkonen et al. (2000)'s Study for Beam Walking Test.

Score	Description
0	Unable to maintain balance and falls down from the beam
1	Unable to traverse the beam but remains perched on the beam
2	Falls down while walking
3	Able to traverse the beam by dragging foot on the affected side
4	Traverses the beam with more than 50% foot slips
5	Crosses the beam with less than 50% foot slips
6	Crosses the beam with no foot slips

2.3.2.3 THE FOOT FAULT TEST

The foot fault test is used to assess sensorimotor integration of the rodents' forelimbs and hindlimbs (Ding et al., 2004). The apparatus is composed of an elevated grid surface (10 × 110 cm^2, 1.0 m high from ground; a square opening: 9 cm^2; diameter of the grid wire: 1.0 mm) which is connected to a platform (15 × 20 cm^2) at each end (Ding et al., 2004). Noise or a manual prod is often used to encourage rodents to cross the grid surface. Limb displacements are considered as foot faults. The number of foot faults over one minute is collected to evaluate the limb function.

2.3.2.4 THE CORNER TEST

The corner test was first designed for rats to assess sensorimotor and postural asymmetries (Schallert et al., 1982) and later applied in mouse studies (Li et al., 2004; Bouët et al., 2007). A $30°$ corner is made by two vertical cardboards with the same dimension ($30 \times 20 \times 1\ cm^3$) with a small opening at the corner (Li et al., 2004). A rat or mouse would instinctively enter the corner until their vibrissae or skin touched the board, at which time they would immediately turn either right or left. After tens trials, the ratio of right and left turns is used as a measure of asymmetry.

2.3.2.5 THE CYLINDER TEST

The cylinder test measures the asymmetries of limb use by placing the experimental rat or mouse into a clear cylinder with sufficient height and width (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000; Woodlee et al., 2005; Li et al., 2004; Hewlett & Corbett, 2006). The number of wall contacts using either affected or unaffected forelimb is collected and used as a parameter for asymmetry analysis.

2.3.2.6 THE STAIRCASE TEST

The staircase test assesses skilled paw use, specifically for reaching and grasping. It is less labour-intensive than other tests and achieves the same goal (Hewlett & Corbett, 2006; Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991; Abrous & Bunett, 1994). Montoya et al. (1991) initially designed the apparatus by using a plexiglass box combined with a removable baited double staircase. Food pellets

were placed on staircases. Independent paw reaching and grasping food measures are collected and analysed.

2.3.2.7 THE LOCOMOTOR TESTS

The locomotor tests used by De Ryck et al. (1989) have been widely used in other studies (Ke et al., 2011; Ohlsson & Johansson, 1995; Heo & Kim, 2013). This test consists of 8 subtasks for postural and locomotor reactions. Six out of the eight subtasks assess forelimb function while the rest assess hindlimb function. To fulfil these tasks, a rat must:

- Be suspended 10 cm over a table; this test is used to observe the stretch ability of affected forelimb.
- Be placed in front of a table edge to observe whether the affected forelimb is placed on the table top as the unaffected forelimb.
- Be prohibited from seeing or touching the table by gently raising its head up to a 45-angle to observe whether the affected forelimb is placed on the table top as the unaffected forelimb.
- Be placed on a table edge and gently pushed from behind towards the edge, allowing one to observe the gripping ability of the affected forelimb.
- Be placed towards a table with affected limbs just over the edge in order to examine lateral placement of the affected forelimb and hindlimb.
- Be positioned along a table edge and gently pushed towards table edge, allowing one to observe the ability of both affected forelimb and hindlimb from slipping off.

The grading system for each task has 3 levels with 0, 1, and 2 points assigned for each level. 0 points indicate complete disability of locomotor function, 1 point indicates delayed (2s) or incomplete performance, and 2 points are given to a rat if it performs normally. The summed score of all 8 subtasks ranging from 0 (maximum deficit) to 16 (normal) is a parameter to measure locomotor ability.

2.3.3 COGNITIVE ASSESSMENT

Commonly used cognitive tests include: 1) passive avoidance and 2) maze (radial arm maze (RAM) and Morris water maze (MWM)) tests (DeVries et al., 2001; Bouët et al., 2007). Commercial passive avoidance boxes are have two compartments: one is wide, white, and illuminated, while the other is small, black, and dark. The dark compartment is separated from the white one by a door between them. Rodents are first placed in the white compartment. Once they enter the dark compartment, they will get an electrical foot shock; eventually they will stop visiting the dark compartment, indicating that they have formed a memory of the dark compartment and the shock. Since MCAO stroke may lead to cognitive deficits, the latency before rats enter the dark one can be used to evaluate their memory.

Typical RAM and MWM tasks are shown in Figure 2.14 (n.d., 2013). In the RAM task, food is placed at the end of each arm. After training, normal rodents will learn and remember the food location and quickly find it. In the MWM task, rodents are trained to remember the position of a platform placed just under the water's surface. Through RAM and MWM tests, long-term learning and cognitive deficits of stroke rodent models can be assessed (Modo et al., 2000;

DeVries et al., 2001). Compared to MWM system, the RAM task is more difficult to learn due to its requirements for extensive training, motor coordination, and food restriction for motivation (DeVries et al., 2001).

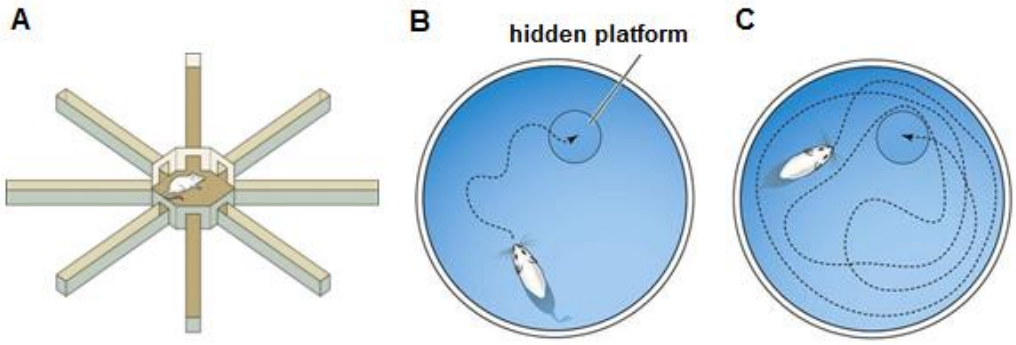

Figure 2.14 Typical radial arm maze task (A) and Morris water maze task (B, C). B) normal rat after learning; C) rats with cognitive deficits. (Reprinted and modified from Hormones and Behavior Chap 12, In *StudyBlue*, n.d., Retrieved 7 Jan 2014 from http://www.studyblue.com/notes/note/n/hormones-and-behavior-chap12/de ck/4620295. Copyright 2013 by StudyBlue, Inc.).

2.3.4 SUMMARY OF FUNCTIONAL ASSESSMENTS

These functional tests can be catergorized into 3 aspects: neurological deficit, sensorimotor, and cognitive asssessments. Differences among these commonly used assessments are described in Table 2.8.

For neurological deficits assessment, both Bederson's and Longa's grading system can be used; the Logna's system has an additional level compared to Bederson's (Section 2.3.1).

Sensorimotor assessments can be broadly divided into 3 catergories: motor coordination and balance, sensorimotor integration, and limb use assessments. The rotorad test requires labor-intensive training and it is also a stressful exercise;

beam walking and balance tests may be less stressful compared to the rotorad test. Petullo et al. (1999) seperately assessed motor coordination and balance using beam walking and beam balance, while Jolkkonen et al. (2000) integrated walking and balance tests in one grading system, possibly reducing labor. The length of time that rodents spend traversing the beam is the basis of scoring in the Petullo's protocol, while the ratio of foot slips the rodents experiences when traversing the beam is the basis in Jolkkonen's protocol. Stroke rodents models in the subacute phase may be less able to move, so Jolkkonen's test may be more appropriate for this study.

The foot fault test of senorimotor integration assessment usually uses noise or a manual prod, possibly inducing unwanted stress. The corner, cylinder, and staircase tests in limb use assessment require rodents to be active to move. Moreover, the staricase test requires a rodents' desire for food so that fasting prior to testing is nessessary. De Ryck et al (1989)'s tasks assess rodents' locomotor activity with no stress and no requirement for movement, and are less labor-intensive and easy to conduct; thus, it may be the most appropriate to use this test in this study.

For cognitive assessments, the passive avoidance test and the MWM test can both cause extra stress to rodents. Additionally, the RAM test demands rodents to strongly desire food and, similar to the staircase test, fasting is nessessary before training. Fasting can be a source of stress and should be avoided during rehabilitation.

In conclusion, Longa's grading system for neurological deficits assessment, Jolkkonen's grading system for beam walking assessment, and De Ryck's tasks for locomotor activity assessment may be appropriate for this study and require further investigation on their sensitivity to intervention outcomes.

2.4 HISTOLOGICAL EVALUATIONS

It is important to determine the location and size of infarct tissue in the brain after ischemic stroke in animal models in order to confirm the success of surgery and evaluat new neuroprotective agents or therapeutic interventions. Two commonly used techniques in the field of histological assessments are hematoxylin-eosin (H&E) staining (Schmued, 1990; Chen, Raman, Bodendiek, O'Donnell, & Wulff, 2011; D ́az-Ru ́z et al., 2010) and 2'3'5' triphenyltetrazolium chloride (TTC) staining (Bederson et al., 1986a; Isayama, Pitts, & Nishimura, 1991; Sato et al., 2013). Both of these techniques can effectively determine the lesion area via brain slice staining and have been widely used; however, it is important to choose appropriate technique because there are differences between H&E and TTC staining.

Table 2.8 Summary of Commonly Used Functional Assessments.

Items		Comparisons or Key Characteristics
Neurological Deficit Assessments		
	Bederson's grading system Longa's grading system	Both present neurogical deficits; the Longa's exhibits one more level of deficits.
Sensorimotor Assessment		
	motor coordination and balance	
		Rotorad Petullo's Jolkkonen's
	Sensorimotor integration	
		Foot fault
	Limb use	
		corner cylinder
		staircase
		De Ryck's locomotor tasks
Cognitive Assessments		
	passive avoidance	
	maze	
		radial arm maze (RAM) Morris water maze (MWM)

Comparisons or Key Characteristics
Forced movements are needed, probaly inducing stress; Require much time to train rodents running on the central rods; Labor-intensive.
The Jolkkonnen's integrates mortor coordination and balance in one task, wile the Petullo's separatly assesses them. The ability of motor coordination depends on traverse time in the Petullo's, while it is determined by the ratio of foot slips in the Jolkkonen's.
Noise or prod is usually used, possibly inducing stress.
Both for testing asymmestry use of forelimb; Need rodents to be active to move.
Tests reaching and graspping ability; Need rodents to be active and eager for food.
Tests natural reaction of both forelimb and hindlimb; No stress; Less labor-intensive; Easy to conduct.
Need electric foot shock, possibly inducing stress
Both need rodents to be active; The MWM is a forced exercise, probably inducing stress; The RAM requires rodents have strong desire for food, so that fasting is needed before testing, possibly influecing rodents recovery.

2.4.1 H&E STAINING

H&E staining has commonly been used to image human and animal tissues (Harter, Hsu, & Rose, 1967; Sicard, Henninger, Fisher, Duong, & Ferris, 2006; Ono et al., 1997). H&E staining is a complex process. Typically, the targeted tissue is stored in 4% paraformaldehyde solution for fixation and then dehydrated through 25% sucrose solution, followed by coronal cryosection, and staining (Hofmeijer et al., 2004; Hiraki, Baker, & Greenberg, 2012). The stained slices are observed under a microscopy and digitized for further analysis. In ischemic brain areas, irreversibly damaged neurons display different morphology compared to intact neural cells; the cytoplasm becomes intensively eosinophilic, while the nucleus becomes pyknotic in damaged neurons (Figure 2.15) (Zhang, Deng, Ruan, & Xu, 2008; Garcia et al., 1993). The infarct area becomes pallor because of losing affinity for hematoxylin (Figure 2.16(left)) (Bederson et al., 1986a). The size of the infarct area and the number of dead neurons in the area can then be calculated.

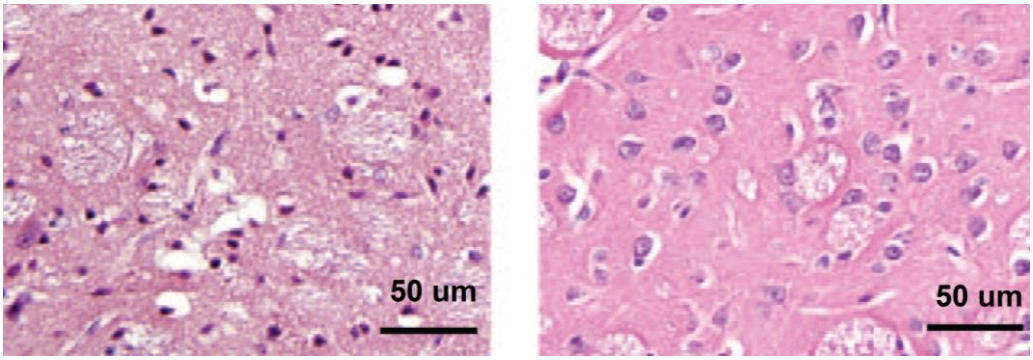

Figure 2.15 H&E staining shows morphology of dead (left) and intact (right) cells in the striatum after transient focal ischemia in rats (Reprinted and modified from Y. Zhang et al., 2008, *Stroke*, *39*, pp. 2375. Copyright 2008 by American Heart Association, Inc.).

2.4.2 TTC STAINING

TTC staining has been proved to be reliable for the detection of ischemic infarction, and it is thus widely used in animal studies (Bae et al., 2013; Shokunbi, Gelb, Peerless, Mervart, & Floyd, 1986; Türeyen, Vemuganti, Sailor, & Dempsey, 2004). TTC is a colorless and water-soluble salt, not a dye, and staining depends on the activity of mitochondrial enzymes. In normal tissue, TTC is broken down by mitochondrial enzymes in brain regions appearing brick red, after staining while in infarct tissues, there is no reaction, which appears white after staining (Figure 2.16(right)) (Benedek et al., 2006; Bederson et al., 1986a). Several studies have reported that TTC staining can reproducibly, reliably, and clearly identify infarct brain tissue 24 hours after the onset of brain ischemia, which is consistent with H&E staining (Isayama et al., 1991; Burnett, Shimazu, & Szabados, 2006; Coyle, 1987). Mitochondrial changes can reveal morphological and biochemical changes within one hour after brain ischemia; however, TTC staining via perfusion at 6 hours post-MCAO does not clearly delineate infarct or ischemic tissues from adjacent normal tissues because the border zone show pale red (Isayama et al., 1991). TTC staining via immersion results in even staining, making it more difficult to distinguish the infarct from normal tissues than via perfusion (Isayama et al., 1991). Compared to H&E staining, TTC staining is a more rapid, less expensive, and easier technique that can be used to evaluate infarct brain area; however, it cannot be used to microcosmically investigate cell changes.

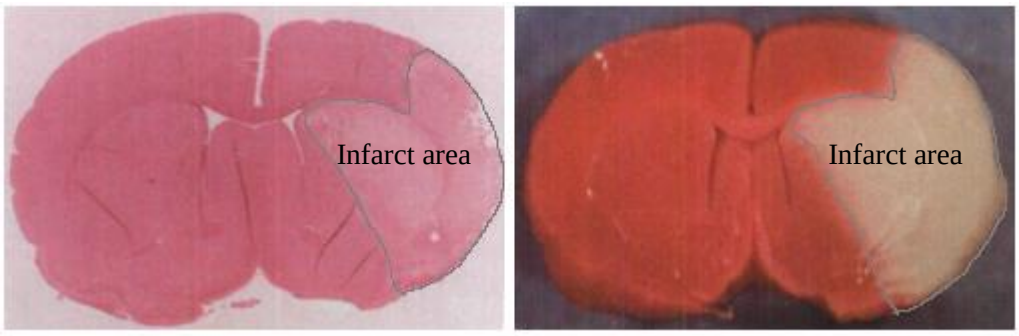

Figure 2.16 Stained brain slices from MCAO induced ischemic rats via H&E (left) and TTC (right) staining (Reprinted and modified from J. B. Bederson et al., 1986a, *Stroke*, *17*, pp. 1305. Copyright 1986 by American Heart Association, Inc.).

2.4.3 SUMMARY OF HISTOLOGICAL EVALUATION TECHNIQUES

Both H&E and TTC staining techniques can be used to identify brain infarct location and size. Infarct size can be reliably obtained 24 hours after brain ischemia. H&E staining technique can be used to calculate brain infarct size, and also can be used to observe morphological changes of cells after ischemia; however, it is time-consuming, labor-intensive, expensive, and complicated compared to TTC staining. In this study, location and size of brain lesion are targeted instead of observing cell morphology; thus, the TTC staining technique is more appropriate.

2.5 CHEMICAL EVALUATIONS

2.5.1 BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

BDNF is a protein discovered in the early 1980's which is encoded by BDNF gene and expresses broadly in the central and peripheral nervous systems (Barde et al., 1982). BDNF is one member of the "neurotrophin" family of growth

factors believed to support neuron survival and encourage new neurons and synapses to grow and differentiate (Weishaupt et al., 2012). Studies have explored the associations of BDNF with human diseases; for example, psychopathological symptoms in patients with eating disorders like anorexia and bulimia nervosa could be modulated by variability of the BDNF gene (Gamero-Villarroel et al., 2013). Patients with depression have lower serum BDNF levels (Li et al., 2013). Normal adolescents (14.0 ± 2.2 years) who exercise have significantly higher plasma BDNF levels compared to those who do not exercise (Pareja-Galeano et al., 2013).

BDNF is widely used as a therapeutic agent in animal studies to verify the effect of interventions. BDNF treatment in cats with optic nerve trauma significantly increases the rate of recovery of the entire central visual pathway (eye and cortex) than BDNF treatment only to the eye; however, both treatments lead to better recovery compared to those not treated with BDNF (Weber & Harman, 2013). Combination use of BDNF and mesenchymal stem cell (MSC) transplantation could significantly improve motor recovery two weeks after MCAO-induced stroke in neonatal rats compared to treatment with only MSC (Van Velthoven et al., 2013).

