INVESTIGATING THE EFFECTS OF LENTI-VIRUS MEDIATED GDNF EXPRESSION ON FUNCTIONAL RECOVERY, BRAIN PLASTICITY AND NEUROGENESIS AFTER TRANSIENT FOCAL CEREBRAL ISCHEMIA IN MICE

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ABSTRACT

INVESTIGATING THE EFFECTS OF LENTI-VIRUS MEDIATED GDNF EXPRESSION ON FUNCTIONAL RECOVERY, BRAIN PLASTICITY AND NEUROGENESIS AFTER TRANSIENT FOCAL CEREBRAL ISCHEMIA IN MICE

Stroke affects lives of many people adversely, although they survive after an attack. Administration of anticoagulants, tissue plasminogen activators (tPA) and statins are current medications for ischemic stroke, clinically. These treatments mediate to prevent further damage, but not sufficient for restoration of functional deficiencies. Numerous different therapeutic factors providing partial recovery have been identified, including neuroprotective molecules, neuromodulators, neurotrophic agents, growth factors, hormones, cell-based systems, etc. Glial cell-line derived neurotrophic factor (GDNF) is one of the powerful candidate therapeutics for stroke treatment. Direct administration of recombinant GDNF protein or upregulation of its expression via genetic manipulation was applied to animal models of brain ischemia by many researchers. In this thesis, prolonged expression of GDNF was achieved by lenti-viral vectors which was administered intracerebrally. Due to its capability to provide sustained expression by integrating host genome and transduce non-dividing cells, lenti-viral vectors were used. This was the first time usage of lenti-viral mediated GDNF gene transfer as a therapeutic purpose for stroke. Measurements of infarct size, swelling and neuronal survival showed an acute improvement triggered by means of GDNF's neuroprotective effects. Also, neuroplasticity was analysed at functional level by behavioral tests and at a molecular level by axonal projection analysis which showed an apparent recovery at post-ischemic day 55. Additionally, DNA fragmentation analysis, neurogenesis analysis, glial scar analysis and protein expression analysis of growth inhibiting factors were performed and showed improvements in neuronal recovery.

ÖZET

FAREDE GEÇİCİ FOKAL SEREBRAL İSKEMİ SONRASI LENTİ-VİRAL VEKTÖR ARACILI GDNF EKSPRESYONUNUN FONKSİYONEL İYİLEŞME, BEYİN PLASTİSİTESİ VE NÖROGENEZ ÜZERİNE ETKİLERİNİN İNCELENMESİ

Beyin felci, hayatta kalmalarına rağmen birçok insanın hayatını kötü bir şekilde etkilemektedir. Pıhtılaşmayı önleyici ajanlar, doku plazminojen aktivatörü ve statinler, klinikte kullanılmakta olan tedaviler olarak sayılabilir. Bu tedaviler, hasarın daha ileri boyutlara ulaşmasını engellerken fonksiyonel eksikliklerin geri kazanılması için yeterli olamamaktadır. Kısmi iyileşme sağladığı gösterilen, nöroprotektif moleküller, nöromodülatörler, nörotrofik ajanlar, büyüme faktörleri, hormonlar, hücre temelli sistemler, vs gibi çeşitli terapötik stratejiler keşfedilmiştir. GDNF (Glial hücre hattından türetilmiş nörotrofik faktör) beyin felci tedavisinde terapötik olarak kullanılmaya aday en güçlü moleküllerden biridir. Rekombinant GDNF molekülünün direkt infüzyonu veya genetik manipulasyonlar ile ekspresyon artışının sağlanması yöntemleri daha önce bazı araçtırmacılar tarafından beyin felci modeli oluşturulmuş hayvanlara uygulanmıştır. Bu tezde, beyin içine uygulanan lenti-viral vektörler vasıtasıyla devamlı bir GDNF ekspresyonunu sağlanmıştır. Genoma entegre olabilme ve bölünmeyen hücreleri enfekte edebilme özellikleri sayesinde lenti-viral vektörlerden yararlanılmıştır. Bu çalışma, lenti-viral aracılı GDNF gen transferinin beyin felcinde terapötik bir amaçla kullanıldığı literatürdeki ilk çalışmadır. Hasar alanı, ödem ve nöronal sağ kalım analizleri, GDNF'in nöroprotektif etkisi sayesinde tetiklenen iyileşme sürecine dair ipuçları vermektedir. Ayrıca, fonksiyonel seviyede davranış deneyleri ve moleküler seviyede aksonal projeksiyon analizleri ile nöroplastisite analiz edilmiş ve iskemi sonrası 55. günde iyileşme açıkça gözlenmiştir. Ek olarak DNA fragmantasyon analizi, nörogenez analizi, glial yara analizi ve büyümeyi inhibe edici faktörlere ait protein ekspresyon analizi gerçekleştirilmiş ve nöronal iyileşmeye dair bulguları gözler önüne sermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

C_n	Total number of cells infected
Df	Virus dilution factor
F	Frequency of GFP expressing cells
mm	Milimeter
ng	Nanogram
V	Volume of the inoculum
U	Unit
µg	Microgram
µl	Microliter
AMPA	2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate
AP	Anterior posterior
6-OHDA	6-hydroxydopamine
BBB	Blood brain barrier
Bcl-2	B cell lymphoma-2
BDA	Biotinylated dextran amine
BDNF	Brain derived neurotrophic factor
BL	Baseline
Bp	Base pair
BrdU	5'bromo-2 deoxyuridine
CBF	Cerebral blood flow
CDNF	Cerebral dopamine neurotrophic factor
^oC	Celcius degree
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
CO_2	Carbon dioxide
DA	Dopaminergic
DAPI	4'-6-diamidino-2-phenylindole
ddH_2O	Double distilled water

DMEM	Dulbecco's modified media
DNA	Deoxyribonucleic acid
DV	Dorsal ventral
ECL	Enhanced chemiluminescence
eNOS	Endothelial nitric oxide synthase
ERK	Signal-regulated kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
GABA	Gamma-Aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFLs	GDNF family ligands
GFP	Green fluorescent protein
GFRα	GDNF family receptor alpha
GPI	Glycosyl-phosphatidylinositol
HCl	Hydrochloric acid
HRP	Horse radish peroxidase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin- 10
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
Kb	Kilobase
KO	Knockout
LDF	Laser doppler flowmetry
Lv	Lenti virus
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
ML	Medial lateral
MMP	Matrix-metalloproteinases

MOI	Multiplicity of infection
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
N	Normal
NCAM	Neural adhesion molecule
NCBI	National center for biotechnology information
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
N_2O	Nitrous oxide
NOS	Nitric oxide synthase
NTFs	Neurotrophic factors
O_2	Superoxide
OD	Optical density
OH	Hydroxyl
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PVDF	Polyvinylidene fluoride
RET	Rearranged during transformation
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
Rpm	Revolution per minute
RT	Room temperature
RTK	Receptor tyrosin kinase
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TUNEL	Terminal transferase fluorescein-dUTP nick end labeling

1. INTRODUCTION

1.1. ISCHEMIC STROKE

According to the data of World Health Organization, stroke is the second leading cause of death accounting for almost 6 million of people's death in 2016 [1]. This number contributes to 11.8 per cent of total deaths. Although the incidence of stroke is higher for people above the age of 60 years, 15 million people are estimated to be suffered from stroke throughout the world each year. The incidence of stroke shows differences depending on the factors such as geography, age, sex, ethnicity, and socioeconomic status. Also, having high blood pressure, diabetes mellitus, high blood cholesterol, heart rhythm disorders, and smoking history, are the risk factors for stroke. More than 50 per cent of the people having an ischemic attack to brain die whereas others remain disabled permanently. Those disabilities may include paralysis, defects in motor coordination, loss of vision, confusion, etc. [2, 3].

Basically, stroke is the blockade of blood flow through the brain in which brain becomes unable to supply required glucose and oxygen. This pathological situation can be triggered mainly by occlusion of vessels via blood clots (embolisms) or narrowing of the vessels (thrombosis). This is named as "ischemic stroke". Also, cerebral hemorrhage or broken arteries can lead stroke, as well. In those cases of "hemorrhagic stroke", leakage of blood into the brain causes swelling and pressure [4]. The only clinical medication for stroke patients is the treatment with tPA within first 4 hours following ischemic onset [5]. Thousands of experimental and hundreds of clinical studies aiming to identify any drugs, components or devices have been completed with promising results but, they haven't been implemented yet [6].

1.1.1. Pathophysiology of Stroke

The human brain needs a non-stop supply of energy (almost as glucose) to fulfill the metabolic requirements. Energy spent by brain for the constitution of ionic fluctuations in order to maintain synaptic potentials and action potentials is approximately 20 per cent of the resting energy of the body. That is why the brain is one of the most energy consuming

organs in spite of comprising only 2 per cent of the body mass [7-9]. The brain is the most energy needing organ as it uses almost 80 per cent of the glucose incoming to the body. Energy production is provided by oxidative phosphorylation of pyruvate, completely. So, together with glucose, oxygen is another factor whose absence destroys ongoing system of the physiology of the brain. Energy failure because of deprivation of glucose and oxygen at the time of ischemia causes a major damage [4, 10, 11].

Ischemia induces a successive period of damage named as ischemic cascade which proceeds as a complicated mechanism ending up with disintegration of cell membranes and neuronal death within seconds to minutes. Ischemic cascade generally continues for hours but can also be terminated after days. Reperfusion, the restoration of blood circulation, is crucial for functional recovery and repairment. On the other hand, it induces a secondary damage called ischemic/reperfusion injury. After the injury, the damaged area is separated into two main regions pathophysiologically; core region and penumbra. The core region is the primarily suffered place where oxygen and glucose transports to the cells are completely halted. Therefore, necrotic cells are abundantly localized here. Peri-infarct region which is called as penumbra resides periphery of the core region. This part of the damaged zone is relatively more intact. By the help of collateral blood vessels that continue to support this region, cells localized in penumbra can be protected from sudden cell death. Those cells are damaged but not dead immediately after ischemia, so rather than necrotic ones apoptotic cells are observed in penumbra dominantly (Figure 1.1). Because of that, therapies aiming to rescue brain tissue mainly focus on penumbra [12-15].

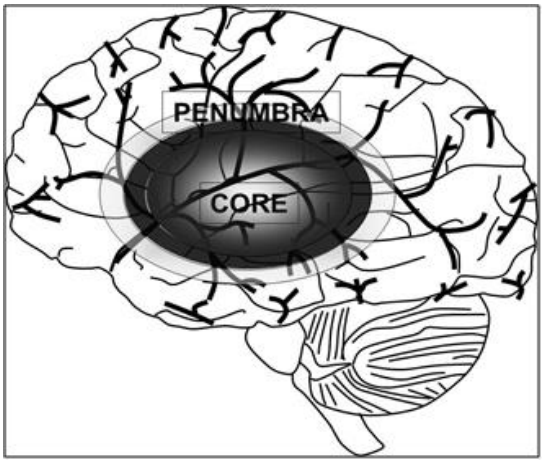

Figure 1.1. Ischemic core and penumbra regions after an ischemic stroke [11].

