THE EFFECT OF EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS ON THE PROLIFERATION AND DIFFERENTIATION OF ENDOGENOUS NEURAL STEM CELLS IN RATS WITH CEREBRAL ISCHEMIA

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The Effect of Extremely Low Frequency Electromagnetic Fields on the Proliferation and Differentiation of Endogenous Neural Stem Cells in Rats with Cerebral Ischemia

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Certificate of originality

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Abstract

Traumatic brain injury (TBI) is the head injury caused by a direct or indirect external force, which is very common in disaster victims hitting by falling of heavy objects. Cerebral ischemia is discussed as one secondary injury mechanism that may participate in TBI. In recent decades, a focus on the neuroplasticity of the brain provides a brand-new research direction with regard to the recovery of brain damage. Neural stem cells (NSCs) which are mainly generated in the subgranular zone (SGZ) and the subventricular zone (SVZ) play an important role in the process of self-repair and neuroplasticity of the brain and represent a main line of current research. The Notch signaling pathway regulated the proliferation and differentiation of NSCs. When the Notch signaling pathway is upgraded, the expression of the key factors of the pathway including Hes1, Hes5 and Notch1 will be increased, the number of NSCs might be increased.

A number of studies have found that extremely low frequency electromagnetic fields (ELF-EMF) can promote the differentiation of NSCs in vitro or in healthy adult rats. Experimental results also show that ELF-EMF could become an important treatment strategy in regenerative medicine. However, reports on the effect of ELF-EMF in cerebral ischemia are still lacking, and the underlying mechanism remains unclear.

In this experiment, Longa's MCAO surgery was performed to establish the animal model of ischemia brain injury. Sprague-Dawley (SD) rats were divided into

sham-surgery group, control group and experiment group randomly, the last two groups received Longa's MCAO surgery. The experiment group further received ELF-EMF (50Hz, 1mT) intervention. Then in different time points, mNSS, De Ryck's Scale and Morris water maze were used to assess neurological function, BrdU labeling method was applied to observe the NSCs' proliferation and differentiation and the new born neurons, RT-PCR and Western blot analysis were used to analyze gene and protein expression of Notch1, Hes1 and Hes5 in the SVZ and SGZ.

The results showed that ELF-EMF (50Hz, 1mT) : 1) significantly improved learning ability and memory, however had no significant effect on mNSS and De Ryck's scores in rats with cerebral ischemia; 2) significantly promoted the proliferation and differentiation of NSCs, and increased the number of new born neurons in SVZ and SGZ in rats with cerebral ischemia; 3) significantly increased the expression of Hes1, Hes5 and Notch1 in the SVZ and SGZ and 4) probably promoted the proliferation and differentiation of NSCs by regulating the Notch signaling pathway in cerebral ischemia.

These findings provide a theoretical basis for the application of ELF-EMF in cerebral ischemia. ELF-EMF may be benefit for TBI and ischemic diseases in central never system.

Key Words: extremely low frequency electromagnetic fields, cerebral ischemia, neural stem cells, Notch signaling pathway, Notch1, Hes1, Hes5

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Abbreviations	Full name
ELF-EMF	extremely low frequency electromagnetic field
NSCs	neural stem cells
Hes	hairy and enhancer of split
CVA	cerebrovascular accident
TBI	traumatic brain injury
DG	dentate granule
SGZ	subgranular zone
LV	lateral ventricle
SVZ	subventricular zone
RMS	rostral migratory stream
OB	olfactory bulb
EGF	epidermal growth factor
FGF	fibroblast growth factor
PDGF	platelet-derived growth factor
BDNF	brain-derived neurotrophic factor
NICD	notch intracellular domain

bHLH	basic helix-loop-helix
MCAO	middle cerebral artery occlusion
mNSS	modified neurological severity score
MWM	morris water maze
BrdU	5-Bromo-2-deoxyUridine
DCX	doublecortin
GFAP	glial fibrillary acidic protein
NeuN	neuronal nuclear antigen
DAPI	4', 6-diamidino-2-phenylindole

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Acquired brain injuries

Acquired brain injury (ABI) refers to the brain damage caused by various reasons after birth (Castellanos-Pinedo et al., 2012). Trauma, hypoxia, infection, tumour, degenerative neurological disorders or stroke are known causes of ABI which is largely associated with dual or multiple disabilities (March et al., 2013). Among all ABI patients, traumatic brain injuries (TBI) and cerebrovascular disease both have a portion of above 40%, all the other injuries such as anoxic brain damage and degenerative neurological diseases take the portion of less than 20% (Avesani et al., 2013).

1.1.1 TBI

TBI is the head injury caused by a direct or indirect external force. It's often happened in accident, war or a kind of disasters. TBI caused by falling of heavy objects are very common problems in earthquake victims (Sutiono et al., 2012). TBI patients account for a large portion of all the injuries even during the early period of earthquake (Gu et al., 2010).

The classification of TBI can be based on different features such as severity, injured locations and mechanisms. Based on the localized injured area or large injured area, TBI can be classified into focal brain injury and diffuse brain injury (Nolan, 2005). According to whether the brain tissues are exposed to the outside after the injury, TBI can be classified into open and closed injury (Maas et al., 2008). The most common classification of brain parenchymal injuries in clinical practice are cerebral concussion, intracranial hematoma, cerebral contusion and brain stem injury.

Cerebral concussion is the lightest one of closed brain injury. Loss of consciousness within half an hour and transient amnesia are the main characteristics of this kind of brain damage. The patients may have slight headache, dizziness, nausea or vomiting. Vital signs are normal, no positive findings are detected in the nervous system (Powell, 2001, Riggs, 2001).

Intracranial hematoma refers to the intracranial hemorrhage caused by brain damage, and the blood accumulates in brain tissue or certain parts of the cranial cavity. It can be divided into (1) epidural hematoma: the external force or the skull fracture tears or hurts dural blood vessels; (2) subdural hematoma: the bleeding sites are in the subdural area (Gomez-Rios and Kuczkowski, 2012, Ryan et al., 2012).

Cerebral contusion is common in traumatic brain injury and generally occurs in the striking site or the opposite site (also called the coup injury or contracoup injury). This kind of injuries can cause severe damage to the deep brain structures. The hemorrhage, rupture, edema and necrosis are showed in different points of the injured brain tissue (Kawamata and Katayama, 2007). It often causes the focal or diffuse cerebral edema, swelling and intracranial hematoma in the adjacent cerebral tissue.

Brain stem injury refers to the injuries in midbrain, pons and medulla oblongata. It can be classified into primary brain stem injury (direct injuries of the brain stem) and secondary brain stem injury (caused by the increased compression pressure on the brain stem secondary to intracranial hematoma and cerebral edema after traumatic brain injury) (Shukla et al., 2007). Studies showed interests on the prognostics factors

of the brain stem injury as its poor prognosis and high mortality rate (Chew et al., 2012, Kim, 2012).

Blunt TBI may cause the changes of cerebral microvessel which may in turn lead to cerebral ischemic damage. Graham (Graham and Adams, 1971) reported 38 cases of autopsy in brain trauma, 21 cases had ischemic brain injury or cerebral infarction, accounting for 55%. Early after TBI, the capillaries around the injured area collapsed due to the edema of surrounding tissue, or the oppression of small blood clots (Kochanek, 1993). Cerebral ischemia is recognized as one secondary injury mechanism that may participate in brain trauma (Mazzeo et al., 2006).

1.1.2 Stroke

Stroke, also known as cerebrovascular accident (CVA), is a sudden loss of brain function due to cerebral blood circulation disorders. Various predisposing factors including brain artery stenosis, occlusion or rupture can cause a acute disturbance on cerebral blood supply (Sims and Muyderman, 2010). Clinical manifestations are symptoms and signs of transient or permanent brain dysfunction. Stroke is usually classified into ischemic and hemorrhagic stroke.

Ischemic stroke accounts for about 80% of all stroke cases (Thrift et al., 2001). It refers to the ischemia, hypoxia and necrosis of local brain tissue due to the insufficiency or blockage of cerebral blood supply. It is mainly due to the emergence of atherosclerosis or thrombosis in the cranial arteries; or abnormal objects (solid, liquid, gas) flowing along the blood circulation into the cerebral artery (Donnan et al., 2008). Hemorrhagic stroke is largely due to the rupture of small cerebral artery caused

by high blood pressure (Burns and Manno, 2008). It can be divided into two subtypes: intracranial hemorrhage and subarachnoid hemorrhage (Donnan et al., 2008). Severity and mortality rate of hemorrhagic stroke are much higher than ischemic stroke.

1.1.3 Functional disorders of the ABI

Brain function related to human activities including motor, sensory, language, memory, emotion, location, computing and logic are very complex and sophisticated. When the brain is injured for either traumatic or non-traumatic reasons, the function of the injured brain tissue will be affected. From the perspective of rehabilitation medicine, the functional disorders of ABI are mainly the following aspects.

Motor dysfunction. The motor control and motor behavior are complex processes including organization and coordination among the multiple neural networks and musculoskeletal system (Buschges et al., 2011, Takeuchi et al., 2012). The neural networks in central neural system including pyramidal system and extrapyramidal system are involved in motor control. Weakness, posture instability, dystonia, ataxia and tremor are common motor dysfunctions caused by the central nervous system lesion (O'Suilleabhain and Dewey, 2004). Dystonia (Tanabe et al., 2009) and synergic patterns (Naghdi et al., 2010) are specific characteristics of the upper motor neuron injury in TBI and stroke.

Somatosensory disturbance. The normal somatosensory can be divided into superficial sensibility, deep sensibility and synaesthesia. Superficial sensibility mainly refers to pain, temperature and touch sensation. The deep sensibility are the sensation of proprioception, vibration and position. When the primary somatosensory area in

the cortex or the sensory tracts are injured in the brain, the sensory dysfunction may appear. Common sensory impairments are hypersensitivity (the feeling is abnormally high to the external stimuli), hypoesthesia (the feeling is decreased or totally loss to external stimuli), paralgesia (the false feeling of the external stimuli) and senestopathia (the strange and discomfort feeling come from the inside of the body). Hypoesthesia or sensory loss is the most common type of somatosensory disturbances in TBI or stroke patients (Sullivan and Hedman, 2008).

Aphasia. It is an acquired impairment following brain damage on understanding and expression in human, especially in pronunciation, vocabulary, grammar and other ingredients of the language. Aphasia could be classified into expressive aphasia, receptive aphasia, conduction aphasia or anomic aphasia. The expressive aphasia is the common type of aphasia in stroke (Mylius et al., 2012, Hoffmann and Chen, 2013). The expressive language center is located in the inferior frontal gyrus in left frontal lobe (Broca's area); while receptive language center is located in the superior temporal gyrus (Wernicke's area) (Rotte, 2005). The main symptoms of expressive aphasia are individuals can understand other's language but are difficult to express themselves; the symptoms of receptive aphasia are patients can not understand the words of others.

Cognitive impairment. Cognition is the body awareness and the mental process involving a series of psychological and social behavior including learning, memory, language, thinking, mental and emotional. Cognitive impairment refers to deficits in the cognition process, accompanied by disturbance in executive function and other

disorders. Cognitive impairment is a common disorder secondary to ABI and related to the recovery and prognosis (Whyte et al., 2011).

These impairments will further cause the disability and handicap which related to the patients' self care and social participation (Ustun et al., 2003). Moreover, these impairments not only affect the patients' performance personally, but also impose a heavy burden on the whole society. How to improve the recovery of these function has been a hot research point in ABI in recent decades.

1.1.4 The functional recovery and brain plasticity

The loss of neurons caused by ABI can result in further functional deficits of the affected area. After the injury, nerve regeneration processes will lead to some extent of functional recovery (Wieloch and Nikolich, 2006). In the early stage of the brain injury, dealing with cellular oxidant stress as well as inflammation and edema in the penumbra is essential. Neurons in the penumbra can recover rapidly and are involved in the repair process. In the post-acute injury stage, the repair process can be divided into the following stages: the phase of dead cells absorption, cell repair, cell metabolism and neural function recovery; the phase of axonal growth, dendritic remodeling and activation; the phase of new neural networks formation and functioning (Wieloch and Nikolich, 2006). The injury triggers the neurogenersis in the brain to a limited extant, however, external interventions also can accelerate the repair processes (Nudo, 2011).

Mechanism of the brain injury recovery are still under exploration. In the recent decade, the brain plasticity, also known as neuroplasticity, has opened a new research

direction in mechanisms of brain injury recovery, and has provided fundamental support for neurorehabilitation. Brain plasticity occurs at different levels, from subtle changes in cells to massive changes in cortical remapping. The role of brain plasticity is widely recognized in functional recovery of motor, sensory and cognitive abilities in rehabilitation medicine.

The brain plasticity theory points out that the brain can recover from function deficits including paralysis, hypoaesthesia, aphasia, aphagia, cognitive impairment after the brain damage. When brain injury happens, the damaged synapses and the surrounding cells of the dead neurons will have a series of changes to accommodate this injury and functional deficits. Axonal sprout, cell genesis, growth factors, brain activation, enriched environment and neural stem cells (NSCs) are all important factors in the process of brain functional recovery (Wieloch and Nikolich, 2006).

1.2 Neural stem cells (NSCs)

1.2.1 The discovery of NSCs

The traditional view of neurology was that mammalian neurons in the central nervous system (CNS) could only be produced in the embryonic stage or a short period after birth, and adult mammalian neurons did not have the ability to self-renew which meant that neurons could not regenerate once damaged. In 1992 however, Reynolds and Weiss (Reynolds and Weiss, 1992) found that cells separated from adult mice's striatum could continue to proliferate and differentiate into other cells, then the concept of neural stem cells (NSCs) was born. NSCs can self-renew and differentiate into various types of cells in the central nervous system. The discovery of NSCs has dispelled the misunderstanding that the matured adult central nervous system no longer has the ability to self-renew and self-repair. The discovery of NSCs also provides a new idea for the treatment of neurological diseases such as traumatic brain injury and stroke (Decimo et al., 2012).

1.2.2 The characteristics of NSCs

NSCs are defined as the pluripotent cells which can produce major cell types of the CNS in adult animals and have the capability of self-renewal (Clarke et al., 2000). The basic characteristics of NSCs are: (1) the potential of multipotent differentiation. NSCs can differentiate into neurons, astrocytes and oligodendrocytes which are the three main cell types in the CNS. (2) Self-maintenance and self-renewal. NSCs have the ability to maintain a certain number in a certain location and to proliferate when necessary. NSCs proliferate in two ways: symmetric division which produces two

stem cells or two progenitor cells, and asymmetric division which generates one new stem cell and one progenitor cell. Progenitor cells only exist for a short time due to their continued migration and differentiation or death after proliferation, while NSCs divide slowly to maintain the stability of stem cell bank and to replenish progenitor cells (Weng and Lee, 2011). The new born cells can be continuously added to the neural networks in the restricted location.

1.2.3 The location of NSCs

The two major neurogenic regions of NSCs in the adult mammalian brain are the subgranular zone (SGZ) which is below the hippocampus dentate granule (DG) and the subventricular zone (SVZ) near the adjacent lateral ventricle (LV) (Yao et al., 2012). Apart from the two well studied regions, a number of brain regions, such as amygdala and cotex, were also reported to have the function of neurogenesis. However, the most progenitors in amygdala would not differentiate into mature neurons or become functional; the evidence is very limited to support the massive cortical neurogenesis (Landgren and Curtis, 2011).

New granular neurons are generated in the SGZ while new neuroblasts are developed continuously in the SVZ and migrate to the olfactory bulb (OB) as well as the neocortical areas through the rostral migratory stream (RMS) (Trujillo et al., 2009, De Filippis and Binda, 2012, Bellenchi et al., 2013). These anatomical structures are shown in Figure 1.1.

The process of NSCs' migration in the adult brain under both normal and injured conditions was investigate in recent years. Neural cells demonstrate two unique types

of mobility: radial migration and tangential migration. In radial migration, which is seen mainly during embryonic cortical development and in restricted areas of the cerebellum and the olfactory bulb, newly born neurons in the neocortex migrate along radial glial processes. Tangential migration is found throughout developing brains, it only persists in the RMS of adult brains. A change of migration pattern from tangential to radial migration also happens in response to cortical injury to adapt migration patterns in response to changes in their surrounding environment (Barkho and Zhao, 2011).

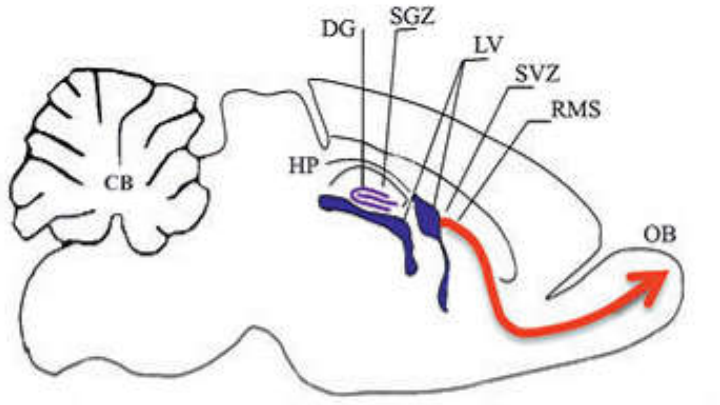


Figure 1. 1 The sagittal plane of the mouse's brain (Bellenchi et al., 2013)

1.2.4 The differentiation of NSCs

The proliferation, migration and differentiation of NSCs are closely related to the microenvironment including the surrounding neural cells, stromal cells and extracellular matrix. The molecular mechanisms to control the developmental processes and determine the cell fate are complex as multiple signaling pathways, transcription factors and interactions are involved (Trujillo et al., 2009).

There are two mechanisms to regulate the differentiation of NSCs: self regulation and exogenous signal regulation. The regulation by the transcription factors and other

factors inside the NSCs in the development of NSCs are specified as self regulation. For example, when the Notch signaling is activated, the NSCs would be in the status of proliferation and thus increase the number of NSCs. In addition, other transcription factors or pathways such as the Wnt signaling pathway and Sonic hedgehog pathway are also involved in the self regulation of NSCs (Faigle and Song, 2013). Exogenous signal regulation refers to the regulation of the developmental processes by the cytokines and other neurological factors in the microenvironment outside the NSCs. Now it's widely recognized that epidermal growth factor (EGF) (Cooke et al., 2011) and basic fibroblast growth factor (FGF) (Sirko et al., 2010) play crucially in the proliferation and differentiation as well as self renewal of NSCs. Platelet-derived growth factor (PDGF) (Xu et al., 2013) and brain-derived neurotrophic factor (BDNF) (Bath et al., 2012) also participate in the differentiation of NSCs. Exogenous signal regulation is important for the development of NSCs, NSCs from the same source can differentiate to various of nerve cells when growing in different environments.

It was reported that the ischemia-stimulated newborn neurons could differentiate into GABAergic and cholinergic neurons and functionally integrate into neural networks in the ischemia-injured striatum of the adult rat from the fourth week after the MCAO surgery (Hou et al., 2008). Song et al described the process of synaptic integration in new born granule neurons (Song et al., 2012): "1) Initial activation of newborn neurons is non-synaptic, which is mediated by ambient GABA present in the local milieu; 2) Newborn neurons become activated by synaptic transmission from local interneurons through input-specific GABAergic signaling; 3) GABAergic inputs

are converted from excitatory to inhibitory, and concomitantly, excitatory glutamatergic dendritic inputs begin to activate the developing neurons; 4) Finally, inhibitory GABA synaptic inputs begin to appear at perisomatic synapses to complete the mature granule cell innervation pattern".

1.2.5 NSCs and diseases

NSCs are usually in relatively quiescent status in the niche in mammalian brain (Lugert et al., 2010). However, neurogenesis with NSCs will be enhanced in various pathological conditions. Many studies have reported enhanced neurogenesis in stroke, TBI, Parkinson's disease, Alzheimer's disease, Huntington's disease and epilepsy (Decimo et al., 2012, Ziemka-Nalecz and Zalewska, 2012, Bellenchi et al., 2013). In pathological conditions, inflammatory processes or transcription factors can stimulate NSCs to activate from both self regulation and exogenous signal regulation to repair the damaged brain.

As it is such a critical disease and imposes a heavy burden in the society, the neurogenesis on ABI has been widely studied in recent decades. Among these studies, the focal cerebral ischemia model appears more popular than other ABI models. Since 1989, Longa has invented the focal cerebral ischemia model induced by the occlusion of middle cerebral artery, resulting in the infarction of cerebral cortex and subcortical tissue (Longa et al., 1989). This model is still used by researchers now due to its stability and reliability. In rodents the peak of cell proliferation induced by ischemic stroke in the SVZ and SGZ is around 1-2 weeks after injury, accompanied by an increasing number of immature neurons at the infarction site (Ziemka-Nalecz and

Zalewska, 2012). Some of the immature neurons will differentiate into the certain type of neurons dying in the insult, however, the majority of the new born neurons will not survive 2-5 weeks after the cerebral ischemia. These may be caused by an inappropriate microenvironment or lack of trophic factors. Only 0.2% of dead neurons in striatum will be replaced by newly differentiated NSCs (Arvidsson et al., 2002, Ziemka-Nalecz and Zalewska, 2012).

1.2.6 The application of NSCs in cerebral ischemia

NSCs play an important role in the self-repair of the damaged brain tissue. Currently, there are two ways to apply NSCs treatment for ischemic brain injury: transplantation of exogenous NSCs and activation of endogenous NSCs (Dibajnia and Morshead, 2012). In contrast to immune rejection, ethical, and other issues of exogenous NSCs (Gincberg et al., 2012), endogenous NSCs have broad research and application prospects. Cerebral ischemia can activate NSCs' proliferation and differentiation, however, the small scale cell proliferation is not enough for significant self-repair. Only a very small amount of newborn neurons can alternate the function of necrotic neurons in the brain. Therefore, increasing endogenous NSCs' proliferation and differentiation has important value and significance for the plasticity of the brain and functional recovery of neurological diseases such as cerebral ischemia.

1.3 Notch signaling pathway

Neural regeneration is a plastic process triggered by external stimuli (Lugert et al., 2010) and affecting the proliferation and differentiation of NSCs through particular mechanisms. The Notch signaling pathway represents such mechanism. It is an important signaling pathway in stem cells which plays an extremely important role in the regulation of NSCs' proliferation and differentiation (Oya et al., 2009, Aguirre et al., 2010). The core components of the Notch signaling pathway are Notch receptors, ligands, and DNA-binding proteins. The mRNA and protein including Hes1, Hes5, mash1, mash3, Ngn2 and Notch intracellular domain (NICD) are essential in Notch signaling pathway.

1.3.1 Hes factors

Kageyama (Kageyama et al., 2008) defined the Hes gene as 'Hes genes are mammalian homologues of Drosophila hairy and Enhancer of split (Hes), which encode basic helix-loop-helix (bHLH) transcriptional repressors'. Hes gene family has so far been found seven homologues which have a conserved domain. Research shows that Hes1 and Hes5 are involved in the Notch signaling pathway as inhibitory bHLH genes in the proliferation and differentiation of NSCs, so Hes1 and Hes 5 are the negative transcription factor in the bHLH gene family.

Hes factors have a specific conserved bHLH domain which is the main functional area and can bind with some promoters such as Mash1 to perform its role of negative transcription. They also have a mild conserved domain called orange domain, which may mediate the interaction with other proteins such as they can bind

competitively with E47 to antagonize Mash1. Additionally, Hes factors have a specific conserved domain named WRPW in addition to bHLH domain, this domain can function as a repression domain to inhibit transcription (Iso et al., 2003, Kageyama et al., 2005, Kageyama et al., 2008). The structure and function of Hes factors are shown in Figure 1.2.

In the developing CNS, the high expression of Hes1 and Hes5 are found in NSCs. Both Hes1 and Hes5 are regulated by Notch signaling. The function of Hes factors in the developing nervous system could be the maintenance of NSCs, the promotion of gliogenesis and the maintenance of boundary cells (Kageyama et al., 2008).

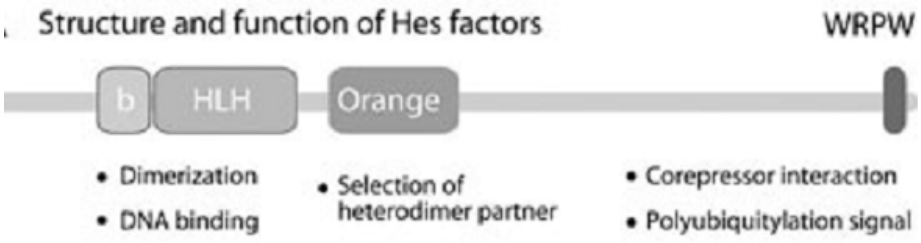


Figure 1. 2 The structure and function of Hes factors (Kageyama et al., 2008)

1.3.2 The process of Notch signaling pathway

Kageyama (Kageyama et al., 2005) had described the process of Notch pathway in detail as below: 'Notch, a transmembrane protein, is activated by the ligands Delta and Jagged, which are also transmembrane proteins expressed by neighboring cells (Figure 1.3, step (1)). Upon activation, Notch is processed to release the NICD, which is transferred into the nucleus and forms a complex with the DNA-binding protein RBP-J (Figure 1.3, step (2)). RBP-J itself is a transcriptional repressor and represses Hes1 and Hes5 expression by binding to their promoters (Figure 1.3, step (4)).

However, when RBP-J forms a complex with NICD, this complex becomes a transcriptional activator and induces Hes1 and Hes5 expression (Figure 1.3, step (2))'.

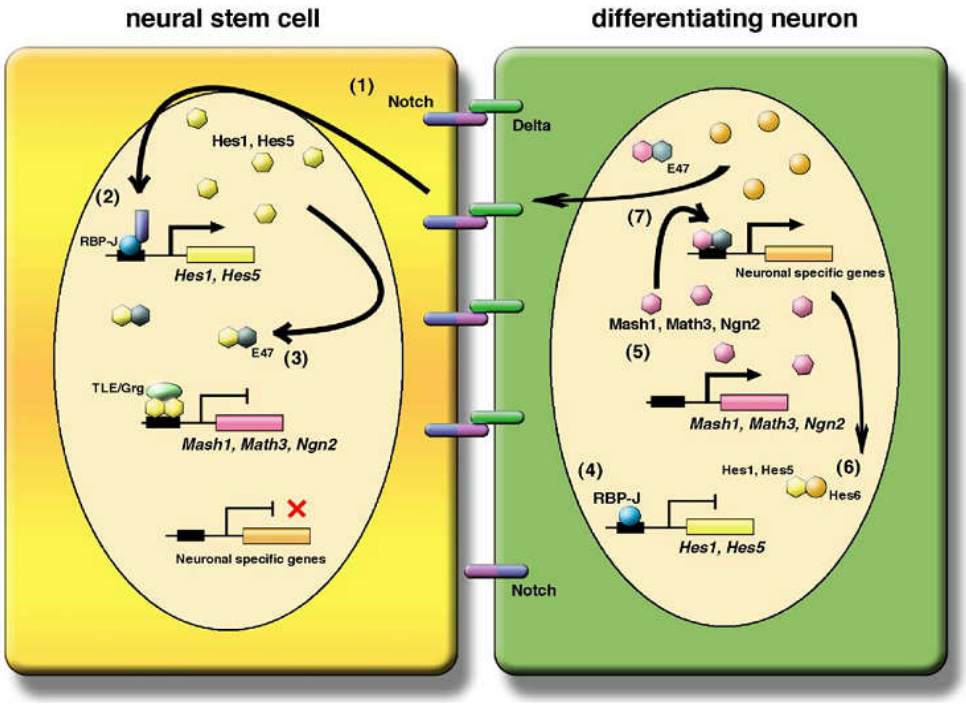


Figure 1. 3 Notch signaling pathway (Kageyama et al., 2005)

Thus, the activation of Notch may upregulate the expression of Hes1 and Hes5. Hes1 and Hes5 are required in Notch signaling as Notch fails to inhibit neuronal differentiation in the absence of Hes1and Hes5 (Ohtsuka et al., 1999). When the Notch signaling pathway is activated, it will promote the proliferation of NSCs; when the Notch signaling pathway is inhibited, the proliferation of the NSCs will be decreased (Kageyama et al., 2005, Hansen et al., 2010, Hoeck et al., 2010, Kato et al., 2010). If the Notch signaling pathway was completely suppressed, there would be a transient increase of neurogenesis, and then followed by the depletion of NSCs and totally lost of the neurogenesis. Notch signaling pathway is essential to maintain

NSCs and to properly regulate neurogenesis both in the embryonic or adult brain (Imayoshi et al., 2010).

1.3.3 Notch signaling pathway and cerebral ischemia

The characteristics of the Notch signaling pathway still work under pathological conditions such as cerebral ischemia. Recent studies (Oya et al., 2009, Wang et al., 2009a, Wang et al., 2009b) showed that, NICD protein and Hes1, Hes5 gene expression changed in the SVZ after cerebral ischemia, and the values changed with time, so that the Notch signaling pathway may regulate the proliferation and differentiation of NSCs in cerebral ischemia. Inhibiting the Notch signaling pathway can block the neural cell proliferation induced by ischemia in the SVZ (Wang et al., 2009b), and also, can promote immature neurons migrate and eventually lead to an increase of newborn neurons in cerebral ischemia (Oya et al., 2009). For the treatment of stroke and related neurodegenerative conditions, Notch signaling may be a therapeutic target (Arumugam et al., 2006).

1.4 ELF-EMF

ELF-EMF is the electromagnetic field with the frequency of 0 ~ 300Hz (Funk et al., 2009). Electrical currents can create electromagnetic fields. In previous studies, ELF-EMF devices were designed almost in the same way (Vazquez-Garcia et al., 2004, Gutierrez-Mercado et al., 2008, Varro et al., 2009). The most important part of these devices are coils which directly generate EMF. One of the best choices are the Helmholtz coils, as these coils create a very uniform field in the space between them.

1.4.1 The effects of ELF-EMF

EMF can penetrate into cell membrane, muscles, bone tissues, nerves or even the whole body (Funk et al., 2009). Many experiments have shown that, ELF-EMF can affect cellular activities through various ion channels, receptors or enzymes on cell membrane or inside the cell. Basic scientific research on ELF-EMF is mainly focused on a series of physiological and pathological processes, such as signaling pathway conduction (Manikonda et al., 2007), cell differentiation (Ayse et al., 2010), apoptosis (Jimenez-Garcia et al., 2010) as well as gene expression (Reyes-Guerrero et al., 2010). Clinical studies of ELF-EMF are mainly in the fields of pain (Robertson et al., 2010), fracture healing (Griffin et al., 2008), osteoarthritis (Ganesan et al., 2009) and osteoporosis (Huang et al., 2008).