BDNF has also been commonly used as a biomarker in animal studies. Upregulated or high BDNF levels in the brain are associated with neural repair or neuroprotection based on preclinical studies of therapeutic interventions (Lee et al., 2013; Hung, P. L. Huang, C. C. Huang, Tu, & Chang, 2013; Heurteaux et al., 2013). BDNF levels in the brain have also been commonly used as an indicator

of rehabilitation outcomes in animal studies with stroke. BDNF is related to neuroplasticity which contributes to motor learning, recovery and neural rehabilitation after stroke (Hosp & Luft, 2011). BDNF is highly expressed in the hippocampus, a region vital to learning, memory, and higher thinking (Tyler et al., 2002). Rehabilitation is the process of relearning; thus, higher BDNF concentration in the brain tissues could imply learning and neural rehabilitation (Soya et al., 2007). Treadmill training after stroke induces upregulation of brain BDNF levels, which presumably is a reflection of better motor recovery (Ferreira et al., 2011). Early treadmill training in hemorrhagic stroke mice can also induce better brain repair associated with high BDNF expression (Chen, Qin, Su, Liu, & Yang, 2012). Brain BDNF levels, thus, are important parameters in determining the effect of post-stroke rehabilitation interventions.

2.5.2 CORTICORSTERONE (CORT): RESPONSE TO STRESS

Stress leads to a series of physical changes within the body and it can have a damaging effect. Moberg and Mench (2000) defined stress as a biological response induced by a threat, "stressor," to its homeostasis. A stress response starts with the central nervous system, followed by each or combination of four general biological defence responses: behavioural response, autonomic nervous system response, neuroendocrine response, and immune response. Figure 2.17 shows a model of the biological responses of animals to stress (Moberg & Mench, 2000).

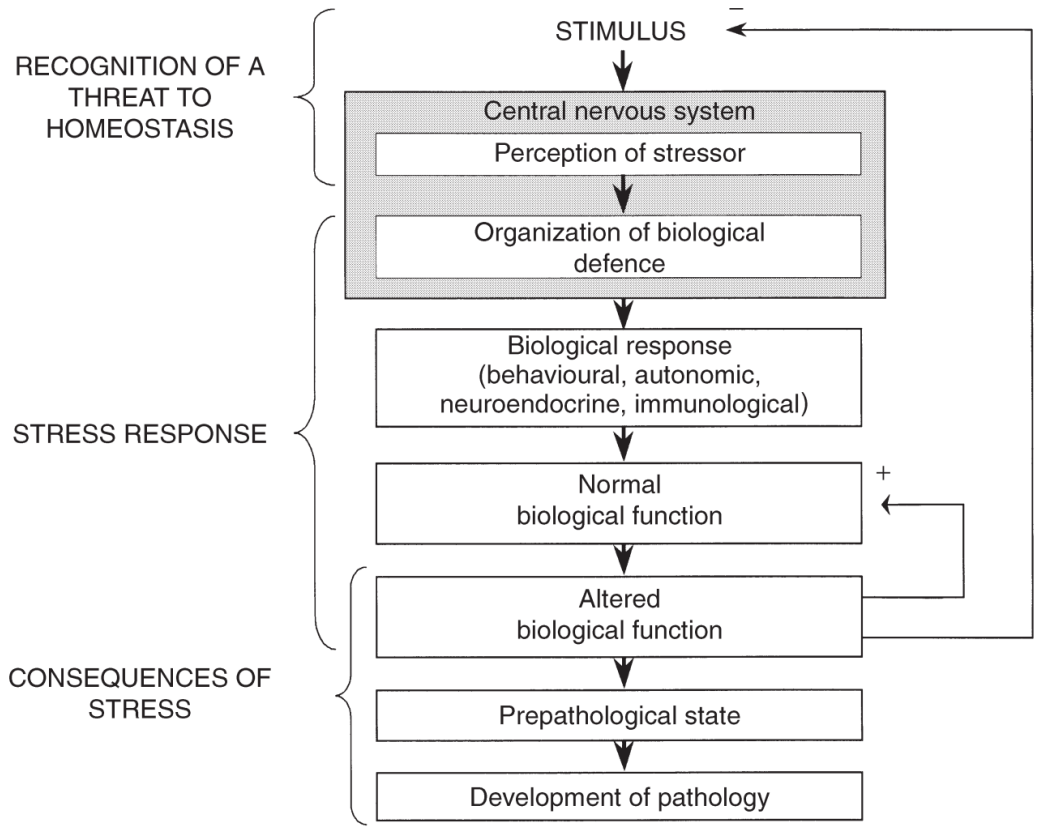

Figure 2.17 A model of biological responses of animals to stress (Reprinted from *The Biology of Animal Stress: Basic Principles and Implication for Animal Welfare* (pp. 4) by G. P. Moberg & J. A. Mench, 2000, UK, Cambridge Press. Copyright 2000 by CAB International).

Theoretically, stress can be measured across the four response systems; however, it's difficult to use behaviour response as a standard means for stress measurement due to individual differences and lack of understanding of animal behaviour during stress. The autonomic nervous system affects diverse biological systems, resulting in a change of heart rate, blood pressure, and gastrointestinal activity; however, it's difficult to monitor stress in acute experiments because autonomic responses are generated within a relatively short duration. During stress, the central nervous system will stimulate endocrine glands to release hormones as signals, which is called neuroendocrine response. It is invasive and difficult to directly detect cerebral hormone levels; however, the immune system,

which is modulated by stress-responsive central nervous systems (especially the hypothalamic-pituitary-adrenal (HPA) axis) (Matteri, Carroll, & Dyer, 2000), provides a way to measure stress. Specifically, when a stressor occurs, the HPA axis releases a hormone called "gulcocorticoid" into blood. Metabolites of gulcocorticoids are voided via feces and urine (Figure 2.18) (Möstl & Palme, 2002).

CORT is a type of the secreted gulcocorticoids, and is potent in rodents (Joëls & Karst, 2012). Stress levels can be studied by measuring CORT concentrations in the blood (R. V. L. Contarteze et al., 2008; R. Contarteze et al., 2008) or by measuring various gulcocorticoid metabolites in urine or feces (Pappano, Roberts, & Beehner, 2010; Palme, 2005). Plasma CORT concentration is widely used as a biomarker of stress in animal models (Ke et al., 2011; R. V. L. Contarteze et al., 2008). Serum CORT concentration in rats after treadmill exercise displays more sensitivity compared to other stress biomarkers, such as adrenal gland cholesterol and ascorbic acid (R. Contarteze et al., 2008). Overloaded and forced exercise is an intensive stressor in post-stroke physical training in stroke rats, and is associated with higher blood CORT levels (Ke et al., 2011).

2.5.3 SUMMARY OF CHEMICAL EVALUATIONS

BDNF levels in the brain can indicate neural recovery in animal studies of stroke rehabilitation. Levels of brain BDNF may be associated with motor function recovery induced by post-stroke treadmill training. BDNF levels in brain tissues, thus, can be adopted as a key parameter in determining the effect of post-stroke rehabilitation interventions.

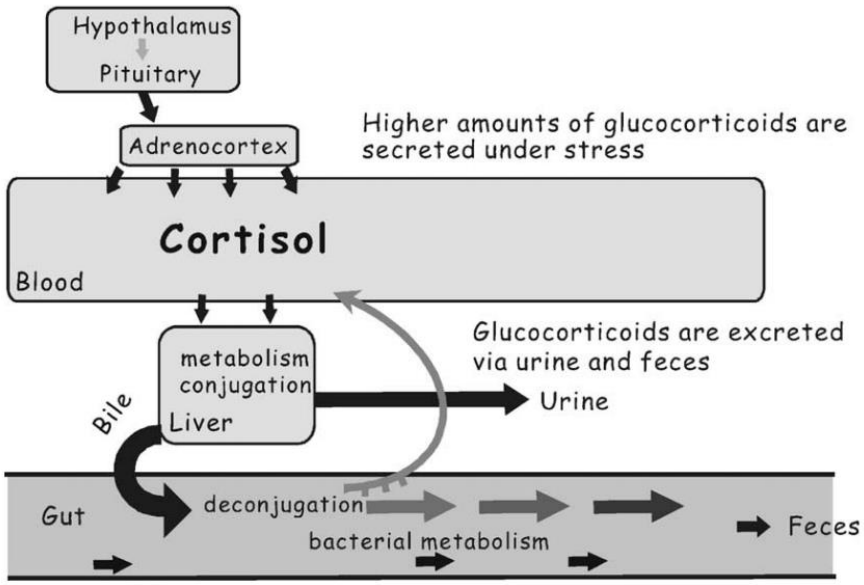

Figure 2.18 Scheme of the secretion, metabolism, and excretion of glucocorticoids (Möstl & Palme, 2002).

Plasma CORT level is also a reliable indicator of stress induced by exercise. High-speed treadmill training can induce high CORT levels in a stroke rat model (Soya et al., 2007); however, stress endurance could also be enhanced by exercise (Steiner et al., 2011). Thus, the relationship between treadmill training and stress needs further investigation. Stress-induced CORT upregulation is indicative of suppressed of BDNF synthesis in the hippocampus (Schaaf, De Jong, De Kloet, & Vreugdenhil, 1998; Castrén, Võikar, & Rantamäki, 2007); therefore, the correlation between stress and neural rehabilitation might be achieved by comparing plasma CORT levels and brain BDNF levels.

CHAPTER 3 METHODOLOGY

3.1 STRUCTURE OF THE STUDY

This study consists of two experiments: a pilot study and a training intensity study. The pilot study was designed to evaluate neural and motor function assessments, and to investigate the effects of treadmill training on motor function recovery, brain infarct areas, and stress levels in the subacute stroke phase by using a focal ischemic stroke rat model. Appropriate assessment methodologies for post-stroke deficits would be selected after comparison. The training intensity study was designed to explore the effects of treadmill training intensity on rehabilitation outcomes in subacute stroke phase by using a focal ischemic stroke rat model. Outcomes from this pilot study have been published through an international conference paper (Sun et al., 2013), while a paper (Sun et al., 2014) related to the effects of training intensity has been published in a Science Citation Index journal "*BioMed Research International*" and is currently under review. Structure of the overall study is shown in Figure 3.1.

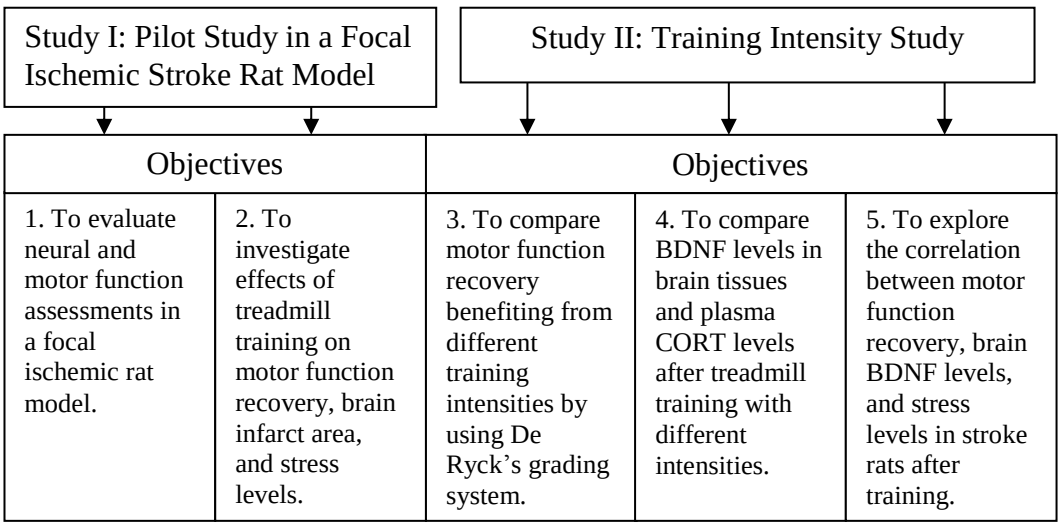

Figure 3.1 Structure of the overall study.

3.2 ETHICAL CONSIDERATION

All procedures were approved by the "Animals Subject Ethics Sub-committee" of The Hong Kong Polytechnic University and conformed to the guidelines on the care and ethical use on experimental animals (Olfert, Cross, & Mcwilliam, 1993). Animal license has been granted from the Department of Health before this study (license No: Rev (12-24) in DH/HA&P/8/2/4 Pt.5) (Appendix 1).

3.3 STUDY I: PILOT STUDY IN A FOCAL ISCHEMIC STROKE RAT MODEL

The pilot study was performed to evaluate neural and motor function assessment tools, and to investigate the effects of treadmill training on motor function recovery, brain infarct areas, and stress levels in the subacute stroke phase by using a focal ischemic stroke rat model. First, motor function assessments included neurological deficit assessment, De Ryck's test, and beam walking test. Effective and appropriate tests were chosen after comparing these motor function tests. Second, the effects of treadmill training on motor recovery, brain infarct area, and stress levels were measured after a 7-day treadmill training intervention 24 hours after brain ischemia.

Forty male Sprague-Dawley (SD) rats (between 2 and 3 months) weighing 280-320g were used in this pilot study. Rats were provided by the Central Animal Facility of The Hong Kong Polytechnic University, and fed individually in standard cages and in a standard room with controlled environment (20 – 22℃; 12-hour day/night cycle). Rats freely accessed to food and water. All procedures

and treadmill training interventions were completed between 9:00 am and 13:00 pm to minimize the effect of the circadian clock on the rodent's behaviors.

Detailed procedures in this pilot study were shown in Figure 3.2. Briefly, rats were trained on a 3-day accommodation to a treadmill (KN-73, Natsume Ltd., Japan). Thirty-seven rats passed screening requirements (section 3.3.1) and received MCAo/r surgery (section 3.3.2). Twenty-four rats were successfully induced with stroke via Longa's grading system of neurological deficits (Longa et al., 1989) (section 3.3.4.1) 24 hours after brain ischemia, and then equally and randomly assigned into two groups: control (Con) and treadmill training (TG). Rats in each group received daily neurological assessment (Longa's test), motor function (De Ryck's test; section 3.3.4.2), and beam walking (Jolkkonen's test; section 3.3.4.3) tests that started at 24 hours after MCAo/r surgery and lasted for 7 consecutive days. Scores of the three tests were analysed to evaluate their sensitivity for accurately detecting functional improvement. TG rats experienced treadmill training treatment (section 3.3.3) over this experimental period, while Con rats were fed in standard cages individually. Immediately after the last intervention, rats were decapitated under deep anesthesia to obtain trunk blood and harvest the brain for plasma CORT measurement (section 3.3.6) and TTC staining (section 3.3.5), respectively. Motor function recovery, brain infarct area, and stress levels were compared to investigate the effects of treadmill training in the subacute stroke phase.

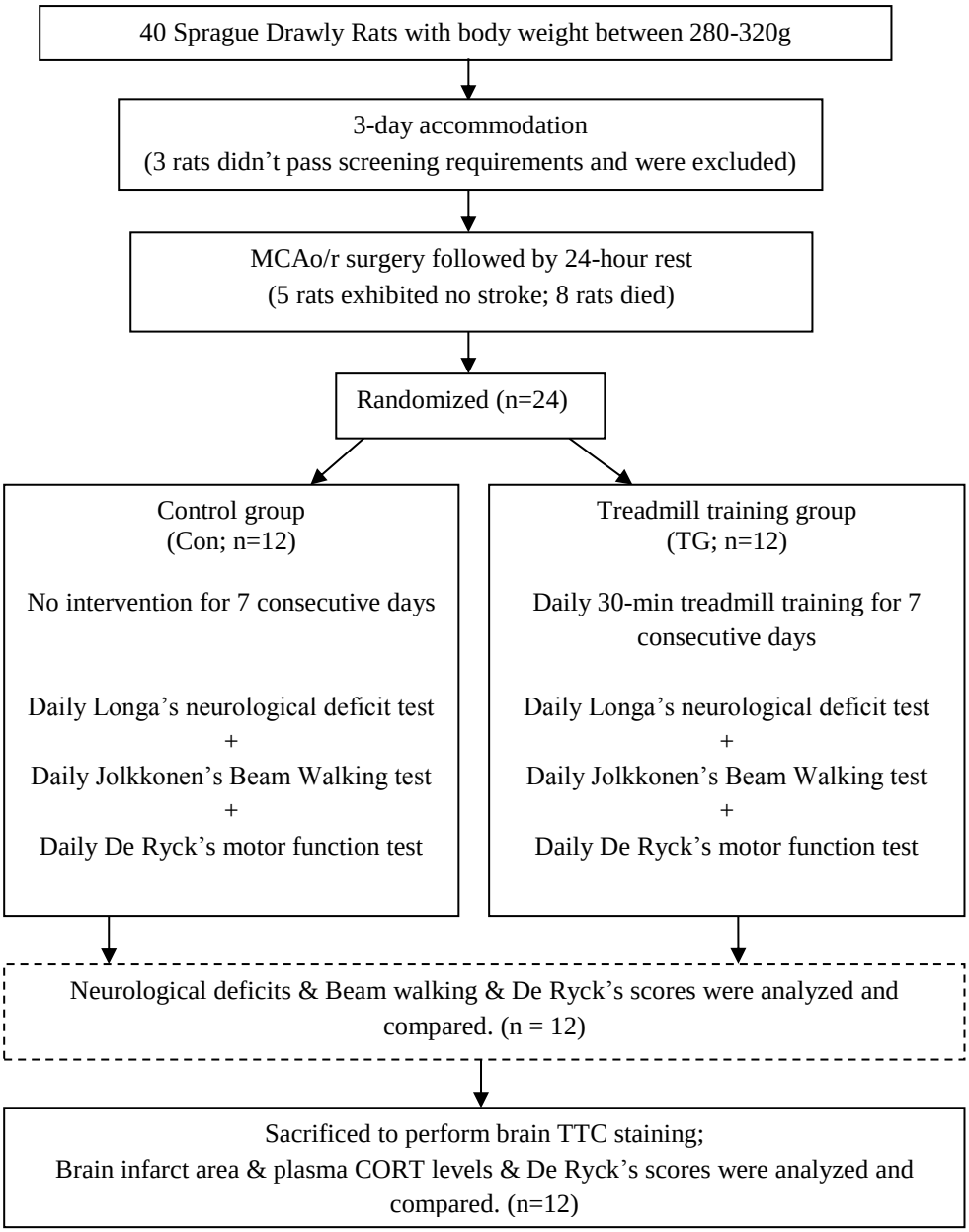

Figure 3.2 Detailed procedures of the pilot study.

3.3.1 ACCOMMODATION INTERVENTION

Rats contributing to this study were trained on a treadmill (KN-73, Natsume Ltd., Japan; Figure 3.3) for three consecutive days to become accustomed to treadmill exercises. This was aimed to minimize the stress rats would meet when placed in a new situation and to exclude rats unable to run on the treadmill over the 3 training days (Ke, 2012). The treadmill was placed in a horizontal position with

no slope. Daily training program included 3 sections with 10-minute rest between each two sections, and each section lasted for 10 minutes (Nomura et al., 2005). Treadmill running speed for each training section over the 3 days was shown in Figure 3.4. Rats with occasional stops during running would receive a gentle nudge to help their running (Ke, 2012). Rats would be excluded if they could not complete all these training sections even with a nudge help. After accommodation, 3 rats did not meet the screening requirements and were excluded. The pass rate was around 92% in this pilot study.