Ischemic stroke begins with a violent focal hypo-perfusion, which induces a process of excitatory and oxidative damage causing microvascular injury, BBB (blood brain barrier) impairment and inflammation. The severity of damage depends on a number of factors; the degree and the duration of ischemia and the ability of the brain to repair itself [15].

Permeability of BBB reduces at some extent according to the duration and severity of ischemia. Within minutes to hours after initiation of ischemia, internal width of capillaries shortens due to shrinkage. Swelling of endothelial cells together with neurons and astrocytes causes this shrinkage. Moreover, activated proteases (i.e. tPA, matrix-metalloproteinases (MMPs), cathepsins, and heparanases) distributed from necrotic cells contribute to BBB and extracellular matrix degradation. Expression of tight junction proteins has been shown to reduce under hypoxic conditions [16]. Another mechanism affecting BBB permeability is elevated amounts of cytokines secreted by neurons and astrocytes at the site of injury. It is known from previous studies that upregulation of cytokines impairs BBB permeability [17].

Energy failure because of retarded blood flow makes neurons loose their resting membrane potentials. Most of the ATP produced in neurons is spent by ion carrying membrane proteins (ion pumps) for maintenance of membrane potential. Thus, inability of providing energy, causes neuron and glia membranes to depolarize. That provides leakage of excitatory glutamate neurotransmitters to extracellular and synaptic space. Failure of glutamate retrieval by pre-synaptic neurons contributes to accumulation of excessive amount of glutamates in extracellular matrix (Figure 1.2). This process is named as glutamate neurotoxicity (excitotoxicity) [11, 18, 19].

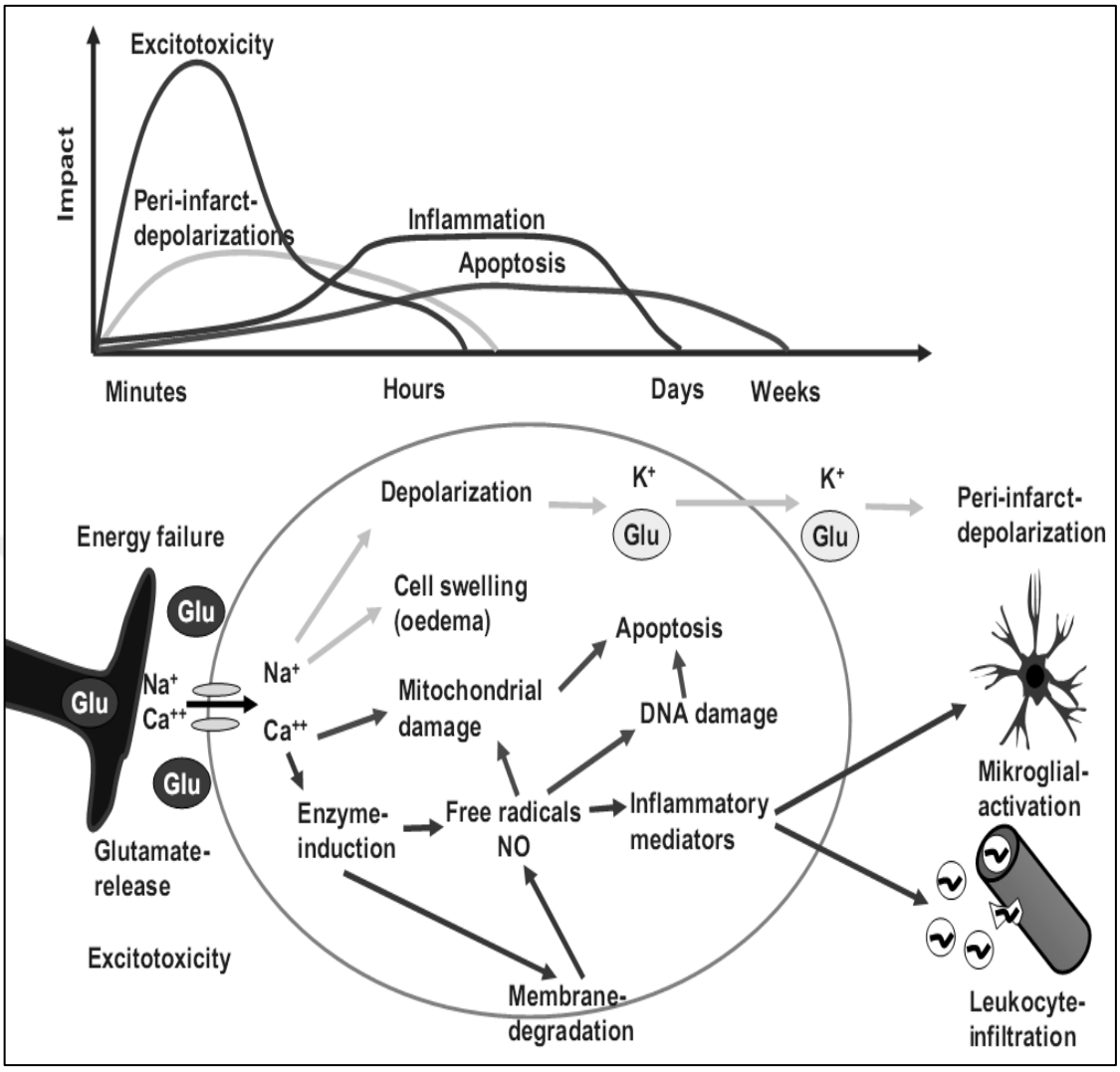

Figure 1.2. Pathophysiology of ischemic cascade [11].

Glutamate is the main excitatory neurotransmitter in the mammalian brain. It mediates intracellular signaling, synaptic plasticity, growth and differentiation. Glutamate released from pre-synaptic neurons initiates postsynaptic signaling through distinct ionotropic and metabotropic glutamate receptors. The ionotropic glutamate receptors involve N-methyl-D-aspartate (NMDA) receptor, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptor, and kainate receptor subtypes. Under normal conditions, pre-synaptic membrane become permeable against Na^+, K^+ and Ca^{2+} ions with the activation of glutamate receptors. In case of ischemia, stimulation of post-synaptic neurons by an excessive amount of glutamate in the synaptic space induces a stable depolarization with the flow of Na^+ and Ca^{2+} inward whereas glutamate and K^+ outward [20]. Repeated release of glutamate and spreading waves of depolarization expand from the core region through

penumbra. This peri-infarct depolarization provokes neuronal death at the surrounded region of injury where energy failure is partially adequate for survival. Increased numbers of depolarization cause enlargement of the infarct size [21] It is assumed that peri-infarct depolarization is responsible for approximately 20 per cent of ischemic damage [22].

The influx of Na^+ together with Cl^- after overstimulation causes water molecules to follow these ions towards inside of the cell. Water molecules are gathered and form cytosolic edema. As mentioned, the concentration of Ca^{2+} ions increase during glutamate neurotoxicity. Ca^{2+} is a significant secondary messenger molecule which coordinates activity of many proteins and enzymes. Even little oscillations in the amount of Ca^{2+} are enough for promotion of distinct Ca^{2+} dependent mechanisms. Ca^{2+} activates lipases, proteases, endonucleases which mediate membrane oxidation, breakdown of genomic DNA and proteins, production of free radicals [11].

Reactive oxygen species (ROS) are the most dangerous radicals which contribute to pathogenesis of stroke. Physiological balance between oxidants and antioxidants becomes destroyed because of ischemia and reperfusion injury. This imbalance causes oxidative stress with many harmful outcomes. Superoxide (O_2^-), hydroxyl (OH) and nitric oxide (NO) are primary species derived from oxygen. NO, a secondary messenger being used as an excitatory factor by some neurons is produced in high amounts. All three types of nitric oxide synthase (NOS) enzymes (nNOS; neuronal NOS, eNOS; endothelial NOS, iNOS; inducible NOS) are upregulated and mediate the formation of ROS. The rise in oxygen radicals promotes the expression of transcription factor Nrf-2 which primarily regulates antioxidant response element mediated transcription of antioxidant genes encoding proteins like catalase, superoxide dismutase and glutathione reductase. Since oxidative stress is one of the important factors contributing to subsequent damage, several compounds having antioxidant properties have been used and shown to alleviate stroke-related brain damage [15, 23, 24].

Oxygen radicals trigger generation of transcription factors, including Nuclear Factor Kappa B (NF-κB), hypoxia inducible factor-1, interferon regulator factor 1 and signal transducer and activator of transcription 3 which later activate various pro-inflammatory genes. As a result, cytokines are overproduced followed by expression of adhesion molecules on

endothelial cell surface in the injured cerebral tissue. In addition, intercellular adhesion molecule-1, P-selectin and E-selectin are major adhesion molecules which collect leukocytes to surface of endothelia and mediate their infiltration to the brain [15, 25, 26].

Further outcome of the pathological process is ignition of inflammation after microglial activation and leukocyte infiltration. Microglia is the firstly invited cell to injury area. Microglial proliferation peaks at 48-72 hours at the post-ischemic period and proceeds for several weeks. However, the arrival time of leukocytes to the brain tissue can reach to days [15, 27]. Astrocytes and microglia become activated after ischemia and migrate to the injury site. On the activated form, glial cells undergo some morphological and functional alterations. They extend protrusions and stimulate expression of intermediate filament, glial fibrillary acidic protein (GFAP) for a better structural support. Astrocytes and microglia can release plentiful substances such as cytotoxins, cytoprotectives and neurotrophic factors for initiating further signals. Tumor necrosis factor alpha (TNF-α) , interleukin-1β (IL-1β), interleukin-6 (IL-6), and prostanoids are pro-inflammatory whereas TGF-β and interleukin-10 (IL-10) are neuroprotective among the cytokines secreted by glia [15, 28, 29]. Several approaches aiming to inhibit cytotoxic cytokines or activating neuroprotective ones as a potential therapy for stroke are under investigation both pre-clinically and clinically. Activated astrocytes covering around the lesion site within first days after ischemia form a “glial scar”. This scar consists of extracellular matrix proteoglycans heparan sulfate, dermatan sulfate, keratan sulfate, and chondroitin sulfate together with astrocytes. Although glial scar determines the border of the lesion by inhibiting expansion, it limits post-ischemic regeneration by preventing axonal growth [30].