1.4.2 The effects of ELF-EMF on neurogenesis

In neurological research, studies of ELF-EMF are mainly in vitro, featuring various cells separated from different organisms. The in vitro experiments showed that ELF-EMF can promote the differentiation of NSCs (Piacentini et al., 2008),

affect Ca^{2+} concentration in the synapses and neurons (Morabito et al., 2010), promote the growth of axons in chromaffin cells (Hernandez-Hernandez et al., 2009) and increase the density of cutaneous nerve fiber in the distal limb (Weintraub et al., 2009). ELF-EMF can significantly enhance the activity of synapses, and the effect is strongly dependent on the structure of the synapses and neural networks (Varro et al., 2009). ELF-EMF can change the shape of the membrane of the neurosecretory cell, increase the synaptophysin of the synaptic vesicles, and promote cell differentiation (Lisi et al., 2006). ELF-EMF can excite or inhibit electrical activities in the hippocampus, and influence physiological activity in this area (Ahmed and Wieraszko, 2008).

There are only few studies observing the effects of ELF-EMF on neurogenesis in vivo. Cuccurazzu (Cuccurazzu et al., 2010) applied ELF-EMF (50 Hz, 1 mT) to the adult male mice and found that the expression of **Hes1**, Mash1, NeuroD2 and calcium channel protein promoted significantly in the dentate gyrus (DG), and ELF-EMF significantly promoted the differentiation of NSCs to become mature neurons; through the analysis of the excitatory postsynaptic potentials in the DG, the authors believed that the newborn mature neurons had been integrated into the granule cell layer of the DG, and had enhanced the synaptic plasticity in the hippocampus.

Recently, Tasset (Tasset et al., 2012a) investigated the neuroprotective effects of ELF-EMF (60Hz and 0.7mT) in a rat model of Huntington's disease for 21 days. The results showed ELF-EMF improved neurological scores of the rats, increased the levels of BDNF in brain tissue and reduced both oxidative damage and neuronal loss

in the brain of rats model. These results showed that the ELF-EMF may become an important therapeutic tool in regenerative medicine.

1.5 Summary

In summary, available information supports the following three perspectives:

First, one of the hotspots of cerebral ischemia research is to promote the regeneration of neurons and enhance the plasticity of the brain, which have a very closely relation to the proliferation and differentiation of the NSCs.

Second, a large number of studies suggest that ELF-EMF can promote the regeneration of neurons and synapses, but these studies were mainly in vitro and normal animal experiments, one study also showed the neuroprotective effects in a Huntington's disease model, but there is no report about the effect of ELF-EMF in cerebral ischemia up until now.

Third, the Notch signaling pathway plays an important role in the recovery from cerebral ischemia. The activation of Notch signaling pathway can inhibit the NSCs differentiation and promote proliferation. Hes1, Hes5 and NICD are key components of the Notch signaling pathway.

We can conclude that, ELF-EMF could be an effective tool for increasing neurogenesis, but since now there is no report on nerve regeneration after cerebral ischemia. The purpose of this study is thus to examine whether ELF-EMF can improve endogenous NSCs proliferation or differentiation by regulating the Notch signaling pathway after cerebral ischemia.

1.6 Objectives and hypothesis

1.6.1 Objectives

The overall goal of this project is to examine the effects of ELF-EMF on the recovery of neurological function in rats with cerebral ischemia, in order to detect a potential new way to treat acquired brain injury.

The specific objectives of this study are to observe whether ELF-EMF with a frequency of 50Hz and an intensity of 1.0mT can:

1. affect the expression of Hes1, Hes5 and Notch1 in the SGZ and SVZ,
2. improve the proliferation and differentiation of the neural stem cell in the SGZ and SVZ, and
3. improve neurological function of rats with cerebral ischemia

1.6.2 Hypothesis

This study is based on the following hypothesis:

1. The expression of Hes1, Hes5 and Notch1 in the SGZ and SVZ will increase significantly in the ELF-EMF group as compared to the non-treated control group.
2. The proliferation and differentiation of NSCs in the SGZ and SVZ will increase significantly in the ELF-EMF group as compared to the non-treated control group.
3. The ELF-EMF group will show greater functional improvement than rats with cerebral ischemia in the non-treated control group.

1.6.3 Project significance and value

The project has significant implications for the discovery of innovative ways to treat cerebral ischemia. Moreover, better understanding the mechanisms underlying ELF-EMF in rats with cerebral ischemia will contribute to a theoretical basis for ELF-EMF in the treatment of cerebral ischemia. Eventually, the present study may inform future studies on the effects of ELF-EMF in animal models of cerebral ischemia and other diseases such as Parkinson's disease or Alzheimer’s disease.

CHAPTER 2 THE EFFECTS OF ELF-EMF ON NEUROLOGICAL AND COGNITIVE FUNCTION IN RATS WITH CEREBRAL ISCHEMIA

2.1 Introduction

ELF-EMF is the electromagnetic field with the frequency of 0 ~ 300Hz (Funk et al., 2009). Studies have shown that, ELF-EMF can affect cellular activities through various ion channels, receptors or enzymes on cell membrane or inside the cell. ELF-EMF had been applied on cells or animals and was found it could affect signaling pathway conduction (Manikonda et al., 2007), cell differentiation (Ayse et al., 2010), apoptosis (Jimenez-Garcia et al., 2010) as well as gene expression (Reyes-Guerrero et al., 2010). ELF-EMF has also been studied in clinical studies, it has the effect of alleviating pain (Robertson et al., 2010), accelerating the process of fracture healing (Griffin et al., 2008), protecting chondrocytes in osteoarthritis (Ganesan et al., 2009) and increasing BMD in osteoporosis (Huang et al., 2008).

The effect of ELF-EMF on neurological function such as motor, sensory and cognitive function have also been investigated. Tasset et al (Tasset et al., 2012a) reported that after 21 days' intervention of ELF-EMF, neurological function scores were improved in Huntington rats. Das et al (Das et al., 2012) reported that 6 weeks of ELF-EMF intervention could significantly improve motor and sensory function in rats with spinal cord hemisection. ELF-EMF also could be benefit on cognitive and memory function. Two hours per day and 9 days' exposure of ELF-EMF could improve social recognition memory in normal adult rats (Vazquez-Garcia et al., 2004). These presented studies showed that the ELF-EMF may become an important therapeutic tool in promoting the neurological function. However, the effect of ELF-EMF on the neurological function in cerebral ischemia is still not clear.

This chapter describes the effects of ELF-EMF on neurological function and cognitive function in rats with cerebral ischemia. Rats were assigned into sham-surgery group, control group and experiment group. The control group and the experiment group received Longa's middle cerebral artery occlusion surgery to establish cerebral ischemia, only the experiment group was exposed to ELF-EMF. For the assessment of neurological function, mNSS and De Ryck's behavioral test were performed. All rats used in Chapter 3 and Chapter 4 of this project were assessed at different time points. For assessment of cognitive function, acquisition training and the probe-trial of the Morris water maze test were used. Data were processed in Excel 2013, GraphPad Prism 5 and SPSS19.0.

2.2 Materials and methods

2.2.1 Animals and ethical approval

Healthy, male, specific pathogen free Sprague-Dawley (SD) rats, aged 12 weeks were used. The rats were kept in controlled conditions regarding temperature (20±2℃) and illumination (12h light and 12h dark cycle, 8:00 to 20:00) with food and water made available ad libidum throughout the experiments.

All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health, United States of America) and were approved by the Ethics commission of Sichuan University.

2.2.2 The establishment of rats' cerebral ischemia model

A focal cerebral ischemia model was established in the control group and the experiment group by the Longa's brain artery embolization method (middle cerebral artery occlusion, MCAO) (Longa et al., 1989). Rats were anesthetized by abdominal injection of 10% chloral hydrate in the dosage of 0.3 mL/100g) and then fixed on the operation table. First, the proximal parts of the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were ligated. Next, a fishing line was used to insert into ICA for about 2cm, from the origin of the ECA. The incision was sutured and the rat was kept warm until it recovered from anesthesia. In the sham-surgery group, only an incision was made in the neck skin but no vascular occlusion was performed. Rectal temperature was kept at 37.0-37.5℃ during the procedure.

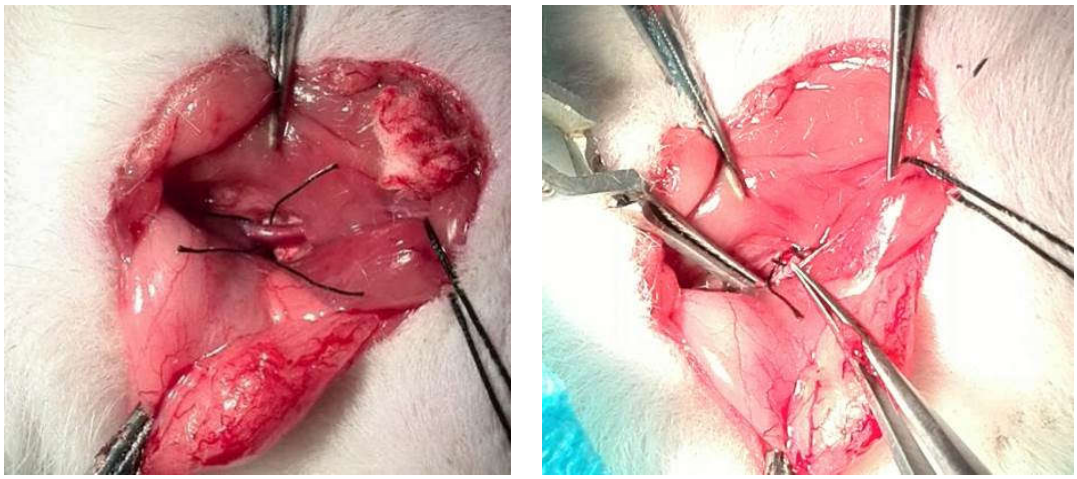


Figure 2. 1 The MCAO surgery for the rats

2.2.3 Inclusion criteria

The model was considered successful if the rats awoke and presented one of following symptoms 24h after the surgery: (1) flexion and internal rotation of the left forelimb; (2) weakness of the left forelimb, or falling down to the left side; (3) circling towards the left side. Rats will be excluded if they have: (1) subarachnoid hemorrhage observed in dissection; (2) died before sacrifice.

2.2.4 Grouping

(1) The functional deficits assessment: all the rats which were used in the third part and the fourth part of this thesis were used to evaluate functional deficits. The detailed grouping plans can be found in the third part and the fourth part of this thesis. All rats were tested at the beginning of the grouping (the one day after the MCAO) and every time point (at the 3^{rd}, 7^{th}, 14^{th} and 28^{th} day of intervention) before sacrifice.

(2) Morris water maze test: rats were randomly assigned into sham-surgery group (Sham group) and MCAO group. All the rats in MCAO group accepted the MCAO surgery. After surgery, successful modeled rats were randomly assigned into

control group (Con group) and experiment group (Exp group) using a random number table.

The flow charts for the functional deficits assessment are shown in Chapter 3 (Figure 3.1, Figure 3.2) and Chapter 4 (Figure 4.1). The flow charts for Morris water maze test is shown in Figure 2.2.

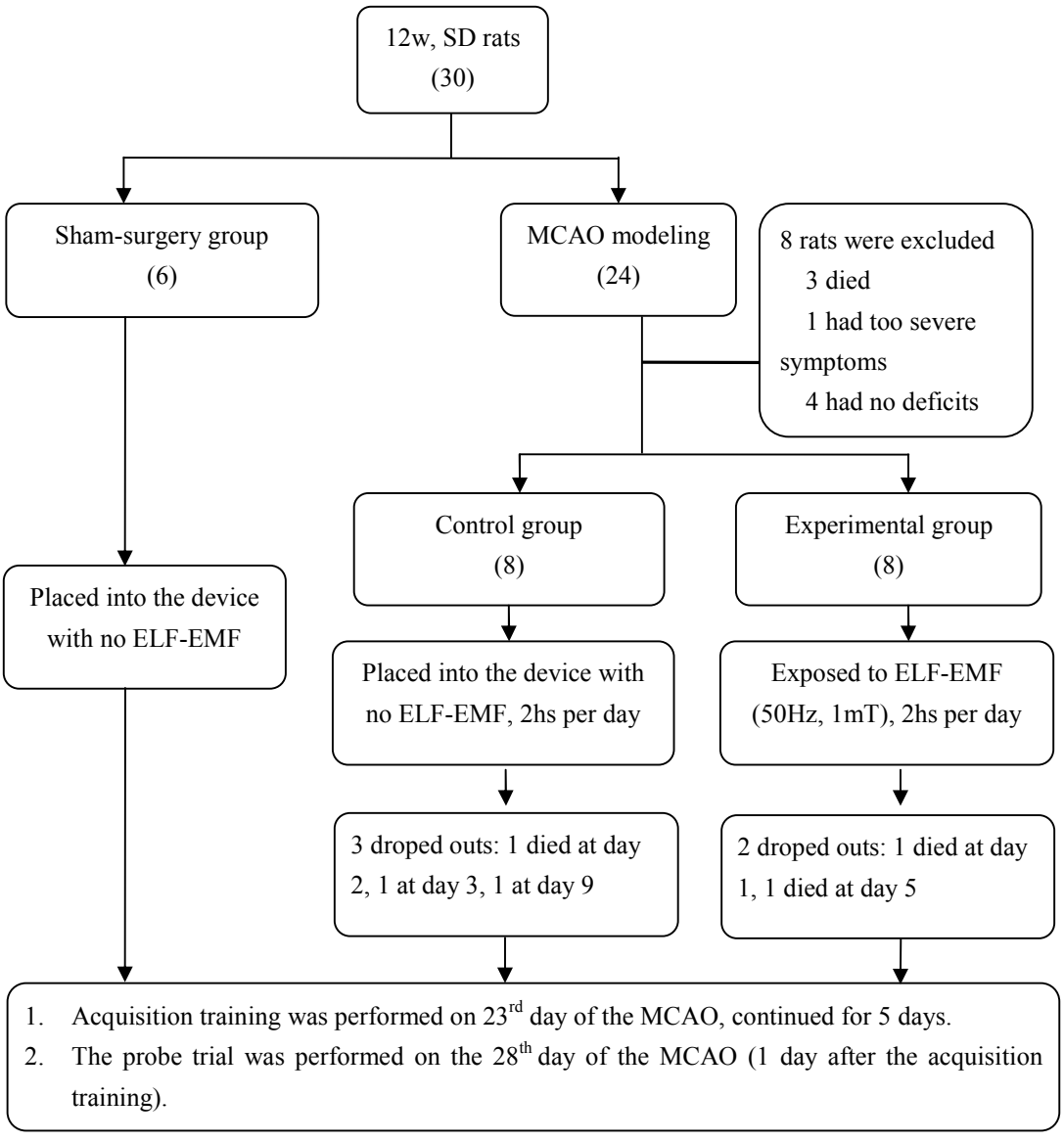


Figure 2. 2 The flow chart of the Morris water maze test

2.2.5 Interventions

(1) An ELF-EMF device was constructed following previous studies (Vazquez-Garcia et al., 2004, Gutierrez-Mercado et al., 2008, Varro et al., 2009). The parameters of the ELF-EMF device including the size, the weight, the intensity and frequency of the magnetic fields were set by our group. Then the device was designed and manufactured by the College of Manufacturing Science and Engineering in Sichuan University. Intensity and frequency were tested using a hand-held detector to confirm the parameters of the EMF generated by the device before the delivery.

The device consisted of the computer, power amplifier and Helmholz coils (two solenoids (diameter: 40cm) with a common axis and 20cm's distance between the two coils) (Figure 2.3-1, figure 2.3-2). Helmholz coils can generate a homogenous electromagnetic field in the space between two coils. Both of the coils were surrounded by 300 turns of copper wire with a diameter of 0.8mm. A 50Hz audio signal was generated by a computer program and then the power amplifier turned the audio signal into an electrical current which drives the coils. This device could generate the required ELF-EMF with a homogeneous distribution (Wang et al., 2014b) (Figure 2.4). Intensity was tested by a hand-held EMF detector every 15min during the experiments. The value was fixed at 1.0±0.05mT (Figure 2.5).

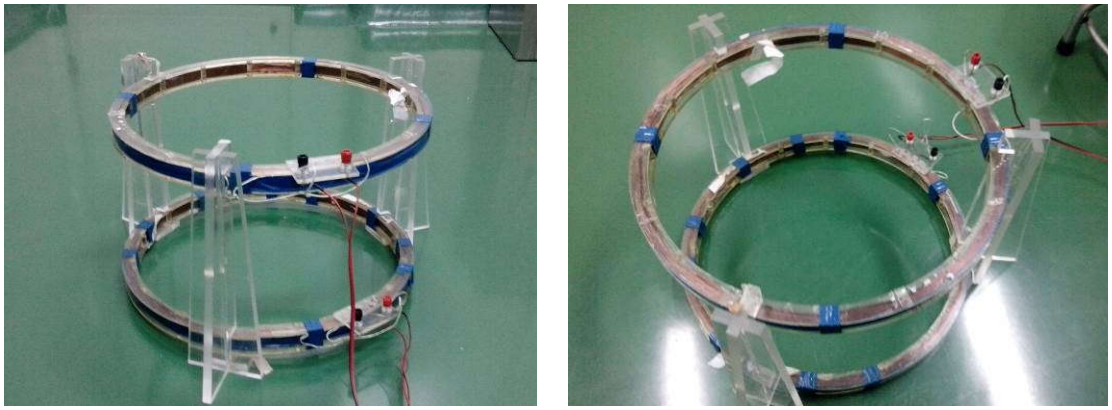


Figure 2.3-1 The coils of the ELF-EMF device

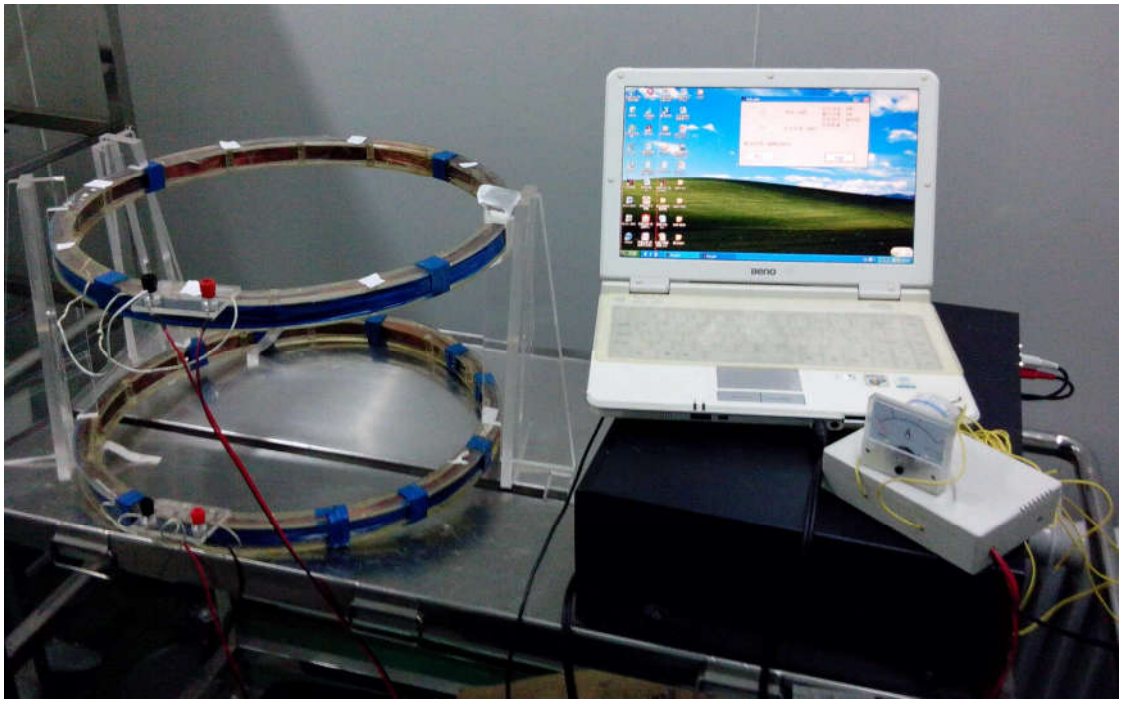


Figure 2.3-2 The consist of ELF-EMF device

Figure 2.3 The ELF-EMF device

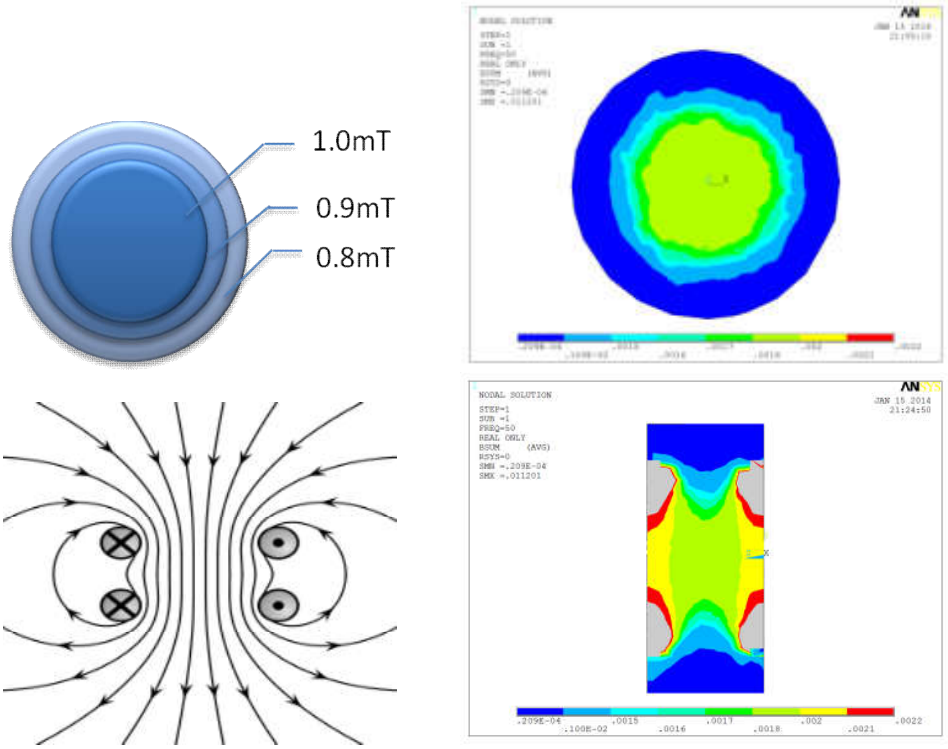


Figure 2. 4 The distribution and intensity of the EMF generated by the device (Wang et al., 2014b)

(2) Interventions given to the rats started 24h after the surgery. For the intervention, the rats were placed in a plastic cage, and then the cage was placed into the coils of the ELF-EMF device. For the experiment group, the device was turned on and an electromagnetic field detector was used to make sure the frequency and intensity was 50Hz, 1mT in the plastic cage. The sham-surgery group and the control group were also put into the plastic cage which was placed into the coils but the device was kept turned off. According to the aforementioned literature, intervention time was 2h per time, 1 times per day.

Figure 2. 5 The intensity of ELF-EMF measured by a hand-held EMF detector

(3) Rats were tested at four time points, which were 3d, 7d, 14d and 28d after commencement of the ELF-EMF intervention. Six to seven rats were sacrificed at each time point in every group according to the remaining number of the rats. Rats to

be sacrificed next time were randomly chosen using a random number table and marked with permanent markers 3 days before the respective time point.

2.2.6 Behaviour assessment

2.2.6.1 Neurological evaluation

The modified neurological severity score (mNSS) (Chen et al., 2001) and De Ryck's behavioral tests (Deryck et al., 1989) were used to evaluate the neurological function of every rat at the beginning of the first ELF-EMF intervention and before sacrifice at every time point.

(1) The mNSS evaluates the motor, sensory, balance, reflex and abnormal movements of the rats (Chen et al., 2009). The mNSS score ranges between 0-18 points (normal is 0, the most serious injury is 18 points). Regarding severity of injury, scores 1-6 are classified as mild damage; 7-12 are moderate damage and 13-18 are severe damage (Table 2.1).

Motor tests	
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 s)	3
Attempts to balance on beam but falls off (>40 s)	4
Attempts to balance on beam but falls off (>20 s)	5
Falls off; no attempt to balance or hang on to beam (<20 s)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

Table 2. 1 The mNSS scale (Chen et al., 2001)

(2) The De Ryck's behavioral test is widely used to examine the sensorimotor functions in rodent stroke models. It is a 16-point scale which includes 8 tests with each scored from 0 (maximum deficit) to 2 (no deficit). It tests the rats' visual, tactile, proprioception and postural reflexes. Six tests are for forelimbs and the other 2 are for

the hind limbs (Ke et al., 2011). Some pictures of De Ryck's behavioral tests are shown in Figure 2.6.

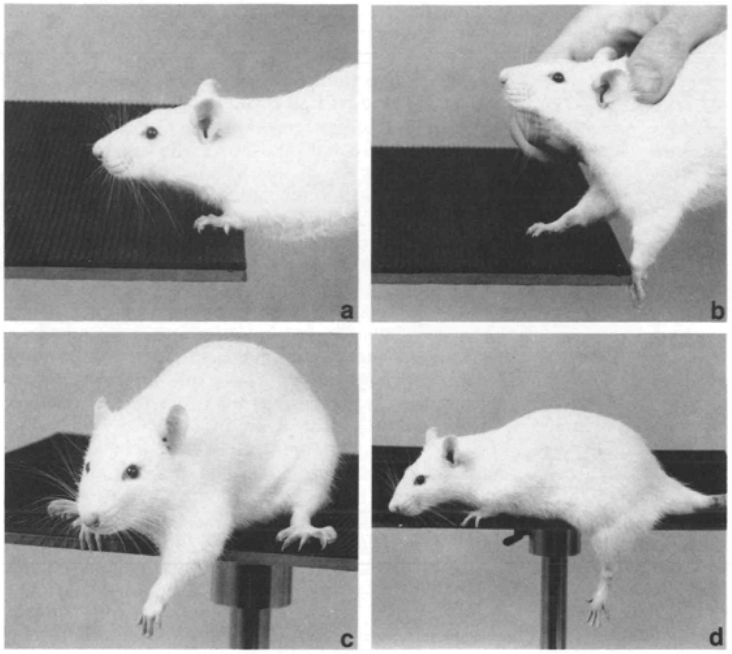


Figure 2. 6 The De Ryck's behavioral tests (Deryck et al., 1989)

2.2.6.1 Cognitive evaluation

The Morris water maze (MWM) was used to evaluate cognitive function of the rats (Morris, 1984). The MWM test is an experiment that forces the animal to swim and learn to find a platform hidden in the water. MWM is widely used in evaluating the cognitive function in rats with cerebral ischemia (Diederich et al., 2014, Lee et al., 2014, Qu et al., 2014).

The Morris water maze used in this experiment (RWD, China) was a round pool made by stainless steel, with a diameter of 1.3m, and a height of 0.6m. There were 4 points marked on the pool wall, which divided the pool into 4 quadrants. A platform with 10cm's diameter and 30cm's height was placed in one of the quadrant. The

temperature was maintained at 24~25℃ in the laboratory and the water's temperature was controlled at 23±2℃. The water was 2cm higher than the platform and was dyed into black water, so that the underwater platform was invisible. The objects around the pool were unchanged to provide a reference for rats to orientate towards the platform. A camera at the top of the water maze was connected with a computer, to record the rats' behavior in the water. Then the video was analyzed by the SMART behavior analysis system (Panlab, Spain) to receive parameters including swimming track, distance, latency and speed.

One day before the first time of the water maze test, the cued version/ visible platform trial was used to eliminate the influence of motor and sensory dysfunction. The procedure was performed as below: place the platform above the water surface so that it is visible, if rats can easily swim towards the platform in the water, then the rats can see the platform and have the ability to swim; otherwise, the rats should be excluded. The acquisition and probe-trial were used for the formal water maze test.

For the acquisition training, the rat was put into the water with its head towards the pool wall, the position was randomly selected from the four quadrants of the pool. The time was recorded when the rat found the platform hidden in the water, if the rat did not find the platform in 60s, the rat was guided to the platform and left to stay on the platform for 10s, then it was put into water again for training.

The acquisition training started on the 23rd day of the intervention, 4 times per day and was continued for 5 days. Recorded were latency (the duration from the rat put into the water to finding the platform), swimming distance and swimming speed.

For the probe-trial, the platform in the water was removed, then rats were put into the water from the opposite quadrant of the platform, and the time that the rat spend in the former platform quadrant was recorded and the times that the rat crossed the platform area.

The probe-trial was performed on the 2nd day after the acquisition training was finished. Recorded were latency (the time from the rat put into the water to reaching the position of the former platform), number of crossing (the times that rat crossed the former platform area) and time of stay (the time that rat stayed in the former platform quadrant).

2.2.7 Statistics

All descriptive data are presented as mean ± standard deviation (x ± SD). Chi-square tests were used to compare mortality in the different groups. For mNSS, De Ryck's behavioral score and the data of the acquisition training in Morris water test, two-way repeated measure ANOVA was performed for comparing differences between the 3 groups at different time points. For the results of the probe-trial in MWM test, one-way ANOVA was employed for comparing the differences between the 3 groups. Significant ANOVA results were further analyzed with post-hoc LSD tests. Alpha errors were set at 0.05. SPSS 19.0 was used to perform the above statistical analysis.

2.3 Results

Rats woke up at about 0.5h~1.5h post operation. After 24h, according to the inclusion criteria, rats subjected to the cerebral ischemia model which had no symptom or too severe symptoms as well as dead rats were excluded. Then all rats with cerebral ischemia were randomly assigned to two groups. After grouping, the experiment group and the control/sham-operated group immediately received experimental and control intervention, respectively. Rats were sacrificed at different time points, i.e. at the 3rd day, 7th day, 14th day and 28th day after commencement of the intervention. Behavioral assessments were performed before sacrifice, and the total number of death was cumulatively calculated for each group.

2.3.1 Death rate

A total of 240 rats was used in this projects, 24 rats were assigned to the Sham group 216 rats to the group receiving MCAO surgery. One hundred and thirty seven rats were successfully modeled and then randomly assigned to Control (Con) group (68 rats totally) or Experimental (Exp) group (69 rats totally). One day to 3 day after surgery, most of the rats were apathic, acquired reduced food and showed reduced activity. The number of deaths was highest in 3 days after surgery. The overall mortality was 0% in the Sham group, 20.59% in the Con group and 18.84% in the Exp group. Compared with the Sham group, the Con group and the Exp group had significantly higher mortality ($p<0.05$); compared with the Con group, the mortality rate in the Exp group did not differ significantly ($p>0.05$), Table 2.2.