Figure 3.3 Treadmill (KN-73, Natsume Ltd., Japan) used in the study with a speed adjustment from 0 to 30 m/min.

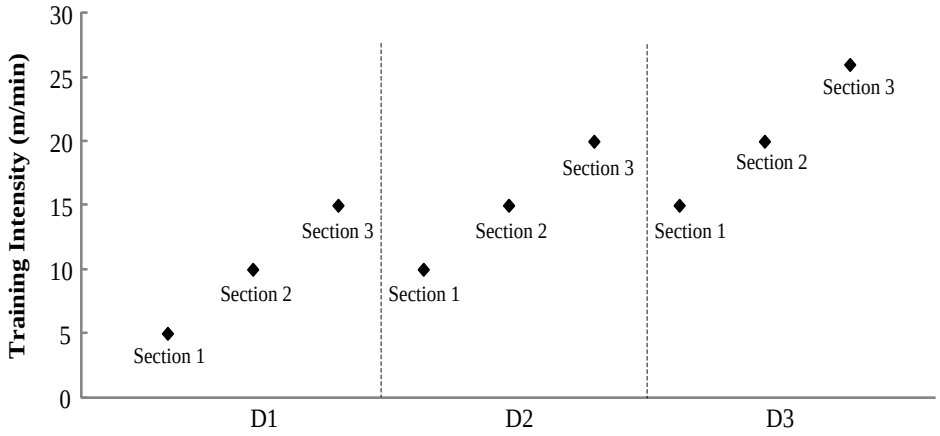

Figure 3.4 Treadmill speed setup of each section during 3-day accommodation. Each section lasted for 10 minutes with 10-minute rest between two sections.

3.3.2 MIDDLE CEREBRAL ARTERY OCCLUSION/REPERFUSION (MCAO/R) SURGERY

The MCAo/r surgery induced focal ischemic stroke rat model by Koizumi et al. (1986) was employed in this study. Surgery mimicked practices by Ke et al. (2011). Briefly, rats in all groups were anesthetized with 10% chloral hydrate (0.4 mg/kg for induction and 0.02 mg subsequently). Incisions were made at the neck midline to expose the common carotid artery (CCA), and then the external carotid artery was ligated. Subsequently, a commercial filament with a tip diameter of 0.39 ± 0.02 mm (Beijing Sunbio Biotech, China) was inserted into the CCA and advanced along the internal carotid artery until the tip of the filament reached the middle cerebral artery. Occlusion lasted for 60 minutes after which the filament was then withdrawn to allow reperfusion. Twenty-four hours after MCAo/r surgery, rats were examined for neurological deficits by using Longa's test (Longa et al., 1989). After surgery, eight rats died, and five exhibited no observable neurological deficits and were excluded. The rest 24 rats were induced with successful stroke with neurological scores between 1 and 3. The success rate of MCAo/r surgery was around 65%, while the mortality rate was around 22%. Brain damaged area after the MCAo/r surgery involves ipsilateral striatum and sensorimotor cortex, similar to previous study (Figure 3.5) (Ke et al., 2011).

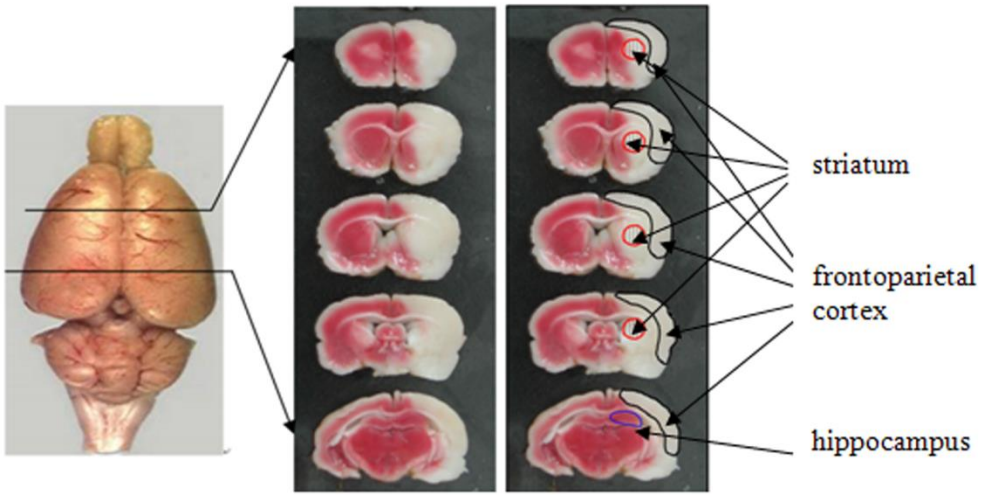

Figure 3.5 TTC stained brain slices shows the damaged brain regions including ipsilateral striatum and sensorimotor cortex. Red and white colors indicate intact and dead brain tissues, respectively (Reprinted from Z. Ke et al., 2011, *PLoS ONE, 6*, e16643, pp. 4. Copyright 2011 by Ke et al.).

3.3.3 TREADMILL TRAINING INTERVENTION

Rats in the treadmill training group obtained daily training intervention at the velocity of 25 m/min for 30 minutes with the same rest regimen as in the accommodation protocol (section 3.3.1). The intervention started at 24 hours after MCAo/r surgery and lasted for 7 consecutive days, as early treadmill training after stroke showed better motor function recovery (Ke et al., 2011; Yang et al., 2003); moreover, motor function recovery may reach a relative platform after 7 consecutive intervention days, based on previous studies (Chen, et al., 2012; Ke et al., 2011).

3.3.4 EVALUATION OF POST-STROKE FUNCTIONAL ASSESSMENTS

After reviewing commonly used assessment tools (table 2.8) for post-stroke functional evaluation, Longa's grading system for neurological deficits

assessment, Jolkkonen's grading system for beam walking assessment, and De Ryck's tasks for locomotor activity assessment might be appropriate for this study and were investigated to evaluate their sensitivity to outcomes of treadmill treatment. Appropirate assessment tools would be selected for the following studies.

3.3.4.1 NEUROLOGICAL DEFICIT ASSESSMENT

Longa's grading system (Longa et al., 1989) for neurological deficit assessment was adopted for this pilot study. Detailed description of grading is shown in Table 3.1. Using this grading system, 0 points indicate that there is no neurological deficit, while 4 points indicate a severe deficit. In the pilot study, all rats surviving the MCAo/r surgery received this neurological assessment at 24 hours after ischemia. Rats displaying no neurological deficit were excluded. To evaluate whether this grading system was appropriate for assessment over the 7 consecutive intervention days, rats from each group (Con and TG) received daily assessment until the end of the intervention period by a skilled researcher blind to group assignment. Each assessment was repeated 3 times, and the mean value was recorded as the daily neurological score. Neurological scores over intervention period were compared between Con and TG rats.

Table 3.1 Assessments of Neurological Deficits (Longa et al., 1989).

Grade	Neurological deficits
0	No neurological deficit
1	Failure to extend the impaired forepaw fully
2	Circling to the impaired side
3	Falling to the impaired side
4	Do not walk spontaneously and have a depressed level of consciousness

3.3.4.2 DE RYCK'S MOTOR FUNCTION ASSESSMENT

De Ryck's tasks for locomotor function assessment (De Ryck et al., 1989) was employed due to its no stress, convenience, less labor, sensitivity, and preciseness. The detailed description of this grading system is shown in section 2.3.2.7. The testing procedures are shown in Figure 3.6. Briefly, rats must complete 8 tasks. Among the 8 tasks, six are used to test the forelimb function, while the rest assess hindlimb function. Each task has 3 levels of scores: 0, 1, and 2. 0 points indicate complete inability, while 1 point indicates incomplete or delayed (> 2s) placement. 2 points indicate that the affected limbs can accomplish a task as well as the unaffected limbs. A normal rat has a maximum score of 16, while a stroke rat may have a score between 0 and 16. In this pilot study, rats in both Con and TG groups received daily motor function tests via De Ryck's tasks over the 7-day intervention period. Each test was performed 3 times daily by a skilled researcher blind to group assignment. Mean values were recorded as motor function scores. Daily motor function scores were then analyzed to evaluate the sensitivity of the De Ryck's test and to compare motor recovery between groups.

3.3.4.3 BEAM WALKING TEST

Jolkkonen's beam walking test (Jolkkonen et al., 2000) was adopted for this pilot study. The apparatus consisted of a beam (2 cm wide * 130 cm long) and a home cage being connected with the beam at one end, and was elevated horizontally 110 cm above the ground. Foot slips were counted when a rat traversed the beam from one end to the home cage. The scoring system includes 7 levels of scores ranging from 0 to 6 (Table 2.6); 0 points indicate that a rat is unable to keep

balance and falls down from the beam, while 6 points indicate that a rat can traverse the beam normally. In this pilot study, rats from each group (Con and TG) received daily assessment over the 7-day intervention period by a skilled researcher blind to group assignments. Each test was repeated 3 times. Mean values were then analyzed to evaluate the sensitivity of this beam walking test.

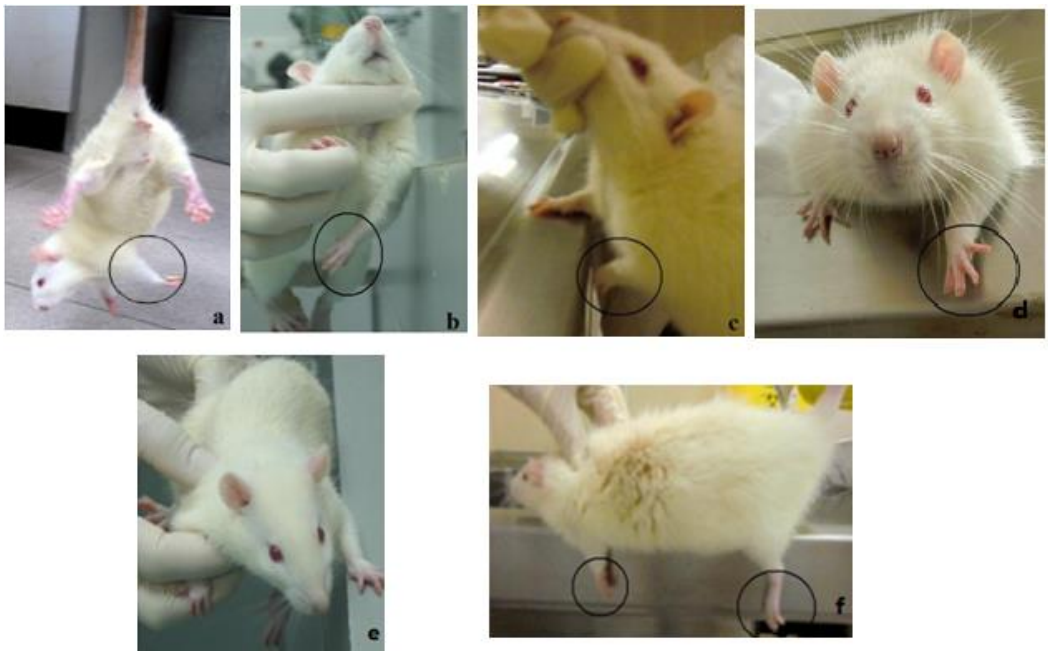

Figure 3.6 De Ryck's tasks for locomotor function assessment. A stroke rat was A) suspended 10 cm over a table with the affected forelimb unable to stretch; B) placed in front of a table edge with the affected forelimb unable to be placed on the table; C) prohibited from seeing or touching the table via vibrissae by gently raising its head up to a 45-angle, with the affected forelimb unable to land on the table; D) placed on a table edge and gently pushed from the back towards the edge, showing no gripping of the affected forelimb; E) placed towards a table with affected limbs just over the edge, showing placement of both the affected forelimb and hindlimb; F) positioned along a table edge, and gently pushed towards table edge, showing no gripping of both affected forelimb and hindlimb.

3.3.5 TTC STAINING

Rats were sacrificed after the last intervention via decapitation under deep anesthesia. The brain was immediately and carefully extracted from the skull,

and then cut into 2 mm coronal sections by using a brain matrix (RBM-4000C; ASI Instruments Inc., Houston, Texas, USA; Figure 3.7A). Brain slices were immersed in phosphate buffered 2% TTC (Sigma-Aldrich Co., St. Louis, Missouri, USA) solution at room temperature for 30 minutes, followed by fixation with 10% formaldehyde solution. Fixed brain sections were digitally photographed via a camera. Brain infarct size was then offline calculated by using freeware (ImageJ, National Institutes of Health). Figure 3.7B shows an example of TTC stained brain slices from a stroke rat in this pilot study. The infarct brain tissues (appearing white) were apparently distinguished from the intact tissues (appearing red).

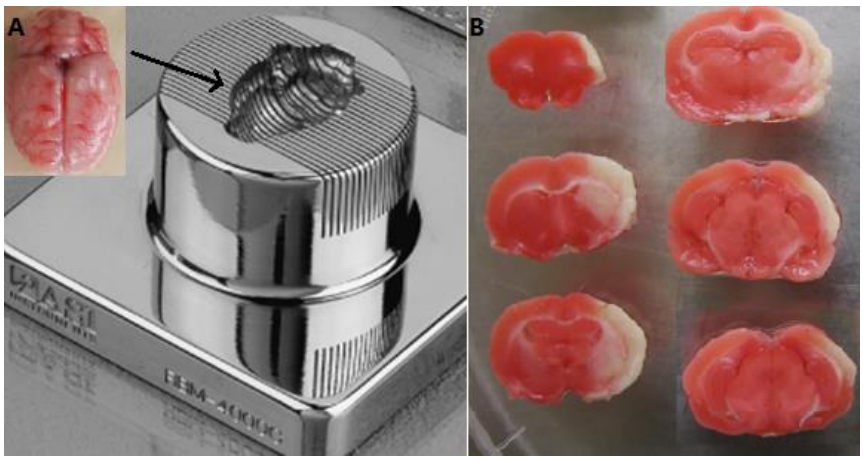

Figure 3.7 A brain matrix (A) (RBM-4000C; ASI Instruments Inc., Houston, Texas, USA) and an example of TTC stained brain slices of a stroke rat (B). Red color: intact tissue; white color: infarct tissue.

3.3.6 PLASMA CORTICOSTERONE (CORT) DETECTION

Plasma CORT concentrations were measured by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Hayes et al., 2008; Ke et al., 2011; Quiri é et al., 2012; Hawley, Morch, Christie, & Leasure, 2012). Rats were anesthetized within two hours after the last intervention and then sacrificed via

decapitation. Trunk blood was immediately collected and centrifuged to acquire plasma. Plasma CORT concentrations were then quantified via CORT EIA Kit (Cayman, USA) based on manufacturer's instructions, followed by comparison between plasma CORT levels of Con and TG rats via one-way analysis of variance (ANOVA) method.

3.3.7 STATISTICAL ANALYSIS

All results were expressed as means ± standard deviations. SPSS (IBM, version 20) was used for data analysis and the level of statistical significance was set at $p = 0.05$. Two-way repeated measures ANOVA with baseline as covariate was used to compare 7-day neurological deficit scores, De Ryck's motor function scores, and beam walking scores between Con and TG rats, respectively. One-way ANOVA test was used to compare plasma CORT levels and infarct volumes between the two groups, respectively.

3.4 STUDY II: EFFECTS OF TREADMILL TRAINING WITH DIFFERENT INTENSITIES ON MOTOR FUNCTION RECOVERY AND NEUROREHABILITATION

This study was designed to explore the effects of treadmill training intensity on rehabilitation outcomes including motor function recovery and neurorehabilitation in the subacute stroke phase. Daily motor function was assessed using De Ryck's test, and neurorehabilitation outcome was determined by measuring brain BDNF levels. Stress levels induced by treadmill training with different intensities were also determined by measuring plasma CORT levels.

Thus, in this study, motor function recovery, brain BDNF levels, and stress levels were assessed and potential relationships among these parameters were subsequently assessed. A paper (Sun et al., 2014) of these studies has been published on *BioMed Research International*, a Science Citation Index journal.

This study totally utilized 94 male SD rats (between 2 and 3 months; 280-350g) provided by the Central Animal Facilities of The Hong Kong Polytechnic University. Rats were fed individually in standard cages and in a standard room with controlled environment (20 – 22℃; 12-hour day/night cycle). Rats had free access to food and drink. The study consisted of two continuous parts (Figure 3.8). First, 20 rats were used to estimate the minimal sample size to reach significant differences in motor function recovery across different training protocols. The procedures are shown in Figure 3.9A. Another 74 rats were then recruited based on the minimal sample size, and were also assessed using the same procedures as the previous 20 rats (Figure 3.9B). Rehabilitation outcomes of all rats were analyzed to compare motor function recovery, brain BDNF levels, and plasma CORT levels across groups.

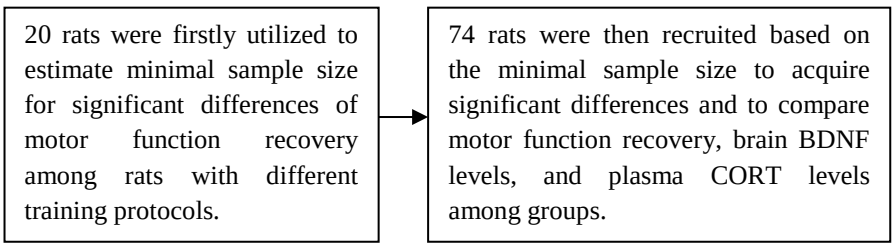

Figure 3.8 Experimental setup in Study II.

This study designated stroke rats into four groups: control (CG), low training intensity (LG), gradually increasing training intensity (GIG), and high training

intensity (HG). The detailed procedures are shown in Figure 3.9. Briefly, rats received accommodation training in the same manner as the pilot study (section 3.3.1). Rats passing initial training received MCAo/r surgery (section 3.3.2). Neurological deficits were assessed at 24 hours after surgery using Longa's test. Rats without observable signs of stroke were excluded, while the remaining stroke rats were randomly assigned into the 4 groups (CG, LG, GIG, and HG). Rats in CG were fed in standard cages for one week, while the rest underwent daily treadmill training with different training intensities. LG rats were allowed to run on the treadmill at a velocity of 5 m/min, while HG rats ran at 26 m/min with the same training and rest regimens as in the pilot study. Rats in GIG ran from 5 m/min on the 1st day (D1) up to 26 m/min on the last intervention day (D7). Daily motor function scores were assessed using De Ryck's test. The scoring system was described section 3.3.4.2. On the last intervention day, rats were anesthetized and sacrificed via decapitation within two hours after the last intervention. Trunk blood and brain tissues from the hippocampus, striatum, and sensorimotor cortex were collected. Trunk blood samples were immediately centrifuged to acquire plasma. Brain tissue samples were processed according to a standard BDNF sample preparation protocol (Promega, USA). Plasma and brain tissue samples were used for CORT and BDNF detection, respectively.