Cerebral ischemia activates many different intracellular signaling pathways that are critical for the response of cells to injury. Pro-inflammation, cell survival, proliferation, and apoptosis are key mechanisms determining the fate of cells during post-ischemic period. One such cascade for regulation of survival after ischemia is mitogen-activated protein kinase (MAPK) mediated signaling. Elements of the pathway; signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) activate distinct effector pathways with similar functions. Phosphorylation from threonine and tyrosine residues is necessary for activation of these proteins in order to catalyze subsequent phosphorylation of downstream proteins [20]. Furthermore, apoptosis pathway is activated depending on ischemic injury by a variety of

death signals such as increase of intracellular Ca^{2+}, generation of free radicals, insufficient production of growth factor and neurotrophic factors, damage of DNA, release of cytochrome c from mitochondria, etc. As previously mentioned, these events are expected to occur in the penumbra, where cells and tissue protect their structural integrity and energy failure in penumbra is not as critical as the one in core region. Apoptosis can be initiated via mitochondrial and death receptor (DR) mediated pathways. In each case, B cell lymphoma-2 (Bcl-2) family proteins have significant roles for decision of cell's fate. Bcl-2 family proteins are separated into two groups; first one is anti-apoptotic Bcl-2 like survival factors and second one is pro-apoptotic Bcl-2 like death factors. Mitochondria is a key component for regulation of apoptosis as it stores many kinds of apoptogenic proteins like cytochrome c, apoptosis inducing factor, pro-caspase-2, pro-caspase-3, pro-caspase-8 and pro-caspase-9. Cellular stresses including DNA damage, dissociation of mitochondrial membrane, lack of survival stimuli, increase of free radicals trigger the mitochondrial pathway by releasing apoptotic factors. In DR mediated pathway, extracellular ligands and TNF family cytokines bind to death receptors and promote homotrimerization of them. Then, an intracellular signaling pathway is started with recruitment adaptor protein and caspase cascade is activated [20, 31].

1.1.2. Recovery After Stroke

Depending upon the damaged area; type and degree of functional disabilities after stroke show differences. However, general outcomes are paralysis, inability to control movements, sensory loss, speaking problems, learning and memory problems and emotional disturbances. Functional failures like them can be replaced to some degree according to severity of damage. Underlying mechanisms of the recovery depends on neurogenesis and neuroplasticity [25].

Principally, recovery is all of integrated events that coordinate renewal and repair of damaged tissue after injury. Neural recovery consists of several mechanisms such as reversal of brain functions suffered because of ischemia, cellular renewal (neurogenesis, gliogenesis, angiogenesis) and repair (axonal sprouting, generation of neuronal connections and change of excitatory factors in peri-infarct tissue) and each of them are the result of distinct cellular responses [32, 33]. Neuronal reorganization and plasticity that begin in the very early stages

immediately after stroke, proceed for several weeks and affect regions distant to the injury site [34]. Neural stem cells and transit amplifying cells are promoted for proliferation and initiate neurogenesis. Glia cells are also provoked for dividing and differentiating adjacent to the injury zone. Newborn neurons differentiate into mature neurons during migration to lesioned area where they will reside. They constitute novel synaptic connections and long distance projections. Release of growth factors and cytokines by neurons, astrocytes, microglia and infiltrated leukocytes at the site of injury is a major event for directing immature neurons to the scene. However, most of the newborn neurons die just after their production without achieving to migrate to ischemic area despite the presence of powerful neurogenic signals [33, 35].

Neural plasticity is the capability of the central nervous system (CNS) to accommodate and respond to environmental alterations or injuries [36, 37]. This ability of CNS is considered to evolve in order to overcome encountered challenges such as modifications in cognitive strategies and establisment of novel neuronal networks [38]. In this respect, neuronal communications are regulated by a highly dynamic system which can be changed immediately upon a trauma. In human, reorganization of surviving cells mediated by molecular regenerative mechanisms are prompted by rehabilitation techniques and this leads to spontaneous recovery [39].

Animal studies show neuronal sprouting by cortical projections linked to injury zones and interhemispheric lateralizations both anatomically and physiologically, following a focal damage. Both ipsilesional and contralesional motor cortices are identified to undergo plastic reorganization. Activation of the contralesional hemisphere is seen to be greater in patients with poor motor function in order to deal with motor recovery [40, 41]. Post-ischemic neuronal sprouting is detectable within 2 weeks and can be named as the rapid phase which is followed by a slower phase proceeding for several months [36]. Specific sets of genes such as transcription factor c-fos, the growth cone phosphoprotein GAP-43 and cytoskeletal modifying protein SPRR1 are expressed during the early phase of axonal sprouting whereas cytoskeletal and cell adhesion molecules are expressed during late phases. Presence of growth inhibitory molecules and a lack of growth-promoting factors in the lesioned area restrict axonal pathfinding. The prototypical myelin inhibitors Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) expressed on the

surface of myelin, proteoglycans Versican and Brevican, transmembrane ligands Ephrin-a5, Ephrin-b1 have been extensively characterized as inhibitors of neurite outgrowth *in vitro* and *in vivo* [42, 43].

1.2. NEUROTROPHIC FACTORS

Adult brain is a dynamic factory where new neurons are generated and served for required regions for contribution of them to the existing neuronal circuits or generating new ones. Adult neurogenesis is known to occur in two brain regions; sub-ventricular zone of lateral ventricles and sub-granular zone of dentate gyrus [44, 45]. Many external and internal factors such as stress, aging, glucocorticoid hormones, drug abuse, exercise, neuronal activity, hippocampal-dependent learning, etc. affecting adult neurogenesis have been demonstrated [37]. Neurotrophic factors (NTFs) are the growth hormones that are directly included in the mechanism controlling adult neurogenesis. Many researches indicated NTFs as essential polypeptides for development and maintenance of nervous system in vertebrates. They act as stimulatory hormones for proliferation, differentiation, migration and maturation of neurons. They are important regulators for synaptic transmission [46]. NTFs also contribute to neuronal plasticity by generating and reorganizing neural circuits. In addition, several studies indicated the importance of NTFs for their role on axonal growth of both peripheral and central nervous system neurons [47-50]. Due to these significant characteristics, NTFs have been examined broadly as a potential candidate for nervous system damages and diseases [51, 52]. NTFs have been one of the most promising compounds for many years as a treatment for neurodegenerative diseases [53-57], motor neuron diseases [58-61] and neuronal traumas [62, 63].

After identification of the nerve growth factor (NGF) which is the firstly discovered growth factor and NTF, [64] it had been proposed that neurons compete for restricted amount of NTFs in order to activate survival pathways and sustain the synaptic connections. Apoptosis is the matter of course for those neurons that cannot reach sufficient NTFs [65]. Brain is the major source of NTFs but it was demonstrated that certain tissues also need neurotrophic factors for tissue regeneration [51, 66]. Each NTF shows a different temporal and spatial distribution in the brain. NTFs act on distinct neuronal subgroups which are specifically arranged by genetic competence to respond the signals of NTFs and having an appropriate

cellular organization [65].

Neurotrophic factors can be classified into 3 main groups according to their structural features; neurotrophins, TGF-β (Transforming Growth Factor β) and neuropoietic cytokines (Figure 1.3). NGF, the firstly characterized NTF belongs to neurotrophin family. BDNF (brain derived neurotrophic factor), neurotrophin-3, neurotrophin-4/5, neurotrophin-6 and neurotrophin-7 are the other members of the family [48, 67]. CDNF (cerebral dopamine neurotrophic factor) and MANF (mesencephalic astrocyte-derived neurotrophic factor) are novel proteins discovered as neurotrophic factors [65].

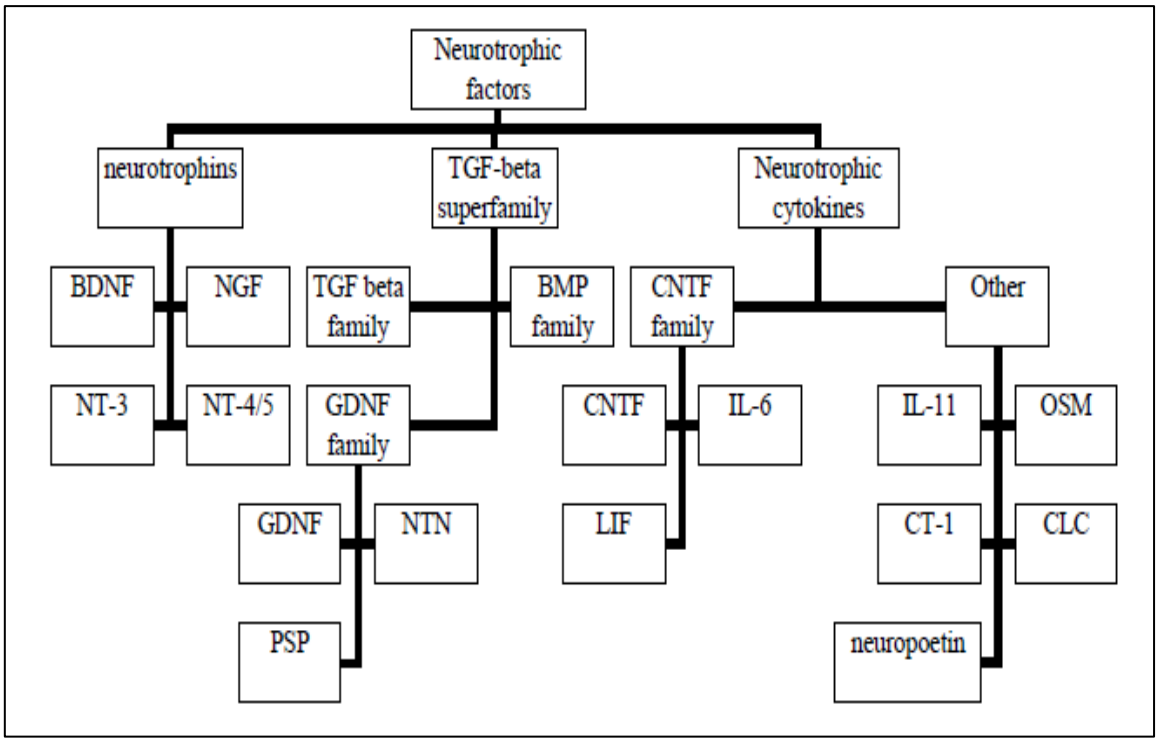

Figure 1.3. Categorization of neurotrophic factors [48].

1.2.1. Glial Cell-line Derived Neurotrophic Factor (GDNF)

GDNF family ligands (GFLs) are subfamily of TGF-β superfamily which also encompasses various cytokines families [68]. Until today, there are four proteins discovered belonging to GFLs; GDNF, artemin, neurturin and persephin. After identification of GDNF; the first member of the family, a new family of cytokines have been incorporated in TGF-β superfamily with the name of GFLs [69]. GFLs have seven conserved cysteine residues and

the capacity for formation of disulfide bonded homodimers which are the structural indicators of TGF-β supefamily [70].