Group	Total No. at the beginning	Total No. of deaths	Total No. of survivors	Death rate (%)
Sham group	24	0	24	0
Con group	68	14	54	20.59*
Exp group	69	13	56	18.84*

*Compared with Sham group, $p<0.05$

Table 2.2 Death and survival rates in each group

2.3.2 Neurological function assessment

All the rats used in WMW did not receive the neurological function assessment. A total of 18 rats were tested with neurological function assessment in the Sham group. At the beginning, 68 rats in the control group and 69 rats in the experiment group were assessed. And at the four time points of the 3rd day, 7th day, 14th day and 28th day, the total number of assessed rats was 12, 12, 13 and 12 in the Con group and 12, 12, 13 and 13 in the Exp group.

2.3.2.1 The mNSS score

All rats in the Sham group were normal, without any neurological deficits, and their average mNSS score was 0. The mNSS score in the Exp group and Con group was higher than in the Sham group, the difference was significant ($p<0.001$). At the 1st day, 3rd day, 7th day, 14th day and 28th day, there were no significant difference ($p> 0.05$) between the Exp group and the Con group (Table 2.3, Figure 2.7).

	1st day	3rd day	7th day	14th day	28th day
Con group	8.49±2.17	6.42±1.83	4.42±1.38	4.00±1.41	2.50±1.45
Exp group	8.28±2.59	6.17±1.59	4.17±1.99	4.15±1.91	2.38±1.26

Table 2.3 The mNSS score at different time points

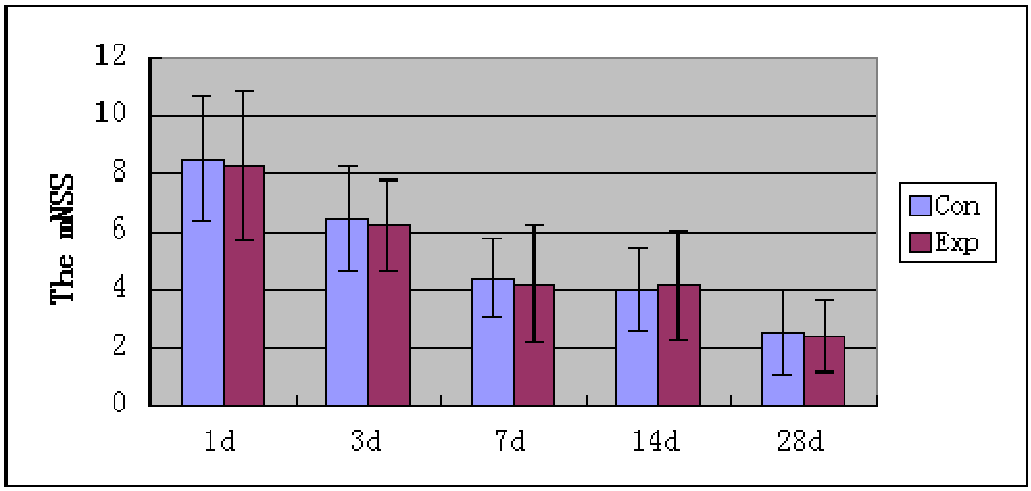


Figure 2.7 The mNSS score in different time points

2.2.2.2 The De Ryck's score

All rats in the Sham group were normal, without any neurological deficit, and average De Ryck's score was 16 points. The De Ryck's score in the Exp group and Con group were lower than in the Sham group, the difference was significant ($p < 0.001$). At the 1st day, 3rd day, 7th day, 14th day and 28th day, there were no significant differences ($P > 0.05$) between the Exp group and the Con group. (Table 2.4, Figure 2.8)

	The 1st day	The 3rd day	The 7th day	The 14th day	The 28th day
Con group	6.57±2.38	7.08±2.11	9.00±2.09	10.69±2.78	11.83±2.62
Exp group	6.69±2.27	7.33±3.11	9.92±2.31	11.08±3.15	12.23±2.39

Table 2.4 The De Ryck's score in different time points

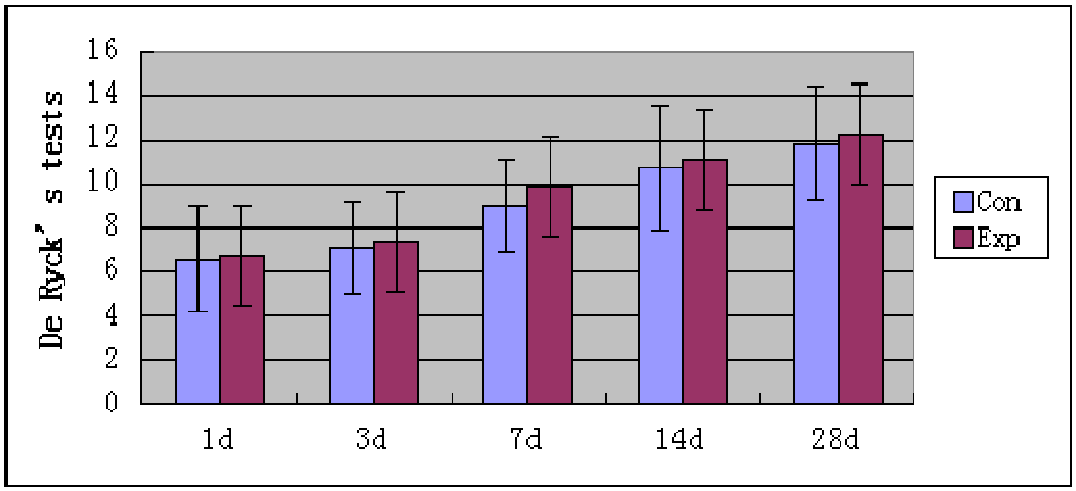


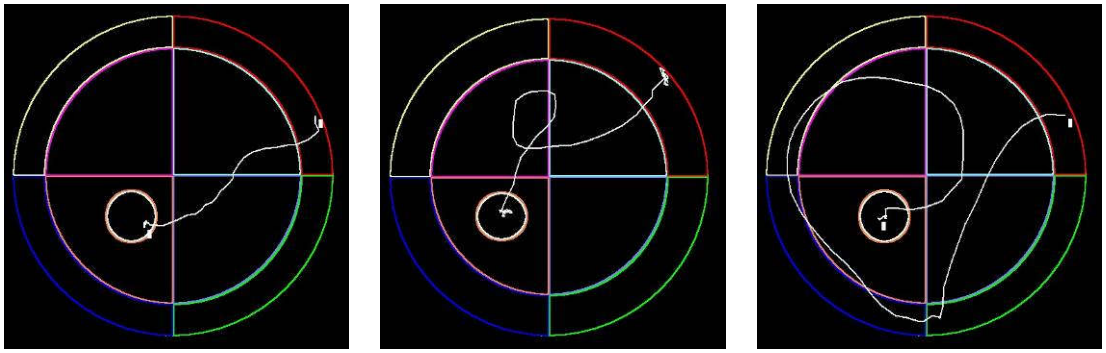
Figure 2.8 The De Ryck's score in different time points

2.3.3 Cognitive and memory function assessment

A total of 30 rats were subjected to the MWM test. Six rats were randomly assigned to the Sham group, and 24 rats to MCAO group. The 16 rats which were successful modeled and met the inclusion criteria were randomly divided into Con group and Exp group. Finally, there were 5 rats in the Con group and 6 rats in Exp group when the experiment was completed. At the 23rd day of the intervention, 5 days of continuous acquisition training were performed for all rats, and at the 6th day (i.e., the 2nd day after the last acquisition training), the probe-trial was performed.

2.3.3.1 Acquisition training

In acquisition training, three indicators including swimming distance, latency and swimming speed were recorded and analyzed. The swimming tracks of the acquisition training at the 5th day are shown in Figure 2.9.



A. Sham group B. Exp group C. Con group

Figure 2.9 The swimming tracks of the acquisition training at the 5th day

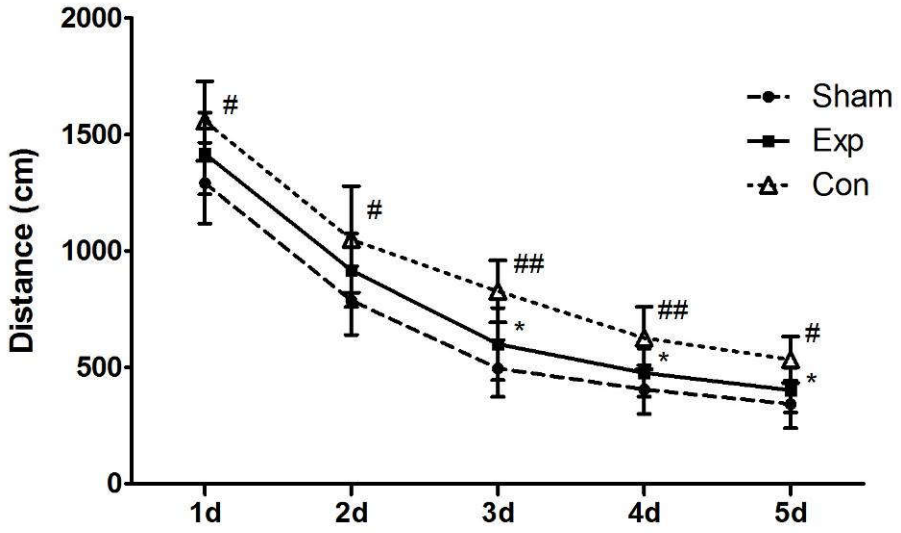
(1) Swimming distance: two way repeated measure ANOVA of the swimming distance in 3 groups showed that: there was no interaction between group and time ($F = 0.715$, $p = 0.677$), but there was a statistically significant main effect of group membership ($F= 0.684$, $p = 0.009$). Results of one-way ANOVA showed that the swimming distance was significantly different between the 3 groups at all the 5 days. Results of the LSD post-hoc tests showed that: at the 1st and 2nd day, the swimming distance in the Con group was longer than the Sham group ($p <0.05$), however, no significance comparing with Exp group ($p> 0.05$). At the 3rd day, 4th day and 5th day, comparing with the Sham group, the swimming distance in the Con group increased significantly ($p<0.01$); comparing with the Con group, the Exp group had

significantly shorter swimming distance ($p<0.05$). The difference between the Exp group and the Sham group were not significant ($p>0.05$). (Table 2.5, Figure 2.10)

	The 1st day	The 2nd day	The 3rd day	The 4th day	The 5th day
Sham group	1291.74±174.06	786.42±147.13	495.34±121.93	404.71±104.93	341.19±102.45
Exp group	1418.30±175.44	917.76±158.34	600.11±154.83*	476.70±102.13*	401.59±95.88*
Con group	1557.78±170.64#	1049.32±227.75#	827.28±133.16##	626.64±133.55##	532.72±100.14#

Compared with Sham group, #p<0.05, ##p<0.01; compared with Con group, *p<0.05

Table 2.5 Swimming distance in acquisition training



Compared with Sham group, #p<0.05, ##p<0.01; compared with Con group, *p<0.05

Figure 2.10 Swimming distance in acquisition training

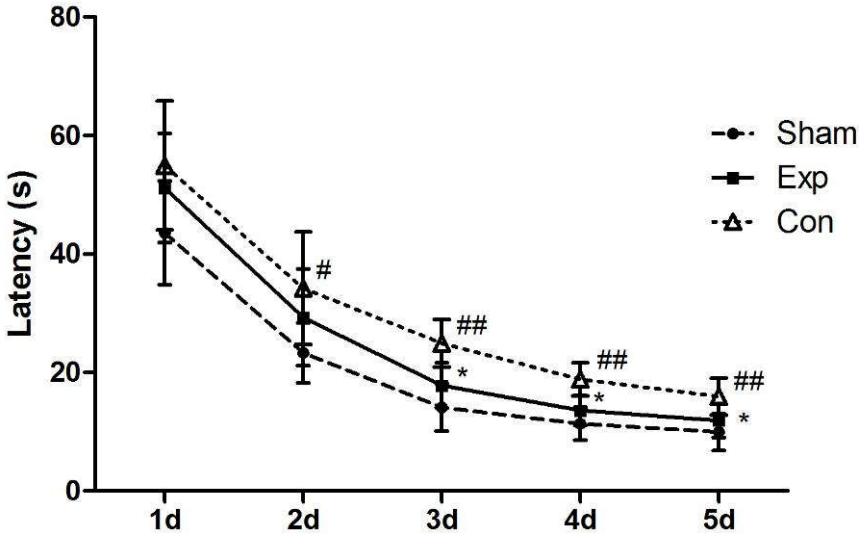
(2) Latency: results of two way repeated measure ANOVA of the latency in 3 groups showed that: there was no interaction between group and time ($F = 0.721$, $p =$

0.672), but there was a statistically significant difference between 3 groups (F= 6.166, p = 0.012). Results of one-way ANOVA showed that the latency was significantly different between the 3 groups at all the 5 days. Results of the LSD post-hoc tests showed that: at the 1st day of the training, no difference was found between 3 groups ($p>0.05$). At the 2nd day, comparing with the Sham group, the Con group had significant longer latency ($p<0.05$), however, no significance between the Con group and the Exp group ($p> 0.05$). At the 3rd day, 4th day and 5th day, comparing with the Sham group, the Con group increased the latency significantly ($p<0.01$); comparing with the Con group, the Exp group had significantly shorter latency ($p<0.05$). The difference between Exp group and the Sham group were not significant ($p>0.05$). (Table 2.6, Figure 2.11)

	The 1st day	The 2nd day	The 3rd day	The 4th day	The 5th day
Sham group	43.57±8.74	23.29±5.07	14.10±4.02	11.33±2.81	9.93±3.13
Exp group	51.15±9.23	29.30±8.18	17.82±3.80*	13.62±2.58*	11.89±2.85*
Con group	54.93±10.90	34.19±9.51#	24.91±4.02##	18.82±2.81##	15.92±3.13##

Compared with Sham group, #p<0.05, ##p<0.01; compared with Con group, *p<0.05

Table 2.6 Latency in acquisition training



Compared with Sham group, #p<0.05, ##p<0.01; compared with Con group, *p<0.05

Figure 2.11 Latency in acquisition training

(3) Swimming speed: results of two way repeated measures ANOVA of the swimming in 3 groups and different time points showed that there was no interaction between group and time (F= 0.454, p= 0.876) and there was no significant difference between groups (F=1.046, p=0.377). (Table 2.7, Figure 2.12)

	The 1st day	The 2nd day	The 3rd day	The 4th day	The 5th day
Sham group	30.45±2.96	33.98±2.65	35.45±3.34	35.68±2.62	34.48±2.86
Exp group	28.05±2.96	32.10±3.91	33.59±3.78	34.92±2.57	32.92±3.73
Con group	28.92±3.84	31.14±2.75	33.24±2.44	33.94±3.69	33.59±3.05

Table 2.7 Swimming speed of acquisition training

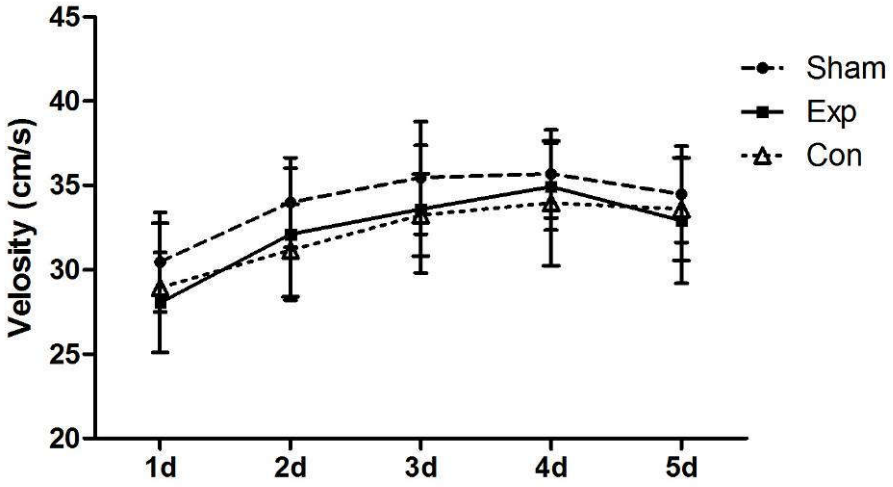
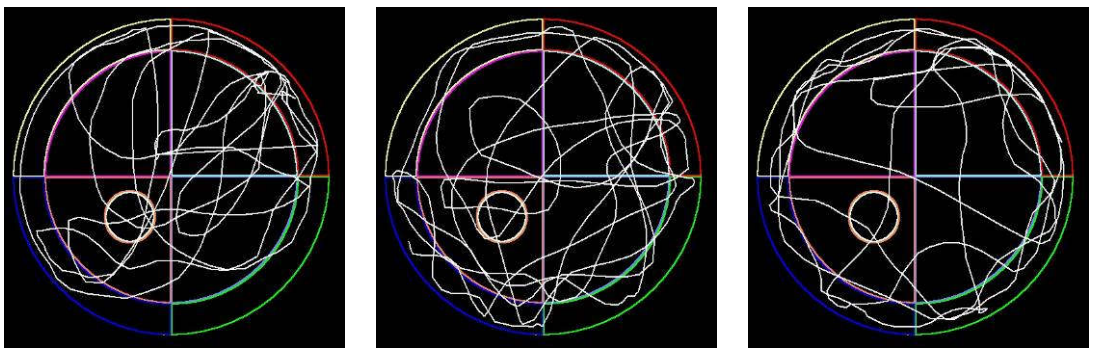


Figure 2.12 Swimming speed of acquisition training

2.3.3.2 Probe-trial

Three indicators were recorded and analyzed: latency that the rat reached the former position of the platform, the number of crossings of the former platform position and the time that the rat stayed in the former platform quadrant. The swimming tracks of the probe-trial are shown in Figure 2.13. The scores of probe-trial are presented in table 2.8.



A. Sham group B. Exp group C. Con group

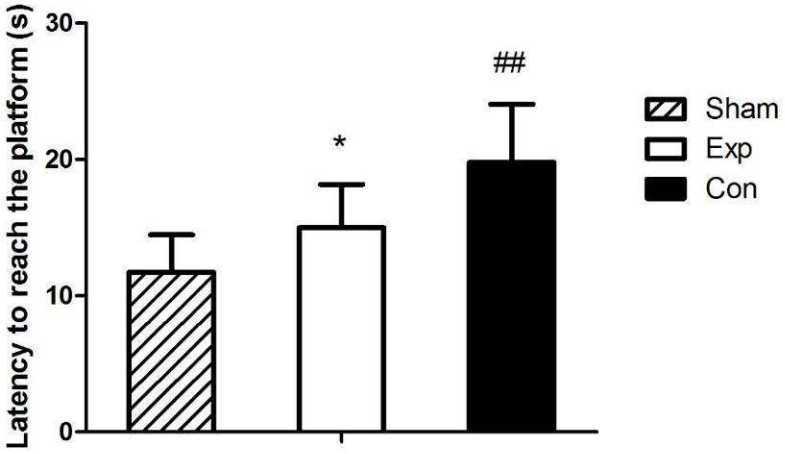
Figure 2.13 The swimming tracks of the probe-trial

	Latency	Number of crossings	Time of stay
Sham group	11.71±2.75	4.17±1.17	25.34±4.17
Exp group	14.98±3.17*	3.17±1.17	23.23±3.63*
Con group	19.76±4.27$^{\#\#}$	1.80±1.30$^{\#\#}$	17.87±3.67$^{\#\#}$

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$

Table 2.8 The score of probe-trial

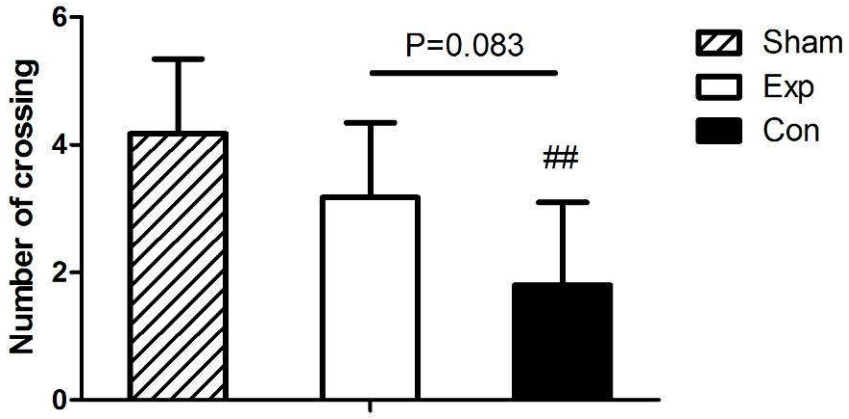
(1) Latency: the results of one-way ANOVA showed that the latency was significantly different between the 3 groups (F = 7.714, p = 0.006). Results of post-hoc results tests showed that, as compared with the Sham group, the latency of the Con group was significantly increased (p = 0.002), no difference was found between the Exp group and Sham group (p = 0.117); compared with the Con group, the latency in the Exp group was significantly reduced (p = 0.035) (Table 2.8, Figure 2.14).



Compared with Sham group, $^{\#\#}p<0.01$, compared with Exp group, $^{*}p<0.05$

Figure 2.14 The latency that the rats reached the position of former platform

(2) Number of crossings: the results of one-way ANOVA showed that the number of crossings was significantly different between the 3 groups (F = 5.232, p = 0.020), results of post-hoc analysis showed that, comparing with the Sham group, the Con group had a significantly reduced number of crossings (p = 0.006). There was no difference between the Sham group and the Exp group (p = 0.174); and the difference between the Con group and the Exp group was not significant (p = 0.083) (Table 2.8, Figure 2.15).

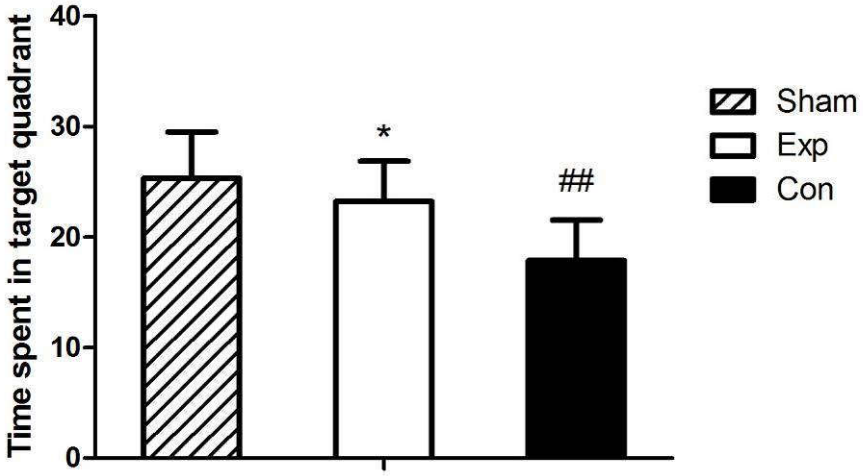


Compared with Sham group, $^{\#\#}p<0.01$

Figure 2.15 Number of crossings of the former position of platform

(3) Time spent in target quadrant: one-way ANOVA showed that the time spent in the target quadrant was significantly different between the 3 groups (F = 5.372, p = 0.019). Results of post-hoc analysis showed that, comparing with the Sham group, the Con group had significantly reduced time spent at target destination (p = 0.006).

Compared with the Con group, the Exp group showed significantly increased time spent in the target quadrant (Table 2.8, Figure 2.16) .



Compared with Sham group, $^{\#\#}p<0.01$, compared with Exp group, $^{*}p<0.05$

Figure 2.16 The time spent in target quadrant

2.4 Discussion

2.4.1 Effect of ELF-EMF on the mortality of rats with cerebral ischemia

There were totally 216 rats accepted the MCAO surgery and 137 rats were successfully modeled and then assigned into Con group or Exp group. The success rate of the MCAO surgery was 63.4% in this project. MCAO is a commonly used model for focal cerebral ischemia and it has been widely used as rats' cerebral ischemia model since Zea Longa first reported in 1989 (Longa et al., 1989). Although this model is stable and reliable, it is still likely to lead to undesired outcome such as intraoperative death, too severe symptoms and failure with too mild or no symptoms. Many reasons can affect the success rate; the physique of rats, feeding and operator's experience may be important factors. Ke Z (Ke et al., 2011) reported that they had 57 successful MCAO models from 117 rats, their success rate was only 48.7%.

During the progression of the pre-experiment, we evaluated and adjusted the whole experimental system and operating procedure. At the same time, we optimized the experimental conditions of the MCAO operation as much as possible and finally got a reliable, stable, repeatable and ideal MCAO model.

In this project, from grouping to the end of the experiment, the overall mortality of each group was low: 0% in the Sham group, around one fifth in MCAO group. Zhou F et al (Zhou et al., 2013) reported that the mortality during 20 hours after MCAO surgery was 18% (8/45). Ke Z et al (Ke et al., 2011) employed the MCAO rats to accept different intensity of exercise training, the mortality rate was obviously varies in each group after 7 days' intervention: 21.4% (3/14) in the involuntary group

of electrical stimulation, 7% (1/14) in the training group with independent running, 26.7% (4/15) in the training group with forced treadmill and 28.6% (4/14) in cerebral ischemia control group. Therefore, it is difficult to compare the mortality rate of rats with cerebral ischemia in different environments. Mortality in the Con group and the Exp group were significantly higher than the Sham group, indicating that the mortality rate of rats significantly increased after cerebral ischemia. There was no difference of the mortality rate in the Exp group and Con group, indicating that ELF-EMF intervention did not have any influence on the mortality rate of rats with cerebral ischemia.

2.4.2 Selection of parameters in ELF-EMF

ELF-EMF is the electromagnetic fields with frequency at 0 ~ 300Hz (Funk et al., 2009). In some previous studies, researchers used Helmholtz coils to generate uniform electromagnetic fields(Vazquez-Garcia et al., 2004, Gutierrez-Mercado et al., 2008, Varro et al., 2009). In this project, the generator which was used to produce an uniform magnetic field was composed of a computer, a power amplifier and Helmholtz coils. A program which was designed with Matlab software generated an audio signal, and then the signal was transmitted to the power amplifier to control the Helmholtz coils to produce an uniform magnetic field. This magnetic field generator was able to generate a uniform magnetic field of 2.0mT (less than 10% changes) in the middle space of the coils (Wang et al., 2014b).

In previous studies, the range of parameters of the ELF-EMF was large. In vitro studies of mesenchymal stem cells, parameters of the ELF-EMF were 15 Hz, 1mT

(Song et al., 2014), 50Hz, 5mT (Bai et al., 2013), or 50Hz, 1mT (Seong et al., 2014). All of the experiments received positive results. In vivo experiments, Cuccurazzu et al (Cuccurazzu et al., 2010) applied ELF-EMF of 50Hz, 1mT in normal mice; Tasset et al (Tasset et al., 2012a, Tasset et al., 2012b) used ELF-EMF of 60Hz, 0.7mT in Huntington rats, Das et al (Das et al., 2012) used ELF-EMF of 50Hz, 17.69μT in rats with spinal cord hemisection, Sherafat et al (Sherafat et al., 2012) used ELF-EMF of 60Hz, 0.7mT in rats with multiple sclerosis. Parameters as well as results of these studies provided a reference and basis for the choice of parameters in this study.

2.4.3 Effects of ELF-EMF on the sensation and movement of rats

mNSS and De Ryck's behavior tests are both commonly used in functional test in rats with cerebral ischemia. mNSS mainly assesses the motor, sensory, balance, and reflex functions and abnormal activities while De Ryck's behavioral test assesses vision, touch, proprioception and postural reflexes. This study found that the sham-operated rats did not have any neurological deficits as their mNSS score was 0 and De Ryck's score was 16 points, which showed significant differences from the Exp group and Con group. This indicated that cerebral ischemia leads to dysfunction of motor, sensation, reflex and abnormal movements, as well as abnormality of vision, touch, proprioception and postural reflexes in rats. Some animal experiments reported that some therapeutic interventions including the human placenta stripping adhesion cells (Shehadah et al., 2014), neuroprotective drugs (Zhang et al., 2012), traditional Chinese medicine (Wang et al., 2014a) and treadmill training (Ke et al., 2011) could improve De Ryck's or mNSS score in MCAO rats.

During the experiment, we observed that the cerebral ischemic rats had a very strong potential of self-healing. Most of the rats recovered to walk freely, climb the balance beam stably and eat independently in about 3 days after the MCAO surgery. The results also showed that on the 3rd, 7th, 14th and 28th day of the intervention, there was no significant difference in mNSS and De Ryck's scores between the Exp group and the Con group, indicating that ELF-EMF could not significantly improve the motor, sensory, balance or other behavioral function of rats with cerebral ischemia. Tasset et al (Tasset et al., 2012a) reported that with the intervention of ELF-EMF, neurological function scores were improved after 21 days in Huntington rats. Das et al (Das et al., 2012) reported that 6 weeks of ELF-EMF intervention could significantly improve motor and sensory function in rats with spinal cord hemisection. The short term follow up, or the small sample size might be the explanation of our negative results. We speculate that ELE-EMF may be more beneficial for the recovery of chronic degenerative diseases of the central nervous system.

2.4.4 Effects of ELF-EMF on the cognitive function of rats

The cognitive function may mainly referred to hippocampus. In MWM test, rats located the hidden platform under the water with the reference of the makers on the pool wall, the spatial navigation of hippocampus was functioning during the process. Moreover, learning ability is related mostly in acquisition training, and memory function is immensely required in the probe-trial, which are all dominated by the hippocampus (Martin and Clark, 2007, Foster and Knierim, 2012, Opitz, 2014). Spatial sequences are stored and activated in ways that may support navigational

strategies. The hippocampus stores learned sequences of locations by the plasticity mechanisms, which can predict the future locations based on learned experiences. During the navigational behaviors or tests, these sequences is activated to guide the animal to learn and remember trajectories toward goals (Foster and Knierim, 2012).

In the acquisition training of MWM, since the swimming speed had no significant difference, both swimming distance and latency may reflect the learning ability of experimental animals (Morris, 1984). With the increase of learning times, animals with strong learning ability find the platform more quickly than those which learned slowly. When there is no significant difference between swimming speed, the shorter the swimming distance is, the shorter the latency would be. The study showed that after 3, 4 and 5 days' acquisition training, while swimming speed was not significantly different between groups, swimming distance and latency in the Con group were longer than in the Sham group, indicating that cerebral ischemia could lead to a reduction of learning ability; swimming distance and latency in the Exp group were shorter than the Con group, indicating that ELF-EMF intervention may enhance the learning capacity of cerebral ischemic rats.

In the probe-trial of MWM, 3 indicators of latency, cross times and time spending in the target platform quadrant were recorded to reflect the rats' memory ability. If the rat had good memory, it should have less latency, more crossings and longer stay in the target quadrant. This study found that compared with the Sham group, the Con group showed longer latency, less crossings and less stay in target quadrant, indicating that cerebral ischemia can impair the memory function in rats,

which is consistent with previous reports (Diederich et al., 2014, Lee et al., 2014). Compared with the Con group, the Exp group showed shorter latency and longer time spend in target quadrant. This demonstrated that ELF-EMF may improve memory function of cerebral ischemic rats.