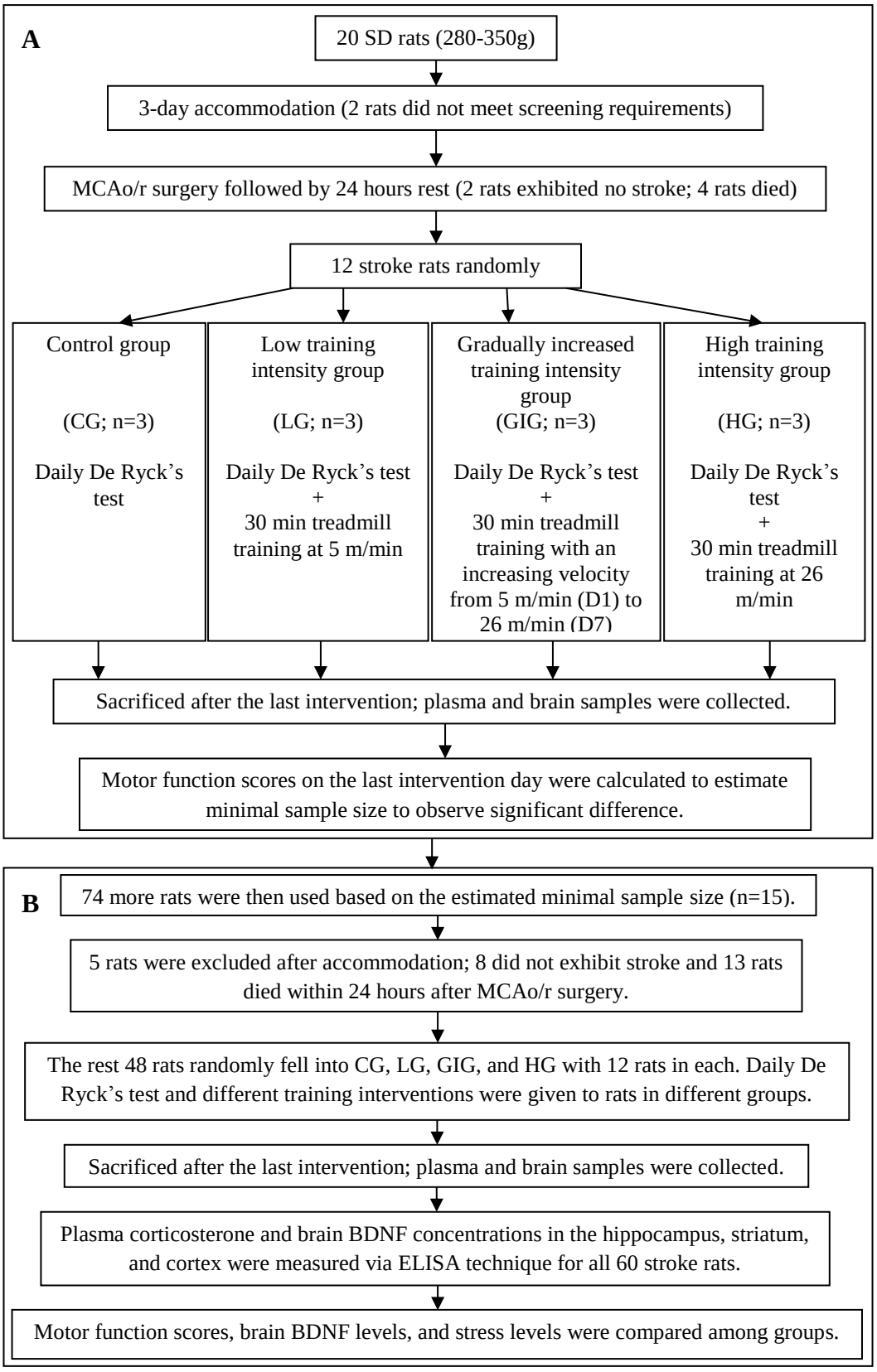

Figure 3.9 Flowchart of experimental procedures in Study II. A) 20 rats were firstly used to estimate the minimal sample size to acquire significant differences of motor function recovery among groups; B) another 74 rats were then recruited based on the minimal sample size.

3.4.1 MINIMAL SAMPLE SIZE ESTIMATION

Twenty rats were employed in order to estimate the minimal sample size needed to achieve significant differences in motor function recovery among the four groups (CG, LG, GIG, and HG). Two rats were excluded after initial training (section 3.3.1), while the other 18 rats received the same MCAo/r surgery as in the pilot study (section 3.3.2). Four rats did not survive the surgery within 24 hours, while two rats exhibited no stroke symptoms and were excluded. Twelve rats were successfully induced with stroke (as determined by using Longa's test (Longa et al., 1989) at 24 hours after the surgery), and were then randomly assigned into the four groups (3 rats per group). The LG, GIG, and HG rats were daily trained with different intensities (section 3.4.2). CG rats were fed individually in standard cages. Daily motor function level was assessed via a skilled researcher blind to group assignments using De Ryck's test. Each measurement was repeated 3 times, and the averages of motor function scores were recorded. After 7 intervention days, rats were sacrificed by decapitation under deep anesthesia within two hours after the last intervention. Plasma samples and brain tissue samples (hippocampus, striatum, and sensorimotor cortex) were then collected and saved for further assessments. Motor function scores over the 7-day intervention period were analyzed, and it was determined that a minimal sample size of 15 was needed to acquire significant differences in motor function recovery across the four groups.

3.4.2 INTENSITY SETUP OF TREADMILL INTERVENTIONS

Different training intensities bring different stress levels to rats (Ke et al., 2011). Velocity is a determining factor in intensity and workload. Different velocities

generated different training intensities. In previous studies, training intensities were mainly set from 2 m/min to 30 m/min and the daily training time length was mainly set at 30 minutes (Hayes et al., 2008; Wang et al., 2001; Shimada et al., 2013). In this study, total training time was the same 30 minutes, and 5 m/min and 26 m/min were chosen as low and high treadmill training velocities, respectively. In LG and HG, rats ran at a constant velocity through 7 days of training at 5 m/min and 26 m/min, respectively. Rats were generally weak at the first several days after stroke but could spontaneously recover (Ke et al., 2011), gaining better motor function with time (Matsuda et al., 2011; Lee et al., 2009). Therefore, this study designated a rat group to gradually increasing intensity from low speed (5 m/min) on the first day to high speed (26 m/min) on the last day. Velocity increased slowly in GIG the first four days, and for the following three, it increased relatively faster. The training set-up for all groups is shown in Figure 3.10.

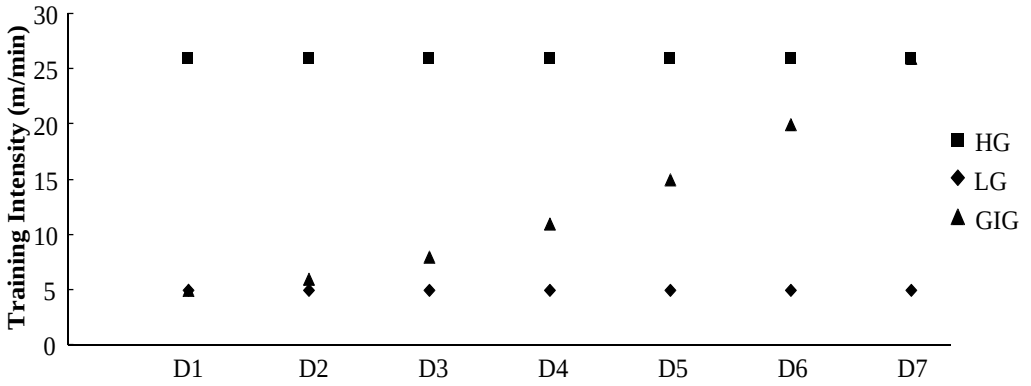

Figure 3.10 Intensities of daily treadmill training started one day after stroke for 7 consecutive intervention days in Study II. HG: high intensity group; LG: low intensity group; GIG: gradually increased intensity group. D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7).

3.4.3 MOTOR FUNCTION TEST

Motor function improvement was evaluated by conducting the De Ryck test (De Ryck et al., 1989) on daily basis throughout the 7-day intervention. Detailed description of the De Ryck's tasks was given in section 3.3.4.2. Briefly, a rat after MCAo/r surgery induced stroke would have a motor function score ranging from 0 (maximum deficit) to 16 (no deficit). A skilled researcher blind to group assignments helped accomplish all the motor function tests over experimental period. Each measurement repeated 3 times, and averages of motor function scores were recorded for statistical analysis.

3.4.4 BRAIN BDNF AND PLASMA CORT DETECTION

Brain BDNF and plasma CORT were evaluated using an enzyme-linked immunosorbent assay (ELISA; protocol in Appendix 2) (Hayes et al., 2008; Ke et al., 2011; Quiri é et al., 2012; Hawley et al., 2012). All rats were deeply anesthetized within two hours after the last intervention and sacrificed via decapitation. Trunk blood was immediately collected and centrifuged to obtain plasma. The brain was carefully extracted from the skull, and brain tissues including hippocampus, striatum, and affected sensorimotor cortex were then obtained immediately. BDNF Emax ® ImmunoAssay System (Promega, USA) was used to measure BDNF concentrations. Plasma CORT concentrations were quantified via Cayman's CORT EIA Kit (Cayman, USA).

3.4.5 STATISTICAL ANALYSIS

All results were expressed as means ± standard deviations. SPSS (IBM, version 20) was used for data analysis and the level of statistical significance was set at $p = 0.05$. A power analysis with effect size (alpha = 0.05 and power = 0.80) was conducted to estimate the minimal sample size necessary to observe a significant difference in motor function scores between each two groups by using G*Power (version 3.1.7). Power is recommended at 0.8 by Cohen (1988), referring to the probability that a statistical test will reject the null hypothesis when the null hypothesis is actually false. The null hypothesis in this study is that the intervention treatments exhibit no effect on experimental rats. If the interventions in this study did affect variables like motor function scores (the null hypothesis is false) and experimental results also showed the differences, the probability of this incidence was 0.8. When sample size becomes larger, power increases. If the null hypothesis is false and the results support it, the probability of this incidence is called type II error.

Intention-to-treat was used for any rat that died during the intervention period. It means that scores on the rest intervention days after rats' deaths are the same as on the last day before deaths. Two-way repeated measures analysis of variance (ANOVA) with baseline as covariate was used to compare motor function scores among groups. Time and group were set as within-subject and between-subject factors, respectively. One-way ANOVA test was used to compare motor function scores at each of the 7 time points over experimental period if significant difference among groups and no interaction of time and group was observed after 2-way repeated measures ANOVA, and also to compare CORT and BDNF levels.

The Bonferroni post-hoc test was employed if a significant difference was observed after the ANOVA analysis.

CHAPTER 4 RESULTS

4.1 STUDY I: PILOT STUDY IN A FOCAL ISCHEMIC STROKE RAT MODEL

This pilot study was designed to evaluate neural and motor assessment tools, and to investigate the effects of treadmill training on brain infarct area and stress levels in subacute stroke phase by using a focal ischemic stroke rat model. First, neural and motor function assessments were evaluated, including Longa's test, De Ryck's test, and beam walking test (section 4.1.2). Second, the effects of treadmill training on motor recovery, brain infarct area, and stress levels were assessed (section 4.1.3).

4.1.1 ACCOMMODATION & MCAO/R SURGERY

Forty male SD rats (between 2 and 3 months; 280-320g) were used in this pilot study. Three rats did not meet the screening requirements (did not run on the treadmill even with a gentle nudge over the 3-day accommodation training) and were excluded after accommodation. The pass rate of accommodation was around 93%. The rest 37 rats received MCAo/r surgery. Among the 37 rats, 8 rats died within one day after surgery, while 5 did not exhibit observable stroke determined by testing neurological deficits at 24 hours after surgery. The mortality rate of MCAo/r surgery was approximately 22%, while no-stroke rate was approximately 13%. Twenty-four rats were induced with stroke and the success rate of MCAo/r surgery in this pilot study was approximately 65%, which was consistent with previous studies (Ke, 2012; Belayev, Alonso, Busto,

Zhao, & Ginsberg, 1996). Table 4.1 provided an overview of accommodation and MCAo/r surgery.

Table 4.1 An Overview of Accommodation and MCAo/r Surgery.

rats amount	After accommodation training	After MCAo/r surgery
40	37 (pass rate around 93%)	5 (no stroke rate around 13%) 8 (died; mortality rate around 22%) 24 (stroke; success rate around 65%)
	3 (drop-out rate around 7%)	

4.1.2 EVALUATIONS OF NEURAL AND MOTOR FUNCTION ASSESSMENTS

To evaluate neural and motor function assessments, stroke rats from each Con and TG group received assessments of neurological deficits using Longa's test, motor function using De Ryck's test, and beam walking function using Jolkkonen's test over the 7-day intervention.

4.1.2.1 LONGA'S TEST

The Longa's test was adopted to assess neurological deficits induced by MCAo/r surgery. Neurological deficits of all experimental rats in this pilot study were assessed on the first intervention day to verify the success of MCAo/r surgery. Five out of 40 rats exhibited a score of 0, indicating no symptoms of stroke. Twenty-four rats achieved a score of 1 and 2, indicating successful stroke modeling.

Changes in neurological deficits of both Con (n=12) and TG (n=12) groups over the 7-day intervention were shown in Figure 4.1. Both Con and TG rats exhibited

apparent stroke symptoms with a mean neurological score of 1.5 ± 0.6 on the first intervention day (D1). Neurological scores spontaneously recovered to point 1 (failure to fully extend the impaired forepaw) for Con rats at the 2nd day (D2), followed by a stable level until the 5th intervention day (D5). Con rats showed a small amount of recovery with scores of 0.75 ± 0.5 on the 6th day (D6), and maintained the same level on the last intervention day (D7). TG rats showed the same trend and level of neurological deficits over the 7-day intervention except for the last two intervention days. On D6 and D7, TG rats showed a lower mean score of 0.5 ± 0.6, indicating improved but not significant neural recovery.

Longa's grading system indicates four levels of stroke; however, most rats surviving the MCAo/r surgery for at least 24 hours and with apparent motor disability only exhibited levels 1 and 2. Rats that survived the MCAo/r surgery but died within 24 hours may have exhibited greater levels of neurological deficits: totally falling to affected body side (level 3) or loss of consciousness (level 4). Thus, rats with moderate neurological deficits mainly could survive stroke, but were limited within 2 levels. The limited scoring scale possibly narrowed the difference between spontaneous and training-induced neural recovery evaluated by Longa's test.

In conclusion, Longa's grading system for neurological deficit assessment can be used to verify the success of stroke modeling; however, it might not be suitable to effectively differentiate spontaneous neural recovery from treadmill training-induced recovery in the subacute stroke phase.

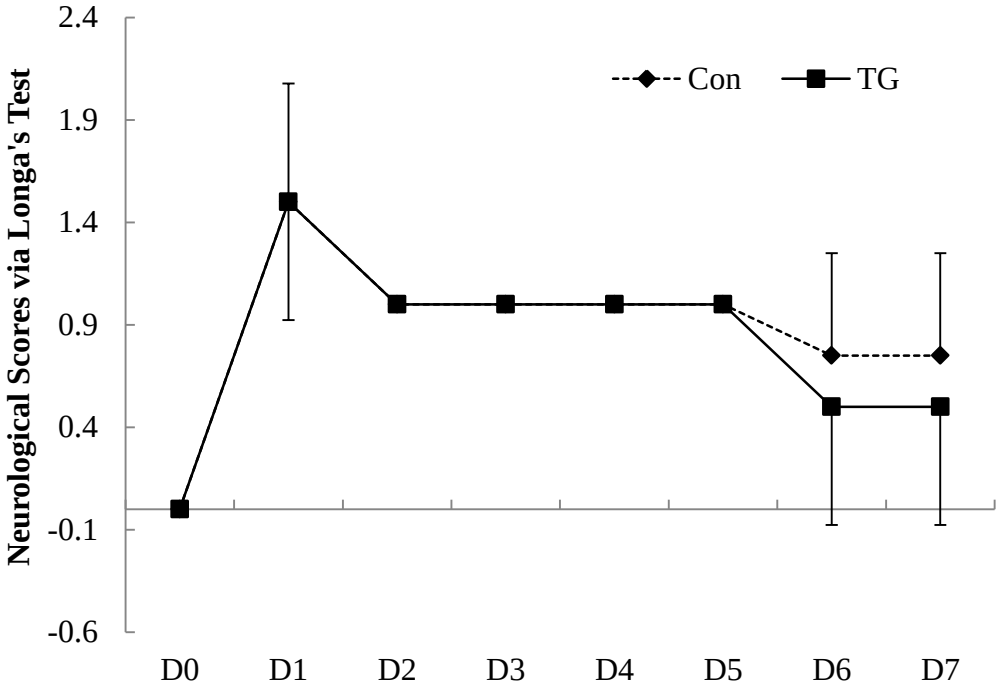

Figure 4.1 Changes in neurological deficits over the 7-day intervention in the pilot study. Con: control group; TG: treadmill training group; D0: right before the MCAo/r surgery; D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7).

4.1.2.2 DE RYCK'S TEST

De Ryck's test was employed to monitor motor function changes over the 7-day intervention. Normal rats exhibit motor function scores of 16, while disabled rats with stroke fall into the scale of points 0 to 16.

Figure 4.2 shows the changes in motor function over the 7 intervention days. All rats partially lost their motor function with scores of 5.4 ± 1.2 on the first day after stroke. Both Con (n=12) and TG (n=12) rats stably recovered their motor function with time; however, rats with daily treadmill training significantly (p<0.05) regained motor function compared to rats without training. Motor function scores from De Ryck's test could more accurately distinguish spontaneous recovery from training-induced recovery over the 7-day intervention

in subacute stroke phase. Advantages of De Ryck's test may include the follwoing: 1) a larger scale which may detect minimal changes; 2) a main focus on sensorimotor function of the forelimbs. Since forelimbs are responsible for more meticulous activities than hindlimbs, there is a larger brain area controlling forelimb function. Through comparing brain functional diagrams (Paxinos & Watson, 2007) with the TTC staining brain sections (Figure 4.3), brain regions controlling the sensory function of left forelimb were affected more than of left hindlimb by the right MCAo/r surgery.