All GDNF family ligands show activity in homo-dimerized forms and all of them have been shown to act as neuroprotective and neurorestorative agents [71]. All of the four GFLs signal via a two-receptor system. GFLs firstly bind to the glycosyl-phosphatidylinositol (GPI) anchored GFRα (GDNF family receptor alpha) receptors 1-4, which is the first receptor of this co-receptor system [72, 73]. GFRαs are co-receptors of GFLs and are not capable to induce a signal transduction alone. Each of GFLs bind to their specific GFRαs and form binary complexes. Then, these complexes associate with RET (Rearranged during Transformation) receptor which is a RTK (Receptor Tyrosin Kinase). Unlike most RTKs that are directly activated by binding to their ligands, the activation of RET by GFLs requires the presence of co-receptors. GFLs are also able to signal in a RET-independent way by acting on NCAM (Neural cell adhesion molecule) as an alternative receptor [71, 74-76].

GDNF was firstly isolated from the supernatant of rat glioma cell-line in 1993 and named as a “potent neurotrophic factor” which promotes survival of dopaminergic (DA) neurons in midbrain. Lin et al. demonstrated that GDNF stimulates growth and morphological differentiation of DA neurons specifically and enhances their dopamine uptake [69].

Human GDNF gene is located in chromosome 5 at p12 and p13.1 where there are two alternatively spliced isoforms encoded as 211 and 185 amino acid residue, respectively [77, 78]. The shorter form of these pre-pro-peptides is deleted and lost 26 aa residue before proteolytic cleavage and therefore, both the long and short isoforms yield a mature GDNF with the same size of 134 aa residue [69, 79]. After removal of targeting sequence and proteolytic cleavage by proteases such as furin endoproteinase, PACE4, and proprotein convertases PC5A, PC5B, and PC7, mature GDNF is N-glycosylated from two residues before secretion out of the cell [80, 81]. This biologically active form of GDNF is an N-glycosylated homodimer in the 33-45 kDa range [82].

GDNF was demonstrated to be transported, retrogradely in the central nervous system [83]. In the adult DA system; striatum, nucleus accumbens, thalamus, hippocampus and cerebellum are the primary regions where GDNF mRNA is detected. These regions are

known to be innervated by axons projecting from midbrain DA neurons [84, 85]. In addition to DA, motor neurons, noradrenergic neurons, Purkinje cells, cholinergic neurons, serotonergic neurons, sympathetic and ciliary neurons, sensory neurons, and neurons of the myenteric plexus are identified as the target cells of GDNF [86]. Also GDNF expression has been detected throughout the body in many non-neuronal tissues, including the kidney, gut, lung, bones and etc.[87].

Neuronal cells rather than glia have been demonstrated to express GDNF in the striatum region of mouse brain. In the striatum, GDNF protein has been found to be expressed largely by fast spiking, parvalbumin-positive GABAergic (Gamma-Aminobutyric acid) interneurons which represent only a small portion of all striatal neurons [88, 89]. Other types of neurons expressing GDNF are somatostatinergic and cholinergic interneurons. Medium spiny neurons are shown as lack of GDNF expression [89, 90].

1.2.1.1. Structure of GDNF

Crystal structure of rat GDNF was firstly figured out by Eigenbrot and Gerber in 1997 [91]. GDNF monomers consist of two stranded finger motifs which are composed of β-sheet loops and an α-helix structure called as heel. Fingers and helix backbones are tied together by a cysteine knot which is constituted with three disulfide bridges [92]. The two GDNF monomers associate in a configuration that the two helices from the heel region and the two cysteine knot motifs belonging to each monomer meet at the center in a “hand shake” like head-to-tail orientation (Figure 1.4) [75]. Finger 2 motif is determined to be critical during interaction with and activation of co-receptors GFRα and RET [93].

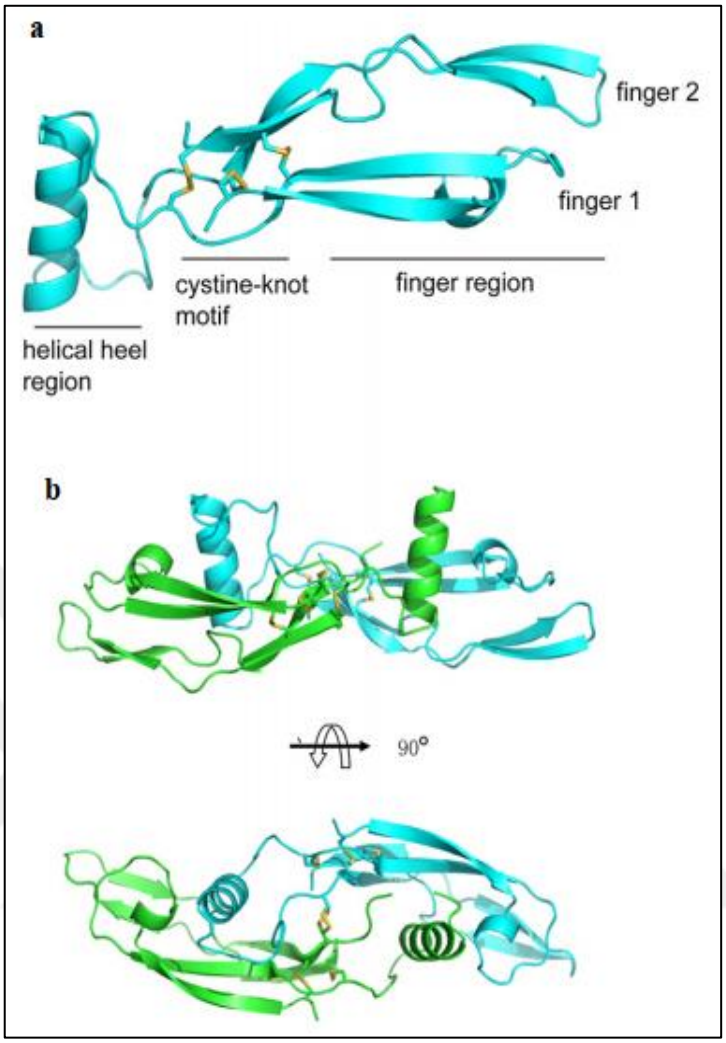

Figure 1.4. Three dimentional simulation of GDNF (a) GDNF monomer, (b) GDNF heterodimer [75].

1.2.1.2. Function of GDNF

Lin et al. demonstrated that recombinant human GDNF induces survival and differentiation of dopaminergic neurons specifically in rat embryonic midbrain cultures. Dopamine uptake by these neurons is also found to be increased. Importantly, GABAergic neurons, serotonergic neurons or astrocytes are not susceptible against GNDF [69].

GDNF knockout (KO) mice were observed to die soon after birth because of undeveloped kidneys. They had a normal body posture, healthy limbs and a regularly developed DA system, which shows GDNF is not fundamental during prenatal development of DA system.

There were no obvious difference in the number of tyrosine hydroxylase (TH) positive dopaminergic neurons in the substantia nigra and the density of dopaminergic axonal projections to the striatum between the wild type and KO mice [94]. In another work focused on the role of GDNF on postnatal DA system development, GDNF decreased the rate of programmed cell death and increased the number of DA neurons transiently which returned to normal in progressive times [95]. These data show that, GDNF doesn't have a significant role during development but have an apparent growth promoting role in adult brain on DA neurons. In addition to growth stimulating effects, GDNF also has restorative, regenerative and protective roles as it induces axonal sprouting and branching of neurons from somewhere by the effect of neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [96] and 6-hydroxydopamine (6-OHDA) [97] which specifically damages dopaminergic neurons. GDNF has been suggested as a possible therapeutic for Parkinson disease which is diagnosed with a continuous loss of midbrain dopaminergic neurons, due to these phenotypic characteristics and high specificity against dopaminergic neurons. GDNF has also been found to have trophic and protective effects on noradrenergic neurons, cholinergic neurons and motor neurons [86]. Spinal motor neurons and forebrain neurons were shown to rescued from axotomy induced cell death after GDNF treatment [98, 99]. In a research on nerve-muscle coculture system, GDNF was shown to stimulate presynaptic transmitter release [100].

1.2.1.3. GDNF Signaling

Binding of GDNF to GFRα-1 triggers dimerization of RET receptor tyrosine kinase, and stimulates activation of kinase domains that causes phosphorylation of intracellular domains. RET is a single transmembrane spanning receptor tyrosine kinase that is responsible for many critical processes during development of central and peripheral nervous system, kidney organogenesis and spermatogenesis [101]. RET is the major signaling receptor for GDNF but it works cooperatively with GFRα co-receptor [102, 103]. This activation initiates several signaling pathways in which MAPK and the phosphatidylinositol 3-kinase (PI3K)/Akt are the ones responsible for the activation of survival and neuroprotection related genes [71, 104, 105]. GDNF can also signal through c-Src kinase to promote neurite outgrowth (Figure 1.5) [106].

Paratcha et. al, discovered neuronal adhesion molecule (NCAM) as an alternative receptor for GDNF apart from RET [107]. NCAM serves as a receptor for all types of GFLs and mediates survival, growth, migration and differentiation of neurons. In the cells that RET expression is absent, GDNF-GFRα-1 complex binds to NCAM and provokes rapid activation of intracellular tyrosine kinases Fyn and focal adhesion kinase (FAK). This information clarifies why RET KO mice have a distinct phenotype from GDNF KO mice [108].

GDNF may prevent apoptosis in the DA neuron population by directly up-regulating the anti-apoptotic proteins Bcl-2 and Bcl-X via PI3K pathway and by suppressing the effector caspase 3 [109, 110].

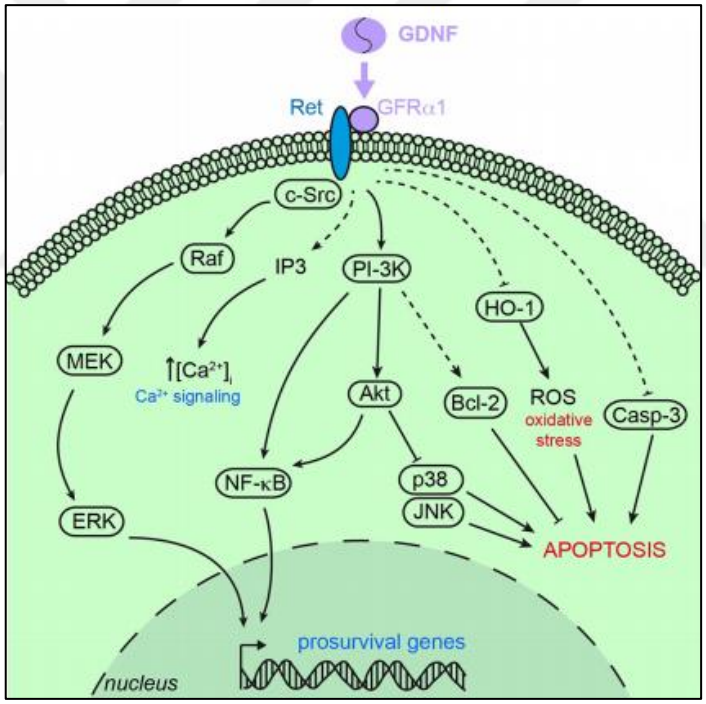

Figure 1.5. GDNF signaling pathways [90].