Effect of ELF-EMF on cognitive and memory function is still controversial. In 2004 Vazquez-Garcia M et al (Vazquez-Garcia et al., 2004) reported that during 2 hours per day and 9 days' exposure of ELF-EMF (60Hz, 1mT), normal adult rats improved their social recognition memory. Twelve weeks' continuous intervention of ELF-EMF (50Hz, 0.1mT) could not improve the memory or change the β-amyloid deposition and the shape of neurons in rats with Alzheimer's disease (Zhang et al., 2015). Duan Y et al (Duan et al., 2014) found that 28 days' high dose of ELF-EMF (50Hz, 8mT) might hurt the rats' memory function by increasing the neurotransmitters such as glutamate and GABA in the hippocampus. Therefore, ELF-EMF may have potential application in the treatment of neurological diseases, but its effect might strongly depend on the parameters such as frequency and intensity (Mahdavi et al., 2014, Masoudian et al., 2015). This study demonstrated that, ELF-EMF (50Hz, 1mT) could significantly improve the learning ability and memory of cerebral ischemic rats and might be a potential treatment for cognitive disorders after cerebral ischemia, and it might also have values in treating some central nervous system diseases which have cognitive and memory disorders such as Alzheimer's disease.

2.5 Conclusion

ELF-EMF had no influence on the mortality rate of cerebral ischemic rats. ELF-EMF could not improve physical function such as motor and sensory abilities. ELF-EMF might have positive effects on cognitive function and memory in cerebral ischemia. Consequently, ELF-EMF might have potential therapeutic value in cognitive disorders of the central nervous system.

CHAPTER 3 THE EFFECT OF ELF-EMF ON THE PROLIFERATION AND DIFFERENTIATION OF ENDOGENOUSE NSCS IN RATS WITH CEREBRAL ISCHEMIA

3.1 Introduction

The results in Chapter 2 demonstrates that the ELF-EMF intervention have positive effects on the learning and memory ability in rats with cerebral ischemia. These changes may be related to the enhancement of the regeneration or the repair of the ischemic brain which was also known as neuroplasticity. The plasticity is the fundamental mechanism in functional recovery of motor, sensory and cognitive abilities.

NSCs have the ability to self-renew and differentiate into various types of cells in the central nervous system to maintain the number of themselves or to repair the injured brain tissues. Thus, NSCs play a crucial role in the process of neuroplasticity. NSCs in the adult mammalian brain are mainly located in the subgranular zone (SGZ) which is below the hippocampus dentate granule (DG) and the subventricular zone (SVZ) near the adjacent lateral ventricle (LV) (Yao et al., 2012). NSCs are usually in relatively quiescent status in the niche in mammalian brain (Lugert et al., 2010). However, NSCs will be activated in various pathological conditions in stroke (Decimo et al., 2012, Ziemka-Nalecz and Zalewska, 2012, Bellenchi et al., 2013).

In rodents, the peak of cell proliferation induced by ischemic stroke in the SVZ and SGZ is around 1 to 2 weeks after injury, accompanied by an increasing number of immature neurons at the infarction site (Ziemka-Nalecz and Zalewska, 2012). The proliferation and differentiation of NSCs could be labeled by some experimental methods. The immunofluorescence method with the BrdU single or double labeling method is commonly used to label the status of the NSCs. BrdU single-labeled cells

represented new proliferated NSCs, BrdU/DCX double-labeled cells newborn NSCs differentiating into neurons, BrdU/GFAP double-labeled cells newborn NSCs differentiating into neuroglias, and BrdU/NeuN double-labeled cells new mature neurons (Landgren and Curtis, 2011, Zhang et al., 2012, Low et al., 2013).

This chapter describes the effects of ELF-EMF on the proliferation, differentiation into different cell lines and maturation of endogenous NSCs. Grouping and intervention were identical with the ones described in Chapter 2. In this experiment, the NSCs' proliferation and differentiation were observed at three time points: the 3rd day, 7th day and 14th day after commencement of the intervention. The maturity of new born neurons was observed on the 28th day of the intervention. The BrdU injection method was used to label the endogenous NSCs. The immunofluorescence method was used to process brain tissue slices. These NSCs and neurons were counted in both SVZ and SGZ, the two regions in the rat's brain within which NSCs are generated. Data and images were processed with Excel 2013, GraphPad Prism 5, PhotoshopCS3 and SPSS 19.0.

3.2 Materials and methods

3.2.1 Animals and grouping

Animals and grouping were described in chapter 2. There were two different experiments in this chapter. One aimed to observe new born NSCs' proliferation and differentiation at day 3, day 5, day 7, day 14 and day 28. Another aimed to observe the survival of the newly differentiated neurons.

SD rats were randomly assigned into sham-surgery group (Sham group) and MCAO group. Successfully MCAO modeled rats were further randomly divided into control group (Con group) and experiment group (Exp group).

(1) To observe the NSCs' proliferation and differentiation: rats in Sham group, Con group and Exp group were sacrificed at 3 time points, i.e. at the 3rd day, the 7th day and 14th day of the invention.

(2) To observe the new born neurons: the remaining rats from the Sham group, Con group and Exp group were sacrificed on the 28th day of the intervention.

3.2.2 Modeling and intervention

Modeling and intervention were described in chapter 2. Flow chart of this experiment is shown in figure 3.1 and figure 3.2.

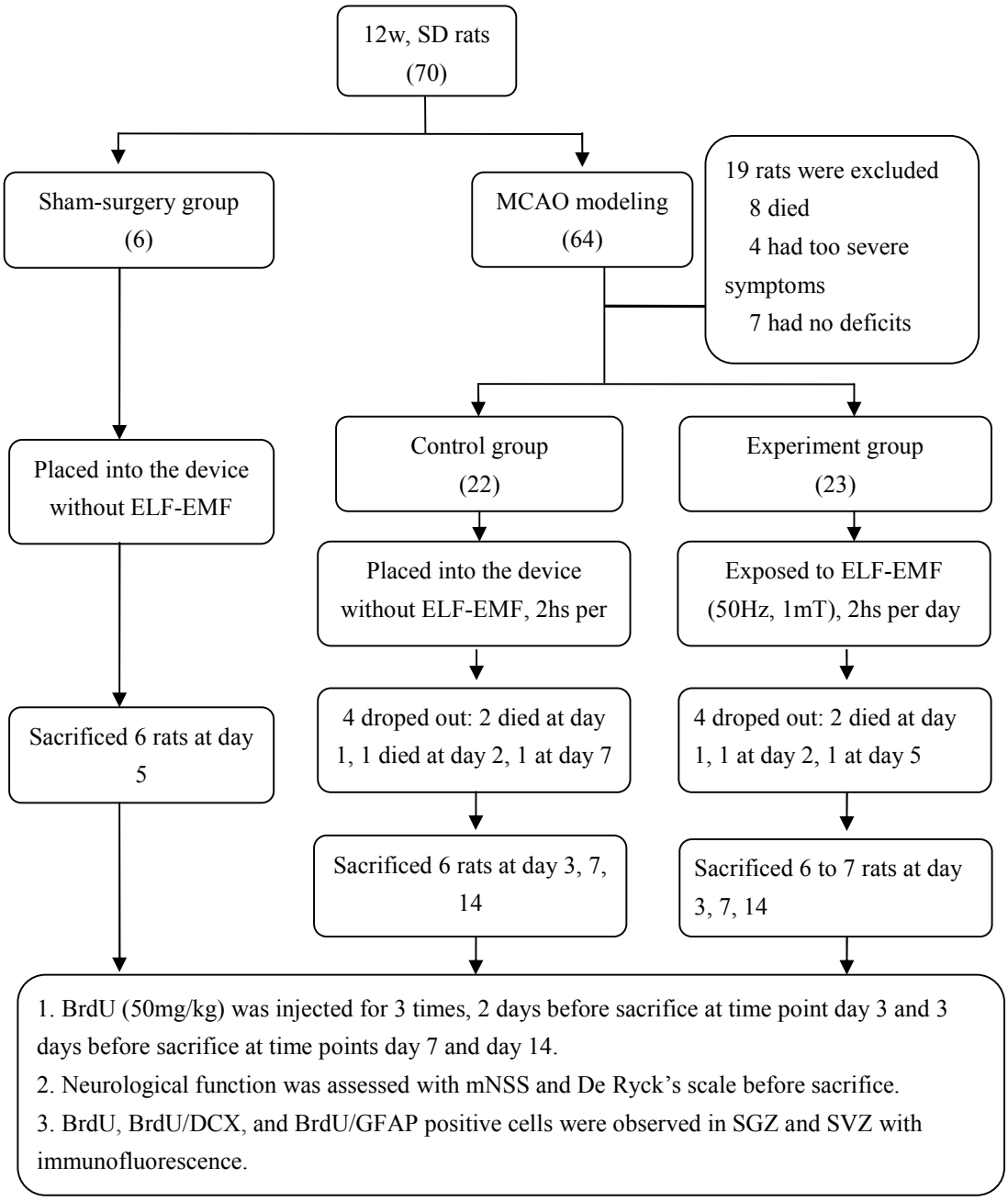


Figure 3.1 Flow chart of experiment on the proliferation and differentiation of NSCs

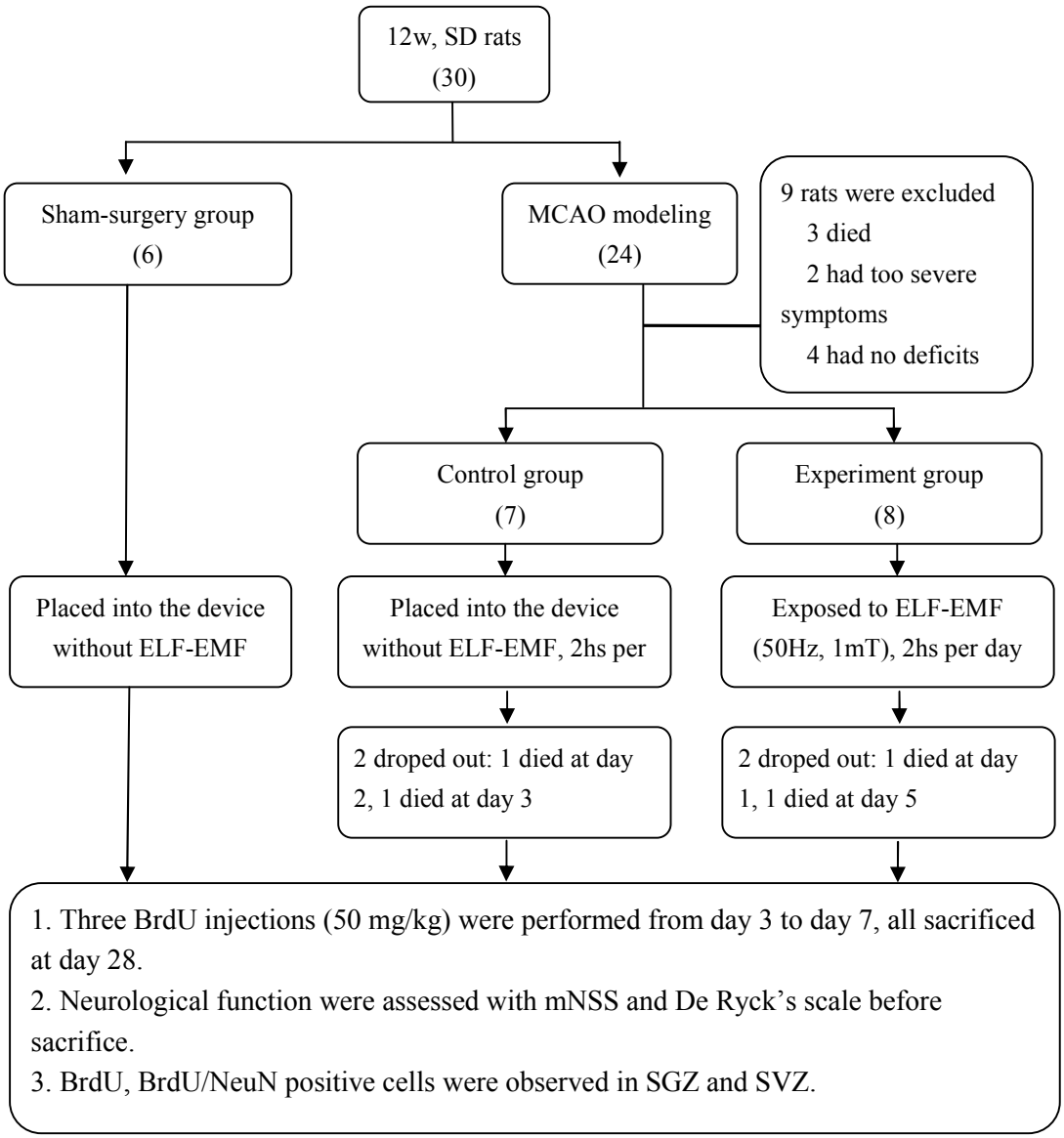


Figure 3.2 Flow chart of experiment on new born neurons

3.2.3 BrdU injection

The BrdU injection method was used to label NSCs. BrdU was dissolved in saline a short time before injection and stored in a dark place with a temperature of 4℃. Intraperitoneal injection of BrdU was given to rats in the dosage of 50mg / kg.

3.2.3.1 Injection to observe the proliferation and differentiation

For the rats at the time point of the 3rd day (the 4th day of the cerebral ischemia), intraperitoneal injection of BrdU was used two days before sacrifice. Injections were performed 3 times, in intervals of 12h. At other time points, BrdU was injected intraperitoneally 3 days before sacrifice, 3 times at intervals of 24h. Rats at each time point were to be executed at 12 hours after the last injection.

3.2.3.2 Injection to observe the survival of newborn neurons

Four continuous intraperitoneal injections of BrdU were performed to all rats at an interval of 24h, started from the 3rd day to the 7th day of the ELF-EMF intervention (the 4th day to the 8th day of the cerebral ischemia). Only the rats in the Exp group received the ELF-EMF exposure 24h after the MCAO surgery. All rats in the three groups were sacrificed on day 28 of the ELF-EMF intervention (day 29 of the MCAO).

3.2.4 Tissue harvest

Rats were sacrificed at the corresponding time points after the ELF-EMF intervention. The rat was anesthetized with 10% chloral hydrate in a dosage of 0.3mL/100g. Then its chest was opened, blunt dissection of the pericardium and soft tissue with forceps was performed in order to fully expose the heart. The needle was penetrated from the left ventricle into the aorta cannulation, and right atrial appendage was snipped off, then the perfusion could be started. Approximately 100mL of 0.9% saline was used firstly for the perfusion until the outflow of a colorless liquid in the heart was observed, and then about 200mL of 4% paraformaldehyde solution was

used to continue the perfusion. After all the paraformaldehyde was completely perfused and the limbs' twitching was stopped, the rat was decapitated, and the brain tissue was taken out from the skull (Figure 3.3).

The perfused brain tissue was placed into a stainless steel adult rat brain slicer matrix, three blades were used for cutting the brain tissue in the coronal planes. The first blade cut in about 4mm anterior to optic chiasma, the second blade cut right on the hypothalamus (near the anterior edge of the pituitary gland handle), the third blade cut in the boundary of the brain and cerebellum. The brain tissue between the first and third blade was taken for further processing. The brain tissue between the first and second blade (optic chiasma to the handle of pituitary gland) was used to observe SVZ; the brain tissue between the second and third blade (the handle of pituitary gland to the cerebellum) was used to observe the SGZ (Figure 3.4).

The two tissue samples were placed in 4% paraformaldehyde for further fixation. After placing the two tissue blocks in paraformaldehyde for 6hs at 4℃, the tissue samples were then placed into 30% sucrose solution for dehydration. After sinking to the bottom, the two brain tissue samples were rinsed with PBS and dehydrated with ethanol. The xylene was used to make the tissues transparent. Then the tissues were embedded with paraffin and stored for slicing.

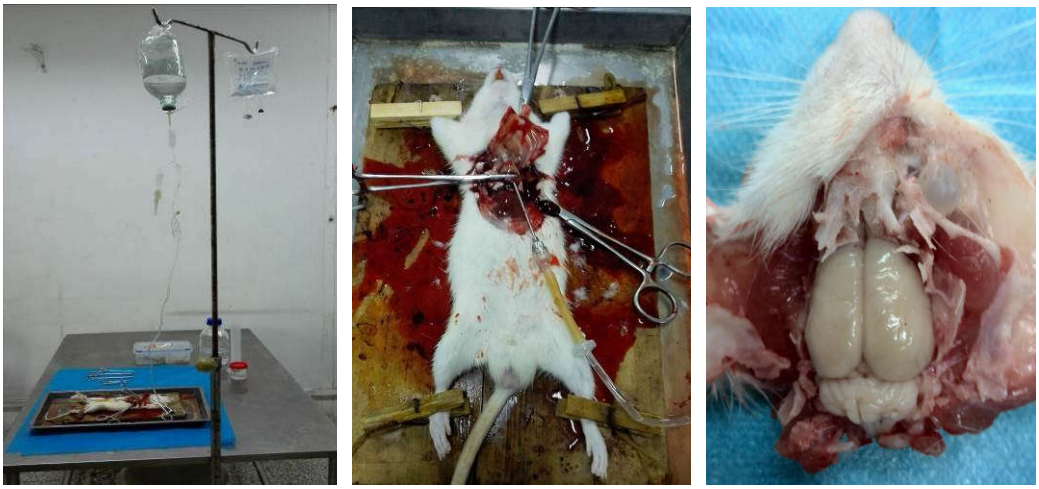


Figure 3.3 The perfusion and the perfused brain

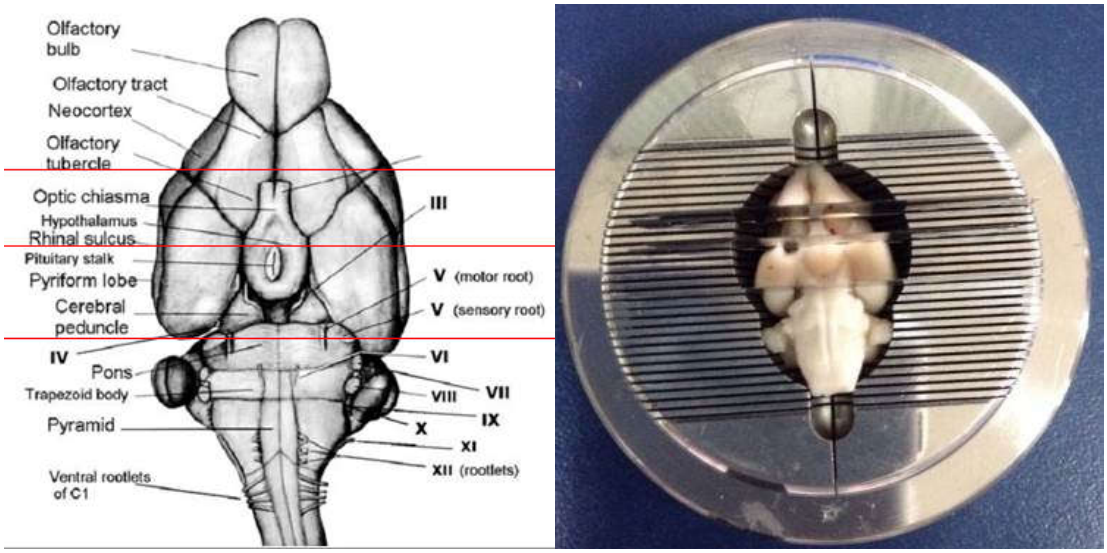


Figure 3.4 The coronal section that cut the brain tissue

The section between Interaural 10.64-8.64mm was taken from the first brain tissue sample to observe the SVZ. The section between Interaural 2.20-7.20mm was taken from the second brain tissue sample to observe the SGZ. One slice in sixth series of sections was collected. In the groups within which we were observing proliferation and differentiation of NSCs, each brain slice had a thickness of 10μm,

three sets of slices were collected: slices in sets 1 were used for single BrdU immunofluorescence, slices in sets 2 were used for BrdU/GFAP double staining immunofluorescence, slices in sets 3 were used for BrdU/DCX double immunofluorescence. All slices were kept at 4℃ in the refrigerator. In the groups selected to observe the survival of the new born neurons, each brain slice had a thickness of 10μm, 1 set of slices was taken to observe BrdU/NeuN double-labeling immunofluorescence.

3.2.5 Process of immunofluorescence

3.2.5.1 Devices used in the procedure

Devices	Company	Country
Rotary Microtome (Leica RM2135)	Leica	German
Paraffin Embedding Station (Leica EG1150H)	Leica	German
Automatic Dehydrator (Leica ASP300S)	Leica	German
Constant Temperature Incubator (Binder BD 115)	Binder	German
Leica Microsystems CMS GmbH	Leica	German
Confocal Microscopy	Nikon	Janpan

3.2.5.2 Agents

Agents	Company	Country
BrdU	Sigma	Missouri, USA

Rabbit polyclonal to GFAP – Astrocyte Marker cat.ab7260	Abcam	Cambridge, UK
Rabbit polyclonal to FOX3/NeuN cat.ab104225	Abcam	Cambridge, UK
Rabbit polyclonal to Doublecortin – Neuronal Marker cat.ab18723	Abcam	Cambridge, UK
BrdU(Ab-3),Mouse MAb cat.MS-1058-P0	NeoMarkers	California, USA
Alexa Fluor 594 Donkey Anti-Mouse IgG (H+L) Antibody cat.A21203	Molecular Probes	Oregon, USA
Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) cat.#4412S	CST	Massachusetts, USA
EDTA cat.0322	Amresco	Ohio, USA
DAPI cat.D9542	Sigma	Missouri, USA
Triton X-100 cat.T9284	Sigma	Missouri, USA

3.2.5.3 Protocol

The immunofluorescence staining process of the paraffin embedded tissue sections.

(1) Dewaxing and rehydration:

1) Place slices in an oven at 60℃ for 0.5h.

2) Transfer slices to xylene for 2 changes, 10min each.

3) Transfer slices to 95% ethanol for 2 changes, 5 min each.

4) Transfer one time through 95%, 85% and 75% ethanol respectively for 5min each time.

5) Rinse in double distilled water temporarily.

(2) Antigen retrieval:

1) Place slices into the immunohistochemical box.

2) Add an appropriate amount of antigen retrieval solution EDTA (Amresco cat.0322) and cover the box.

3) Place the box into a water pot of 97℃ for 40min.

4) Close the heater of the water pot, wait until the natural cooling.

5) Leave the antigen retrieval completed slices into the immunohistochemical box, wash with PBS for 3 times, 5min each time.

6) Draw a circle surrounding the tissue on the slices with crayon.

7) Add 50μL 2N HCL at 37℃ for 30min.

8) Wash slices in PBS for three times, 5min each time.

(3) Antibody incubation:

1) BrdU primary antibody incubation

a. Add 50μL Mouse anti-BrdU (Brdu(Ab-3), Mouse MAb (NeoMarkers cat.MS-1058-P0)) diluted 1: 200 in PBS.

b. Incubate at 4℃ for a whole night.

c. Wash in PBS for three times, 5min each time.

2) BrdU secondary antibody incubation

a. Add 50μL Donkey Anti-Mouse IgG (Alexa Fluor 594 Donkey Anti-Mouse IgG (H+L) Antibody (Molecular Probes cat.A21203)) diluted 1: 200 in PBS.

b. Incubate for 1h at room temperature.

c. Wash in PBS three times, 5min each time.

3) Antibody incubation for double staining

a. Add 50μL one of the antibody diluted 1: 300 in PBS according to the requirement: ①Rabbit polyclonal to GFAP - Astrocyte Marker (Abcam cat.ab7260); ②Rabbit polyclonal to Doublecortin - Neuronal Marker (Abcam cat.ab18723); ③ Rabbit polyclonal to Fox3 / NeuN (Abcam cat.ab104225). Incubate overnight at 4℃, and then wash in PBS three times, 5min each time.

b. Add 50μL second antibody of Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) (Cell Signaling Technology cat.#4412S) diluted 1: 300 in PBS. Incubate for 1h at room temperature, and then wash in PBS three times, 5min each time.

4) Incubating the DAPI for nuclear staining.

a. Add DAPI 50μL, place at room temperature for 5min.

b. Wash in PBS three times, 5min each time.

5) Mount with glycerol buffer.

6) Store slices in the dark at 4℃ for observation.

3.2.6 Cell count and analysis

Cells count and image acquisition were performed with fluorescence microscopy, blinded method was used, namely, the assessors did not know the grouping. There

were 4 series of slices of BrdU +, BrdU+/GFAP+, BrdU+/DCX+ and BrdU+/NeuN+ for SVZ or SGZ for each rat, and 5 to 7 slices for each series. Slices were observed in 10 × 40 or 10 × 20 magnification, four fields were randomly selected in each slice for cell count. The BrdU+ single or double-labeled positive cells were counted in each field of each slice on the right side (ischemic side) of the brain, then the average number of positive cells in each field for each rat was calculated.

3.2.7 Statistical analysis

Descriptive data are presented as mean ± standard error ($\bar{x}$± SE). SPSS 19.0 was used for all data processing. Two-way ANOVA was performed for the comparison of different groups and different time points. One-way ANOVA was used for the comparison of the number of BrdU+ single-labeled cells, BrdU+/DCX+, BrdU+/GFAP+ and BrdU+ /NeuN+ double labeled cells in the SGZ and SVZ in the Sham group, the Con group and the Exp group. LSD tests were used for post-hoc analysis. Alpha error was set at $p<0.05$.

3.3 Results

3.3.1 Proliferation and differentiation of NSCs in SVZ

3.3.1.1 BrdU single-labeled positive cells (BrdU+ cells)

BrdU+ cells represented newly proliferated NSCs. A remarkable proliferation of NSCs was found in both the Exp group and the Con group at the 3rd day of the intervention (the 4th day of ischemia). The most active proliferation happened on the 7th day of the intervention (the 8th day of ischemia). However, on the 14th day of the intervention (the 15th day of ischemia), proliferation decreased remarkably and was close to the level of the Sham group.

On the 3rd day of the intervention, the number of BrdU+ cells were significantly increased in both the Con group and the Exp group as compared with the Sham group ($p<0.01$); compared with the Con group, the number of BrdU+ cells were significantly increased in the Exp group ($p<0.05$). On the 7th day of the intervention, compared with the Sham group, the number of BrdU+ cells were significantly increased in both the Con group and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+ cells were significantly increased in the Exp group ($p<0.01$). On the 14th day of the intervention, the number of BrdU+ cells were significantly increased in the Exp group as compared with the Con group and Sham group ($p<0.05$); the number of BrdU+ cells was greater in the Con group than the Sham group, but the difference was not statistically significant ($p>0.05$) (Figure 3.5-1 ~ Figure 3.5-7, Table 3.1, Figure 3.6).

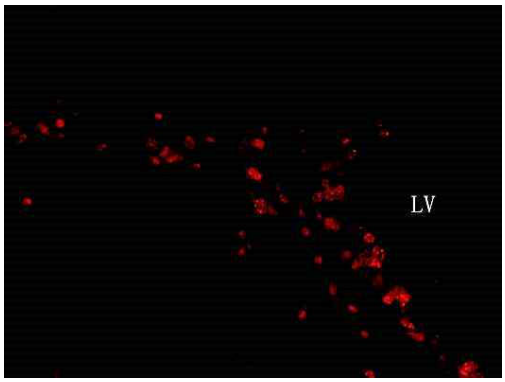


Figure 3.5-1 BrdU+ cells in Sham group

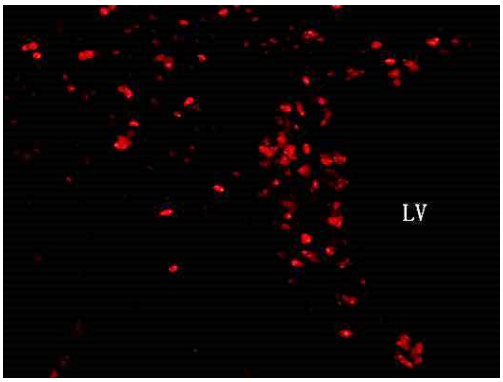


Figure 3.5-2

BrdU+ cells in Con group on day 3

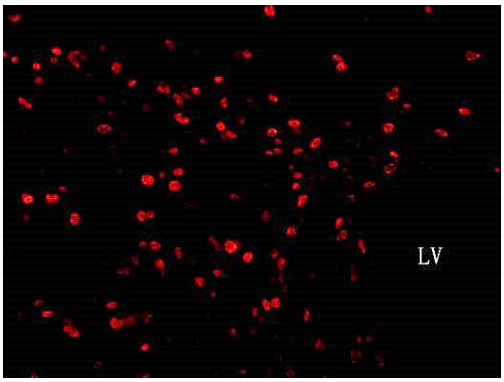


Figure 3.5-3

BrdU+ cells in Exp group on day 3

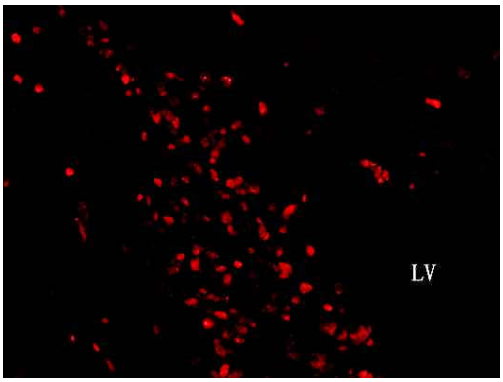


Figure 3.5-4

BrdU+ cells in Con group on day 7

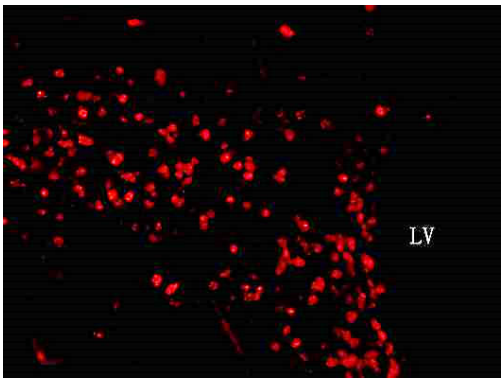


Figure 3.5-5

BrdU+ cells in Exp group on day 7

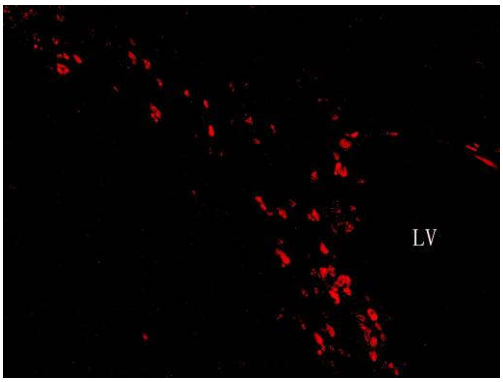


Figure 3.5-6

BrdU+ cells in Con group on day 14

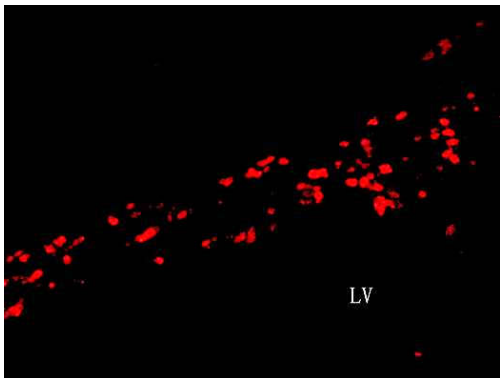


Figure 3.5-7

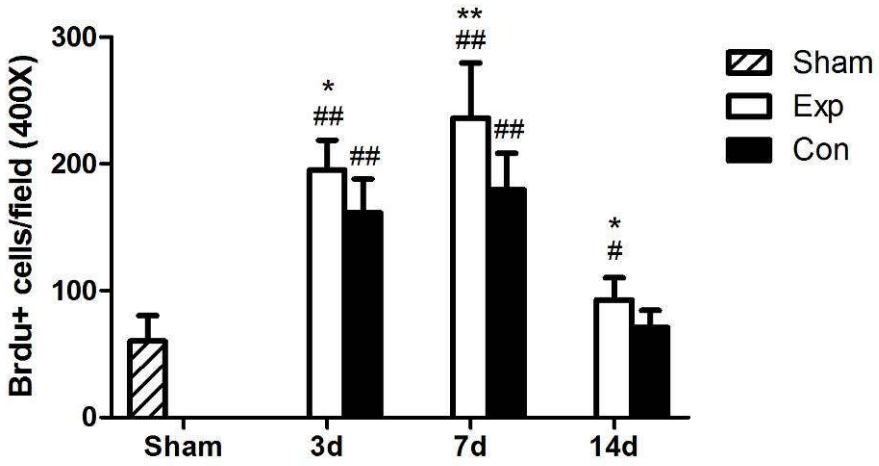
BrdU+ cells in Exp group on day 14

Figure 3.5 BrdU+ cells in the SVZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		60.17±20.13	
Con group (n=6)	161.25±26.97$^{\#\#}$	179.50±28.86$^{\#\#}$	70.83±13.56
Exp group (n=6)	195.00±23.66$^{\#\#*}$	236.17±43.47$^{\#\#**}$	92.86±17.33$^{\#*}$ (n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.1 Number of BrdU+ cells at different time point in SVZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.6 Number of BrdU+ cells at different time points in SVZ (n/400 magnification)

3.2.1.2 BrdU/DCX double-labeled positive cells (BrdU+/DCX+ cells)

BrdU+/DCX+ cells represented newborn NSCs which were differentiating into neurons. On the 3rd day of the intervention (the 4th day of the ischemia), the BrdU+/DCX+ cells were remarkable increased in both the Exp group and the Con group. The greatest amount of BrdU+/DCX+ cells was observed on the 7th day of the intervention (the 8th day of the ischemia). On the 14th day of the intervention (the 15th day of the ischemia), neural differentiation decreased significantly.