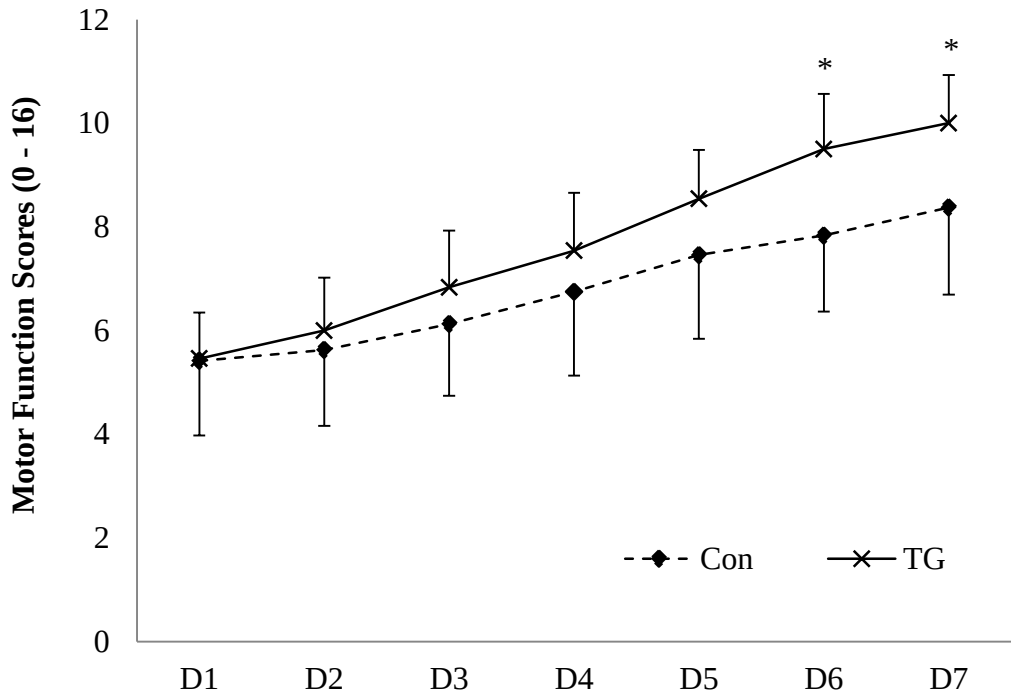

Figure 4.2 Changes of motor function in both control (Con, n=12) and treadmill training (TG, n=12) groups by using De Ryck's test over the 7-day intervention. D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7). *: significant difference observed between groups (p<0.05) at the time point.

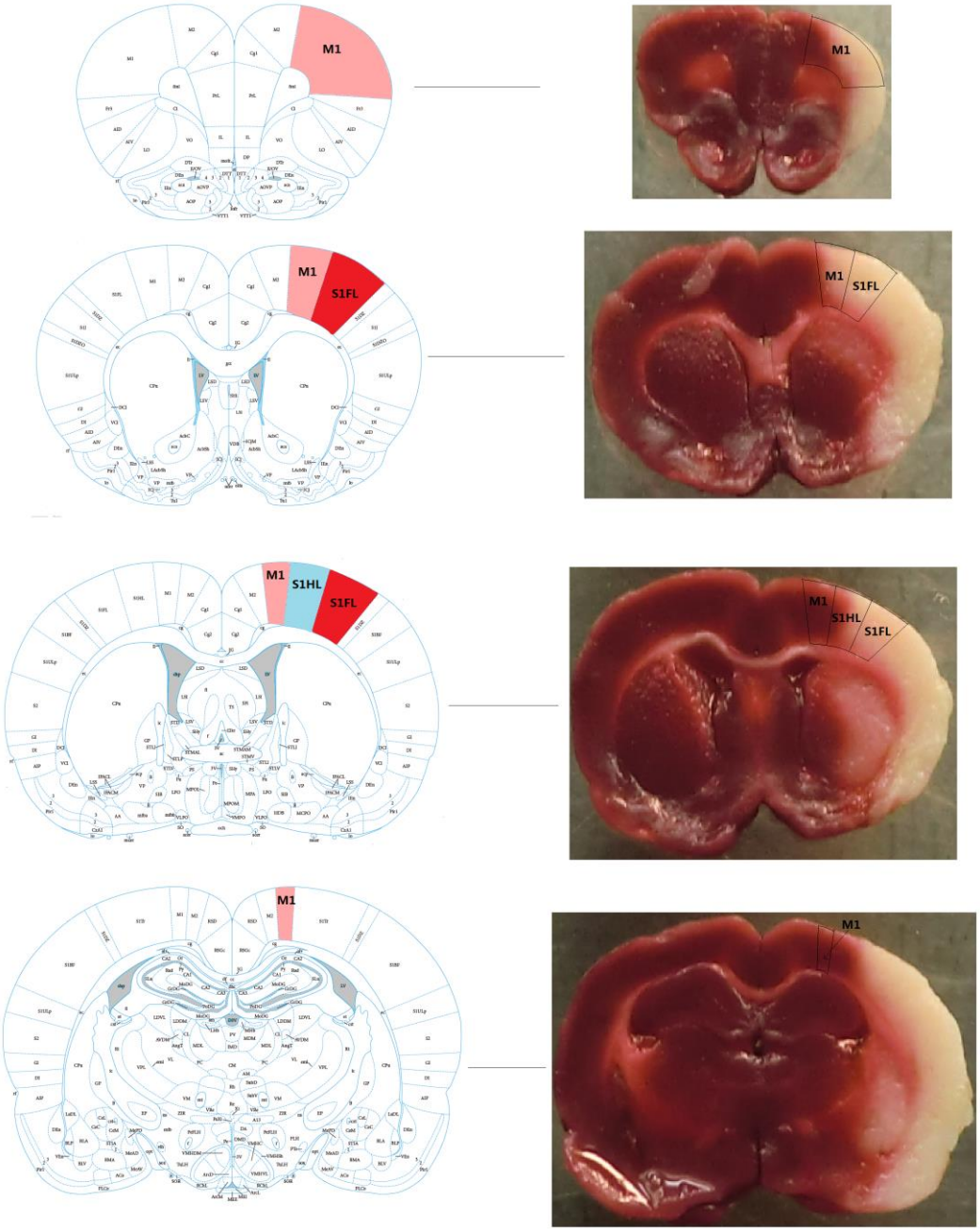

Figure 4.3 Comparison between brain functional schematic diagrams (left column (Paxinos & Watson, 2007)) and corresponding TTC stained brain sections (right column). M1: primary motor cortex (pink); S1FL: primary somatosensory cortex of left forelimb (red); S1HL: primary somatosensory cortex of left hindlimb (blue).

4.1.2.3 BEAM WALKING

Jolkkonen's scoring system for the beam walking test was used in this pilot study.

The score scale ranges from 0 to 6 points, indicating severe motor disabilities to

normal motor function. Daily beam walking scores were recorded and analyzed over the 7-day intervention. All stroke rats exhibited beam walking scores of 2.0 ± 0.5 one day after brain ischemia, followed by a gradual recovery over the following intervention days. Figure 4.4 shows the beam walking score changes of Con (n=12) and TG (n=12) rats over the 7-day intervention. Minor differences were observed between spontaneous and training-induced recovery, possibly as a result of main concern on the hindlimb function in this scoring system. Hindlimb function, however, was less affected compared to forelimb function based on the comparison of rat brain functional atlas (Paxinos & Watson, 2007) and TTC stained brain slices (Figure 4.3).

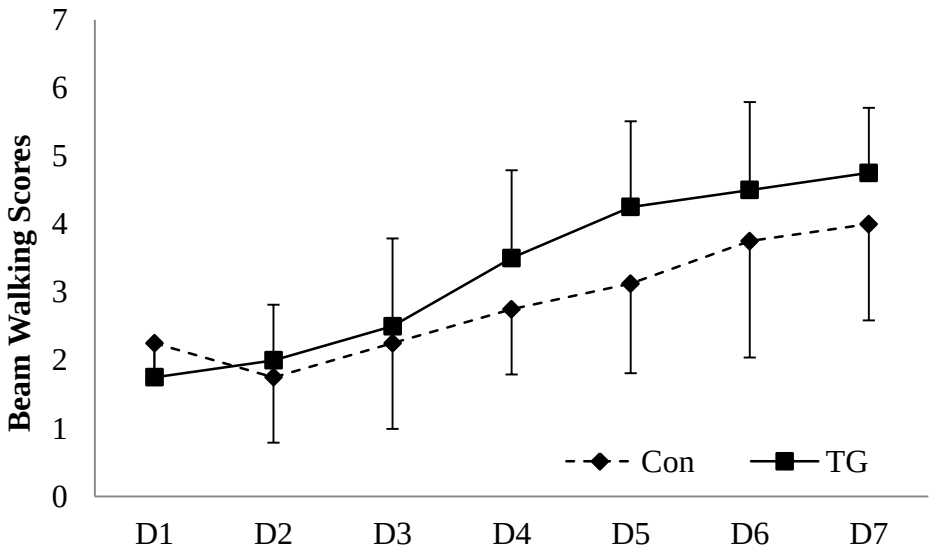

Figure 4.4 Beam walking scores over the 7-day intervention. Con: control group (n=12); TG: treadmill training group (n=12). D1-D7: 7 consecutive intervention days on the first intervention day (D1) to the last intervention day (D7).

4.1.2.4 SUMMARY: KEY FINDINGS OF ASSESSMENT EVALUATIONS

In this part of pilot study, three assessment methods including Longa's test, De Ryck's test, and beam walking test, were evaluated over 7 intervention days.

After comparing spontaneous and treadmill training-induced recovery, De Ryck's test showed optimized sensitivity to present minor discrepancy over this intervention period. Longa's test could effectively detect neurological deficits in stroke rat models, but was not sensitive to differentiate the differences of neurological deficits between the Con and TG rats over the experimental period. Beam walking test was also insensitive to distinguish motor recovery between rats with or without treadmill training.

In conclusion, Longa's test can be used to determine if a rat is successfully induced with stroke after MCAo/r modeling, while De Ryck's test can be employed to monitor motor function recovery over the 7 intervention days. The beam walking test, however, could not distinguish the minor discrepancies between spontaneous and training induced motor recovery in this study.

4.1.3 EFFECTS OF TREADMILL TRAINING ON MOTOR RECOVERY, BRAIN INFARCT AREA, AND STRESS LEVELS

This part of pilot study was designed to investigate the effects of treadmill training on motor recovery, brain infarct area, and stress levels over 7 consecutive intervention days. This study totally had 40 rats involved. Sixteen rats were excluded after initial training (3 rats) and MCAo/r surgery (5 rats with no stroke; 8 rats died). Twenty-four rats with stroke were randomly assigned into Con and TG groups with 12 rats in each. The success rate of rats modeling in this study was higher than in previous studies (Ke et al., 2011; Zhang, Ke, Li, Yip, & Tong, 2013). Daily body weight and motor function were monitored. All rats survived the 7-day intervention. Cerebral infarct sizes after intervention were

obtained via TTC staining. Stress levels were acquired through plasma CORT concentration detection after the last intervention.

Figure 4.5 shows the changes of body weight in both groups over the 7 intervention days. All rats had a body weight of 306.6 ± 22.2 g right before MCAo/r surgery, dropping sharply down to 279.4 ± 23.7 g with an average weight loss of 27.2 g. Rat from both Con and TG groups exhibited similar body weight trends and no significant difference were found between groups.

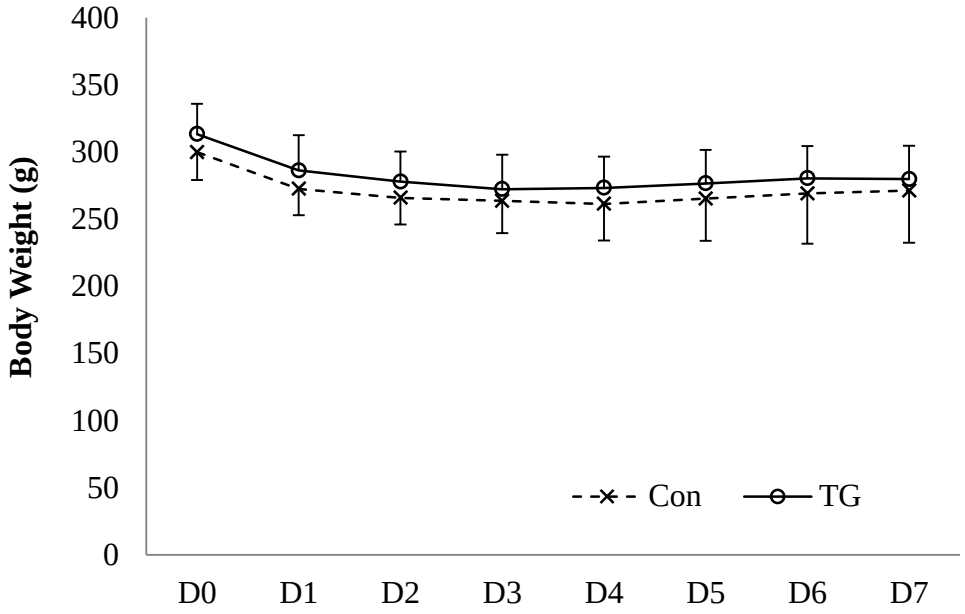

Figure 4.5 Body weight changes of control (Con) and treadmill training (TG) rats before and after stroke. D0: right before the MCAo/r surgery; D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7).

4.1.3.1 MOTOR FUNCTION RECOVERY

Rats in both control (Con; n=12) and treadmill training (TG; n=12) groups exhibited similar level of motor disability one day after MCAo/r surgery with motor function scores of 5.4 ± 1.4 and 5.5 ± 0.9, respectively. Both Con and TG

rats gradually recovered their motor function over the 7-day intervention (Figure 4.2). Difference of motor function between groups occurred on the 5th day and become significant from the 6th day on (p<0.05). On the last intervention day, motor function scores of rats were 8.4 ± 1.7 and 10.0 ± 0.9 in Con and TG groups, respectively. Results indicated that treadmill training could facilitate motor function recovery after 7-day intervention.

4.1.3.2 TTC STAINING & CEREBRAL INFARCT SIZE

TTC staining successfully distinguished intact and infarct brain regions (Figure 4.6A) and clearly showed the damaged striatum and sensorimotor cortex (Figure 4.6B). Figure 4.7A provided examples of TTC stained brain slices from Con and TG rats, respectively. The sizes of brain infarct area were 262.8 ± 37.8 mm^3 and 221.9 ± 17.4 mm^3 in Con and TG groups, respectively (Figure 4.7B). TG rats showed significantly smaller infarct sizes than Con rats (p<0.05), demonstrating that post-stroke treadmill exercise in the subacute phase may contribute to the neural protection or facilitate neural repair.

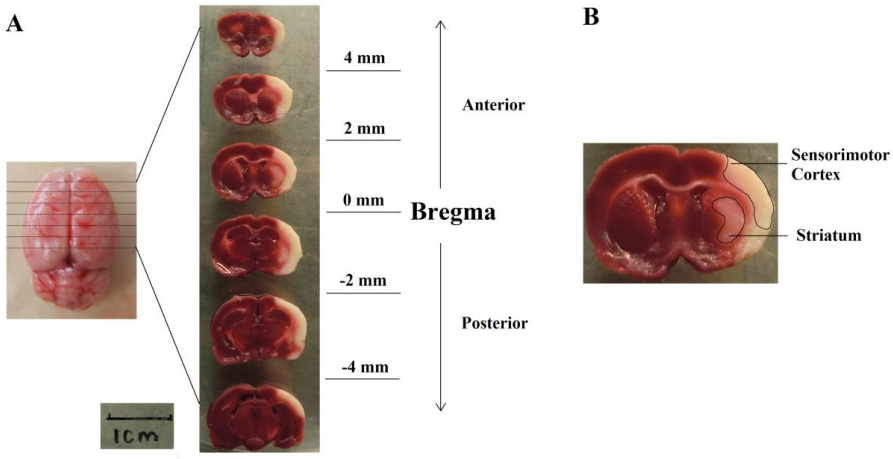

Figure 4.6 TTC stained brain slices. White regions: infarct area; red regions: normal tissue. A) Corresponding brain regions of TTC stained brain slices; B) Part of affected striatum and sensorimotor cortex.

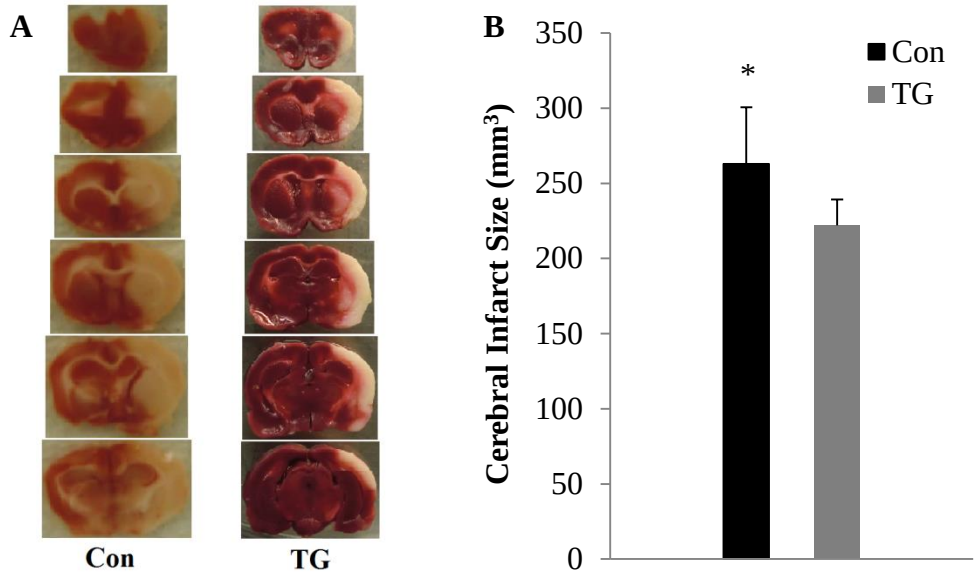

Figure 4.7 Examples of TTC stained brain slices of two stroke rats from control (Con) and treadmill training (TG) groups (A); cerebral infarct sizes in Con (n=12) and TG (n=12) rats (B). *: significant difference observed between groups (p<0.05).

4.1.3.3 PLASMA CORT CONCENTRATION

Figure 4.8 shows the results of plasma CORT concentration measurements. The plasma CORT concentrations of Con and TG rats were 360.6 ± 191.3 nM/L and 730.6 ± 167.2 nM/L, respectively. Rats with treadmill training exhibited significantly higher (p<0.05) plasma CORT levels, indicating that TG rats were stressed when trained on the treadmill.

4.1.3.4 SUMMARY: KEY FINDINGS OF THE EFFECTS OF TREADMILL TRAINING ON STROKE RECOVERY

In this part of the pilot study, stroke rats with treadmill training still exhibited significantly greater motor function recovery and smaller infarct volumes compared to spontaneously recovered rats, even if treadmill training was stressful. Stress inhibits stroke rehabilitation and should be minimized during rehabilitation. Given the high stress level that treadmill training caused in this

study (almost twofold higher than Con rats), there may be a better training protocol which could decrease stress level while increasing or at least keeping the same motor function recovery as in the pilot study.