1.3. ROLE OF GDNF IN ISCHEMIC STROKE

Impacts of ischemia on the mRNA and protein expression of the GDNF signaling pathway have been examined using animal models of transient focal and global cerebral ischemia by many researchers. Abe and Hayashi showed neuroprotective role of GDNF during ischemia for the first time [111].

In one study, as a result of pathophysiologic process, expressions of GDNF, GFRs and RET have been altered with induction of ischemia. They were upregulated just after cerebral ischemia and their expressions were observed to be kept in high levels at the end of 8 days following permanent MCAO [112]. In another study, upregulation of RET and GFRα1 was detected as early as first hours after reperfusion in striatum [113]. In case of 30 minutes of MCAO, GDNF mRNA levels were found to increase only transiently in a few hours after perfusion in regions of striatum and cortex [111]. Similarly, a marked decline was observed in amount of RET receptor 48 hours after 45 minutes of MCAO [114]. This temporary rise of endogenous GDNF expression in acute stage of ischemia was thought to be related with post-ischemic recovery but the mechanism of action has not clearly shown yet [115]. Evenly, these fluctuations in the expression of GDNF dragged many researchers behind the question of "Can GDNF become an efficient therapeutic agent for stroke?" GDNF was initially shown to be protective in Parkinson's disease but it was revealed that GDNF also promotes neuroprotection against cerebral ischemia.

Exogenous GDNF administration mainly focuses on several methods; delivery of the recombinant GDNF protein directly, delivery of the GDNF gene via viral vectors or delivery of GDNF expressing cells. Wang et al., firstly used purified GDNF protein as a potential therapeutic agent in an animal model of stroke. Intraventricularly applied GDNF was shown to reduce infarct size and NO production [116]. Topically applied GDNF was found to reduce lesioned area and numbers of apoptotic cells in ischemic cortex 12 hours after permanent ischemia [117] and 24 hours after 90 minutes of MCAO [118]. GDNF was also administered within a gel foam [119] and fibrin glue [120] instead of single application. These studies stated that GDNF decreased expression of Hsp-70 in animals 24 hours after induction of 90 minutes of MCAO [119] and provided behavioral recovery [120]. Comparatively longer periods of GDNF treatment (14 days) with a presoaked sponge after induction of MCAO served comprehensive data about the effect of GDNF on neurogenesis, angiogenesis and synaptogenesis [121]. GDNF increased markers of cell proliferation and synaptogenesis. Although topical application is a simple and practicable technique, increasing penetration of GNDF through deep forebrain administration of GDNF via direct injections was preferred by lots of researchers. By this way, damaged area can be targeted and accessed directly. When GDNF was applied intraventricularly before MCAO in rats, the infarct size was shown to be reduced [116]. In another study, GDNF was administered via

mini osmotic pump for stable infusion before MCAO in rats. Neuronal survival and neurogenesis was found to be rised [122]. Moreover, GDNF fusion proteins were used as a potent administration way to facilitate passage through BBB for GDNF in stroke treatment, experimentally. TAT which is a cell penetrating peptide when fused with GDNF and administered to mice before ischemia, number of apoptotic cells were declined and number of viable neurons were raised [123]. Furthermore, transplantations of stem cells such as bone marrow stem cells, mesenchymal stem cells, neural stem cells which were modified for GDNF overexpression were also reported to be neuroprotective and neurorestorative after MCAO [124-126]. Lastly, viral vector mediated GDNF gene therapy is a plausible method used by many researchers. Adeno-associated virus and lenti virus are mostly utilized ones. Studies involving execution of viral GDNF vectors in both striatum and cortex after ischemia revealed beneficial roles of GDNF onto neuronal survival and neurogenesis [127, 128].

1.4. OBJECTIVE OF THE STUDY

In the present project, lenti-viral mediated GDNF gene delivery was accomplished as a purpose of therapeutic approach for cerebral ischemia. By using GDNF which is a higly known neurotrophic factor, and used for treatment of not only ischemia, but also many neurodegenerative diseases, post-ischemic regenerative mechanisms were aimed to be induced. Improvement triggered by GDNF on the molecular and functional levels were already shared by previous researches. Important points making this study different from others are usage of a lenti-viral vector system which enabled long term expression of GDNF and focusing on neuronal plasticity in addition to generalized analysis representing post-ischemic recovery such as infarct size analysis, neuronal survival analysis, cell death analysis, etc. Currently, diseases of central nervous system are known to be overwhelmed only by considering long term therapeutic strategies for modulating neuronal plasticity.

Although intracerebral administration of lenti-viral vectors is a more invasive technique than others, lenti-viral mediated gene delivery is clinically applicable. This thesis unraveled some of the absent information about GDNF's action on detailed functional improvements which are the result of a complex recovery mechanism primarily involving plasticity related molecular and structural changes.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL SETUP AND GROUPS

All experimental procedures were accomplished with the approval of the government according to local basis for care and use of laboratory animals (Bezmialem Vakif University, Local Ethical Committee of Experimental Animals, 2016/252). Experiments were performed using 88 male C57/Bl6 mice (8-10 weeks, 20-25 g). All animals were kept under regular lighting conditions as 12 hours darkness and 12 hours light.

Animal studies were designed as 3 experimental sets demonstrated in Figure 2.1, Figure 2.2 and Figure 2.3. In set 1, healthy animals were used for defining expression pattern of Lv-GDFN-GFP vector. Set 2 was planned for observing acute effects of Lv-GDNF-GFP in animals which were exposed to 90 minutes or 30 minutes focal cerebral ischemia followed by 24 hours or 72 hours reperfusion, respectively. Lastly, in set 3, a comprehensive study scheme was determined for investigating long term effects of Lv-GDNF-GFP. In this set, functional recovery, neuronal plasticity and neurogenesis were examined in animals after 30 minutes middle cerebral artery occlusion (MCAO).

2.1.1. Determination of Lv-GDNF-GFP Expression in Healthy Animals

Eight adult, male C57/Bl6 mice were anesthetized with 400 mg/kg chloral hydrate (intraperitoneally) (23100; Sigma-Aldrich, USA). Viral vectors were applied as a concentration of 10^8 particle in 2 µl PBS according to titer calculations (section 2.2.4.) [128]. Skull of the mice was drilled and intrastriatal (AP: Bregma level, ML: -2.5 mm, DV: +3 mm) Lv-GDNF-GFP or Lv-GFP injections were applied under a stereotactic frame via micro-syringe pump controller (Micro 4; World Precision Instrument).

Animals were decapitated after 10 days (n=4 for Lv-GDNF-GFP, n=4 for Lv-GFP) and their brains were frozen on dry ice following removal (Figure 2.1). Striatum regions of each brain were dissected and pooled by categorizing them. Expression of GDNF was determined on

mRNA level with quantitative real-time PCR (qPCR) and on protein level with western blot.

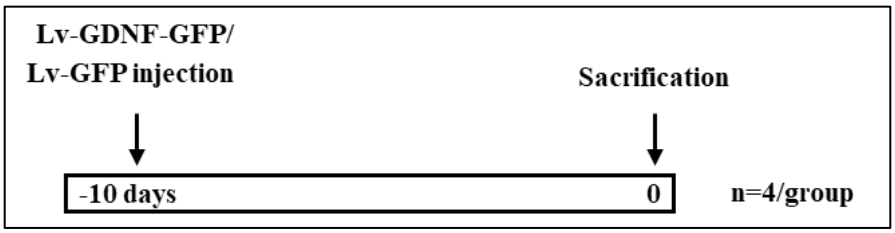

Figure 2.1. Schematic representation of experimental set for examination of exogenous GDNF expression.

2.1.2. Acute Effects of Lv-GDNF-GFP Treatment

For studying acute effects of GDNF, mice were treated with Lv-GDNF-GFP or Lv-GFP vectors before induction of MCAO for 30 or 90 minutes. Animals were treated with lentiviral GFP or GDNF 10 days before onset of ischemia. At day 0, experimental ischemia was induced. For 30-minute MCAO, reperfusion was completed after 72 hours (n=7) whereas for 90 minutes MCAO reperfusion was completed after 24 hours (n=7). Lv-GFP treatments were used as controls (n=7 for Lv-GFP) (Figure 2.2).

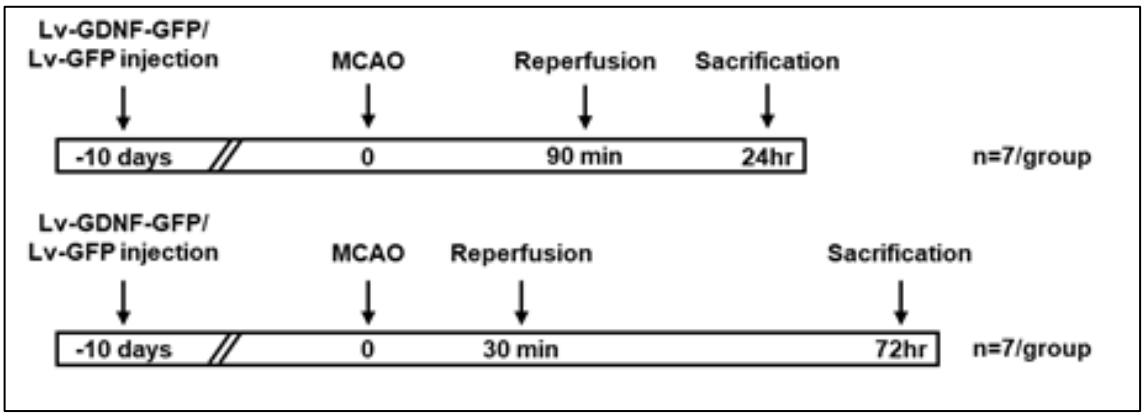

Figure 2.2. Schematic representation of experimental set for investigating acute effects of GDNF treatment.

2.1.3. Effects of Lv-GDNF-GFP on Functional Recovery, Neuroplasticity and Neurogenesis

This set of experiments was designed for detailed molecular and functional analysis. Lenti-viral vectors were administered to animals 10 days before ischemia. Three different groups were established separately for i) axonal projection and behavioral analysis (n=12), ii) protein expression analysis (n=7), iii) immunohistochemistry (IHC) analysis (n=7). Functional recovery was observed via behavioral tests which were applied at 5 different time points; before ischemia (as baseline), at day 3, day 14, day 28 and day 45 post-ischemia. Grip strength, rota-rod, open field and light/dark transition tests were applied. Same group of animals were also exposed to tracer injections at day 45 post-ischemia for analyzing axonal projections (Figure 2.3).