On the 3rd day of the intervention, the number of BrdU+/DCX+ cells was significantly increased in both the Con group and the Exp group as compared with the Sham group ($p<0.01$); compared with the Con group, the number of BrdU+/DCX+ cells was significantly increased in the Exp group ($p<0.05$).

On the 7th day of the intervention, compared with the Sham group, the number of BrdU+/DCX+ cells was significantly increased in both the Con group and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+/DCX+ cells was significantly increased in the Exp group ($p<0.01$).

On the 14th day of the intervention, the number of BrdU+/DCX+ cells was significantly increased in the Exp group compared with the Con group and Sham group ($p<0.05$); the number of BrdU+/DCX+ cells was greater in the Con group than the Sham group, but the difference was not statistically significant ($p>0.05$) (Figure 3.7-1 - Figure 3.7-7, table 3.2, Figure 3.8).

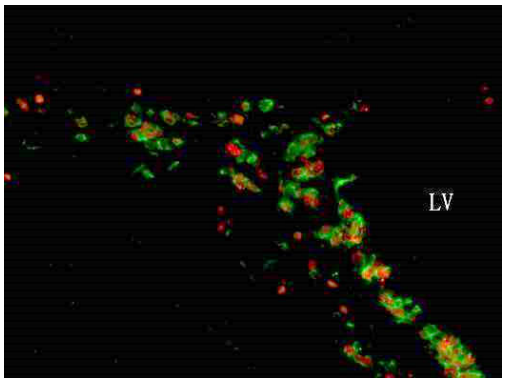


Figure 3.7-1 BrdU+/DCX+ cells in Sham group

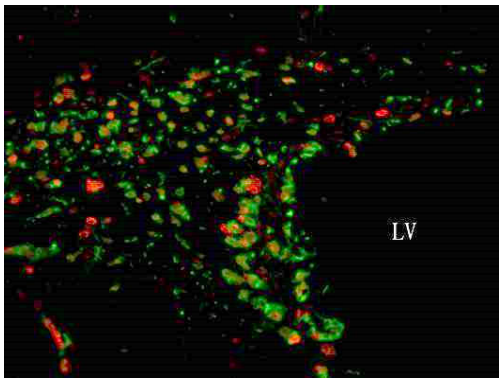


Figure 3.7-2

BrdU+/DCX+ cells in Con group on day 3

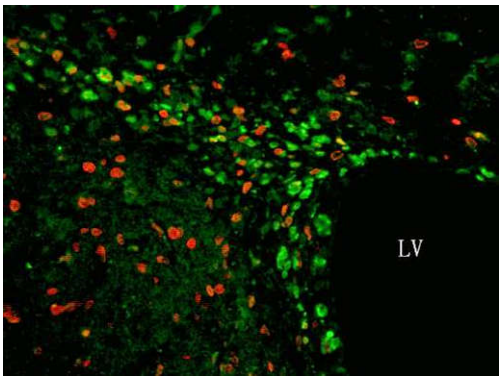


Figure 3.7-3

BrdU+/DCX+ cells in Exp group on day 3

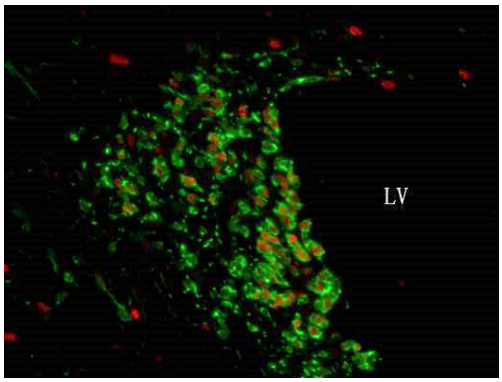


Figure 3.7-4

BrdU+/DCX+ cells in Con group on day 7

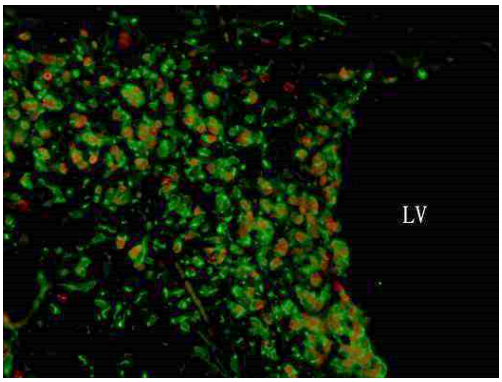


Figure 3.7-5

BrdU+/DCX+ cells in Exp group on day 7

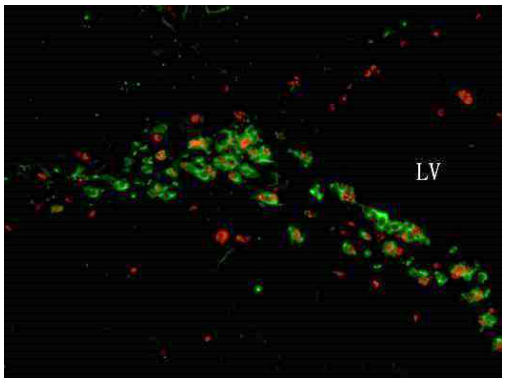


Figure 3.7-6

BrdU+/DCX+ cells in Con group on day 14

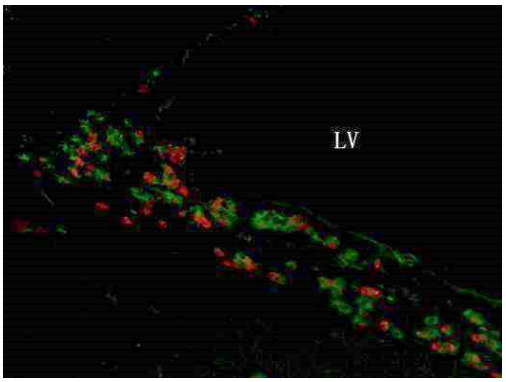


Figure 3.7-7

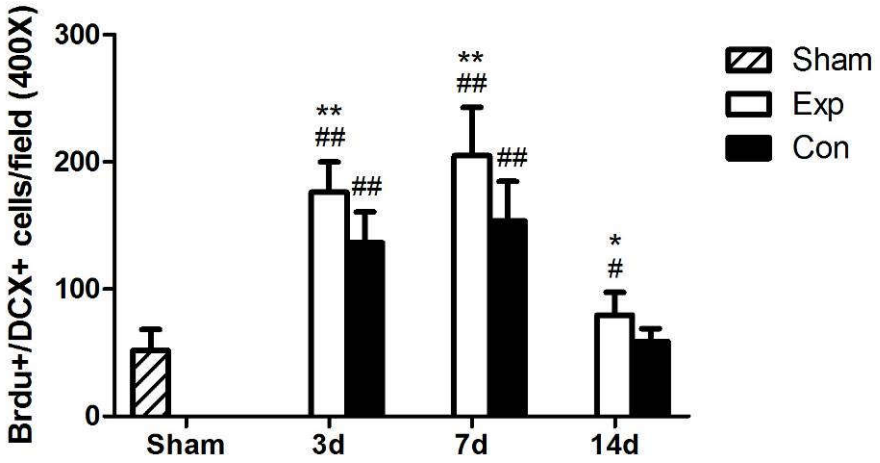
BrdU+/DCX+ cells in Exp group on day 14

Figure 3.7 BrdU+/DCX+ cells in the SVZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		51.67±16.43	
Con group (n=6)	136.33±24.25$^{\#\#}$	153.33±31.29$^{\#\#}$	58.67±10.11
Exp group (n=6)	176.15±23.66$^{\#\#**}$	204.83±38.15$^{\#\#**}$	79.71±17.67$^{\#*}$ (n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.2 Number of BrdU+/DCX+ cells at different time point in SVZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.8 Number of BrdU+/DCX+ cells at different time points in SVZ (n/400 magnification)

3.3.1.2 BrdU/GFAP double-labeled positive cells (BrdU+/GFAP+ cells)

BrdU+/GFAP+ cells represented newborn NSCs which were differentiating into glias. On the 3rd day of the intervention (the 4th day of the ischemia), BrdU+/GFAP+ cells were remarkably increased in both the Exp group and the Con group. The greatest amount of BrdU+/GFAP+ cells was observed on the 7th day of the intervention (the 8th day of the ischemia). On the 14th day of the intervention (the 15th day of the ischemia), the glial differentiation decreased significantly.

On the 3rd day of the intervention, the number of BrdU+/GFAP+ cells was significantly increased in both the Con group and the Exp group as compared with the Sham group ($p<0.01$); compared with the Con group, the number of BrdU+/GFAP+ cells was significantly increased in the Exp group ($p<0.01$). On the 7th day of the intervention, compared with the Sham group, the number of BrdU+/GFAP+ cells was significantly increased in both the Con group and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+/GFAP+ cells was significantly increased in the Exp group ($p<0.05$). On the 14th day of the intervention, no differences were found between 3 groups ($p>0.05$) (Figure 3.9-1 - Figure 3.9-7, table 3.3, Figure 3.10).

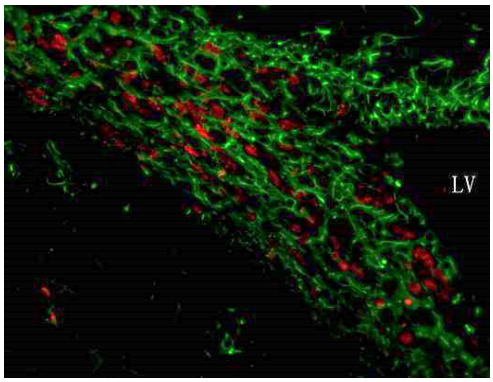


Figure 3.9-1 BrdU+/GFAP+ cells in Sham group

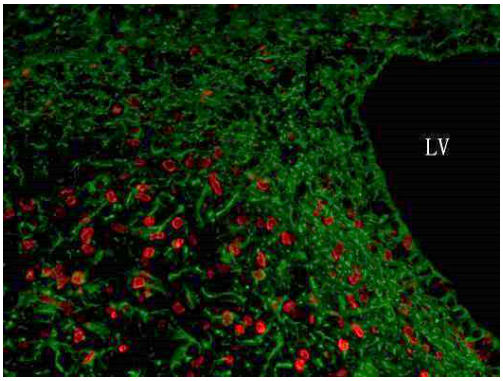


Figure 3.9-2

BrdU+/GFAP+ cells in Con group on day 3

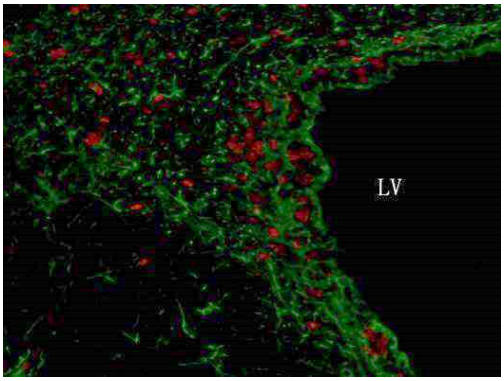


Figure 3.9-3

BrdU+/GFAP+ cells in Exp group on day 3

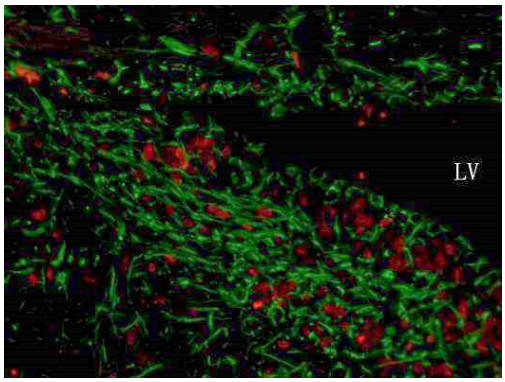


Figure 3.9-4

BrdU+/GFAP+ cells in Con group on day 7

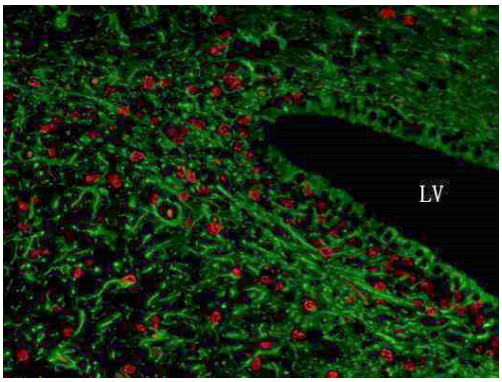


Figure 3.9-5

BrdU+/GFAP+ cells in Exp group on day 7

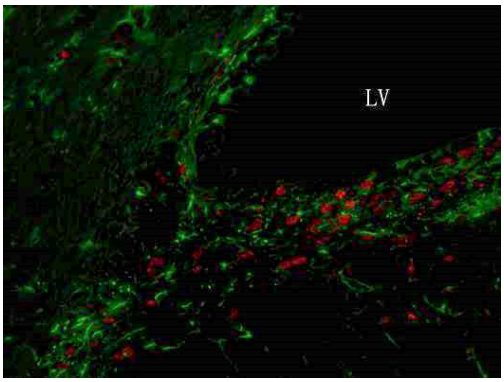


Figure 3.9-6

BrdU+/GFAP+ cells in Con group on day 14

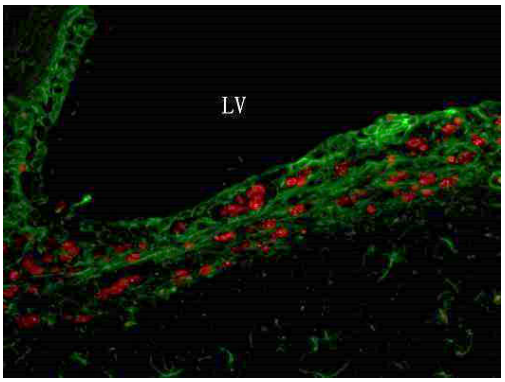


Figure 3.9-7

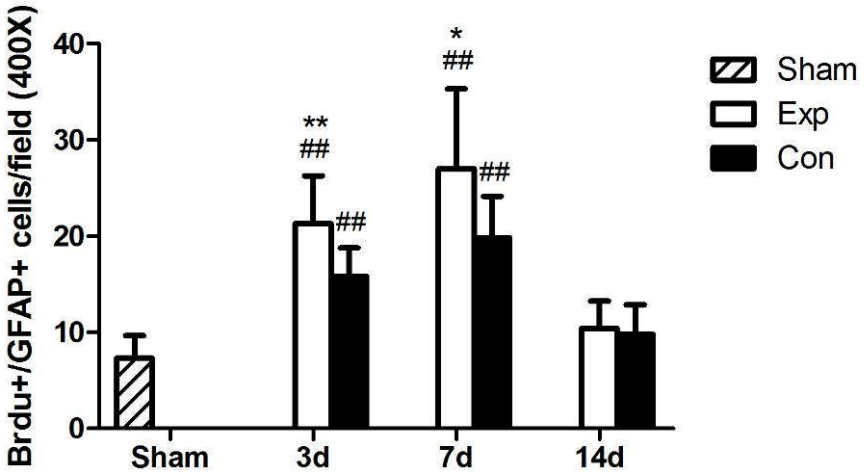
BrdU+/GFAP+ cells in Exp group on day 14

Figure 3.9 BrdU+/GFAP+ cells in the SVZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		6.67±2.16	
Con group (n=6)	15.83±2.94$^{\#\#}$	19.83±4.33$^{\#\#}$	9.83±3.06
Exp group (n=6)	21.33±4.94$^{\#\#**}$	27.03±8.29$^{\#\#*}$	10.43±2.82(n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.3 Number of BrdU+/GFAP+ cells at different time points in SVZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.10 Number of BrdU+/GFAP+ cells at different time points in SVZ (n/400 magnification)

3.3.2 Proliferation and differentiation of NSCs in SGZ

3.3.2.1 BrdU single-labeled positive cells (BrdU+ cells)

There was a consistent trend for the proliferation of NSCs in SVZ and SGZ. A remarkable proliferation of NSCs was observed in both the Exp group and the Con group on the 3rd day of the intervention (the 4th day of the ischemia). The most active proliferation happened on the 7th day of the intervention (the 8th day of the ischemia). However, on the 14th day of the intervention (the 15th day of the ischemia), proliferation decreased.

On the 3rd day of the intervention, compared with the Sham group, the number of BrdU+ cells was significantly increased in the Con group ($p<0.05$) and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+ cells was significantly increased in the Exp group ($p<0.01$).

On the 7th day of the intervention, compared with the Sham group, the number of BrdU+ cells was significantly increased in both the Con group and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+ cells was significantly increased in the Exp group ($p<0.05$).

On the 14th day of the intervention, compared with the Sham group, the number of BrdU+ cells was significantly increased in the Con group ($p<0.05$) and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+ cells was significantly increased in the Exp group ($p<0.05$) (Figure 3.11-1 ~ Figure 3.11-7, Table 3.4, Figure 3.12).

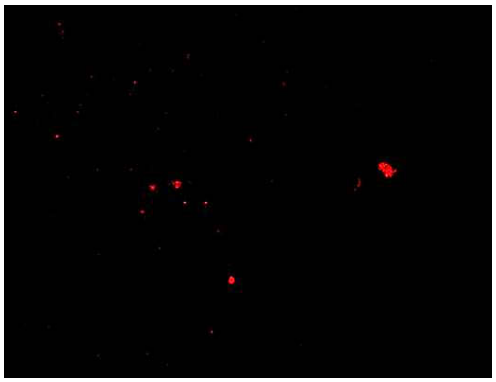


Figure 3.11-1 BrdU+ cells in Sham group

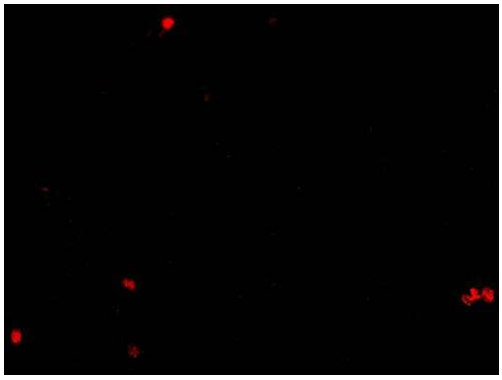


Figure 3.11-2

BrdU+ cells in Con group on day 3

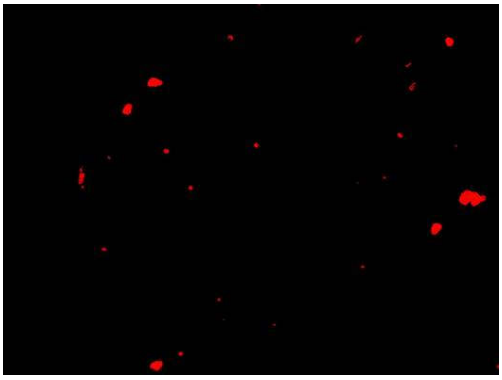


Figure 3.11-3

BrdU+ cells in Exp group on day 3

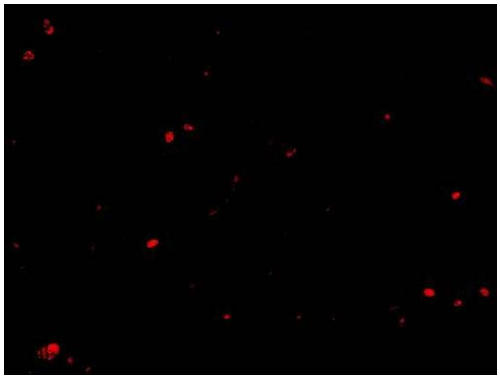


Figure 3.11-4

BrdU+ cells in Con group on day 7

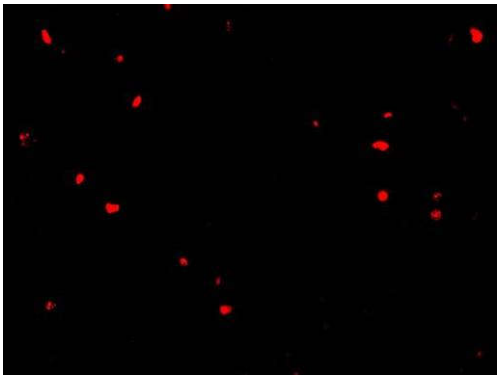


Figure 3.11-5

BrdU+ cells in Exp group on day 7

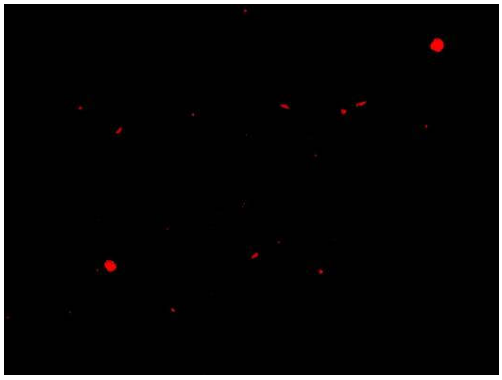


Figure 3.11-6

BrdU+ cells in Con group on day 14

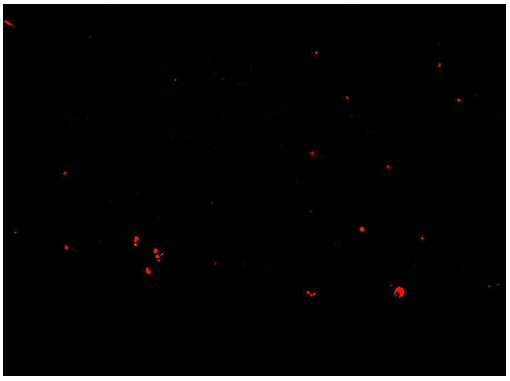


Figure 3.11-7

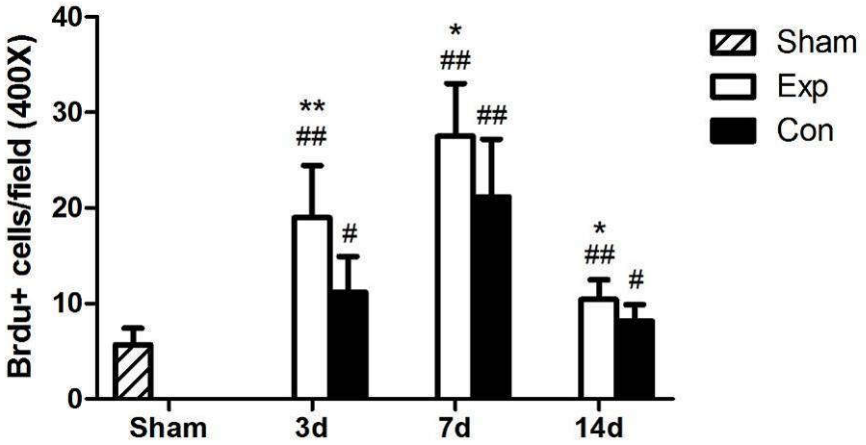
BrdU+ cells in Exp group on day 14

Figure 3.11 BrdU+ cells in the SGZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		5.67±1.75	
Con group (n=6)	11.17±3.76$^{\#}$	21.17±6.05$^{\#\#}$	8.17±1.72$^{\#}$
Exp group (n=6)	19.00±5.44$^{\#\#**}$	27.50±5.54$^{\#\#*}$	10.42±2.07$^{\#\#*}$ (n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.4 Number of BrdU+ cells at different time points in SGZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.12 Number of BrdU+ cells at different time points in SGZ (n/400 magnification)

3.3.2.2 BrdU/DCX double-labeled positive cells (BrdU+/DCX+ cells)

There was a consistent trend for BrdU+/DCX+ cells in the SVZ and SGZ. On the 3rd day of the intervention (the 4th day of the ischemia), the BrdU+/DCX+ cells were remarkably increased in both the Exp group and the Con group. The greatest amout of BrdU+/DCX+ cells was observed on the 7th day of the intervention (the 8th day of the ischemia). On the 14th day of the intervention (the 15th day of the ischemia), neural differentiation decreased significantly.

On the 3rd day of the intervention, compared with the Sham group, the number of BrdU+/DCX+ cells was significantly increased in the Con group ($p<0.05$) and the Exp group ($p<0.01$); compared with the Con group, the number of BrdU+/DCX+ cells was significantly increased in the Exp group ($p<0.01$).

On the 7th day of the intervention, compared with the Sham group, the number of BrdU+/DCX+ cells was significantly increased in both the Con group and the Exp group ($p<0.05$); compared with the Con group, the number of BrdU+/DCX+ cells was significantly increased in the Exp group ($p<0.01$).

On the 14th day of the intervention, the number of BrdU+ cells was significantly increased in the Exp group as compared with the Con group ($p<0.05$) and Sham group ($p<0.01$) (Figure 3.13-1 - Figure 3.13-7, table 3.5, Figure 3.14).