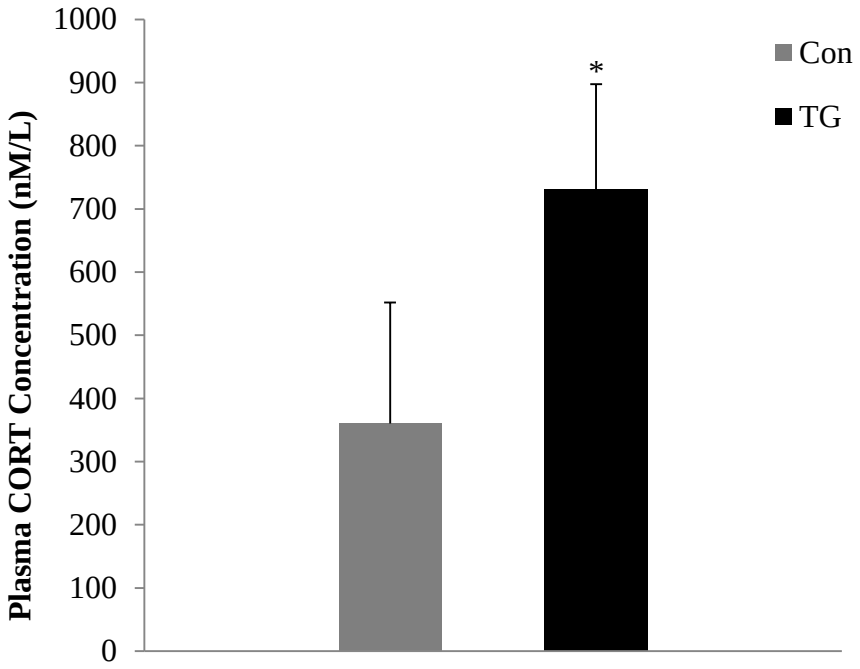

Figure 4.8 Plasma CORT concentrations of Con (n=12) and TG (n=12) rats. CORT: corticosterone; Con: control group; TG: treadmill training group. *: significant difference observed between groups (p<0.05).

4.1.4 SUMMARY: KEY FINDINGS OF THE PILOT STUDY

The pilot study included two parts: 1) evaluation of post-stroke neural and motor function assessments and 2) effects of treadmill training on motor function recovery, cerebral infarct sizes, and plasma CORT concentrations. Results showed that De Ryck's test for motor function assessment was the most sensitive compared to Longa's test for neurological deficit assessment and beam walking test for motor coordination and balance over the 7-day intervention. Longa's test, however, was still suitable to verify the success of MCAo/r modeling. Treadmill

training was found to be a great stressor to stroke rats; however, it could still significantly impact motor function recovery and decrease infarct size. These findings suggested ways in which optimizing these protocols might reduce stress level while improving motor function recovery.

4.2 STUDY II: EFFECTS OF TREADMILL TRAINING WITH DIFFERENT INTENSITIES ON MOTOR FUNCTION RECOVERY AND NEUROREHABILITATION

This study aimed to explore the effects of treadmill training intensity on rehabilitation outcomes in the subacute stroke phase by using a focal ischemic stroke rat model. Data analysis and experiments were conducted by the author of this study (except daily motor function assessment). Findings out of this study (Sun et al., 2014) have been published on a Science Citation Index journal "*BioMed Research International*", and are currently under review.

A total number of 94 rats were used in this study. First, twenty rats were used to estimate the sample size needed to achieve significant differences in motor function improvement among groups (section 4.2.1). The other 74 rats were then recruited to meet the minimal sample size to investigate the effects of training intensity on stroke recovery over a 7-day intervention. Measurements, including motor function scores, brain BDNF levels, and stress levels, were used to compare the effects of treadmill training with different intensities on post-stroke rehabilitation outcomes.

Among the first 20 rats, two were dropped out after initial training, while six were excluded after MCAo/r surgery (2 rats with no stroke; 4 died). Twelve rats with successful stroke were randomly assigned into 4 groups: control (CG, n=3), low training intensity (LG, n=3), gradually increasing training intensity (GIG, n=3), and high training intensity (HG, n=3). Daily motor function scores were recorded and analyzed to acquire the minimal sample size before the following experiments.

Among the rest 74 rats, five were dropped out after initial training, while 21 rats were excluded due to either no stroke (8 rats) or death (13 rats) after MCAo/r surgery. Forty-eight stroke rats were randomly divided into the same 4 groups (CG, LG, GIG, and HG) with 12 rats per group. Daily motor function scores were also recorded.

In total, 60 out of 94 rats were successfully modeled and were randomly assigned into the four groups (15 rats per group). Throughout the experiment, only one rat in the GIG group died on the 6th day; its motor function scores on the last two days were the same as on the 5th day based on the principal of intention-to-treat. Daily body weights were recorded and analyzed for all groups (section 4.2.2). Results of motor function scores, cerebral BNDF concentrations, and plasma CORT concentrations were analyzed (section 4.2.3 - 4.2.5).

4.2.1 MINIMAL SAMPLE SIZE ESTIMATION

Twelve out of 20 rats were successfully induced with stroke and randomly divided into CG, LG, GIG, and HG with 3 rats per group. Daily motor function

scores over the 7-day intervention were recorded. The minimal sample size required to reach significant difference was calculated based on the mean values and standard deviations of motor function scores on the last intervention day between each two groups by using power analysis (alpha = 0.05, power = 0.80). Table 4.2 shows the means ± standard deviations of motor function scores in each group over the 7 intervention days. The minimal sample size necessary to observe significant difference between LG and CG rats was 14 (Table 4.3). Since the power increases with the increase of sample size, sample size for this study was set at 15 to minimize type II error (section 3.4.5).

Table 4.2 Means ± Standard Deviations of Motor Function Scores over the 7-day Intervention.

	D1	D2	D3	D4	D5	D6	D7
CG(n=3)	4.3±0.6	5.2±1.2	5.8±0.8	6.3±1.2	7.0±1.7	8.2±1.9	9.0±1.0
LG(n=3)	4.7±0.6	6.2±0.3	7.3±0.6	8.2±0.3	9.2±0.3	10.2±0.3	10.2±0.3
GIG(n=3)	5.3±2.3	7.0±2.6	8.0±2.6	9.0±2.0	9.5±1.3	11.0±1.7	11.7±1.0
HG(n=3)	4.7±0.3	6.0±1.0	6.8±1.6	7.7±1.5	8.3±1.2	9.5±0.9	10.3±0.3

Table 4.3 Estimated Minimal Sample Size of Motor Function Scores.

	LG	GIG	HG
CG	14	8	10
LG	--	10	274
GIG	10	--	12

4.2.2 CHANGES IN BODY WEIGHT

The body weight in all groups exhibited similar trends over the 7 intervention days (Figure 4.9); these results are similar to the previous pilot study (Figure 4.5). All rats weighing 313.2 ± 25.6 g right before MCAo/r surgery, dropping sharply down to 285.3 ± 27.2 g with an average weight loss of 27.8 g. There were no significant differences among rats in the 4 groups (CG, LG, GIG, and HG).

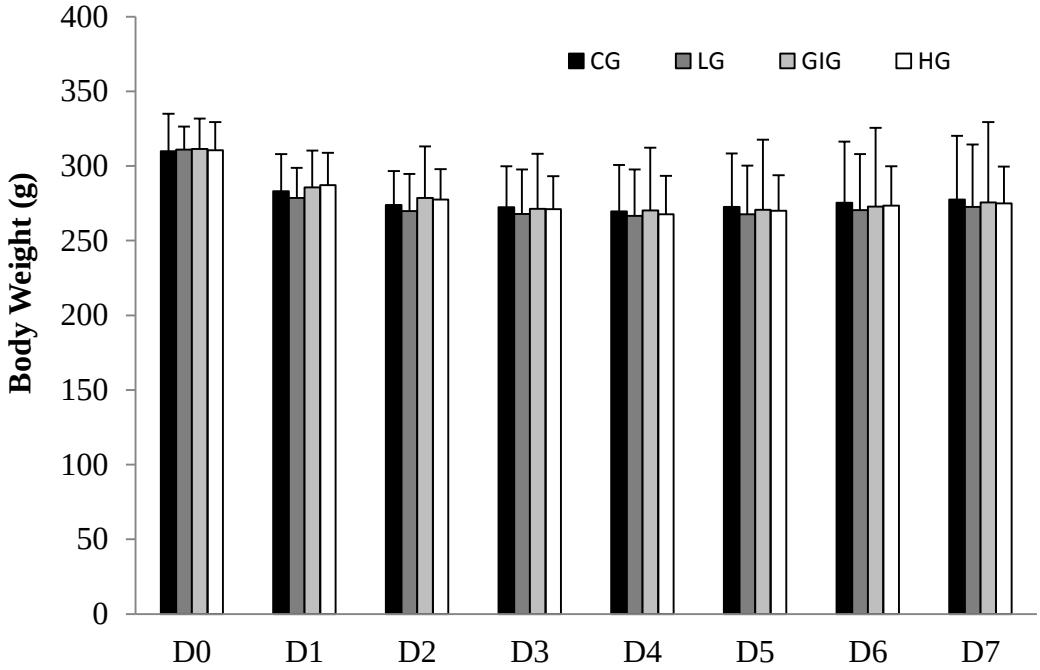

Figure 4.9 Changes in body weights across the 4 groups (CG, LG, GIG, and HG) before and after stroke. CG: control group; LG: low training intensity group; GIG: gradually increasing training intensity group; HG: high training intensity group. D0: right before the MCAo/r surgery; D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7).

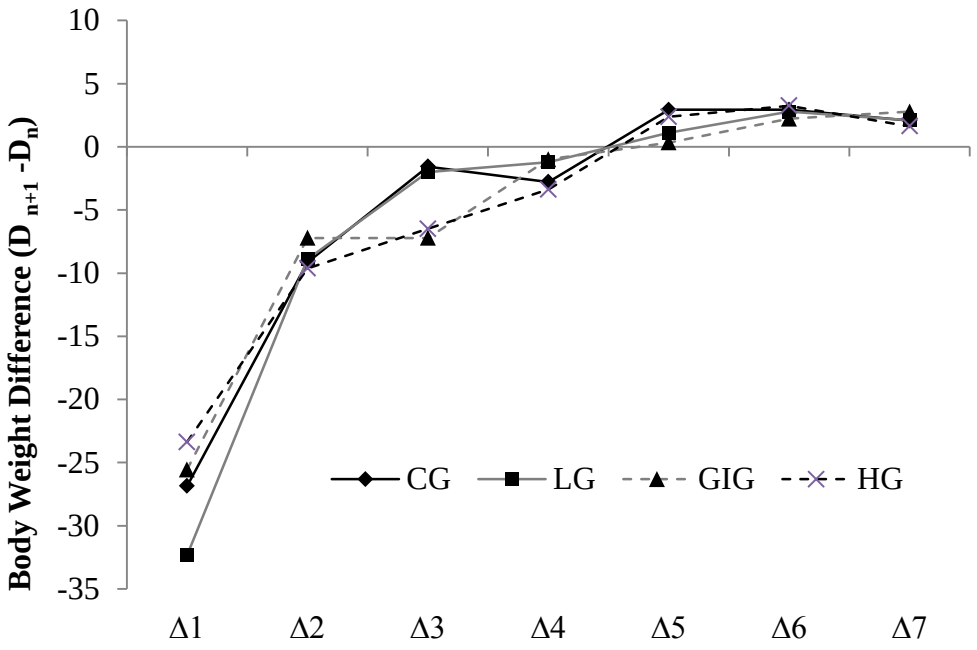

Figure 4.10 Mean body weight differences (data obtained by subtracting weight of the previous day (D_n) from that of the current day (D_{n+1}) in Study II). CG: control group; LG: Low intensity group; GIG: gradually increased intensity group; HG: high intensity group. Δ1: the first intervention day subtracted by the previous day; Δ7: the last intervention day subtracted by the 6th day.

Figure 4.10 shows the mean body weight differences (calculated by subtracting the body weight of the previous day (Dn) from that of the current day (Dn+1) over the 7-day intervention for groups (CG, LG, GIG and HG)), showing that the weight of all groups maintained almost the same levels from the 3rd day on, and exhibited a minor recovery over the final 3 intervention days. No noticeable differences were observed among groups.

4.2.3 OVERVIEW OF REHABILITATION OUTCOMES

Table 4.4 shows an overview of rehabilitation outcomes including motor function recovery, cerebral BDNF levels in the hippocampus, striatum, and cortex, and plasma CORT concentrations in each group (CG, LG, GIG, and HG). Significant differences were observed among groups and Post hoc analysis showed specific differences between each two groups.

Table 4.4 An Overview of Rehabilitation Outcomes of Motor Function, cerebral BDNF Levels in the Hippocampus, Striatum and Cortex, and Plasma Corticosterone (CORT) Levels.

Items	Group	Pre-training	Post-training	Post hoc (p)
Motor Function				CG vs LG (0.018*)
	CG	5.36 ±1.41	8.23 ±1.69	CG vs GIG (<0.001*)
	LG	4.89 ±0.78	10.01 ±0.73	CG vs HG (0.041*)
	GIG	5.37 ±1.64	12.00 ±1.00	LG vs GIG (0.009*)
	HG	5.50 ±0.81	9.64 ±0.90	LG vs HG (1.00)
				GIG vs HG (<0.001*)
BDNF level in hippocampus				CG vs LG (0.044*)
	CG	-	47.68 ±13.25	CG vs GIG (<0.001*)
	LG	-	74.46 ±25.57	CG vs HG (0.032*)
	GIG	-	112.87 ±25.18	LG vs GIG (<0.001*)
	HG	-	76.41 ±34.68	LG vs HG (0.523)
				GIG vs HG (0.001*)
BDNF level in striatum				CG vs LG (1.00)
	CG	-	14.16 ±13.25	CG vs GIG (0.004*)
	LG	-	18.04 ±11.61	CG vs HG (1.00)
	GIG	-	27.77 ±15.57	LG vs GIG (0.044*)
	HG	-	17.94 ±10.26	LG vs HG (1.00)
				GIG vs HG (0.030*)
BDNF level in cortex				CG vs LG (0.980)
	CG	-	11.73 ±7.18	CG vs GIG (0.001*)
	LG	-	14.69 ±3.60	CG vs HG (1.00)
	GIG	-	19.24 ±4.94	LG vs GIG (0.203)
	HG	-	14.64 ±6.50	LG vs HG (1.00)
				GIG vs HG (0.194)
Plasma CORT Level				CG vs LG (0.044*)
	CG	-	347.03 ±181.02	CG vs GIG (0.009*)
	LG	-	508.07 ±161.30	CG vs HG (<0.001*)
	GIG	-	540.63 ±117.40	LG vs GIG (1.000)
	HG	-	716.90 ±156.48	LG vs HG (0.003*)
				GIG vs HG (0.017*)

Values: means ± standard deviations; *p* value: significance level of 2-way Repeated Measures ANOVA multiple comparisons with covariate for motor function scores; significance level of one-way ANOVA for BDNF levels and plasma CORT concentrations.

*: Significant differences observed; post hoc test was conducted to specify the differences between groups.

4.2.4 MOTOR FUNCTION RECOVERY

Motor function scores over the 7-day experimental period were analyzed via 2-way ANOVA with the score on the first day as covariate (Figure 4.11). Significant differences were revealed among the four groups (CG, LG, GIG, and HG). Rats in the GIG had significantly higher (p<0.05) motor function scores from the 3rd day to the last day compared to other three groups. Rats in LG and HG also presented significantly better (p<0.05) motor function recovery from the 6th day than the control group. No significant difference was observed between LG and HG rats. Results suggested that treadmill training with gradually increased intensity could better facilitate motor function recovery during subacute stroke period.

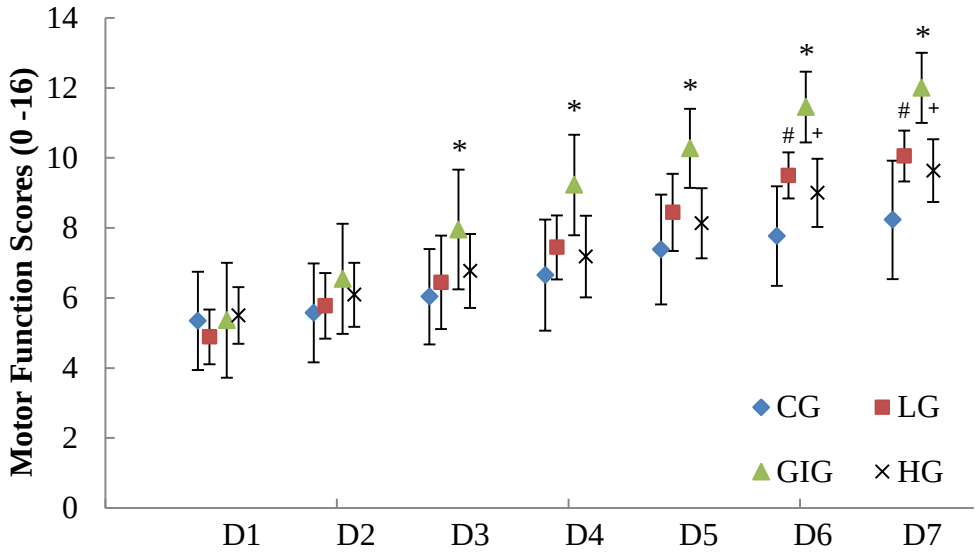

Figure 4.11 Motor function scores over the 7-day intervention. A higher score indicates better motor function. *: significant difference observed at the time point when comparing GIG to the other three groups. $^{\#, +}$: significant difference observed between LG and CG rats, and HG and CG rats, respectively. CG: control group; LG: low training intensity group; GIG: gradually increasing training intensity group; HG: high training intensity group. D1-D7: 7 consecutive intervention days from 24 hours after stroke (D1) to the last intervention day (D7).

4.2.5 CEREBRAL BDNF AND PLASMA CORT CONCENTRATIONS

Cerebral BDNF (hippocampus, striatum, and cortex) and plasma CORT levels were compared among the 4 groups (CG, LG, GIG, and HG) via one-way ANOVA. Significant differences were observed among groups. Post hoc analysis was then undertaken to reveal the difference between each two groups. Figure 4.12 shows the cerebral BDNF levels in the hippocampus, striatum, and cortex for all groups. Results showed that hippocampal BDNF concentrations were significantly higher ($p<0.05$) than in both the striatum and cortex for all groups. GIG rats showed the highest BDNF levels in the hippocampus and striatum. Significantly different cortical BDNF levels were observed between GIG and CG rats. BDNF levels in LG and HG rats were not apparently different but were significantly higher in the hippocampus and striatum than CG rats. Figure 4.13 shows plasma CORT concentrations. Rats in the 3 training groups exhibited significantly higher CORT levels over control. CORT levels in GIG rats were significantly lower than HG, but similar to LG.