Biotinylated Dextran Amine (BDA; D1956; Thermo Fisher Scientific, USA) which has 10,000 dalton molecular weight is transported both anterogradely and retrogradely by neurons and accumulated in cell soma and extensions. It allows finding out how long axons protrude from the area of treatment. Thus, BDA was applied to non-ischemic hemisphere (AP: +0.5 mm, ML: +2.5 mm, DV: +1.5 mm) of animals 10 days before scarification. 2.1 µl BDA dissolved in 0.01 M PBS was applied to animals with dividing 3 equal dosages. These 3 injections were made by 45 degree, 90 degree and 135 degree angles.

For analysis of neurogenesis and gliogenesis, mice were treated with 100 µl 5’bromo-2 deoxyuridine (BrdU; B5002 Sigma Aldrich, USA) solution intraperitoneally beginning from the post-ischemic day 3 with 3 days of intervals. BrdU was dissolved in serum physiologic with a final concentration of 100 mg/kg per mice.

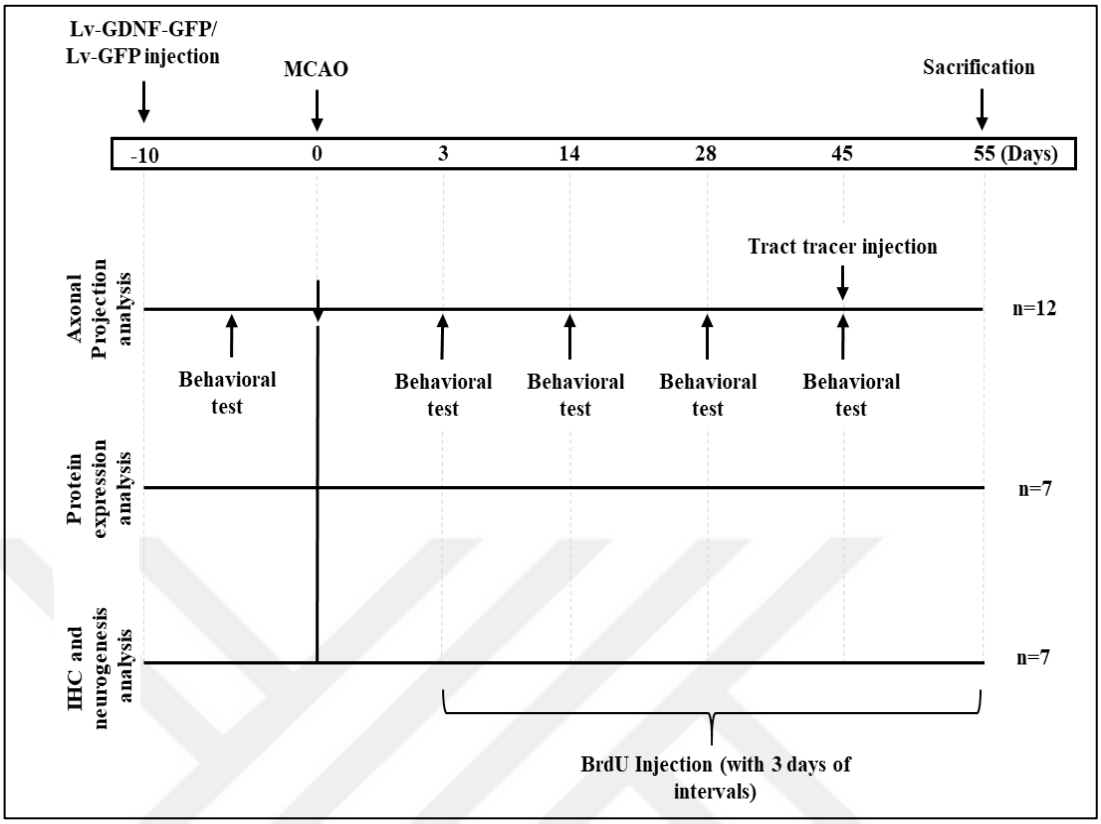

Figure 2.3. Schematic representation of experimental set for analysis of functional recovery, neuroplasticity and neurogenesis.

2.2. LENTIVIRUS PRODUCTION

2.2.1. Production of Expression Vector

2.2.1.1. Total RNA Isolation from Cell Line

SHSY-5Y human neuronal cell line was used for total RNA isolation (11828665001; High pure RNA isolation kit, Roche, Switzerland). Briefly, approximately 10^6 cells including cell suspension was mixed with 500 µl TRIzol reagent (15596026; Thermo Fisher Scientific, USA). After addition of TRIzol cells were mechanically disrupted by pipetting. Chloroform (288306; Sigma Aldrich, USA) was added onto the homogenate with an equal amount. After mixing vigorously, solution was centrifuged at 14,000 rpm for 15 minutes at 4 ºC. Soluble phase at the supernatant was separated and taken to a clean tube. Then, 100 per cent ethanol was added onto this extract with a volume of 600 µl. This mixture was loaded onto DNA

eliminating columns and centrifuged at 14,000 rpm for 3 minutes at RT. Following successive washing and centrifugation steps RNA was eluted with 100 µl nuclease free water. RNA concentration was determined with a spectrophotometer (51119200; Multiskan^{TM} go microplate reader, Thermo Fisher Scientific, USA). RNA (1 µg) was used for cDNA synthesis reaction.

2.1.1.2. cDNA Synthesis

First strand cDNA synthesis reaction was applied in a thermal cycler device (T100TM; Bio-Rad, USA) to RNA samples (04896866001; Roche, Switzerland). Reaction mixtures were prepared with amounts stated in Table 2.1 and reaction was performed according to conditions seen in Table 2.2.

Table 2.1. Volumes of ingredients for cDNA synthesis reaction.

Reagent	Volume
ddH_2O	10,5 µl
5X Reverse transcriptase buffer	4,0 µl
RNase inhibitor (40 U/µl)	0,5 µl
dNTP mix (10 Mm)	2,0 µl
Oligo dT primers (10 µM)	0,5 µl
Random primers (10 µM)	1,0 µl
Reverse transcriptase (200 U/µl)	0,5 µl
RNA (1 µg/ µl)	1,0 µl
Total	20,0 µl

Table 2.2. Reaction conditions for cDNA synthesis reaction.

Temperature (°C)	Time (minutes)
50 °C	60 minutes
85 °C	5 minutes
4 °C	∞

2.2.1.3. Primer Design

Forward and reverse primers for *Homo sapiens* GDNF, transcript variant 1, mRNA was designed with respect to NCBI Reference Sequence: NM_000514.3. Restriction enzymes were chosen according to multiple cloning site of pLenti-CMV-GFP-2A-Puro vector which is the expression vector of the lenti-virus system (pLenti-CMV-GFP-2A-Puro; Applied Biological Materials, Canada) (Figure 2.4). Restriction enzyme cutting sites were added to the primer sequences via polymerase chain reaction (PCR). Features of primers designed and restriction enzyme cutting sites added were stated in Table 2.3.

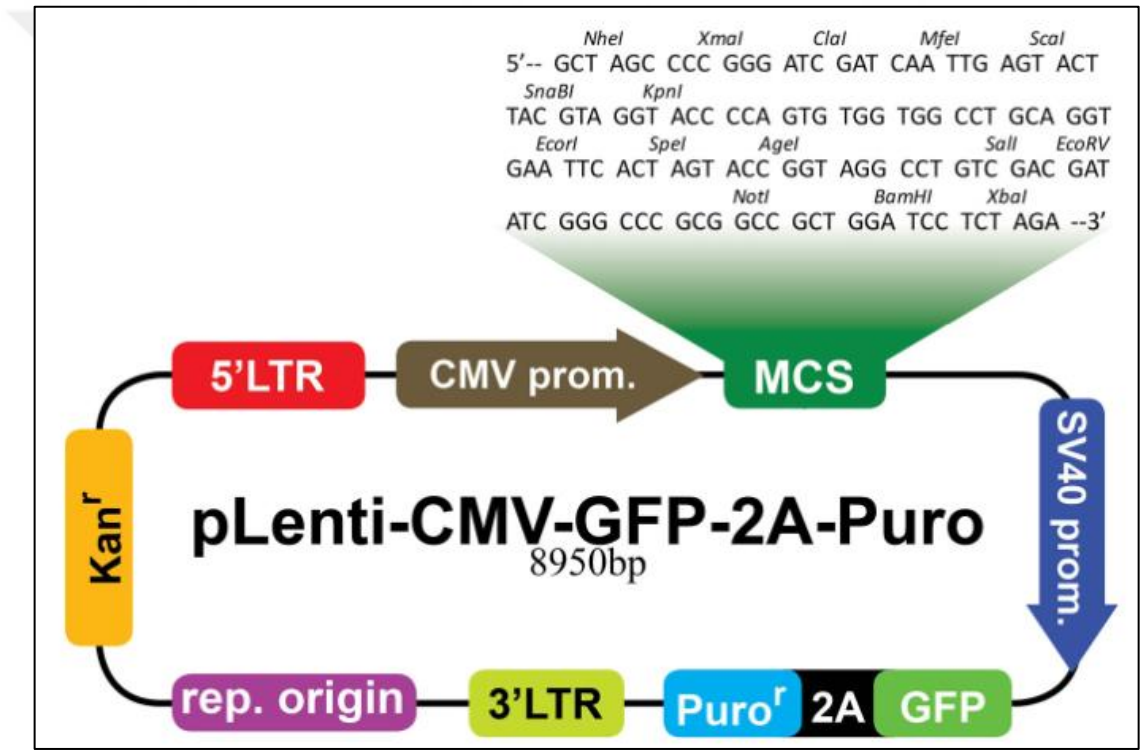

Figure 2.4. Schematic diagram of components of pLenti-CMV-GFP-2A-Puro vector [129].

Table 2.3. GDNF cloning primers.

	Forward Primer	Reverse Primer
Sequence	5' -AGT CAG GTA CCA TGA AGT TAT GGG ATG TCG TGG- 3'	5' -AG TCA GCG GCC GCG GAG TCA GAT ACA TCC ACA CC- 3'
Restriction Enzyme	*KpnI*	*NotI*
Restriction Enzyme Sequence	5'-GGT ACC- 3'	5' -GCG GCC GC - 3'
Total Length (bp)	33	34
GC Content (per cent)	45	52
T_m Value (°C)	64	64

2.2.1.4. Polymerase Chain Reaction (PCR) for GDNF

PCR mixture was prepared by iProof High-Fidelity PCR Kit (172-5330, Bio-Rad, USA). Components of the reaction mixture and conditions are demonstrated in Table 2.4 and Table 2.5. PCR reactions were executed by thermal cycler (T100^{TM}; Bio-Rad, USA).

Table 2.4. Components of PCR reaction.

Reagent	Volume (µl)
cDNA	2,0
Primer forward (10 µM)	0,4
Primer reverse (10 µM)	0,4
iProof Polymerase (2 U/µl)	0,2
5X High Fidelity Buffer	4,0
dNTP mix (10 mM)	0,4
H_2O	12,6
Total	20,0

Table 2.5. PCR reaction conditions.