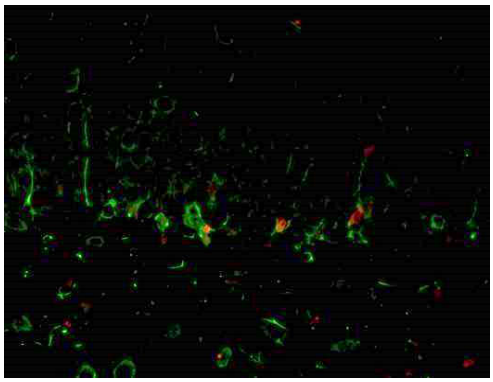


Figure 3.13-1 BrdU+/DCX+ cells in Sham group

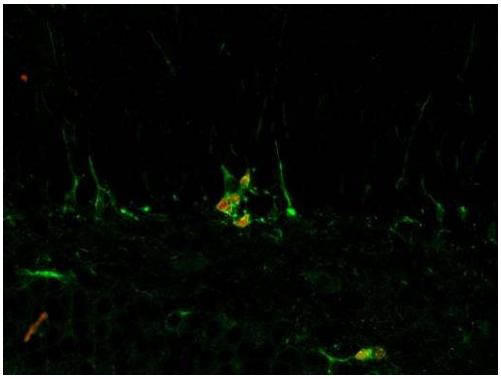


Figure 3.13-2

BrdU+/DCX+ cells in Con group on day 3

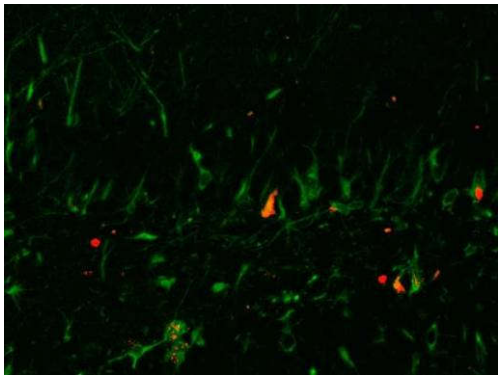


Figure 3.13-3

BrdU+/DCX+ cells in Exp group on day 3

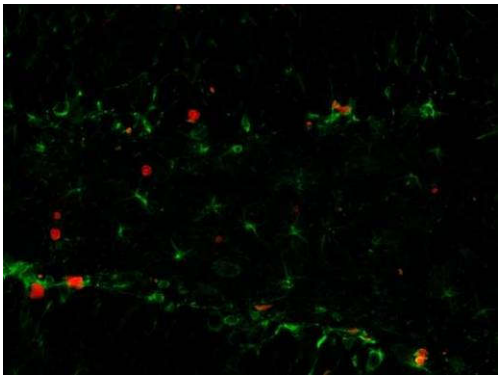


Figure 3.13-4

BrdU+/DCX+ cells in Con group on day 7

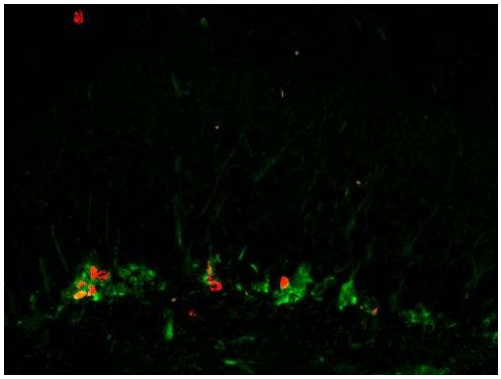


Figure 3.13-5

BrdU+/DCX+ cells in Exp group on day 7

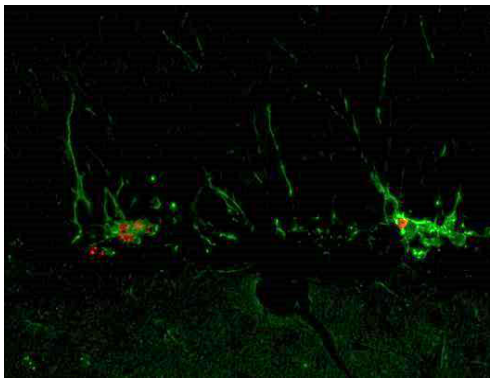


Figure 3.13-6

BrdU+/DCX+ cells in Con group on day 14

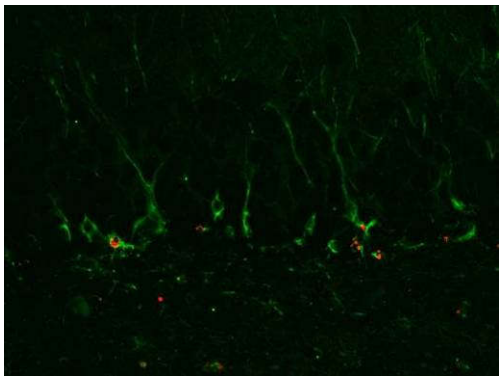


Figure 3.13-7

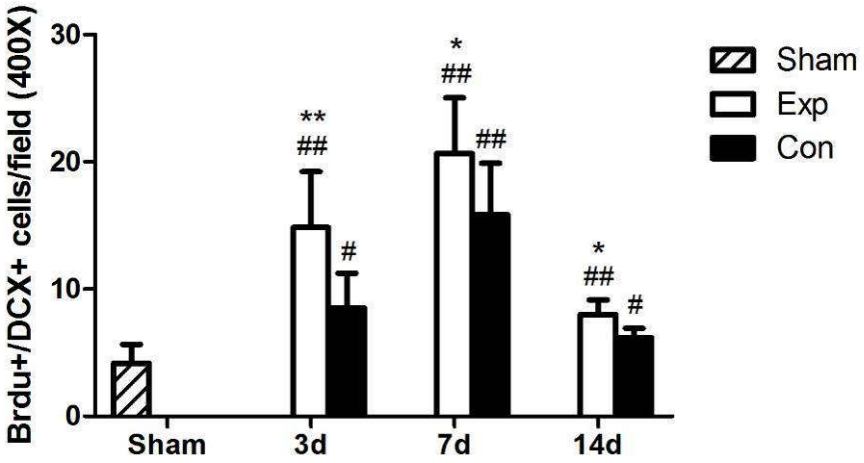
BrdU+/DCX+ cells in Exp group on day 14

Figure 3.13 BrdU+/DCX+ cells in the SGZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		4.17±1.47	
Con group (n=6)	8.50±2.74$^{\#}$	15.83±4.07$^{\#\#}$	6.17±0.75$^{\#\#}$
Exp group (n=6)	14.83±4.40$^{\#\#**}$	20.67±4.37$^{\#\#*}$	8.00±1.15$^{\#\#*}$(n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.5 Number of BrdU+/DCX+ cells at different time points in SGZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.14 Number of BrdU+/DCX+ cells at different time points in SGZ (n/400 magnification)

3.3.2.3 BrdU/GFAP double-labeled positive cells (BrdU+/GFAP+ cells)

On the 3rd day of the intervention (the 4th day of the ischemia), the BrdU+/GFAP+ cells started to increase in both the Exp group and the Con group. The greatest amout of BrdU+/GFAP+ cells was observed on the 7th day of the intervention (the 8th day of the ischemia). On the 14th day of the intervention (the 15th day of the ischemia), the glial differentiation decreased significantly.

On the 3rd day of the intervention, compared with the Sham group, the number of BrdU+/GFAP+ cells were significantly increased in the Exp group ($p<0.01$), but not the Con group ($p>0.05$); no differences were found between the Exp group and the Con group ($p>0.05$).

On the 7th day of the intervention, compared with the Sham group, the number of BrdU+/GFAP+ cells were significantly increased in both the Con group ($p<0.05$) and the Exp group ($p<0.01$); no differences were found between the Exp group and the Con group ($p>0.05$).

On the 14th day of the intervention, no differences were found between 3 groups ($p>0.05$) (Figure 3.15-1 - Figure 3.15-7, table 3.6, Figure 3.16).

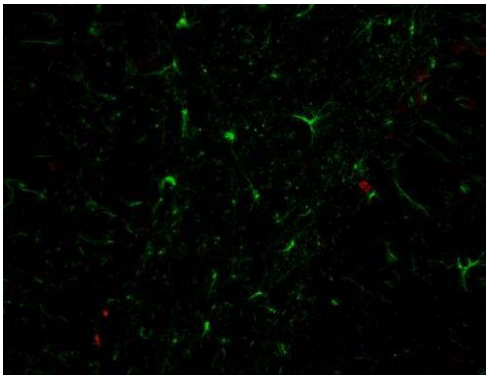


Figure 3.15-1 BrdU+/GFAP+ cells in Sham group

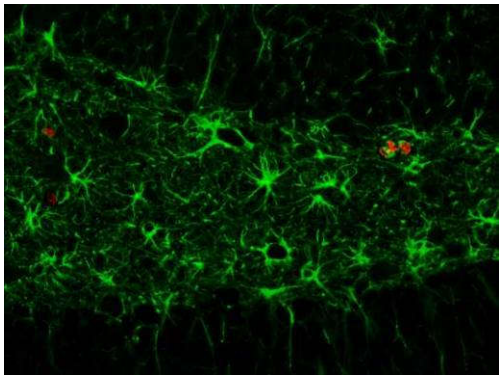


Figure 3.15-2

BrdU+/GFAP+ cells in Con group on day 3

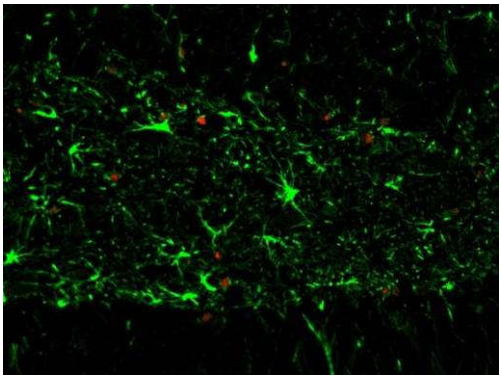


Figure 3.15-3

BrdU+/GFAP+ cells in Exp group on day 3

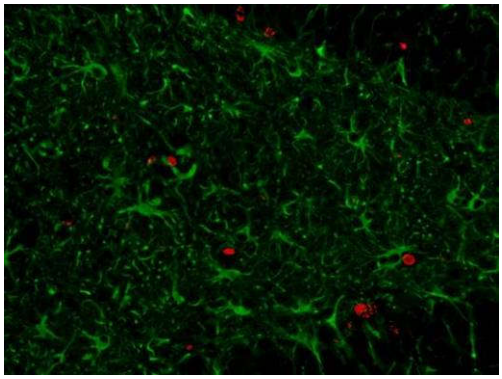


Figure 3.15-4

BrdU+/GFAP+ cells in Con group on day 7

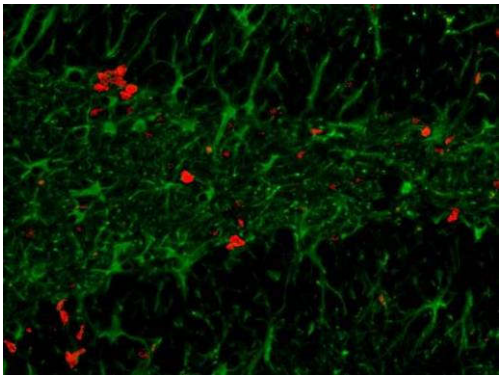


Figure 3.15-5

BrdU+/GFAP+ cells in Exp group on day 7

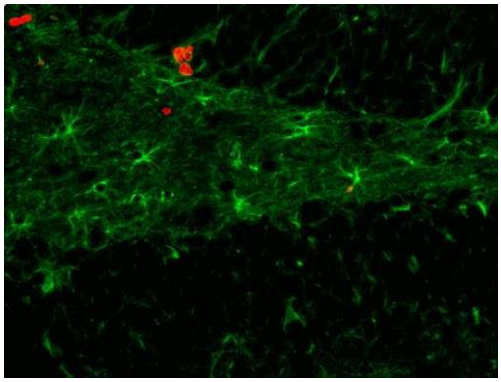


Figure 3.15-6

BrdU+/GFAP+ cells in Con group on day 14

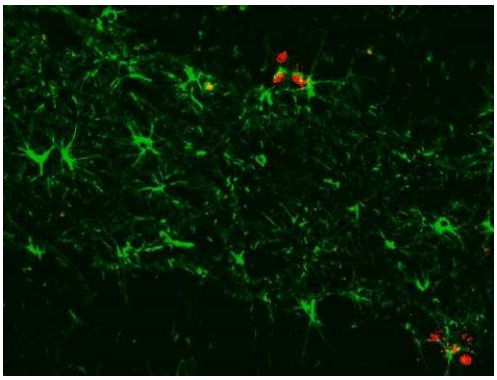


Figure 3.15-7

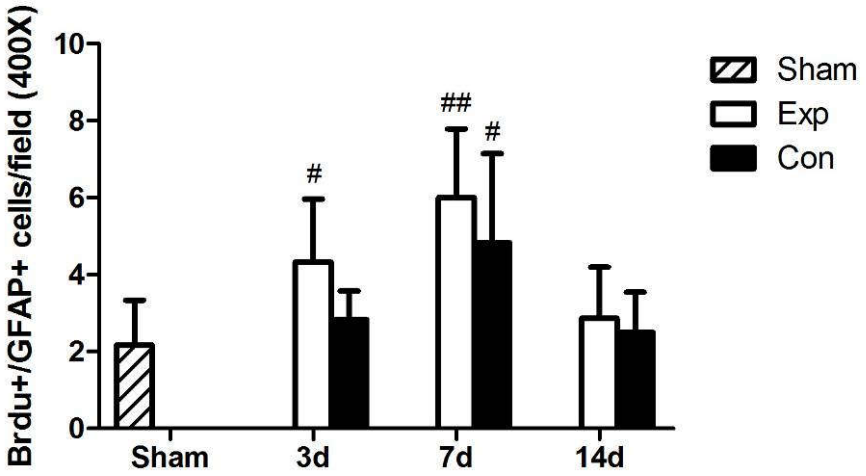
BrdU+/GFAP+ cells in Exp group on day 14

Figure 3.15 BrdU+/GFAP+ cells in the SGZ (×400)

Group	Day 3	Day 7	Day 14
Sham group (n=6)		2.17±1.17	
Con group (n=6)	2.83±0.75	4.83±2.32$^{\#}$	2.50±1.05
Exp group (n=6)	4.33±1.63$^{\#\#}$	6.00±1.79$^{\#\#}$	2.86±1.34(n=7)

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Table 3.6 Number of BrdU+/GFAP+ cells at different time points in SGZ (n/400 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 3.16 Number of BrdU+/GFAP+ cells at different time points in SGZ (n /400 magnification)

3.2.3 The new born neurons in SVZ

After 28 days' intervention, the BrdU/NeuN double positive cells (BrdU+/NeuN+ cells) were detected to observe the newborn neurons which was differentiated from the NSCs. In each slice, BrdU positive cells (nuclear staining,

stained in red) and DAPI (nuclear staining, stained in blue) overlapped well, BrdU+ cells were distributed in the striatum area. NeuN+ cells (stained in green) overlapped with DAPI well, also mainly distributed in the striatum. BrdU+/NeuN+ cells were scattered in those areas.

The results of one-way ANOVA showed that the number of BrdU+/NeuN+ cells was significantly different between the 3 groups, results of post-hoc analysis showed that, comparing with the Sham group, the number of BrdU+/NeuN+ cells was significantly increased in Con group and Exp group ($p < 0.01$). Comparing with the Con group, the e number of BrdU+/NeuN+ cells was significantly increased in Exp group ($p < 0.01$) (Figure 3.17, Table 3.7 and Figure 3.18).

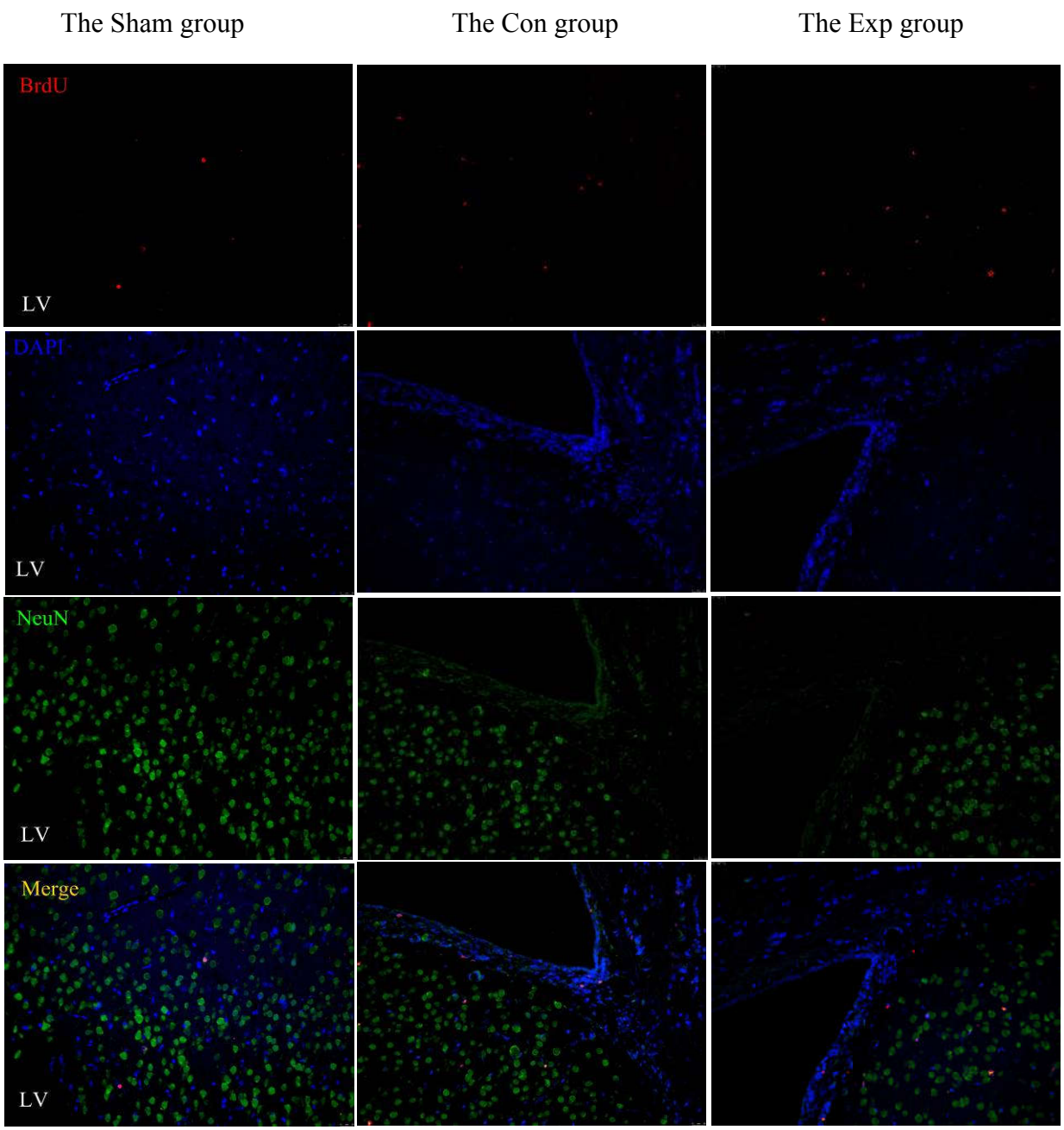
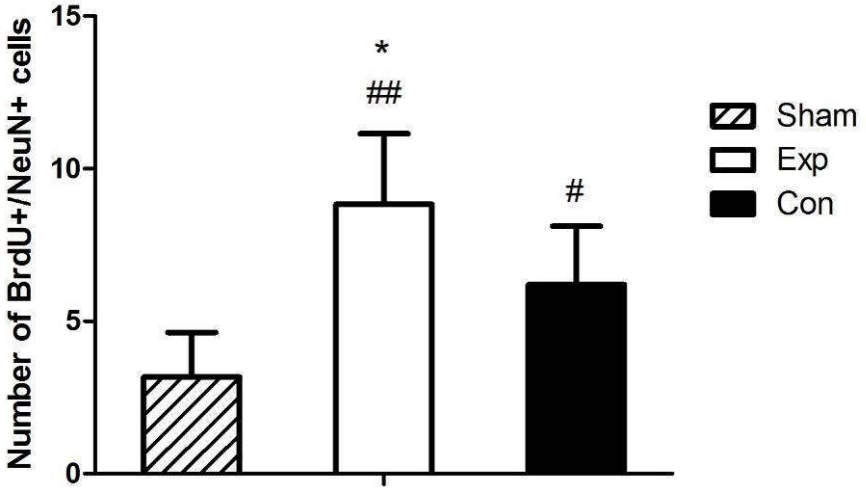


Figure 3.17 BrdU+/NeuN+ cells in the SVZ (×200)

Group	The number of BrdU+/NeuN+ cells
Sham group (n=6)	3.17±1.47
Exp group (n=6)	$8.83\pm2.32^{\#\#*}$
Con group (n=5)	$6.20\pm1.92^{\#}$

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$

Table 3.7 Number of BrdU+/NeuN+ cells at different time points in SVZ (n/200 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$

Figure 3.18 Number of BrdU+/NeuN+ cells at different time points in SVZ (n/200 magnification)

3.2.4 The new born neurons in SGZ

After 28 days' intervention, in each slice, BrdU positive cells (stained in red) and DAPI (stained in blue) overlapped well, BrdU+ cells were distributed in the DG area. NeuN+ cells (stained in green) overlapped with DAPI well, also mainly distributed in the DG. BrdU+/NeuN+ cells were scattered in those areas.

The results of one-way ANOVA showed that the number of BrdU+/NeuN+ cells was significantly different between the 3 groups. Results of post-hoc analysis showed that, comparing with the Sham group, the number of BrdU+/NeuN+ cells was significantly increased in Con group and Exp group ($p < 0.01$). Comparing with the Con group, the number of BrdU+/NeuN+ cells was significantly increased in Exp group ($p < 0.01$) (Figure 3.19, Table 3.8 and Figure 3.20).

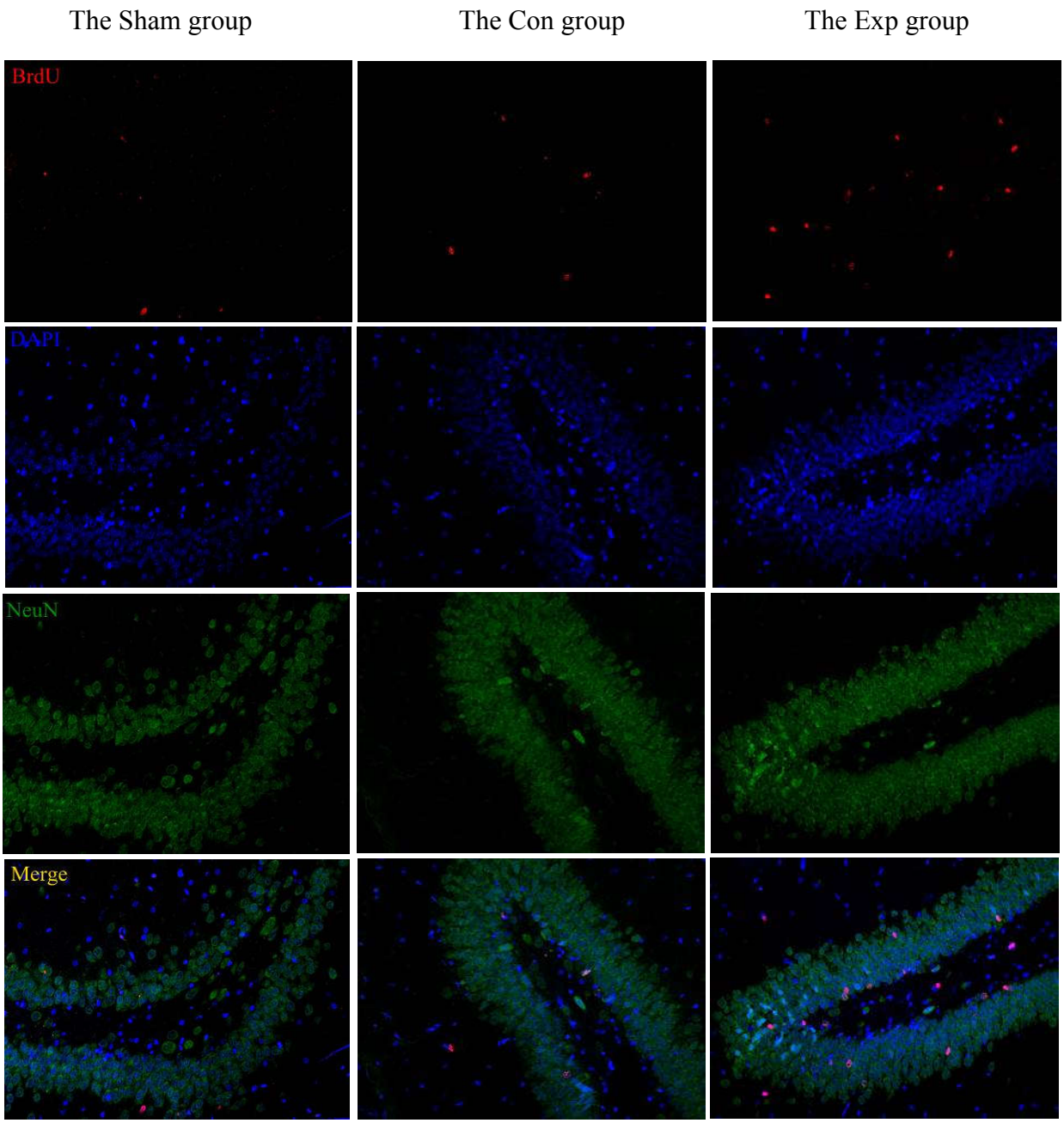
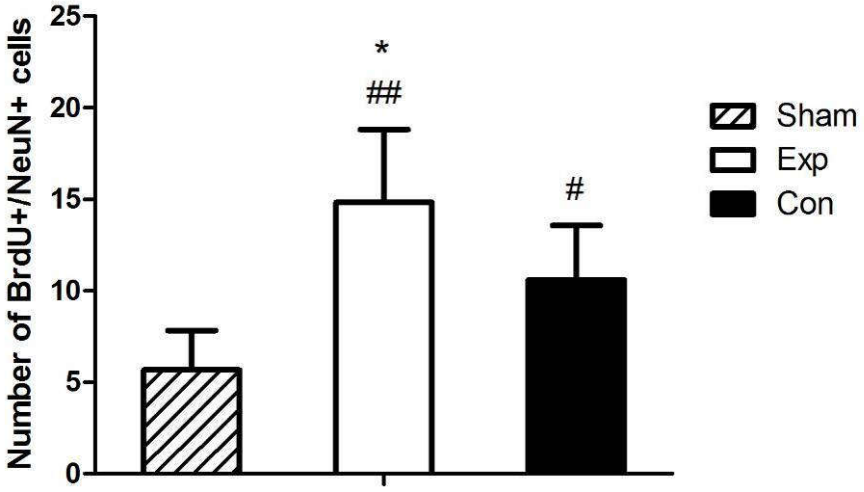


Figure 3.19 BrdU+/NeuN+ cells in the SGZ (×200)

Group	The number of BrdU+/NeuN+ cells
Sham group (n=6)	5.67±2.16
Exp group (n=6)	$14.83\pm3.97^{\#\#*}$
Con group (n=5)	$10.60\pm2.97^{\#}$

Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$

Table 3.8 Number of BrdU+/NeuN+ cells at different time points in SGZ (n/200 magnification)



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$

Figure 3.20 Number of BrdU+/NeuN+ cells at different time points in SGZ (n/200 magnification)

3.4 Discussion

3.4.1 The significance of the promotion of proliferation and differentiation of NSCs

In recent decades, the recovery of neural function after nervous system disease such as cerebral ischemia has been a research hotspot. Brain plasticity or neural plasticity provides a new research direction in brain damage, and provides the relevant theory for neurological rehabilitation. The brain's plasticity occurs at different levels, and the change from the cellular structure to the functional level could be involved. Brain plasticity theory points out that the brain can recover from dysfunction such as paralysis, hypesthesia, aphasia, dysphagia, and impaired cognitive function after brain damage.

The traditional concept assumed that neurons in the central nervous system were solely formed in the embryonic period or shortly after birth, and that neurons do not regenerate in the adult mammalian. Since 1992, the discovery of NSCs (Reynolds and Weiss, 1992) completely changed this theory, and provided new ideas for the treatment for TBI and nervous system diseases such as stroke (Decimo et al., 2012). When the brain is injured, necrotic neurons and their synapses, and the cells surrounding them would produce a series of changes to adapt to the damages and functional deficits. Axonal sprouting, a microenvironment full of a variety of growth factors and the proliferation and differentiation of NSCs are considered to be the important factors in the process of brain functional recovery (Wieloch and Nikolich, 2006). NSCs plays an important role in the self-repair process of the injured brain.

However, in the striatum, only 0.2% dead neurons may be replaced by newly differentiated neurons from NSCs (Ziemka-Nalecz and Zalewska, 2012). Therefore, increasing the proliferation and differentiation of endogenous NSCs has an important significance in brain's plasticity and function recovery after nervous system disease.

3.4.2 BrdU labeling of the NSCs' proliferation and differentiation

In the adult mammalian brain, there are two main areas producing NSCs. One is located in the SGZ which is below the hippocampal dentate gyrus, the other one is the SVZ which is adjacent to the subventricular zone of the lateral ventricle (Zhao et al., 2008, Yao et al., 2012). NSCs have the ability of self proliferation and multi-directional differentiation, and they can maintain the stem cells pool by symmetrical and asymmetrical proliferation (Weng and Lee, 2011). They can also differentiate into three main types of cells in the central nervous system including neurons, astrocytes and oligodendrocytes, and other neurocytes. New neurons that come from NSCs' proliferation can be integrated in the existing neural network and fulfill their special function.

In the process of the proliferation and differentiation of NSCs, newborn cells can be labeled with experimental methods using reagents such as antibodies, and then they can be made visible after the immunofluorescence staining. The cell cycle has four phases including G1, S, G2 and M. Phase S is the period of DNA's synthesis, when the DNA is in semiconservative replication which requires ribonucleotide as raw materials. BrdU is a kind of thymidine analogues. When a cell is in the S phase and BrdU is present at the same time, BrdU will be incorporated into newly

synthesized DNA. BrdU can be kept for a long time in the DNA until the death of the cell. Therefore, BrdU can be used to specifically label newly proliferated NSCs in the brain. With the anti-BrdU monoclonal antibody, the NSCs which BrdU has been incorporated into can be displayed on slices. In this experiment, the BrdU immunofluorescence labeling positive cells were shown in red color.

GFAP (glial fibrillary acidic protein) is a kind of intermediate filament-like protein, usually in the form of monomer. GFAP is mainly distributed in the astrocyte of central nervous system, which involves in the composition of cytoskeleton and maintains its tensile strength. The cartilage cells, fibroblasts, myoepithelial cells, lymphocytes and hepatic satellite cells in peripheral nervous system also express GFAP. However, GFAP in the peripheral system can not be labeled by central GFAP monoclonal antibody, suggesting that the two sources of GFAP may have different structures, and GFAP monoclonal antibody might be specific to the central nervous system. Therefore, BrdU/GFAP double positive cells can be considered newborn glias.

DCX (doublecortin) is a kind of microtubule associated protein, widely expressed in migrating neurons, and it could be observed in immature neurons. Neural precursor cells express DCX when activated immediately and lasting for 2~3 weeks until they differentiate into mature neurons. Generally, the expression of DCX declines after 2 weeks of the differentiation, while the cells begin to express NeuN which is a marker of mature neurons. Therefore, DCX express in the differentiating neurons, which

means it can specifically mark the immature neurons. BrdU/DCX double positive cells can be considered newborn immature neurons.

NeuN (neuronal nuclear antigen) is a specific marker of neurons. It is a kind of protein that expresses in the late stage of the differentiation of neurons and lasts lifelong (Brown et al., 2003). In the hippocampus, NeuN can be detected at the tenth day of the differentiation when new NSCs begin to differentiate into neurons (Brown et al., 2003). The BrdU/NeuN double positive cells can be considered newborn neurons.

During the process of neural regeneration, the newly differentiating cells can be either GFAP positive or DCX positive, and will not appear in the double positive situation. That means GFAP and DCX have an independent direction of differentiation, namely, that GFAP is the direction of glias and DCX is the direction of neurons (Steiner et al., 2004). Brown JP et al (Brown et al., 2003) injected BrdU to the two months old normal rats and found that, 60% of newborn cells begin to express DCX after two hours, and 90% of newborn cells express DCX at the forth day to seventh day, then the expression of DCX declined rapidly. In the process of newborn NSCs' differentiating into mature neural cells, the expression of DCX reached the peak at the 10^{th} day to the 14^{th} day, and then declined at the 21^{st} day. These results provide us with the experimental basis of labeling and observation of neural regeneration.

3.4.3 Effects of ELF-EMF on the proliferation of NSCs

The results of our study found that, in SVZ, on the third day of the intervention (the fourth day of the cerebral ischemia), compared with the Sham group, the Con group and the Exp group showed a significant increase of BrdU positive cells. The proliferation was most active on the 7^{th} day of the intervention (the 8^{th} day of the ischemia). On the 14^{th} day of the intervention (the 15^{th} day of the cerebral ischemia), the proliferation was significantly reduced to the level of Sham group. The trend of the proliferation of NSCs' in SGZ was relatively consistent with that in the SVZ. The results indicate that cerebral ischemia can stimulate the proliferation of the endogenous NSCs, and the most active proliferation period would be about one week after cerebral ischemia. The result that the peak proliferation of NSCs was observed at around 7^{th} day of the cerebral ischemia in SVZ and SGZ was consistent with previous research (Yagita et al., 2001, Tureyen et al., 2004, Lichtenwalner and Parent, 2006, Liu et al., 2013).