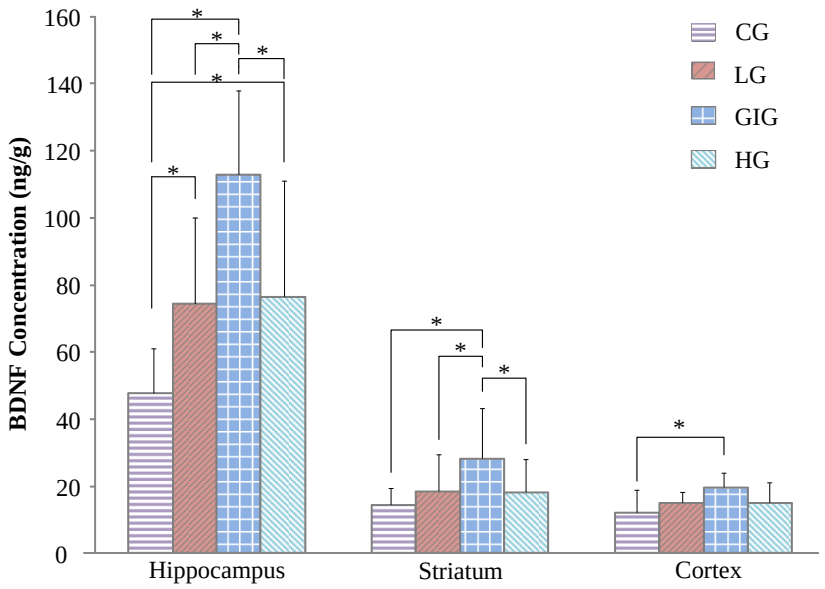

Figure 4.12 BDNF concentrations in the hippocampus, striatum and cortex. *: significant differences between groups acquired via one-way ANOVA with post hoc test. CG: control group; LG: low training intensity group; GIG: gradually increasing training intensity group; HG: high training intensity group.

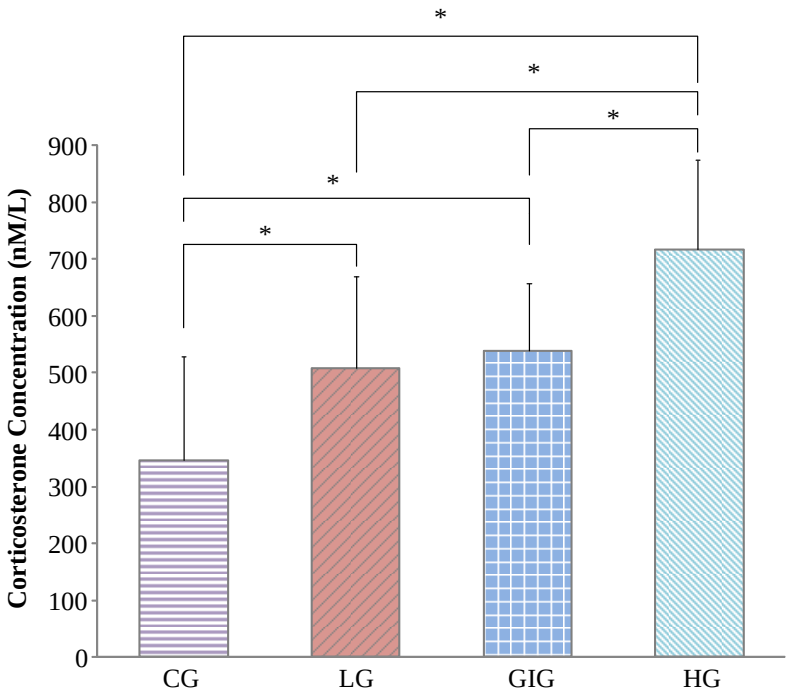

Figure 4.13 Plasma CORT concentrations on the last intervention day. Higher values represent higher stress. *: significant differences between groups acquired via one-way ANOVA with post hoc test. CG: control group; LG: low training intensity group; GIG: gradually increasing training intensity group; HG: high training intensity group.

CHAPTER 5 DISCUSSION

The study included two parts: 1) a pilot study to evaluate neural and motor function assessments and to investigate the effectiveness of treadmill training on motor function recovery, brain infarct size, and stress levels and 2) a training intensity study to explore the effects of treadmill training intensity on motor function and neural rehabilitation.

The pilot study demonstrated that De Ryck's test for motor function assessment was the most sensitive compared to Longa's test for neurological deficit assessment and beam walking test for motor coordination and balance over the 7-day intervention. Longa's test, however, was suitable to verify the success of MCAo/r modeling. In addition, treadmill training showed to be a great stressor to stroke rats; however, it could still be used for motor function recovery and decrease cerebral infarct size. Stress can inhibit stroke rehabilitation and should be minimized during rehabilitation. Findings from the pilot study indicated that an optimized protocol might decrease stress and facilitate better recovery.

Intensity is a determining factor of stress in treadmill exercises. The second study, thus, explored the effects of treadmill training intensity on rehabilitation outcomes after stroke. This study shows that treadmill training intensities for ischemic stroke rats affect motor function recovery, cerebral BDNF concentrations, and stress levels over the 7-day intervention. This study set up three training intensity levels including low, high, and gradually increased intensity from low to high. Gradually increased training intensity (GIG) induced

significantly better motor function recovery. Rats in this group showed similar stress levels in comparison to low training intensity group (LG), but BDNF concentrations in the brain tissues (hippocampus and striatum) were significantly higher than LG. Rats in high training intensity group (HG) were stressed more than LG; however, functional recovery was similar to LG and significantly lower than GIG. Results indicated that rats trained by treadmill with gradually increased intensities better regain motor function.

In addition to the key findings in both the pilot study and the training intensity study, body weights of rats were monitored throughout all the experiments and showed consistent trend (Figure 4.5; Figure 4.9). The following paragraphs describe the findings in this study, including the body weight trend (section 5.1), application of functional assessments in post-stroke treadmill training intervention (section 5.2), the effects of treadmill training on stroke recovery (section 5.3), and effects of treadmill training intensity on rehabilitation after stroke (section 5.4).

5.1 TRENDS IN BODY WEIGHT

In both the pilot and the training intensity studies, body weights of the rats one day after MCAo/r surgery decreased by approximately 27 g and further decreased by approximately 15 g until the 3rd day. Figure 4.10 shows the mean body weight differences of the four groups, showing that the weight of all groups maintained almost the same levels from the 3rd day on, and exhibited a minor recovery over the final 3 intervention days. No noticeable differences were

observed among groups, suggesting that treadmill training regimens in this study may not aggravate complications of stroke (Ke, 2012; Yang et al., 2003).

Brain ischemia-induced body weight loss was also observed in previous animal studies (Petullo et al., 1999; Reglődi, Tamás, & Lengvári, 2003); however, the degree of body weight loss varies across studies. A 90-minute transient MCAO resulted in approximately 80 g of body weight loss at 24 hours post-brain ischemia (Ke, 2012), while in this study, a 60-minute transient MCAO method resulted in an approximately 30 g of body weight loss (when measured at the same time). Body weight loss was positively correlated with occlusion time in previous studies (Petullo et al., 1999; Zhang et al., 1995). Body weight loss 24 hours after brain ischemia also showed a positive correlation with motor function score tested at 24 hours after brain ischemia (Figure 5.1), demonstrating that severe stroke might lead to additional weight loss. This finding indicates that severe neurological deficits could affect metabolism more than mild neurological deficits. Change in body weight over the 7 intervention days in this study was similar to others study which used a similar transient MCAO method (Reglődi et al., 2003). In a study of an embolic stroke rat model, body weight decreased approximately 18.3% at 2 days after ischemia (Zhang et al., 2000), while this study observed an approximately 12.0% of weight loss during this time period. Other potential reasons for differences in body weight reduction may be related to the animals' original weights, species used, and stroke modeling methods. Continuous body weight loss several days after brain ischemia in this MCAO-induced stroke rat model may be due to ingestion restriction which resulted from

mastication and swallowing dysfunction (Dittmar, Spruss, Schuierer, & Horn, 2003) and reduced food intake caused by forelimb disability (Ke, 2012).

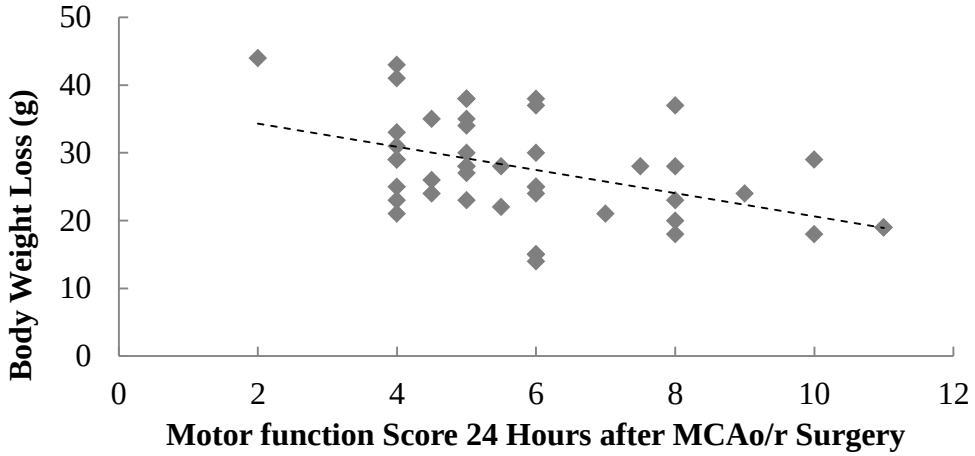

Figure 5.1 Relationship between body weight loss 24 hours after brain ischemia and motor function score tested at the same time.

The mean body weights of all groups (CG, LG, GIG and HG) exhibited the same pattern of weight increase from the 5th intervention day on (Figure 4.10), indicating a possible spontaneous recovery. This spontaneous recovery may result from the recovery of post-operative trauma (Dittmar et al., 2003), the recovery of forelimb grasping function, or the improvement in neurological impairment.

5.2 FUNCTIONAL ASSESSMENTS IN APPLICATION OF POST-STROKE TREADMILL TRAINING INTERVENTION: EVALUATION OF LONGA'S TEST, DE RYCK'S TEST, AND BEAM WALKING TEST

The pilot study evaluated three assessment methods: Longa's test, De Ryck's test, and the beam walking test in the application of post-stroke neural and motor

function evaluations. Longa's test effectively demonstrated neurological deficits in the stroke model, but was not sensitive to differentiate the difference between spontaneous and treadmill training induced recovery over this experimental period. De Ryck's test demonstrated the best sensitivity to indentify the minor discrepancy of motor recovery over the 7-day intervention. The beam walking test was insensitive to distinguish motor recovery between rats with or without treadmill training based on this pilot study.

A six-point neuro-score, which combines assessments of neurological deficits and locomotor function, successfully discriminates post-stroke recovery treated by different neuroprotective agents (Zausinger et al., 2000); however, Longa's test (Longa et al., 1989) only indicates 4 levels of neurological deficits. Most rats who survived the MCAo/r surgery with motor disability exhibited only level 1 and 2 using Longa's test 24 hours after brain ischemia in this study. The actual scoring scale was limited to 2 levels, narrowing the difference between spontaneous and training induced neural recovery over the 7-day intervention. The beam walking test (Jolkkonen et al., 2000) also had a scoring scale of 6 points; however, it did not significantly distinguish training-induced recovery from spontaneous recovery compared to Zausinger et al. (2000)'s 6 point neuro-score. One possible reason is that the tests were designed differently. Compared to Longa's test and the beam walking test, De Ryck's test (De Ryck et al., 1989) has a more than twofold scale, making it possible to detect differences in motor recovery.

Longa's test assesses neurological deficits, while the beam walking test assesses hindlimb function (Nielsen, Samson, Simonsen, & Jensen, 2013). De Ryck's test, however, mainly assesses forelimb function which is most affected in this transient MCAO stroke model. The six-point neuro-score used by Zausinger et al. (2000) also mainly assesses forelimb function. Forelimbs are responsible for more meticulous activities than hindlimbs, resulting in a larger brain area controlling forelimb function (Paxinos & Watson, 2007). Through comparison of brain functional diagrams with the TTC stained brain sections (Figure 4.3), brain regions controlling the function of the left forelimb were affected more by MCAo/r surgery than regions controlling hindlimb function. De Ryck's locomotor function test involves 6 tasks for the forelimb and 2 tasks for the hindlimb, which mostly match the practical loss of motor function induced by MCAo/r surgery.

5.3 EFFECTS OF TREADMILL TRAINING ON STROKE RECOVERY

Treadmill training is a conventional means to facilitate stroke recovery in both clinical and animal studies. Its effectiveness has been studied for many years (Moseley et al., 2003; Macko et al., 2005). Treadmill training before stroke has been shown to demonstrate a neuroprotective effect with improved motor function and reduced calpain expression (Heo & Kim, 2013). Compared to prior-stroke beneficial effects, post-stroke treadmill training could also benefit motor function recovery. Training efficacy varies among studies, possibly due to the use of different protocols, intervention starting point and sustained period, and species. Early intervention of treadmill training after stroke could significantly

reduce brain infarct volume in a study where the effect of treadmill training timing was investigated (Yang et al., 2003). An early and long-term treadmill training intervention after stroke could also result in significant reduction of infarct volume by using a rat model (Matsuda et al., 2011). In addition to the beneficial effect on brain infarct size, treadmill intervention after stroke may also lead to significant recovery of motor function compared to spontaneous recovery (Ke et al., 2011). Consistent with previous studies, this study also shows the positive effect of treadmill training started at 24 hours after ischemia and lasting for 7 consecutive days. Reduced brain lesion and improved motor performance were observed in this study.

Treadmill training attempts to repeatedly move the disabled limbs, which stimulate cerebral neuroplasticity, thus, contributing to motor learning, recovery, and neurorehabilitation after stroke (Hosp & Luft, 2011). Neuroplasticity is the ability of the brain to reshaping cerebral structure, as a result of learning (Richards et al., 2008). Stroke induces brain damage, leading to the loss of the function within the damaged brain regions. Training is a learning process, and post-stroke physical intervention is related to relearning lost motor function and facilitating neural reorganization (Richards et al., 2008). Ploughman also considered exercise brain food that ultimately enhances brain functions like memory and learning (Ploughman, 2008). Additionally, Ploughman et al. (2007) suggests that moderate exercise has positive effects on physically disabled young people aided by their high brain plasticity.

Treadmill training after stroke, however, also created great stress compared to rats that did not receive training. This observation is similar to Soya et al.

(2007)'s study showing that high-speed treadmill training induced high stress levels. Ke et al. (2011) also showed that treadmill training could induce high stress levels, inhibiting motor function and neural recovery compared to wheel training which is associated with lower stress levels. Overloaded training induced high stress level could probably inhibit stroke recovery (Schaaf et al., 1998). In Castrén et al. (2007)'s study, stress also showed negative effects on hippocampal BDNF synthesis, a signature of inhibited neural recovery. Stress could affect structural plasticity of the hippocampus, a region vital to learning, memory and higher thinking (Tyler et al., 2002), by either causing atrophy of CA3 dendrites or by suppressing neurogenesis in dentate gyrus granule cells (McEwen, 1999). Stress can also inhibit neurogenesis in the ventral subiculum, a region of the hippocampus associated with anxiety (Hawley et al., 2012).

Treadmill training after stroke, therefore, may have dual effect on rehabilitation. On one hand, it may facilitate improving motor function recovery and neurorehabiliation; on the other hand, it could increase stress inhibiting its efficacy. Considering the application significance of post-stroke treadmill training, stress should be well controlled during training to maximize the efficacy of training.

5.4 TREADMILL TRAINING INTENSITY AFFECTS MOTOR FUNCTION RECOVERY AND NEUROREHABILITATION

Treadmill training intensity could affect stroke rehabilitation outcomes including motor function recovery, cerebral BDNF levels, and stress levels over the 7-day intervention. Results in this study showed that GIG rats significantly recovered

motor function and produced higher hippocampal BDNF. GIG and LG rats exhibited similar stress levels, which were significantly lower than HG rats. It could be concluded that training with higher intensity did not result in better motor function recovery and resulted in high stress levels. Training with gradually increased intensity achieved a better recovery outcome with lower stress.

Consistent with other studies, BDNF levels were lower in striatum and cortex than in the hippocampus (Ke et al., 2011; Nawa, Carnahan, & Gall, 1995). The hippocampus plays an important role in learning and memory, and rehabilitation is a process of re-learning, making hippocampal neurons active (Tyler et al., 2002)). BDNF level is highly related to neural survival, growth and differentiation (Weishaupt et al., 2012), probably producing a high hippocampal BDNF level. GIG rats showed the highest BDNF concentrations in the hippocampus and striatum and had the best motor function recovery. Importantly, we found a significantly positive relationship (correlation coefficient: 0.537; $p<0.01$) between motor function recovery rate and hippocampal BDNF concentrations (Figure 5.2), demonstrating that BDNF level in the hippocampus could indicate the motor function recovery rate. BDNF has been used to treat photothrombotic stroke rats and improved motor function recovery when compared to spontaneous recovery (Schäbitz et al., 2004). Other studies also show that higher BDNF level in the brain indicates better motor function recovery after stroke (Ke et al., 2011; Zhang & Pardridge, 2006; Ferreira et al., 2011). Our results remain consistent with previous those of studies. Significant higher BDNF levels were found in GIG rats, leading to significantly better motor

function recovery. Similar BDNF levels were observed in LG and HG rats that showed similar motor function recovery.

BDNF is a neurotrophin growth factor, supporting neuron survival and encouraging growth and differentiation of new neurons and synapses (Weishaupt et al., 2012). Since BDNF is related to neuroplasticity contributing to motor learning, recovery and neural rehabilitation after stroke (Hosp & Luft, 2011), BDNF may be highly expressed in the hippocampus, a region vital to learning, memory, and higher thinking (Tyler et al., 2002). Stroke induces the loss of motor function, while rehabilitation is the process of relearning; thus, the higher BDNF concentration in brain tissues could imply learning and neural rehabilitation (Soya et al., 2007). Post-stroke treadmill training, thus, induces higher BDNF production associated with better motor function and neural recovery. GIG training induces more cerebral BDNF production compared to training with fixed low and high intensities, leading to a better motor recovery.