Step	Time	Temperature
Initial denaturation	30 seconds	98 °C
Denaturation	15 seconds	98 °C
Annealing	30 seconds	98 °C
Extension	2 minutes	72 °C
Final Extension	5 minutes	72 °C
Final	∞	4 °C

38 cycle

After amplification, PCR products were mixed with 6X loading dye (R0631; Thermo Fisher Scientific, USA) and loaded on 1 per cent agarose gel. DNA ladder (1 kb) (SM1173; O'GeneRuller-ready to use, Thermo Fisher Scientific, USA) was used as a reference of double-stranded GDNF on agarose gel. The gel was run at 120 V until the dye line was approximately 70-75 per cent of the way down the gel. Then, the gel was visualized via Biorad Chemidoc analyzing system (1708280; Biorad Life Sciences Research, USA) and the GDNF product was confirmed with a size of 636 base pair (bp) (Figure 2.5).

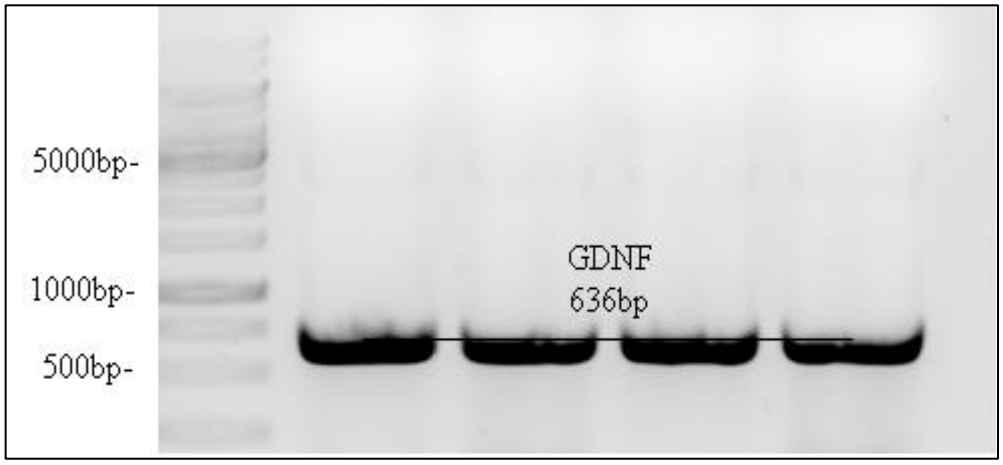

Figure 2.5. Agarose gel electrophoresis of amplified products.

2.2.1.5. Isolation of GDNF DNA from Gel

Bands corresponding to GDNF PCR products that were run under 1 per cent agarose gel electrophoresis were extracted and isolated according to following protocol. Gel pieces were weighed and gel solubilization buffer was added depending on the weight (K2100-12; Thermo Fisher, USA). After incubation at 50 ºC water bath, by flip-flopping occasionally, isopropanol was added onto the mixture with the same volume. This solution was put into columns placed in collection tubes and centrifuged at 14,000 g for 1 minute. Then, columns were cleaned with wash buffer and centrifuged at 14,000 g for 1 minute, two times. Finally, isolation was completed with eluting DNA from the column after addition of elution buffer and centrifuged at 14,000 g for 2 minutes.

2.2.1.6. Double Digestion of Lentiviral Vector and PCR Product

Not*I*-High fidelity (FD0593, ThermoFisher Scientific, USA) and Kpn*I*-High fidelity (FD0524, ThermoFisher Scientific, USA) restriction enzymes were used for digestion of both GDNF PCR product and pLenti-CMV-GFP-2A-Puro vector. Reaction components are indicated in Table 2.6. Reaction mixtures were prepared and incubated at 37°C for 1 hour. At the end of incubation, heat inactivation (5 minutes at 80°C) was performed.

Table 2.6. Components of restriction digestion reaction.

	pLenti	**GDNF**
Product (1µg/µl)	5.0 µl	12.0 µl
10X buffer	2.0 µl	2.0 µl
Not*I* (10,000 U/ml)	1.0 µl	1.0 µl
Kpn*I* (10,000 U/ml)	1.0 µl	1.0 µl
ddH_2O	11.0 µl	4.0 µl
Total	20.0 µl	20.0 µl

2.2.1.7. Purification of GDNF and Plasmid DNA

Restriction endonuclease enzymes and reaction buffers used in the previous step had to be removed (PCR purification kit, K310001; Thermo Fischer, USA). Briefly, binding buffer was added onto the PCR and vector products with an amount which was quarter of the starting volume. Then, solutions were put inside the filtered tubes and centrifuged at 14,000 g for 1 minute behind incubation at room temperature (RT) for 1 minute. Washing steps were applied by treatment of columns with wash buffer and successive centrifugation at 14,000 g was carried out for 1 minute, two times. Lastly, distilled water was used to elute the products following centrifugation at 14,000 g for 1 minute. Concentrations of purified products were measured for further procedures via NanoPhotometer (P300, Implen, Germany). Concentrations of the vector and GDNF can be seen in Table 2.7.

Table 2.7. Concentrations of the vector and GDNF.

	GDNF	**pLenti**
Concentration	150 ng/µl	91 ng/µl

2.2.1.8. Ligation Reaction

Digested GDNF and pLenti-CMV-GFP-2A-Puro were ligated by T4 DNA ligase for 1 hour at RT (M0202S; New England Biolabs, UK). Required insert DNA mass was calculated via Nebiocalculator (Version 1.9.0; New England Biolabs, UK) (Figure 2.6). According to the calculated concentrations required volume of insert DNA was designated (Formula 2.1).

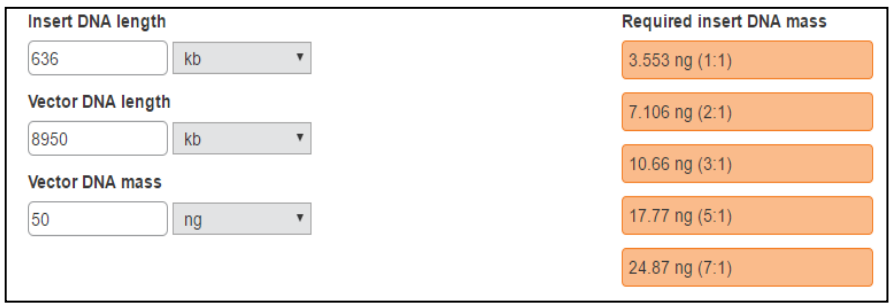

Figure 2.6. Calculations of required insert DNA mass.

$$Required\ Mass\ of\ Insert\ (g) = 3\ x\ Mass\ of\ Vector\ (g)\ x\ \frac{Insert\ length}{Vector\ length} \quad (2.1)$$

According to Nebiocalculator, reagents stated in Table 2.8 were prepared and incubated for 5 minutes at 22°C.

Table 2.8. Components of construction reaction for expression plasmid.

	1:1	**3:1**	**5:1**	**7:1**
pLenti	1,10 µl	1,10 µl	1,10 µl	1,10 µl
GNDF insert	0,47 µl	1,42 µl	2,36 µl	3,31 µl
T4 ligase (400,000 U/ml)	0,50 µl	0,50 µl	0,50 µl	0,50 µl
5X Buffer	2,00 µl	2,00 µl	2,00 µl	2,00 µl
ddH_2O	6,03 µl	4,98 µl	4,04 µl	3,09 µl
Total	10,0 µl	10,0 µl	10,0 µl	10,0 µl

2.2.1.9. Preparation of Competent Bacteria

The thawed *Escherichia coli* cells after taken from -80 ºC were spread into a solid LB agar (A8523, Biomatik, USA) plate without any antibiotics. After 5 minutes incubation at RT, the plate was placed into 37 ºC incubator for overnight incubation. Next day, one colony from the surface of the agar was picked up and put into a 5 ml LB broth (A8523, Biomatik, USA). The cells were incubated at 37 ºC for overnight on a shaker with 150 rpm. From this cell suspension, 1 ml was added into 100 ml fresh LB broth. After incubation at 37 ºC for about 2.5 hours on a shaker with 150 rpm, optical density (OD) at 600 nm was measured and found as 0.3 which is an approvable value. Bacteria suspension was divided into 50 ml falcon tubes which were cooled on ice. The cells were incubated on ice for 5 minutes. Then falcon tubes were centrifuged at 5,000 rpm for 5 minutes. After discarding supernatant, the pellet was pipetted and resuspended in ice-chilled $CaCl_2$ (100 mM) and transferred into a new falcon tube. The suspension was filled up to 20 ml with $CaCl_2$ (100 mM). This cell solution was incubated at +4 ºC for overnight. Next day, the cell solution was centrifuged at 3,000 g for 5 minutes. Supernatant was removed and cell pellet was dissolved in 2 ml of freshly prepared ice-chilled $CaCl_2$ (100 mM).

2.2.1.10. Transformation

Competent bacteria (50 µl) solution and 1 µl of GDNF ligation mixture were added into 1.5 ml eppendorf tubes and incubated on ice for 30 minutes. The cells were heat-shocked at 42 ºC water bath for 60 seconds. Then, they were immediately chilled on ice for 2 minutes. Next, 250 µl LB broth were added on suspension and shaken for 1 hour at 37 ºC in shaker incubator. Later, bacterial suspensions were added on LB agar plates which contain kanamycin (14100; Pan Biotech, Germany) and incubated overnight at 37 ºC.

Next day, 5 positive colonies were selected and added to 5 ml LB Broth which contains kanamycin. Suspension was incubated overnight to increase copy number of bacteria. Next day, plasmid isolation from bacteria was performed by plasmid isolation kit (#11754777001; High pure plasmid isolation kit, Roche, Switzerland).

2.2.1.11. Plasmid Isolation

Bacteria were centrifuged at 5,000 rpm for 5 minutes at +4 ºC. Supernatant was removed and pellet was resuspended in 250 µl suspension buffer (#11754777001; High pure plasmid isolation kit, Roche, Switzerland). Lysis buffer (250 µl) was added onto the cell solution and the solution was transferred into 1,5 ml eppendorf tubes. This solution was flip flopped a few times and incubated at RT for 5 minutes. Then, 350 µl binding buffer was added onto the cell solution, the tube was shaken softly and incubated on ice for 5 minutes. This solution was centrifuged at 14,000 g for 10 minutes at RT. The supernatant was transferred into the columns placed into collection tubes. After 1 minute incubation, it was centrifuged at 14,000 g for 1 minute. The collection tube was cleaned and 500 µl wash 1 buffer was added into the tube. After centrifuging at 14,000 g for 1 minute, the collection tube was cleaned again and washing step was repeated with 700 µl wash 2 buffer. After first centrifugation step at 14,000 rpm for 1 minute, collection tube was cleaned and centrifuged again at 14,000 rpm for 2 minutes. The column binding plasmid DNA was placed into a clean eppendorf tube and 50 µl elution buffer was put into the column. After incubation for 1 minute the column was centrifuged at 14,000 rpm for 1 minute. The solution in the collection tube was added into the column again and it was centrifuged at 14,000 rpm for 2 minutes this time. The collection tube was including plasmid DNA.