After intervention with ELF-EMF of the 3^{rd}, 7^{th} and 14^{th} day, the number of BrdU positive cells in SVZ and SGZ in the Exp group were significantly higher than the Con group which was not exposed to ELF-EMF. This result revealed that ELF-EMF can significantly promote the proliferation of endogenous NSCs in rats with cerebral ischemia.

3.4.4 Effects of ELF-EMF on the differentiation of NSCs

This experiment observed two directions of the differentiation of NSCs interneurons and glias, respectively. BrdU+/DCX+ double positive cells represented

newborn NSCs differentiating into neurons, BrdU+/GFAP+ double positive cells represented newborn NSCs differentiating into glias. In the SVZ and SGZ, compared with the Sham group, BrdU+/DCX+ cells and BrdU+/GFAP+ cells in both the Con group and the Exp group were significantly increased. This results shows that the stimulation of cerebral ischemia not only promoted the proliferation of endogenous NSCs, but also promoted the differentiation of NSCs into neurons and glias at the 3rd day of the intervention (the 4th day of the cerebral ischemia). Differentiation was most pronounced on the 7th day of the intervention (the 8th day of the ischemia). On the 14th day of the intervention (the 15th day of the cerebral ischemia), the differentiation was significantly reduced and closed to the level of the Sham group.

The number of BrdU+/DCX+ and BrdU+/GFAP+ cells in the SVZ and SGZ in the Exp group were significantly higher than the Con group on the 3rd day and the 7th day of the intervention. The results indicate that ELF-EMF can promote endogenous NSCs' differentiation into neurons and glial cells in rats with cerebral ischemia. There is an increasing number of studies reporting the effect of ELF-EMF on promoting cell differentiation. Kim MO et al (Kim et al., 2015) applied ELF-EMF (45Hz, 1mT) on marrow mesenchymal stem cells, 8hs per day for 7 days. Their results showed that the ELF-EMF can promote the differentiation of mesenchymal stem cell and increase the osteogenesis. ELF-EMF (15Hz, 1mT) also had similar effects on bone marrow mesenchymal stem cells (Song et al., 2014). Li Y et al (Li et al., 2002) found 5Hz or 20Hz ELF-EMF could promote the differentiate of NSCs into neurons in vitro.

3.4.5 Effect of ELF-EMF on NSCs differentiating into mature neurons

Recent studies reported that 50Hz of ELF-EMF can promote bone marrow mesenchymal stem cells differentiating into mature neurons (Bai et al., 2013, Kim et al., 2013). Seong Y et al (Seong et al., 2014) found that ELF-EMF (50Hz, 1mT) promoted the bone marrow mesenchymal stem cells differentiating into neurons through the early growth response protein 1, moreover, by transplanting these differentiated neurons to the rats with neurological diseases, the symptoms relieved significantly. Sherafat et al (Sherafat et al., 2012) found that ELF-EMF (60Hz, 0.7mT) could significantly increase NSCs' proliferation and migration and strengthen myelin repair in rats with white matter demyelination. Cuccurazzu and colleagues (Cuccurazzu et al., 2010) reported that ELF-EMF (50Hz, 1mT) could significantly promote NSCs' differentiation into neurons in dentate gyrus in the normal adult rat and new mature neurons could be integrated into the granule cell layer of the dentate gyrus and then enhanced synaptic plasticity of the hippocampus.

We detected BrdU+/NeuN+ positive cells in SVZ and SGZ and found that the number of new matured neurons were increased significantly in the Con group and the Exp group as compared with the Sham group, which indicates that stimulation of cerebral ischemia can significantly promote endogenous NSCs to differentiate into mature neurons in SVZ and SGZ to repair damaged brain tissue. The result was consistent with the previous studies (Arvidsson et al., 2002, Tobin et al., 2014). The number of new matured neurons in SVZ and SGZ areas in Exp group was higher than the Con group, this indicated that the ELF-EMF can promote the endogenous NSCs

differentiating into mature neurons, so as to strengthen the repair of brain tissue and might be of benefit for the recovery of the brain function.

3.5 Conclusion

Cerebral ischemia can stimulate the proliferation and differentiation of endogenous NSCs in the SVZ and SGZ. ELF-EMF can promote the proliferation and differentiation of endogenous NSCs in the SVZ and SGZ and increase the number of newborn neurons in rats with cerebral ischemia. ELF-EMF may promote neural regeneration, and it might have important therapeutic value for neurological diseases and injuries of the central nervous system.

CHAPTER 4 THE MECHANISM OF ELF-EMF ON THE PROLIFERATION AND DIFFERENTIATION OF EDOGENOUSE NSCS ON RATS WITH CEREBRAL ISCHEMIA

4.1 Introduction

The results in Chapter 3 demonstrated that the proliferation and differentiation of the endogenous NSCs in SVZ and SGZ are enhanced in cerebral ischemia, and ELF-EMF can further promote the proliferation and differentiation and increase the number of newborn neurons in rats with cerebral ischemia. However, the possible mechanism of the beneficial effects of the ELF-EMF is not clear.

The Notch signaling pathway plays an extremely important role in the regulation of NSCs' proliferation and differentiation (Oya et al., 2009, Aguirre et al., 2010). The mRNA and protein including Hes1, Hes5, mash1, mash3, Ngn2 and Notch intracellular domain (NICD) are essential in Notch signaling pathway. The activation of Notch leads to upgrading of Hes1 and Hes5 expression. When the Notch signaling pathway is activated, it will promote proliferation of the NSCs (Kageyama et al., 2005, Hansen et al., 2010, Hoeck et al., 2010, Kato et al., 2010). Notch signaling pathway is essential to maintain NSCs and to properly control neurogenesis both in the embryonic or adult brain (Imayoshi et al., 2010).

The Notch signaling pathway still works under pathological condition of cerebral ischemia. Studies (Oya et al., 2009, Wang et al., 2009a, Wang et al., 2009b) showed that the expression of Hes1, Hes5 and Notch changed with time and related with the proliferation and differentiation of NSCs in the SVZ after cerebral ischemia. The inhibition of the Notch signaling pathway can block the NSCs proliferation induced by ischemia in the SVZ (Wang et al., 2009b).

This chapter describes the effect of ELF-EMF on the key factors of Notch signaling pathway in SVZ and SGZ in rats with cerebral ischemia, trying to reveal the mechanism of ELF-EMF in promoting the proliferation and differentiation of endogenous NSCs. The grouping and intervention were discussed in Chapter 2. At the four time points of day 3, day 7, day 14 and day 28 of the intervention, rats were sacrificed and their SVZ and SGZ were harvested. RT-PCR and Western blotting were performed to analyze the mRNA and protein levels of Hes1, Hes5 and Notch1, the key factors of Notch signaling pathway. Data and images were processed in software of Excel 2013, GraphPad Prism 5, PhotoshopCS3, ImageJ 1.48 and SPSS19.0.

4.2 Materials and methods

4.2.1 Animals and grouping

One hundred and ten 12-week-old SD rats were used in this experiment, weighing 220 ~ 280g. Six rats were randomly selected as Sham group using random number table, and the remaining 104 rats accepted the MCAO surgery. The procedure of the MCAO was described in Chapter 2. After the MCAO surgery, 22 rats died, 6 rats had too severe symptoms and 8 rats had no symptom. The other 68 rats which met the including criteria were randomly assigned into the Con group and Exp group.

4.2.2 Interventions

The interventions for each group were carried on as the statement in chapter 2. Rats were killed and their brain tissue was harvested on the 3^{rd} day, 7^{th} day, the 14^{th} day and 28^{th} day of the intervention, respectively. Depending on the number of animals remaining in each group, 6 to 7 rats were randomly selected and sacrificed at each time point. The flow diagram is shown in Figure 4.1.

4.2.3 The brain tissue dissection

The rats were anesthetized with 10% of chloral hydrate in a dosage of 0.3mL/kg, then the rats were decapitated and the brain were taken out quickly. Placed the brain in a petri dish containing buffer solution, cut from the midline of the brain. Bluntly separated the hippocampus tissue of the right cerebral hemisphere with a forceps, and then separated the upper section of the hippocampus with a scalpel to get SGZ; bluntly dissected and fully exposed the right lateral ventricle, cut with a scalpel carefully along the side wall of the lateral ventricle to separate SVZ. The harvested

SGZ and SVZ tissue were equally divided into two parts, one for RT-PCR test and another for Western Blotting test. Then each tissue was placed into one frozen tube, and immediately placed into liquid nitrogen for 1 min and then stored in -80 ℃ refrigerator (Figure 4.2, Figure 4.3).

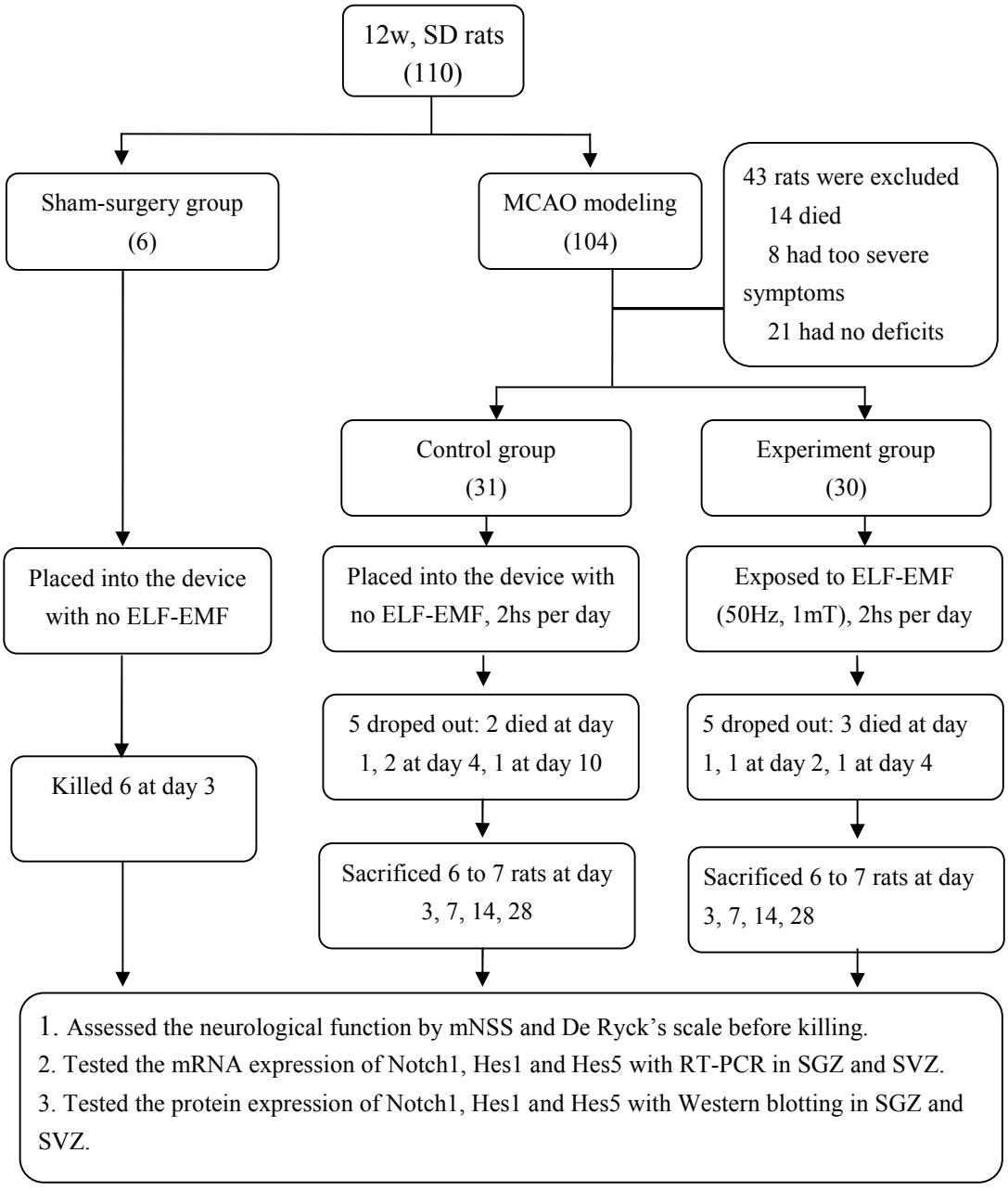


Figure 4.1 The flow chart of the experiment

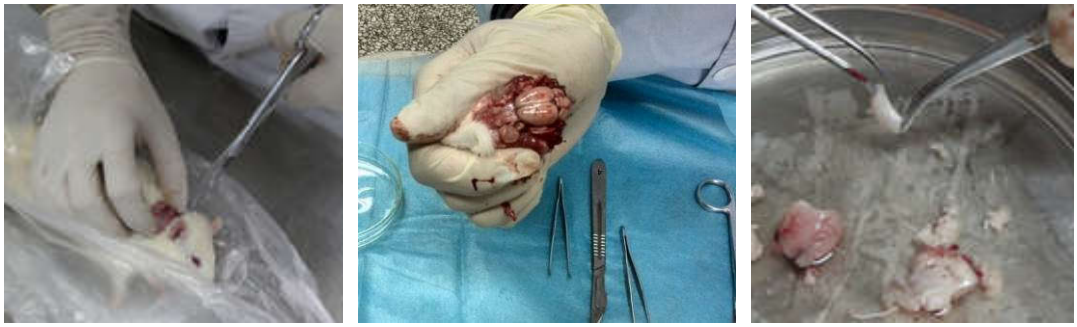


Figure 4.2 Dissected the hippocampus

Figure 4.3 Dissected the SVZ

4.2.4 The procedure for real-time PCR

4.2.4.1 The devices

Device	Company	Country
Real-time PCR detection systems	Bio-Rad	California, USA
MyCycler PCR System	Bio-Rad	California, USA

4.2.4.2 The agents

Agents	Company	Country

Trizol Reagent cat.15596-018	Ambion	Texas, USA
iScript cDNA Synthesis Kit cat.#170-8890	Bio-Rad	California, USA
iQ SYBR Green Supermix cat.#170-8880	Bio-Rad	California, USA
TURBO DNA-free Kit cat.AM1907	Life Technologies	New York, USA

4.2.4.3 Design and synthesize of primer

Gene sequences were identified in the Gene Bank. Then the primer was designed with Prime Primer software and synthesized by the Biological Engineering Company (Shanghai). Gene sequences and the number of nucleotides were shown in the table below.

Primer	Sequences (5'to3')	No. of nucleotides	Fragment (bp)
Hes1-rat-F	GAGTGCATGAACGAGGTGAC	20	108
Hes1-rat-R	CGTTGATCTGGGTCATGCAG	20	
Hes5-rat-F	CCAGCGACACGCAGATGA	18	165
Hes5-rat-R	AGAGGCCGCAGGCAGATT	18	
Notch1-rat-F	TCACCCCTGAACAGCATCAT	20	106
Notch1-rat-R	TTCACAGTGCACATTGGAGC	20	
GAPDH-rat-F	TATGGAATCCTGTGGCATC	19	86
GAPDH-rat-R	GTGTTGGCATAGAGGTCTT	19	

4.2.4.4 The extraction of total RNA from brain tissue

(1) Take out the frozen tube which contain the collected tissue from the -80℃ refrigerator.

(2) Add Trizol 500μL into the frozen tube, the place it on ice.

(3) Blow the tissue repeatedly with a needle until its full cleavage.

(4) Transfer the liquid into a 1.5mL EP tube, place it for 5min at room temperature.

(5) Add 0.2mL chloroform into EP tube, vibrate heavily for 15s and then place at room temperature for 2 ~ 3min.

(6) Centrifugate the EP tube in 12000 rpm for 15min at 4℃.

(7) Transfer the upper aqueous phase into another new 1.5mL RNAse-free EP tube after the centrifugation.

(8) Add an equal volume of isopropanol, place at -20℃ for 0.5h.

(9) Centrifugate in 12000 rpm for 15min at 4℃.

(10) Discard the supernatant, leave the white precipitate in the bottom of the tube.

(11) Add 0.5mL 75% ethanol to wash.

(12) Centrifugate in 7500 rpm for 5min at 4℃.

(13) Discard the supernatant, dry in a biological safety cabinet naturally.

(14) Add 30μL Rnase-free water to dissolve the precipitate after air-drying.

4.2.4.5 The measurement of RNA concentrations

The concentration and purity of the RNA sample were tested with Nanodrop 2000 UV spectrophotometer.

4.2.4.6 Removal of DNA

(all the bellowing reagent were from the TURBO DNA-free kit, Invitrogen cat.AM1907)

(1) Add 3μL 10 × RNA-free kit buffer and 1μL DNAse, incubate for 20 ~ 30min at 37 ℃.

(2) Add 3μL DNAse inaction salt, mix and place at room temperature for 5min, vibrate at least 1 in this period.

(3) Centrifuge in 13000 rpm for 2min at 4 ℃, then transfer the supernatant to another 1.5mL EP tube.

4.2.4.7 Reverse transcriptase

(all the following reagents were from iScript CDNA Sythesis kit, BIO-RAD cat. # 170-8890)

(1) Construction of retroviral systems (assume the total system volume of 20μL)

5× Tscript reaction mix	4μL
Tscript reverse transcriptase	1μL
RNA template	1μg (Adjust the volume according to the concentration of the RNA)
Nuclease-free water	Complement to 20μL

(2) Operational procedure

The fluorescence ration PCR instrument (Biorad chroma 4) was used to complete the following steps.

95℃, 2min

95℃，10s

60℃，30s } 40 cycles

(Initial denaturation in 95℃ for 2 min；denaturation in 95℃ for 10s，Primer annealing in 60℃ for 30s, 40 cycles. Melting curve was from 60℃ to 95℃, reading every 0.5℃, holding for 5s.)

(3) Results and data analysis

2-△△Ct method was employed to calculate relative expression of the genes.

4.2.5 Western blotting operating procedure

4.2.5.1 The devices and agents

(1) The instruments and equipments

Devices	Company	County
Bio-Rad Universal Hood II	Bio-Rad	Hercules, CA
Molecular Imager Gel Doc XR System	Bio-Rad	Hercules, CA

(2) The main agents

Agents	Company	County
RAPI lysis buffer cat.P0013B	Beyotime	Jiangsu, China
cOmplete, Mini cat.04693124001	Roche	Swiss

BCA Protein content detection kit cat.KGPBCA	KeyGen Biotech	Nanjing, China
Pageruler Prestained protein ladder cat.#26616	Thermo Scientific	New Hampshire, USA
SDS-PAGE loading buffer (5×) cat.P0015	Beyotime	Jiangsu, China
30% Acrylamide/Bis Solution,29:1 cat. #161-0156	Bio-Rad	California, USA
Resolving Gel Buffer cat.#161-0798	Bio-Rad	California, USA
Stacking Gel Buffer cat.#161-0799	Bio-Rad	California, USA
Ammonium Persulfate (APS) cat.0486	Amresco	Ohio, USA
TEMED cat.0761	Amresco	Ohio, USA
HES1 (D6P2U) Rabbit mAb cat. #11988	CST	Massachusetts, USA
Notch1 (5B5) Rat mAb cat.#3447	CST	Massachusetts, USA
Mouse Anti-beta Actin Monoclonal Antibody cat.TA-09	ZSGB-BIO	Beijing, China
Peroxidase-Conjugated Goat Anti-Rat IgG (H+L) cat.ZB-2307	ZSGB-BIO	Beijing, China
Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H+L) cat.ZB-2305	ZSGB-BIO	Beijing, China
Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) cat.ZB-2301	ZSGB-BIO	Beijing, China
Immobilon Western Chemiluminescent HRP Substrate cat.WBKLS0100	Millipore	Massachusetts, USA

4.2.5.2 Total protein extraction

(1) Preparation of lysis buffer

Agents	Dosage /200μL
RAPI buffer (Beyotime cat.P0013B)	178μL
NaF 200×	1μL
Na_3VO_4 200×	1μL
Cocktail 10× (ROCHE cOmplete, Mini cat.04693124001)	20μL

(2) Protein extraction

1) Take out the microtube which contain the tissues from the - 80 ℃ refrigerator.

2) Add 100μL lysis buffer in to the tube.

3) Place the tube on ice and blow the tissue repeatedly to make it fully cracking.

4) Transfer the liquid into the 1.5 mL EP tube and incubate for 30 min on ice.

5) Centrifuge the EP tube in 14000 RPM, for 5 min at 4℃.

6) Transfer the supernatant fluid into a fresh 1.5 mL EP tube.

4.2.5.2 protein concentration determination

(1) In 96 - well plates, with the BCA protein detection kit (the KeyGEN Bio TECH the KGPBCA), the protein concentration of the processed liquid in previous steps was determined with the BCA protein assay.

(2) Calculate the required total volume of the liquid which must contain 50μg total protein according to the concentration. Then add the required volume of sample into a 200μL PCR tube, add SDS-PAGE loading buffer (5 x) (Beyotime the P0015) into the PCR tube. Boil the sample in 97℃ for 5 min.

4.2.5.3 Electrophoresis

(1) Preparing the GEL (all reagents are placed in the ice, and shaking before using)

1) Separation gel was prepared according to the following system:

Separation gel (Lower layer)	mL
dd H_2O	1.9
BIO-RAD 30%Acrylamide/Bis Solution,29:1 cat.#161-0156	1.7
Resolving Gel Buffer (BIO-RAD cat.#161-0798)	1.3
10% SDS (Amresco cat.0227)	0.05
10% APS (Amresco cat.0486)	0.05
TEMED (Amresco cat.0761)	0.002

After added separation gel slowly into the mould, the distilled water was used to isolate the air. Placed the separation gel for 30 min, distilled water was poured after the polymerization of the separation gel. Remove any bubbles.

2) Stacking gel was prepared according to the following system:

Stacking gel (Upper layer)	mL
dd H_2O	1.4
BIO-RAD 30%Acrylamide/Bis Solution,29:1 cat.#161-0156	0.33
Stacking Gel Buffer (BIO-RAD cat.#161-0799)	0.25
10% SDS (Amresco cat.0227)	0.02
10% APS (Amresco cat.0486)	0.02
TEMED (Amresco cat.0761)	0.004

Slowly add the stacking gel and placed the dentate template, remove any bubbles, place the gel for 20 min for polymerization. Then take out the dentate template, add the processed samples to the hole in the gel. Add 5μL Pageruler Prestained protein ladder (Thermo Scientific cat. # 26616) into the first hole, add 5μL 1 x SDS-PAGE loading buffer into other empty holes.

(2) SDS-PAGE electrophoresis

1) Run the stacking gel for 30 min at 80V.

2) Run the separation gel for 1h at 100V.

4.2.5.4 Transferring the protein from the gel to membrane

(1) Mark the orientation of the gel from the bottom.

(2) Take the gel out of the device and cut off the unwanted parts after electrophoresis.

(3) Cut a PVDF membrane with the same width of the gel, label the membrane and put it into the methanol for 15s to activate it.

(4) Place the filter paper, gel, PVDF membrane, and the filter paper in turn from cathode to anode on the transfer plate.

(5) Run the transferring for 50 min at 100V. The procedure should be carried out on ice, ice packs were placed into the transfer buffer to keep the low temperature.

4.2.5.5 Antibody incubation

(1) Blocking

1) Rinse the transferred membrane in the TBS for 3 times, each time 5 min.

2) Bloch in the 5% non - fat milk for 1h.

3) Wash the membrane in the TBS for 3 times, each time 5 min.

(2) Incubation of the primary antibody

1) Add the following primary antibody according to the target protein, incubate overnight at 4℃.

Notch1 (5B5) Rat mAb (CTS cat.#3447) 1μL + 1mL 5% non-fat milk

Mouse Anti-beta Actin Monoclonal Antibody (ZSGB-BIO cat.TA-09) 1μL + 1mL 5% non-fat milk

Hes1 (D6P2U) Rabbit mAb (CTS cat.#11988) 1μL + 1mL BSA (5% BSA, soluted with TBS/T)

2) Rinse the blot in TBST for 3 times, 5 min each.

(3) Incubation of the secondary antibody

1) Add the following secondary antibody according to the target protein, incubate for 1h at 37℃.

Peroxidase-Conjugated Goat Anti-Rat IgG (H+L) (ZSGB-BIO cat.ZB-2307) 2μL + 10mL 5% non-fat milk

Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (ZSGB-BIO cat.ZB-2305) 2μL + 10mL 5% non-fat milk

Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (ZSGB-BIO cat.ZB-2301) 2μL + 10mL 5% non-fat milk

2) Rinse the blot in TBST for 3 times, 5 min each.

4.2.5.6 Imaging

1. Mix the A, B liquid in Immobilon Western Chemiluminescent HRP Substrate (MILLIPORE, cat. WBKLS0100) in a proportion of 1:1 to make the chemiluminescent substrate.

2. Apply the chemiluminescent substrate to the well incubated PVDF membrane.

3. Capture the chemiluminescent signals with Bio-Rad Universal Hood II photographic imaging system.

4. Use light density scanning analysis software to measure the color stripe of the protein, get the value of optical density with a β-actin stripe for internal reference.

4.2.6 Statistical analysis

Data are presented as mean ± standard deviation ($\bar{x}$ ± SD). SPSS19.0 was used for all data analysis. One-way ANOVA was used to compare the expression of Nothc1, Hes1 and Hes5 in SGZ or SVZ in the Sham group, the Con group and the Exp group. LSD tests were used for all post-hoc analysis. Alpha error was at 0.05.

4.3 Results

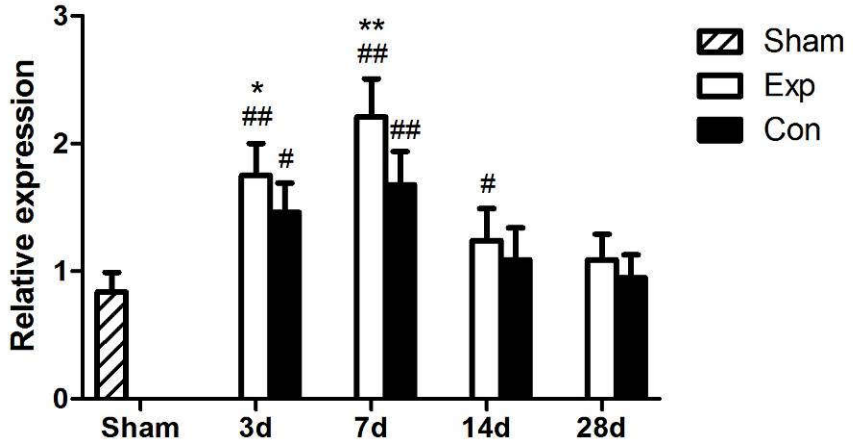
4.3.1 RT-PCR analysis in SVZ

The results of RT-PCR for quantitative analysis of gene expression in SVZ are as follows.

4.3.1.1 Hes1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Hes1 mRNA levels was significantly different between 3 groups ($p<0.05$); on day 28, difference between the 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day, compared with Sham group, Hes1 mRNA level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), Hes1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$); on day 7, compared with the Sham group, Hes1 mRNA level was significantly increased in both the Exp group and the Con group ($p<0.01$), Hes1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.01$); on day 14, Hes1 mRNA level in the Exp group was significantly higher than the Sham group ($p<0.05$), however, no difference was found between the Exp group and the Con group ($p>0.05$) (Figure 4.4).



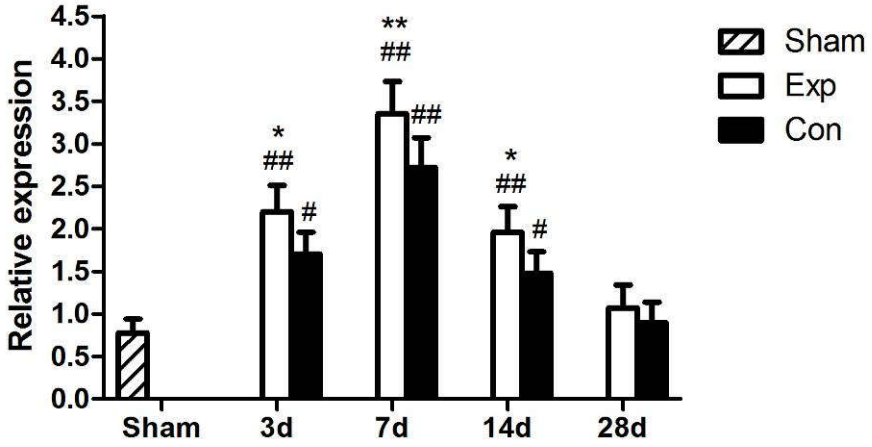
Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.4 The expression of Hes1 mRNA in SVZ

4.3.1.2 Hes5

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Hes5 mRNA levels was significantly different ($p<0.05$) between 3 groups; on day 28, difference between 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day and 14th day, compared with Sham group, Hes5 mRNA level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), Hes5 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$); on day 7, compared with the Sham group, Hes5 mRNA level was significantly increased in both Exp group and Con group ($p<0.01$), Hes5 mRNA level in the Exp group was significantly higher than the Con group ($p<0.01$) (Figure 4.5).



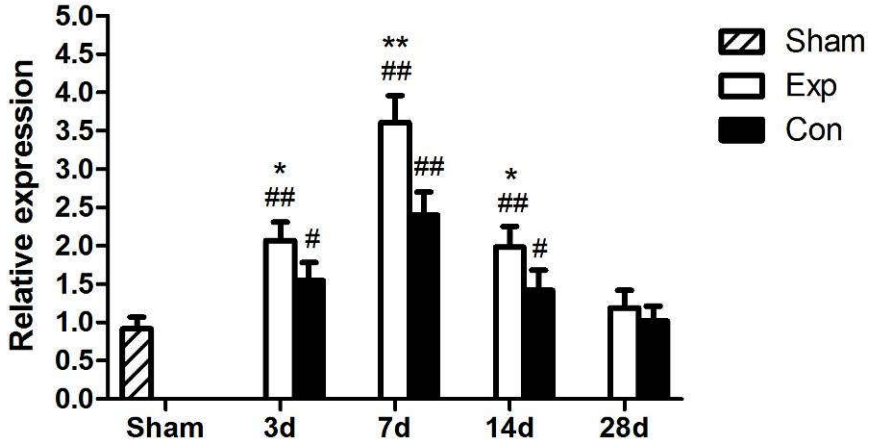
Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.5 The expression of Hes5 mRNA in SVZ

4.3.1.3 Notch1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Notch1 mRNA levels was significantly different ($p<0.05$); on day 28, no significant difference was found between 3 groups ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day and 14th day, compared with Sham group, Notch1 mRNA level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), Notch1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$); on day 7, compared with the Sham group, Notch1 mRNA level was significantly increased in both the Exp group and the Con group ($p<0.01$), Notch1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$) (Figure 4.6).