Ploughman considered exercise brain food that ultimately enhances brain functions like memory and learning (Ploughman, 2008). Additionally, Ploughman et al. (2007) suggests that moderate exercise has positive effects on physically disabled young people aided by their high brain plasticity. Both prolonged and short-term moderate exercise increases hippocampal BDNF levels and brain mitochondrial biogenesis in rats (Ferreira et al., 2011; Ploughman et al., 2005; Ogonovszky et al., 2005). Physical training for stroke rats was reported to facilitate motor function recovery and upregulate BDNF levels (Zhang et al., 2013). 4-week consecutive low-speed treadmill training started on the 4th day post stroke was found to improve hippocampal function in a MCAO induced

stroke rat model (Shimada et al., 2013). Treadmill exercise after stroke, with repetitive attempts to move the paretic limbs, is a relearning process, facilitating neural reorganization. Since BDNF is an indicator of neurogenesis, post-stroke treadmill training may be able to upregulate brain BDNF concentrations, a result supported by this study. GIG training induces the highest BDNF levels, possibly indicating the best learning compared to training with fixed intensities. GIG training could be more appropriate for post-stroke motor rehabilitation than training with fixed intensities.

Physical training facilitates rehabilitation after stroke, but it is also a source of stress that mediates BDNF regulation. CORT is a steroid hormone produced by the hypothalamic-pituitary-adrenal axis and is released into the blood. Adrenalectomized Wister rats were used to investigate the time course and dose-dependency of CORT's effect on BDNF mRNA and protein, with results showing short-term costicosterone concentration changes having transient and dose-dependent down-regulation effects for both hippocampal BDNF mRNA and protein (Schaaf et al., 1998). Forced treadmill training induces stress and has been suggested to lower physical rehabilitation and BDNF levels in the hippocampus compared to voluntary wheel running; yet it still stimulates functional recovery (Ke et al., 2011). Stress can also affect the hippocampus by either causing atrophy of dendrites in the CA3 region or suppressing neurogenesis of dentate gyrus granule neurons (McEwen, 1999). Stress can also inhibit neurogenesis in the ventral subregion, a portion of the hippocampus associated with anxiety (Hawley et al., 2012). Exercise, however, increases muscle and brain mitochondrial biogenesis, strengthening fatigue resistance and

endurance performance (Steiner et al., 2011). In this study, rats in training groups exhibited elevated stress levels compared to control. GIG training, however, induced lower stress level compared to training with high intensity. GIG training, thus, may better improve stress endurance. Lower stress levels in GIG rats reduced the inhibitory effect of stress on BDNF production.

Repeated training is an important tool applied widely in clinics and laboratories to improve recovery after stroke. Intensity in forced training is a critical stress-inducing factor. Thus, this study designed a gradually increasing treadmill training intensity regimen for stroke rats. Results showed that the training intensity should be designed to match recovery rate and minimize stress. Training with gradually increased intensity can produce significantly better motor function rehabilitation compared to stably low and high training intensity by regulation of BDNF and stress level. Observations of this study extended understanding the importance of training intensity on rehabilitation after stroke. A training protocol that includes gradually increasing training intensity should be considered in both animal and clinical human studies.

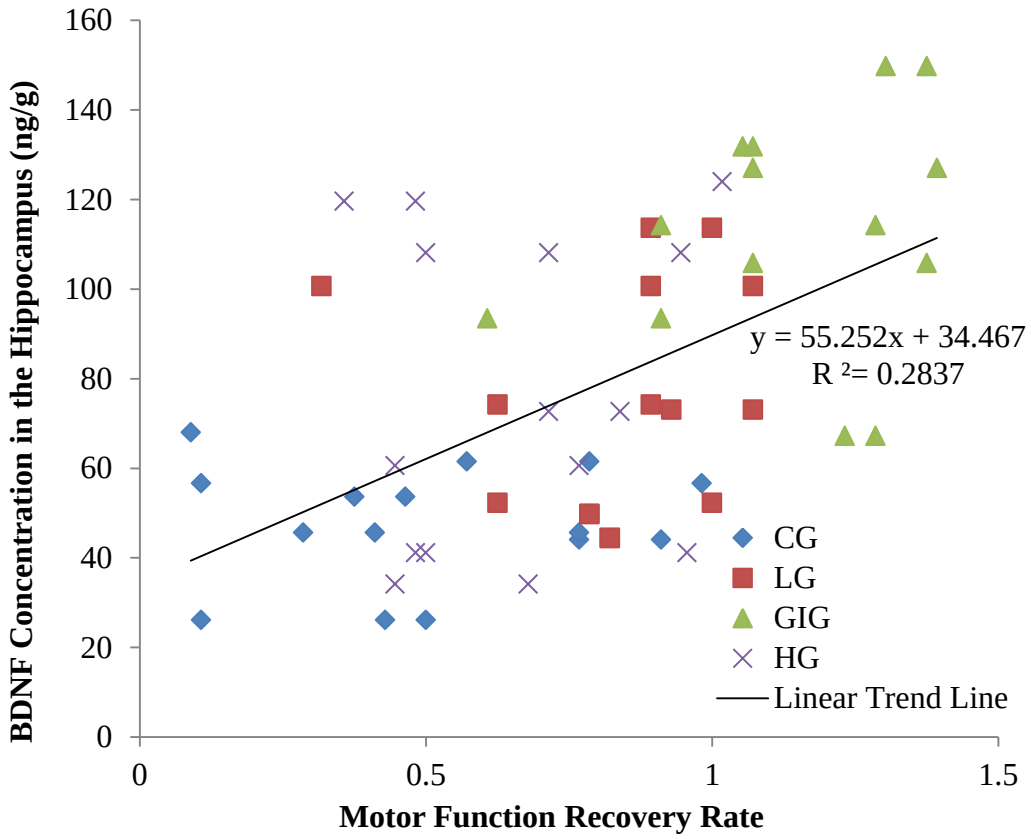

Figure 5.2 Correlation between BDNF concentration in the hippocampus and motor function recovery rate.

CHAPTER 6 CONCLUSIONS

This study first evaluated the sensitivity of post-stroke function assessments including Longa's, De Ryck's, and beam walking tests. De Ryck's test showed the best sensitivity to indentify minor discrepancies in motor function recovery over the 7 early intervention days. Longa's test can effectively indicate neurological deficits after stroke, but was not sensitive to differentiate the differences between spontaneous and treadmill training-induced neural recovery over the experimental period. The beam walking test was also insensitive to distinguish motor recovery between rats with or without treadmill training over this experimental period. Thus, De Ryck's test was selected for daily motor function evaluation, while Longa's test was used for neurological deficit evaluation to verify the success of stroke modeling for the following studies. The effects of treadmill training on motor function recovery, brain infarct size, and stress levels were then investigated, with results showing that treadmill training after stroke induced high stress but still could improve motor function recovery and reduce brain infarct size.

Stress has negative effects on recovery after stroke and should be well minimized during rehabilitation to achieve improved recovery. Eexercise could increase stress level; however, it can also increase muscle and brain mitochondrial biogenesis, strengthening fatigue resistance and endurance performance (Steiner et al., 2011). There may be an optimized training protocol which can lead to lower stress and improve stroke recovery. Intensity is a key factor in treadmill training and associated with stress. High-speed treadmill training induces high

CORT levels in a stroke rat model (Soya et al., 2007). Stress endurance can also be enhanced by exercise (Steiner et al., 2011). Thus, adjusted training intensity may be directly correlated to rehabilitation outcomes.

This study then utilized a standard focal ischemic stroke rat model to explore the effects of treadmill training intensity on stroke recovery in the subacute phase by comparing rehabilitation outcomes of treadmill training with different intensities (control, low training intensity (LG), gradually increasing training intensity (GIG), and high intensity (HG)) over 7 consecutive intervention days. Results showed that training intensity could affect stroke recovery. Specifically, GIG rats significantly recovered motor function and induced higher levels of hippocampal BDNF. GIG and LG rats exhibited similar stress levels, which were significantly lower than HG rats. Training with higher intensity did not result in better motor function recovery and resulted in high stress levels. Training with gradually increased intensity achieved a better recovery outcome with lower stress.

Observations in this study indicate that training intensity influences stroke recovery. Gradually increased training intensity may better promote rehabilitation after stroke than fixed training intensity by upregulating cerebral BDNF levels and downregulating stress levels. For stroke patients, it is easier to monitor stress levels and the degree of recovery after stroke; thus, it may easier to individually design a gradually increased training intensity based on stroke patients' recovery degree and stress levels. This study, however, only investigated one type of gradually increased training intensity. Whether other type of varied training intensity is better or not needs further study. Findings

from this study cannot be directly used for clinical use since physical differences exist between human beings and animals; however, the idea of gradually increasing training intensity can probably be applied to human beings. Gradually increasing intensity should be designed individually based on the recovery of each stroke patient.

For future research, we may: 1) consider a training protocol including gradually increasing training intensity in both animal and clinical human studies for better stroke recovery, 2) dig deep to investigate underlying mechanisms of the effects of intensity on BDNF and/or other biochemical, 3) investigate the correlations between training intensity and stress by real-time stress monitoring, and 4) explore the long-term effects of gradually increasing training intensity on stroke recovery in chronic phase.

Since voluntary wheel training could also lower down stress levels and improve rehabilitation after stroke (Ke et al., 2011), it may be valuable to systematically design a study to observe the difference between voluntary running and treadmill training with gradually increasing intensity in the future.

In this study, high training intensity induced higher stress level but similar motor function recovery and cerebral BDNF levels when compared to low training intensity. In the future study, we may try to increase the high intensity to see if higher intensity would expand neural injury when compared to rats without training, or to decrease the intensity to see if moderate training intensity would better improve rehabilitation in comparison with low training intensity. Those

studies may provide more evidences to understand underlying mechanisms of how training intensity influences rehabilitation.

Young rats were recruited in this study instead of the elderly population who are more vulnerable to stroke. The choice was based on our research question. In this study, we mainly focused on the effects of training intensity on rehabilitation. Moreover, young rats are easier to survive stroke and to feed. This study is the foundation of future studies. In the future, we may study the effects of training intensity on stroke rehabilitation of elderly population and compare the difference between young and elderly population.

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APPENDICES

1. ETHIC APPROVAL

香港特別行政區政府
衞生署
香港灣仔皇后大道東 213 號
胡忠大廈 17 及 21 樓

THE GOVERNMENT OF THE HONG KONG SPECIAL ADMINISTRATIVE REGION
DEPARTMENT OF HEALTH,
WU CHUNG HOUSE, 17TH & 21ST FLOORS,
213 QUEEN'S ROAD EAST, WAN CHAI,
HONG KONG.

本署檔號 OUR REF.: Rev(12-24) in DH/HA&P/8/2/4 Pt.5
來函檔號 YOUR REF.:
電　　話 TEL.: 2961 8645
圖文傳真 FAX.: 2127 7329

20 April 2012

Ms SUN Jing
Department of Health Technology and Informatics
The Hong Kong Polytechnic University

Dear Ms SUN,

Animals (Control of Experiments) Ordinance
Chapter 340

I forward herewith the following licence(s) issued under the above Ordinance:-

Form 2 : Licence to Conduct Experiments

Your attention is drawn to regulations 4 and 5 of the Animals (Control of Experiments) Regulations as excerpted below:-

"4. Records
Every licensee shall keep up-to-date a book in the form set out as Form 6 in the Schedule in which he shall record the particulars therein indicated of all experiments performed by him.

5. Returns
Every licensee shall render to the Director of Health on or before the 1st day of January each year a return in the form set out as Form 7 in the Schedule of all experiments performed by him during the preceding twelve months."

Copies of Form 6 and Form 7 are enclosed for your convenience. Failure to comply with either regulation 4 or regulation 5 is an offence, each offence punishable by a fine of HK$500 and to imprisonment for 3 months. Conviction of an offence against either regulation 4 or regulation 5 or failure to comply with either regulation may result in your licence being cancelled.

2.

Please also be reminded that if you wish to continue your experiments after the specified periods as stated on the above licence / endorsements / teaching permit, you should renew them at least two months before the end-dates. On the other hand, if you have completed or stopped your experiments before the specified periods, you should inform us immediately.

Yours sincerely,

(Dr Kellie SO)
for Director of Health

* *Remarks:-*

A "Code of Practice – Care and Use of Animals for Experimental Purposes" was prepared by the Agriculture, Fisheries and Conservation Department on the advice of the Animal Welfare Advisory Group.

Please visit the Agriculture, Fisheries and Conservation Department's website at <http://www.afcd.gov.hk/english/publications/publications_qua/files/code.pdf> for details of the Code of Practice.

Encl.

Form 2

Licence to Conduct Experiments

Name : SUN Jing [Ref No.: Rev(12-24) in DH/HA&P/8/2/4 Pt.5]
Address : Department of Health Technology and Informatics, The Hong Kong Polytechnic University

By virtue of section 7 of the Animals (Control of Experiments) Ordinance, Chapter 340, the above-named is hereby licensed to conduct the type of experiment(s), at the place(s) and upon the conditions, hereinafter mentioned.

Type of experiment(s)

Rats will be used in the experiment. Neuromuscular stimulation electrodes will be implanted at the hindlimb of the animals under anaesthesia. Analgesics will be given after surgery. Stroke will be induced by middle cerebral artery occlusion under anaesthesia. Analgesics will be given after surgery. Neuromuscular electrical stimulation (NMES) on hindlimb muscles after stroke and functional assessments by De Ryck's test and beam walking test will be conducted. Briefly, in the De Ryck's test, the animals will be subjected to a series of simple behavioural tasks to test for the forelimb and hindlimb functions such as the ability to stretch the ipsilesional forelimb towards a table when being suspended over it, forelimb placement when facing a table edge, lateral placement of forelimb and hindlimb when being placed along the table edge and the ability to keep their grip when being pushed from behind towards the table edge. In the beam walking test, the animals will be placed on a beam and observed for the performance in terms of coordination and integration of motor movement. Conditions of the animals will be monitored. Animals will be euthanized before the end of the study if any serious injury has been inflicted on them. At the end of the experiment, the animals will be sacrificed by overdose of anaesthetics. Brain will be harvested for analyses.

Place(s) where experiment(s) may be conducted

Centralised Animal Facilities, The Hong Kong Polytechnic University

Conditions

1. Such experiment(s) may only be conducted for the following purposes-

   (a) To investigate the neurochemical effects of post-stroke training by NMES correlated with different intervention times and workloads.
   (b) To design a rat model for post-stroke training by NMES with controllable training intensity.
   (c) To find the training schemes that can maximise the motor functional recovery and minimise the loss in brain tissue.

2. This licence is valid from 12 April 2012 to 11 April 2014

Dated 12 April 2012 (Amended on 20 April 2012)

DEPARTMENT OF HEALTH 衞生署 HONG KONG

Licensing Authority

2. PROCEDURES OF BRAIN TISSUE TREATMENT AND ELISA TEST

(*Procedures followed the instructions from the manufacturer, and figures were calculated according to the experimental setup*)

SAMPLE PREPARATION

1. Harvest of cerebral cortex, striatum and hippocampus, immersed in 1 ml lysis buffer.
2. Homogenization.
3. Dilute 200 µl homogenate in 800 µl DPBS.
4. Acidify homogenate to ~3.0 using 1N HCL. (1µl 1N HCl per 50 µl sample)
5. Incubate at room temperature for 15 min.
6. Neutralize with 1N NaOH to pH ~7.6. (1µl 1N NaOH for 50 µl sample)
7. Microfuge at 14000 rpm for 3 min.
8. Collect the supernatant and stored at -20 ^{o}C.

ELISA

1. 10 µl of the Anti-BDNF mAb to 9.99 ml of carbonate coating buffer. Add 100 µl to each well. NOTE: Keep the undiluted Anti-BDNF mAB on ice.
2. Seal the wells and incubate without shaking overnight at 4 ^{o}C.
3. 8.8 ml of Block & Sample 5X Buffer to 35.2 ml of DI water (Aseptic transfer technique).
4. Flick out the contents of the wells and wash with TBST wash buffer.
5. Add 200 µl of Block & Sample 1X Buffer to each well.
6. Incubate at room temperature for 1 hr without shaking.
7. Thaw sample in 4 ^{o}C refrigerator.
8. 2 µl undiluted BDNF Standard to 78 µl Block & Sample 1X Buffer. (Solution A)
    1) 10 µl : 490 µl Block & Sample 1X Buffer (500 pg/ml)
    2) 10 µl : 615 µl Block & Sample 1X Buffer (400 pg/ml)

3) 10 µl : 823 µl Block & Sample 1X Buffer (300 pg/ml)

4) 10 µl : 990 µl Block & Sample 1X Buffer (250 pg/ml)

5) 5 µl : 620 µl Block & Sample 1X Buffer (200 pg/ml)

6) 5 µl : 1245 µl Block & Sample 1X Buffer (100 pg/ml)

7) 50 µl Solution 1) : 450 µl Block & Sample 1X Buffer (50 pg/ml)

NOTE: Keep the undiluted BDNF Standard on ice.

9. Flick out the contents of the wells. Wash once with TBST wash buffer.

10. Designate two columns of wells for the standard cure. (100 µl/well)

	11	12
A	500 pg/ml	500 pg/ml
B	400 pg/ml	400 pg/ml
C	300 pg/ml	300 pg/ml
D	250 pg/ml	250 pg/ml
E	200 pg/ml	200 pg/ml
F	100 pg/ml	100 pg/ml
G	50 pg/ml	50 pg/ml
H	1X B&S Buffer	1X B&S Buffer

11. Add 100 µl of the samples to wells. Seal the wells with a plate sealer and incubate the plate for two hours at room temperature with shaking (400 ± 100 rpm).

12. 16 µl Anti-Human BDNF pAb to 7.984 ml of Block & Sample 1X Buffer.

13. Wash the plate 5 times with TBST wash buffer.

14. Add 100 µl of the diluted Anti-Human BDNF pAb to each well. NOTE: Keep the undiluted Anti-Human BDNF pAb on ice.

15. Seal the wells with a plate sealer and incubate for 2 hrs at room temperature with shaking.

16. Wash the plate 5 times with TBST wash buffer.

17. 41 µl Anti-IgY HRP Conjugate to 8.159 ml of Block & Sample 1X Buffer.
18. Add 100 µl of the diluted Anti-IgY HRP Conjugate to each well. NOTE: Keep the undiluted Anti-IgY HRP on ice
19. Incubate for 1 hr at room temperature with shaking (400 ± 100 rpm).
20. Equilibrate the TMB One Solution to room temperature.
21. Wash the plate five times with TBST wash buffer.
22. Add 100 µl of the room temperature TMB One Solution to each well.
23. Incubate at room temperature with shaking for 10 min. (Blue)
24. Add 100 µl of 1N HCl to the wells in the same order in which substrate was added in the previous step. (Yellow)
25. Record the absorbance at 450 nm on a plate reader within 30 min of stopping the reaction. NOTE: The exterior bottom of the plate must be optically clean for accurate measurement. Wipe the exterior bottom with 70% ethanol if necessary.

---------- END ----------