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.6 The expression of Notch1 mRNA in SVZ

4.3.2 RT-PCR analysis in SGZ

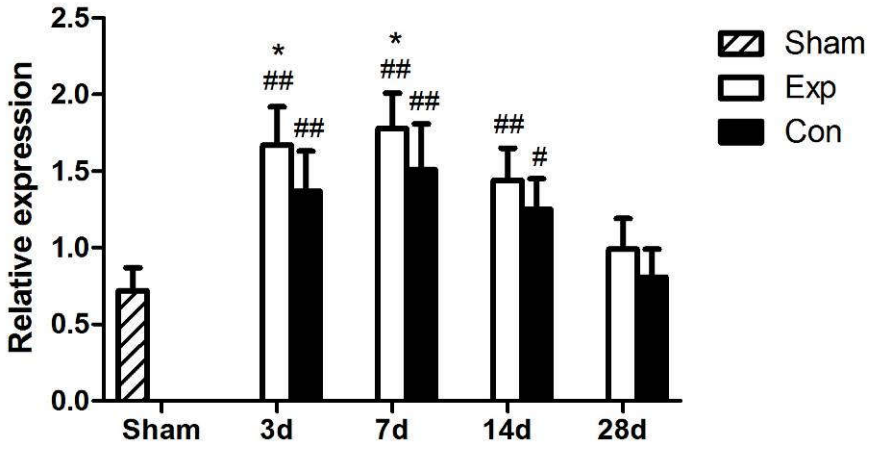
The results of RT-PCR for quantitative analysis of gene expression in SGZ were as follows:

4.3.2.1 Hes1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Hes1 mRNA level was significantly different between 3 groups ($p<0.05$); on day 28, difference between the 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day and 7th day, compared with the Sham group, Hes1 mRNA level was significantly increased in both the Exp group and the Con group ($p<0.01$), Hes1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$); on day 14, Hes1 mRNA levels in the Exp group and the Con group were significantly higher than the Sham group

(p<0.01, p<0.05), however, no difference was found between the Exp group and the Con group (p>0.05) (Figure 4.7).



Compared with Sham group, #p<0.05, ##p<0.01; compared with Con group, *p<0.05, **p<0.01

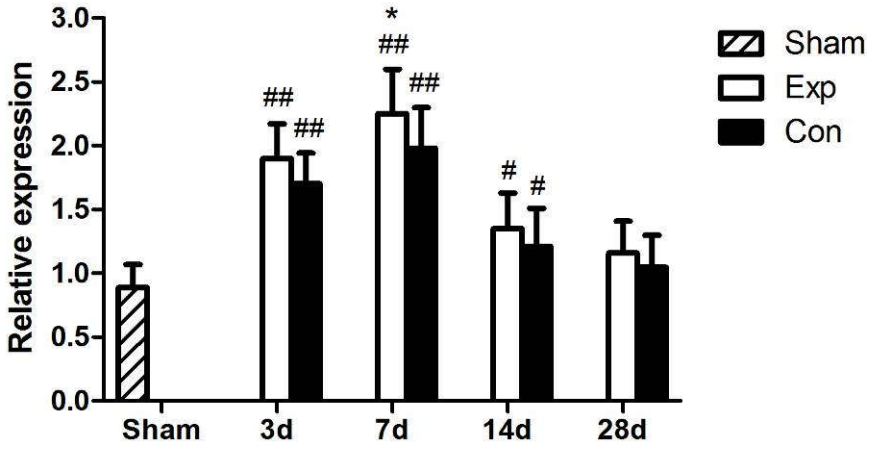
Figure 4.7 The expression of Hes1 mRNA in SGZ

4.3.2.2 Hes5

Results of one-way ANOVA showed that, on day 3, day 7 and day 14 of the intervention, the relative expression of Hes5 mRNA levels was significantly different (p<0.05); on day 28, difference between 3 groups was not significant (p>0.05).

Results of post-hoc analysis showed that: on day 3, compared with the Sham group, Hes5 mRNA level was significantly increased in both the Exp group and the Con group (p<0.01), no difference was found between the Exp group and the Con group (p>0.05); on day 7, compared with the Sham group, Hes5 mRNA level was significantly increased in both the Exp group and the Con group (p<0.01), Hes5 mRNA level in the Exp group was significantly higher than the Con group (p<0.05); on day 14, compared with the Sham group, Hes5 mRNA level was significantly

increased in both Exp group and Con group ($p<0.05$), no difference was found between the Exp group and the Con group ($p>0.05$) (Figure 4.8).



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

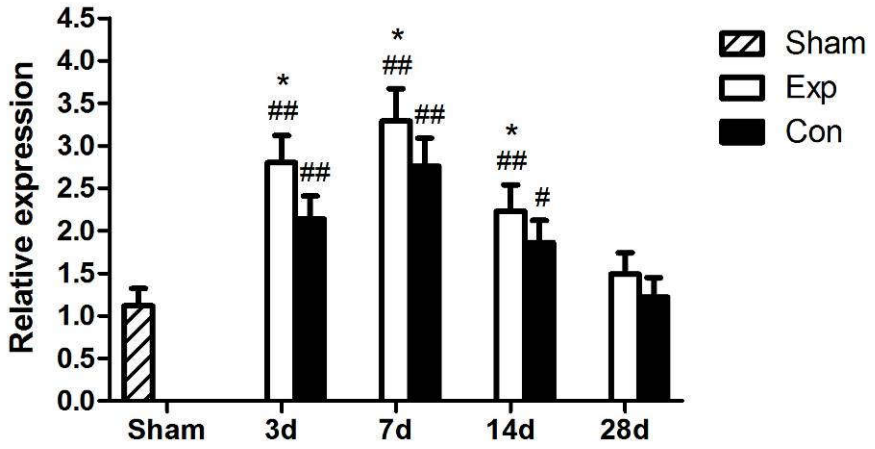
Figure 4.8 The expression of Hes5 mRNA in SVZ

4.3.2.3 Notch1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Notch1 mRNA levels was significantly different ($p<0.05$); on day 28, no significant differences were found between the 3 groups ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day and 7th day, compared with Sham group, Notch1 mRNA level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), Notch1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$); on day 14, compared with the Sham group, Notch1 mRNA level was significantly increased in the Exp group ($p<0.01$) and

the Con group ($p<0.05$), Notch1 mRNA level in the Exp group was significantly higher than the Con group ($p<0.05$) (Figure 4.9).

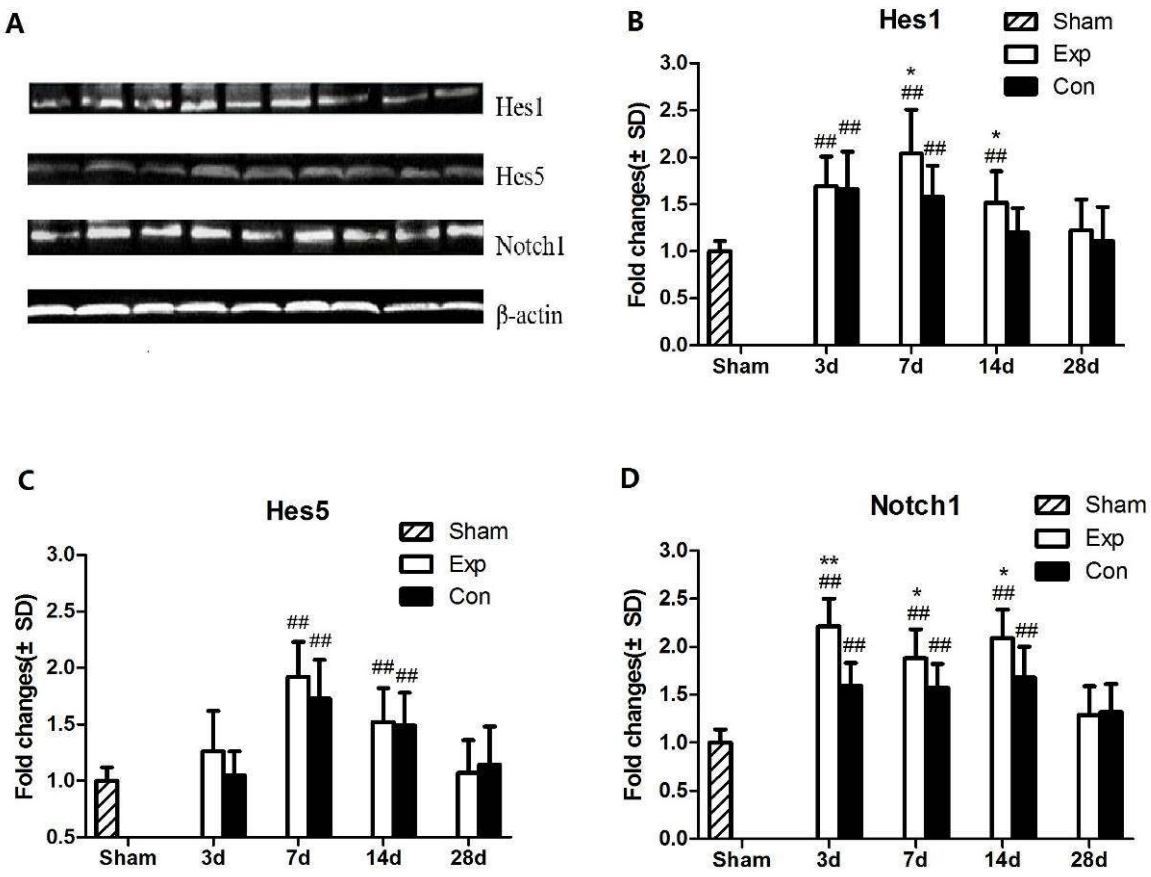


Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.9 The expression of Notch1 mRNA in SVZ

4.3.3 Western blot assay in SVZ

The results of Western blot assay of protein expression in SVZ are shown as follows (Figure 4.10):



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.10 The expression of protein in SVZ

4.3.3.1 Hes1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Hes1 protein levels was significantly different between 3 groups ($p<0.05$); on day 28, difference between the 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day, compared with Sham group, Hes1 protein level was significantly increased in both Exp group and Con group ($p<0.01$), no significant difference between the Exp group and the Con group

($p>0.05$); on the 7th day, compared with Sham group, Hes1 protein level was significantly increased in both Exp group and Con group ($p<0.01$), Hes1 protein level in the Exp group was significantly higher than the Con group ($p<0.05$); on 14th day, Hes1 protein level in the Exp group was significantly higher than the Sham group ($p<0.01$) and the Con group ($p>0.05$) (Figure 4.10B).

4.3.3.2 Hes5

Results of one-way ANOVA showed that, on day 3 and day 28, there were no differences on the relative expression of Hes5 protein between 3 groups ($p>0.05$), on day 7 and day 14 of the intervention, significant differences were observed ($p<0.01$).

Results of post-hoc analysis showed that: on day 7 and day 14, compared with the Sham group, Hes5 protein level was significantly increased in both the Exp group and the Con group ($p<0.01$), no difference was found between the Exp group and the Con group ($p>0.05$) (Figure 4.10C)

4.3.3.3 Notch1

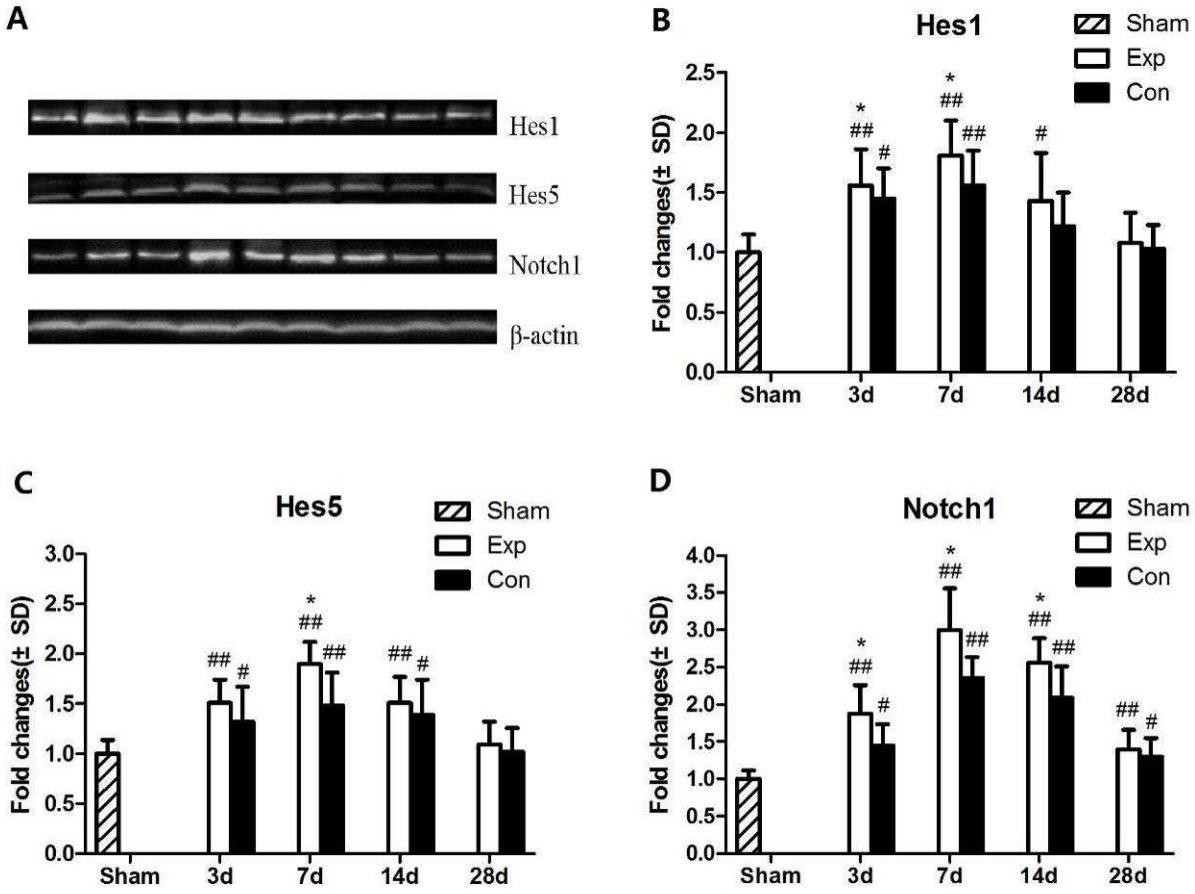
Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Notch1 protein was significantly different ($p<0.05$); on day 28, no significant difference was found between the 3 groups ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day of the intervention, compared with Sham group, Notch1 protein level was significantly increased in both the Exp group and the Con group ($p<0.01$), Notch1 protein level in the Exp group was significantly higher than the Con group ($p<0.01$); on day 7 and day 14, compared with

the Sham group, Notch1 protein level was significantly increased in both the Exp group and the Con group ($p<0.01$), Notch1 protein level in the Exp group was significantly higher than the Con group ($p<0.05$) (Figure 4.10D).

4.3.4 Western blot assay in SGZ

The results of Western blot assay of protein expression in SGZ are shown as follows (Figure 4.11):



Compared with Sham group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with Con group, $^{*}p<0.05$, $^{**}p<0.01$

Figure 4.11 The expression of protein in SGZ

4.3.4.1 Hes1

Results of one-way ANOVA showed that, on day 3, day 4 and day 14 of the intervention, the relative expression of Hes1 protein was significantly different between 3 groups ($p<0.05$); on day 28, differences between 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on the 3rd day, compared with Sham group, Hes1 protein level was significantly increased in both the Exp group and the Con group ($p<0.01$), Hes1 protein level in the Exp group was significantly higher than the Con group ($p<0.05$); on the 7th day, compared with Sham group, Hes1 protein level was significantly increased in both Exp group and Con group ($p<0.01$), Hes1 protein level in the Exp group was significantly higher than the Con group ($p<0.01$); on the 14th day, Hes1 protein level in the Exp group was significantly higher than the Sham group ($p<0.05$), however, no difference was found between the Exp group and the Con group ($p>0.05$) (Figure 4.11B).

4.3.4.2 Hes5

Results of one-way ANOVA showed that, on day 3, day 7 and day 14 of the intervention, the relative expression of Hes5 protein level was significantly different ($p<0.05$); on day 28, difference between the 3 groups was not significant ($p>0.05$).

Results of post-hoc analysis showed that: on day 3 and day 14, compared with Sham group, Hes5 protein level was significantly increased in the Exp group ($p<0.01$), no difference was found between the Exp group and the Con group ($p>0.05$); on day 7, compared with the Sham group, Hes5 protein level was significantly increased in both

the Exp group and the Con group ($p<0.01$), Hes5 protein level in the Exp group was significantly higher than the Con group ($p<0.01$) (Figure 4.11C).

4.3.4.3 Notch1

Results of one-way ANOVA showed that, at each time point (day 3, day 4, day 14 and day 28) of the intervention, the relative expression of Notch1 protein was significantly different ($p<0.05$).

Results of post-hoc analysis showed that: on the 3rd day, compared with Sham group, Notch1 protein level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), Notch1 protein level in the Exp group was significantly higher than the Con group ($p<0.05$); on the 7th day and 14th day, compared with the Sham group, Notch1 protein level was significantly increased in both the Exp group and the Con group ($p<0.01$), Notch1 protein level in the Exp group was significantly higher than the Con group ($p<0.05$); on the 28th day, compared with Sham group, Notch1 protein level was significantly increased in the Exp group ($p<0.01$) and the Con group ($p<0.05$), no difference was found between the Exp group and the Con group ($p>0.05$) (Figure 4.11D).

4.4 Discussion

4.4.1 The significance of promoting the Notch signaling pathway

Notch is an important signaling pathway in NCSs, which plays an important role in the regulation of proliferation and differentiation of NSCs (Oya et al., 2009, Aguirre et al., 2010). Whether Notch signaling pathway is activated could be detected by the expression of Hes1, Hes5 and Notch1. In NSCs, Notch signaling pathway is activated when the Notch receptor binded with ligand, and then the expression of Hes1 and Hes5 is induced. In the process of development and repairment of central nervous system, Hes1 and Hes5 are both expressed in NSCs. In the central nervous system, NSCs continuously produce new nerve cells, this process relies on the regulation of Notch signaling pathway, which makes the proliferation and differentiation of NSCs to be maintained at a relatively equilibrium. When the self-renewal of NSCs reduces or premature neuronal differentiation occurs, the NSCs pool would be depleted and ultimately reduce the differentiation (Imayoshi et al., 2010).

Nerve growth factors are very important in the proliferation of NSCs and other nerve regeneration process (Bath et al., 2012). However, when the Notch signaling pathway is activated, NSCs could self-renewal in the absence of growth factors. This indicates that the Notch pathway also has the silmilar effect of growth factors, or the Notch pathway could promote the expression and production of related growth factors (Yoon et al., 2004). Moreover, Notch pathway inhibitors could significantly reduce the number of NSCs in vitro. And promoting the expression of Notch1, Hes1 and Hes5 could promote the proliferation and self-renewal of neural precursor cells (Hu et al., 2011, Jeon et al., 2011). If Notch signal is completely lost, it would increase the number of NSCs exited the cell cycle and reduce the number of neural precursor cells, then NSCs would be exhausted and nerve regeneration would completely stop (Imayoshi et al., 2010). For the cell fate of NSCs, Notch signaling pathway is a decisive factor, it plays a key role throughout the entire diverse differentiation process of NSCs (Kageyama et al., 2005, Hansen et al., 2010, Hoeck et al., 2010, Kato et al.,

2010).

4.4.2 The effect of Notch signaling pathway in cerebral ischemia

Studies have shown that Notch signaling pathway plays an important role in the regulation of NSCs proliferation and differentiation after cerebral ischemia. Oya S et al (Oya et al., 2009) found that the NSCs proliferated significantly and the Notch signaling pathway was upgrated in the CA1 area of the rats' hippocampus on the 5th day of the cerebral ischemia. Wang X et al (Wang et al., 2009b) found that, after cerebral ischemia, mRNA such as Notch1 and Hes1 were expressed in SVZ. Increasing Notch signaling pathway activation could enhance the NSCs proliferation, and inhibition of Notch signaling pathway could lead to a reduction of the proliferation of the NSCs in SVZ. Wang L et al (Wang et al., 2009a) isolated the cells in SVZ on the 7th day of the rats with cerebral ischemia and cultured them in vitro, They found that in the process of the cells' proliferation, Notch1 and Hes1 was expressed, and the levels of expression were closely related to the extent of the cells' proliferation.

Our study shows that, in SVZ and SGZ, the expression of Hes1, Notch1 and Hes5 followed such a law: after cerebral ischemia, all the expressions were increased. The results of quantitative RT-PCR showed that, in SVZ, the expression of Hes1 increased significantly on the 4th day (the 3rd day of the ELF-EMF intervention） and the 8th day (the 7th day of the ELF-EMF intervention) of the cerebral ischemia. The expressions of Hes5 and Notch1 increased on day 4, day 8 and day 15 of the cerebral ischemia. In SGZ, the expressions of Hes1, Hes5 and Notch1 all increased on day 4, day 8 and day 15 after cerebral ischemia. The results of Western blot showed that, in SVZ, the expression of Hes1 protein increased on day 4 and day 8 of the cerebral ischemia. The expression of Hes5 protein increased on day 8 and day 15 after cerebral ischemia. The expression of Notch1 protein increased on day 4, day 8 and day 15 after cerebral ischemia. In SGZ, the expression of Hes1 increased on day 4 and day 8 after cerebral ischemia. The expression of Hes5 protein increased on day 4, day 8 and day 15 after cerebral ischemia. The expression of Notch1 protein increased on day 4,

day 8, day 15 and day 29 after cerebral ischemia.

In the third part of this project, we observed that cerebral ischemia could significantly stimulate the proliferation of endogenous NSCs cells. In SGZ and SVZ, significant proliferation of NSCs could be observed on the 4th day of the ischemia. However the most active period of proliferation was about 1w after cerebral ischemia. Two weeks after cerebral ischemia, the proliferation of NSCs was significantly decreased. With the increase of the proliferation, the endogenous NSCs began to differentiate, and the trends of the differentiation were consistent with proliferation. The results proved once again that the proliferation of endogenous NSCs cell was closely related to the Notch signaling pathway in cerebral ischemia.

4.4.3 Effects of ELF-EMF on the Notch signaling pathway

In recent years, studies about the effect of ELF-EMF on signaling pathways were increasing. Park JE et al (Park et al., 2013) found that ELF-EMF (50Hz, 1mT) could activate the EGFR pathway to promote the human marrow mesenchymal stem cells to differentiate into neurons, however, Song M et al (Song et al., 2014) found these effects could be generated by ELF-EMF (15Hz, 1mT) through MEK / ERK pathway. Soda A et al (Soda et al., 2008) reported that ELF-EMF (60Hz, 3mT) could affect the collagen synthesis of the mouse's osteoblast-like cells through p38 MAPK pathway and PI13 pathway. Vianalae G et al (Vianale et al., 2008) reported that ELF-EMF (50Hz, 1 mT) could inhibit NF-kappaB signaling pathway to regulate the production of chemokine and the growth of glial cells, and to inhibit the inflammatory process. Song HS et al (Song et al., 2010) found that ELF-EMF (60Hz, 0.1mT or 1mT) did not affect the phosphorylation signaling pathway in cultured macrophages and mast cells. Those studies showed that the effect of ELF-EMF on cellular signaling pathways may be differed, it may be promotion, inhibition or have no effect.

The results of RT-PCR in this study found that: in SVZ, on day 3 and day 7 of the ELF-EMF exposure, the expressions of Hes1, Hes5 and Notch1 were significantly increased; and on day 14 of the ELF-EMF exposure, the expressions of Hes5 and Notch1 were increased significantly compared with the cerebral ischemia group

which did not receive the exposure. In SGZ, compared with ischemic group, on day 3 of the ELF-EMF intervention, the expressions of Hes1 and Notch1 were significantly increased; on day 7 of the ELF-EMF exposure, the expression of Hes1, Hes5 and Notch1 were significantly increased; on day 14, the expression of Notch1 was significantly increased. On day 28, the expressions of Hes1, Hes5 and Notch1 were all increased in SVZ and SGZ, but there was no statistically significant difference.

Results of the expression of protein by Western blot assay showed that: in SVZ, on day 3 of the ELF-EMF exposure, the expression of Notch1 was significantly increased; on day 7 and day 14 of the ELF-EMF intervention, the expressions of Hes1 and Notch1 were increased significantly compared with the cerebral ischemia group which did not accept the exposure. In SGZ, compared with ischemic group, on day 3 of the ELF-EMF exposure, the expressions of Hes1 and Notch1 were significantly increased; on day 7 of the ELF-EMF exposure, the expressions of Hes1, Hes5 and Notch1 were significantly increased; on day 14 of the ELF-EMF exposure, the expression of Notch1 was significantly increased. On day 28, the expression of Hes1, Hes5 and Notch1 were all increased in SVZ and SGZ, but there was no statistically significant difference.

In the third part of this study, we observed that on the 3rd day, 7th day and 14th day of the ELF-EMF intervention, the number of BrdU+ cells in SVZ and SGZ were significantly increased in the ischemic rats after the exposure of ELF-EMF. This proved that the ELF-EMF intervention could significantly increase the endogenous NSCs proliferation after cerebral ischemia. On the 3rd day, 7th day and 14th day of the ELF-EMF intervention, the number of BrdU+/DCX+ cells in SVZ and SGZ were significantly increased in the ischemic rats after the exposure of ELF-EMF. This proved that the ELF-EMF intervention could significantly increase the differentiation of NSCs into neurons in cerebral ischemia. After 28 days' ELF-EMF exposure, the number of newborn neurons was significantly increased, this might be related to the continuous high expression of the Notch signal pathway. The proliferation and differentiation of endogenous NSCs was consistent to the expression of Notch signaling pathway in SVZ and SGZ, suggested that the proliferation and

differentiation of endogenous NSCs cells after cerebral ischemia was closely related to the regulation of Notch signal pathway.

4.5 Conclusion

The ELF-EMF intervention could significantly promote the expression of Hes1, Hes5 and Notch1 in the cerebral ischemic rats, and upgrade the Notch signaling pathway. Probably by regulating the Notch signaling pathway, ELF-EMF could promote the proliferation and differentiation of endogenous NSCs, and increase the number of new neurons in cerebral ischemia.

CHAPTER 5 CONCLUSIONS AND SUGGESTIONS FOR FUTURE REASERCH

5.1 Conclusions of this study

To our knowledge, we have not yet found reports on the effect of ELF-EMF on the proliferation and differentiation of endogenous NSCs in cerebral ischemia in vivo. In this study, the effect of ELF-EMF on motor and sensory deficits as well as the cognitive impairments in rats with cerebral ischemia was investigated. The effect of ELF-EMF on proliferation and differentiation of NSCs in SVZ and SGZ was observed, the expressions of the key factors of Notch signaling pathway were explored.

Our findings showed that 1) ELF-EMF has no influence on the mortality rate of cerebral ischemic rats, ELF-EMF could improve the learning and memory function but not the physical function such as motor and sensory; 2) ELF-EMF could promote the proliferation and differentiation of endogenous NSCs in the SVZ and SGZ and increase the number of newborn neurons in rats with cerebral ischemia; 3) The ELF-EMF intervention could significantly promote the expression of Hes1, Hes5 and Notch1 in the cerebral ischemic rats, and upgrade the Notch signaling pathway.

This project initially revealed the effect and the mechanism of ELF-EMF on ischemic stroke. In the condition of this experiment, the following conclusions were obtained.

1. ELF-EMF might have positive effect on cognitive and memory disorders in cerebral ischemia. Moreover, ELF-EMF might have potential therapeutic values in cognitive disorders in central nerve system.

2. ELF-EMF could promote the neural regeneration, and it might have important therapeutic value to the neurological diseases injuries in central nervous system.

3. Probably by regulating the Notch signaling pathway, ELF-EMF could promote the proliferation and differentiation of endogenous NSCs, and increase the number of new neurons in cerebral ischemia.

This project not only observes the effect of ELF-EMF on neurological function in cerebral ischemia, but also explores the fundamental mechanisms in cells level and molecules level. Our findings have primarily provided a theoretical basis for the application of ELF-EMF in rehabilitation of cerebral ischemia and has discovered an innovative way to treat cerebral ischemia. Our study also provides a vast mind of applying ELF-EMF in other diseases in central never system such as Parkinson's disease or Alzheimer’s disease. Most important, this project has approved that ELF-EMF has the potential to be a new therapeutic intervention in rehabilitation medicine.

5.2 Limitations and suggestions for future research

Although some important results have been observed in this projects, there are still some limitations need to be considered.

1. The project only observed the ELF-EMF with the frequency of 50Hz and the intensity of 1mT. The parameter setting was mainly based on the existing literature reports, it might not the best parameter for promoting the proliferation and differentiation of NSCs in cerebral ischemia.

2. Our results show that the ELF-EMF intervention had no significant improvement on mNSS score and De Ryck's score in rats with cerebral ischemia. During the experimental process, we observed that the recovery of the motor and sensory function was very quick even without any intervention in rats after cerebral ischemia. The sensitivity and specificity of mNSS and De Ryck's tests might be the reason that no obvious differences were found with or without the ELF-EMF intervention.

3. In the process of proliferation and differentiation of NSCs, the expression of Notch signaling pathway changes dynamically. While the Notch signal is activated in one part of the NSCs, the Notch signal might be inactive in another part of the NSCs. Notch1, Hes1 and Hes5 were just some key factors of Notch signal pathway, they could only initially reflex the change of Notch signaling pathway.

4. This result of study initially showed that there would be some collections of proliferation and differentiation of NSCs with the activation of Notch signaling pathway after cerebral ischemia. However, due to the restrictions of funding and time, no further experiments such as observing the proliferation and differentiation of NSCs in condition of blocking the Notch signal pathway in vivo or vitro were conducted.

Based on the shortages and uncompleted work of this project, the future work could be considered in the following aspects:

(1) Set different parameters such as frequencies, intensities and time of exposure of the ELF-EMF.

(2) Find more sensitively assessment methods to test the functional changes in rats with cerebral ischemia.

(3) Test more details and more systematic observation of the factors in Notch signaling pathway.

(4) Block the Notch signaling pathway in vivo and vitro, and then observe the proliferation and differentiation of NSCs.

In addition, in the practical application of rehabilitation treatment, the clinical practitioners seldom only provide one single treatment for the patients. Therefore, combine the ELF-EMF with drugs, exercises or other treatment methods, also could be considered as the expansion of this project.

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