2.2.1.12. Confirmation of DNA with Restriction Enzymes

In order to validate cloning of *Homo sapiens* GDNF, transcript variant 1 into pLenti-CMV-GFP-2A-Puro, double digestion was performed. Reactions indicated in Table 2.9 were prepared for 5 samples;

Table 2.9. Components of restriction digestion reaction for confirmation.

Reagent	Volume
Plasmid	6.0 µl
Kpn*I*	0.5 µl
Not*I*	0.5 µl
10X Buffer	2.0 µl
ddH_2O	11.0 µl
Total	20.0 µl

After incubation for 1 hr, samples were mixed with 6X loading dye (R0631; Thermo Fisher Scientific) and loaded on 1 per cent agarose gel. DNA ladder (1 kb) (Thermo Scientific O'GeneRuller- ready to use) was used as a reference of double stranded GDNF, transcript variant 1and pLenti-CMV-GFP-2A-Puro on agarose gel. Gel was run at 120 V until the dye line was approximately 70-75 per cent of the way down the gel. Then, gel was visualized via Biorad Chemidoc analyzing system (1708280; Biorad Life Sciences Research, USA). Results demonstrated in Figure 2.7 confirm that all 5 plasmids had correct gene of interest into pLenti-CMV-GFP-2A-Puro.

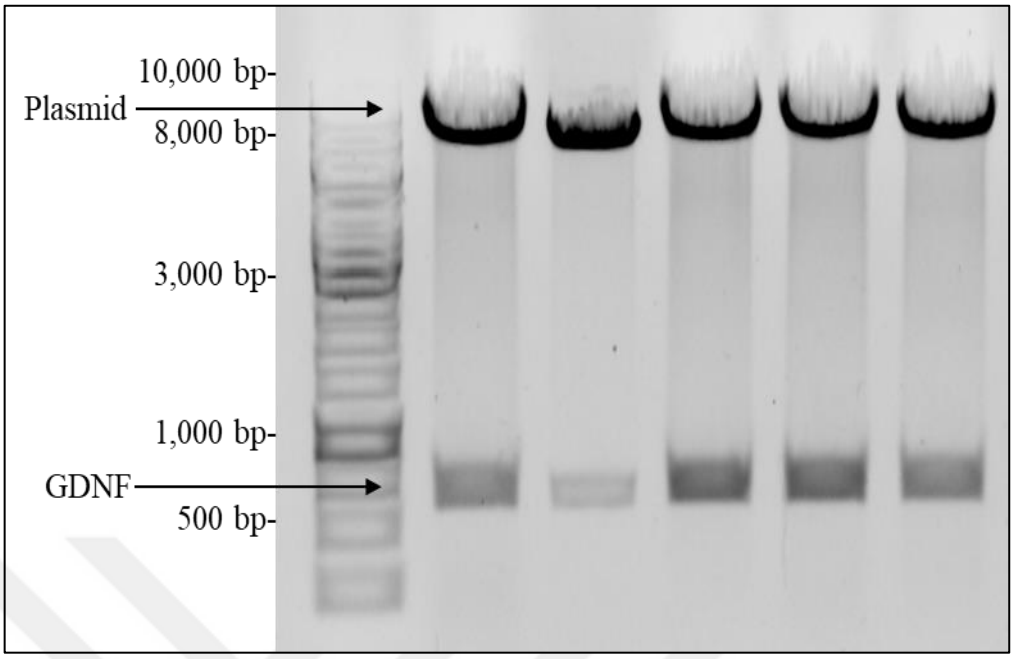

Figure 2.7. Confirmation of the accuracy of vector construction.

2.2.2. Amplification of Plasmids

Before packaging of lenti-virus, high throughput production of expression and envelope plasmids were needed. pMD2.G which encodes VSV-G envelope protein and psPAX which has only regulatory regions within an empty backbone were used as a lenti-viral packaging systems (12259; 12260, Addgene, UK). Bacteria containing p-Lenti vectors confirmed to be ligated accurately were grown and proliferated in high-volume media. Same procedure was applied for other packaging plasmids. Then, high scale plasmid isolation was performed with the same protocol indicated in section 2.2.1.11.

2.2.3. Packaging of Lenti-virus

In the first day, 3.5 x 10^6 HEK293T cells were seeded on 10 cm tissue culture in 12 ml Dulbecco's modified medium (DMEM; P04-01158, Pan Biotech, Germany). Cells were incubated overnight at 37 ºC, with 5 per cent CO_2. Next day, transfection was performed using Lipofectamine 3000 reagent (3000001; Thermo Fisher Scientific, USA). Reaction mixture prepared in Opti-MEM cell culture medium (31985062, Thermo Fisher Scientific, USA). Tube A and Tube B described in the Table 2.10 and Table 2.11 were prepared.

Table 2.10. Ingredients of Tube A for Lipofectamine 3000.

Reagent	Volume
Lipofectamin 3000 (L3000) reagent	41.0 µl
Optimem	1,459.0 µl
Total	1,500.0 µl

Table 2.11. Ingredients of Tube B for Lipofectamine 3000.

Reagent	Volume (µl)
pMD2.G (0.5 µg/ µl)	7,0 µl
psPAX (1.0 µg/ µl)	7,0 µl
pLenti (1.0 µg/ µl)	7,0 µl
P3000 reagent	35 µl
Optimem	1444 µl
Total	1500 µl

In order to prepare lipid-DNA complexes, Tube A was transferred into Tube B and mixed well, then incubated for 20 minutes at RT. Six ml medium from 10 cm tissue culture plate was removed prior to addition of lipid-DNA complex. Later complex was added drop by drop by gently agitating. Plate was incubated for 6 hours at 37 ºC, with 5 per cent CO_2. Six hours after transfection, medium was changed with 12 ml fresh DMEM medium. Plate was returned to incubator and incubated overnight at 37 ºC, with 5 per cent CO_2. After 24 hours, transfection medium was collected and stored at 4 ºC. Medium was changed with fresh 12 ml DMEM medium. Approximately 52 hours after post-transfection medium was collected and combined with the first collection. All suspension centrifuged at 2,000 rpm for 10 minutes than filtered with 0.45 µm low binding filter to discard cellular debris. Medium was added to ultracentrifuge tubes and centrifuged at 100,000 g for 2 hours. Pellet which contains the virus particles were dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS; P04-3650, Pan Biotech, Germany) without calcium and magnesium.

2.2.4. Calculation of Virus Titer

293T cells (4 x 10^5) were inoculated per well in a 12-well plate, incubated at 37°C with 5 per cent CO_2 for 18-20 hours. Next day, 10-fold serial dilutions of virus particles (different dilutions for each Lv-GFP and Lv-GDNF-GFP) ranging from 10^{-1} to 10^{-4} with 2 ml total volume were prepared in conical vials. They were mixed gently by inverting the tubes several times. One ml of each of diluted virus particles were added to wells and incubated at 37°C for 18-20 hours. Medium was replaced in every 2-3 days by the time GFP expression was observed. After having sufficient GFP signaling, cells were trypsinized, inactivated with culture media, spun and resuspended in cold phosphate buffer saline (PBS), respectively, for fluorescence activated cell sorting (FACS) analysis (Becton Dickson Influx cell sorter, USA).

Ice cold 2 per cent formaldehyde solution (500 µl) was added onto cells and mixed properly. Cells were incubated at 4 ºC for 10-60 minutes and cells were centrifuged at 1,000 rpm at 4 ºC for 5 minutes. Supernatant was separated, and cells were washed with PBS. Ethanol (1 ml, 70 per cent) was added onto cell suspension and incubated at 4 ºC for overnight. Next day, cells were analyzed for GFP expression via flow cytometry and the percentage of cells that were GFP positive was recorded.

In order to determine the titer of the virus particles, wells having cells expressing GFP between 1 and 20 per cent were used. According to the formula (2.2), multiplicity of infection (MOI) was calculated as 10^8. In this formula F is the frequency of GFP expressing cells, C_n is the total number of cells infected ($4x10^5$ for this study), V is the volume of the inoculum (1 ml for this study), and Df is the virus dilution factor. The 293T cells expressing GFP reporter protein which was coded in pLenti-GDNF vectors can be seen in Figure 2.8.

$$Virus\ titer\ (TU/ml) = \frac{FxCn}{V(ml)}\ x\ Df \qquad (2.2)$$

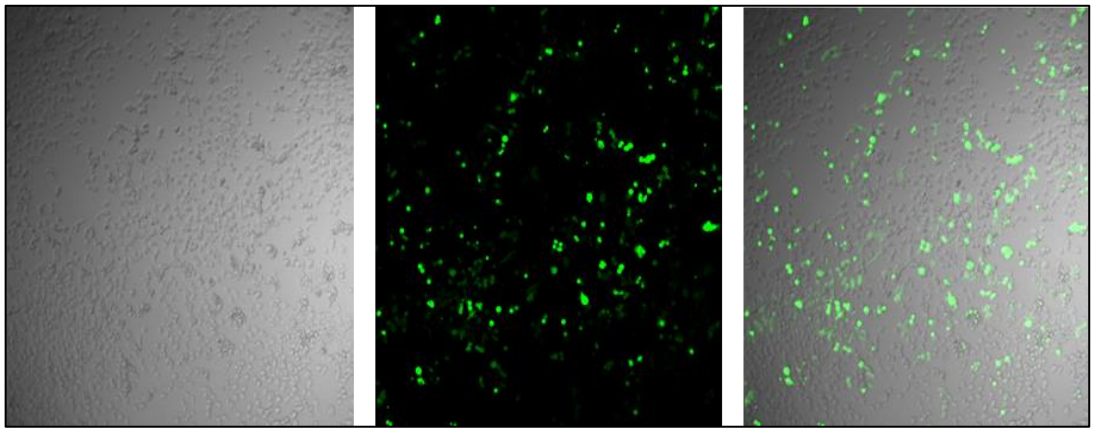

Figure 2.8. pLenti-GDNF vector expression in 293T cells.

2.3. INDUCTION OF CEREBRAL ISCHEMIA AND REPERFUSION

Adult, 88 male C57Bl6 mice (8-10 weeks, 20-25g) were anesthetized with 1 per cent isoflurane (30 per cent O_2, 69 per cent N_2O). Rectal temperature which had to be approximately 36.5-37.0^oC was controlled using a feedback-controlled heating system (MAY instruments, Ankara, Turkey). During MCAO and reperfusion, cerebral blood flow (CBF) was monitored via Laser Doppler Flowmetry (LDF). For this, a flexible 0.5 mm fiber optic probe (Perimed, Sweden) was attached to the intact skull above the MCA territory (AP: +2 mm and ML: +6 mm from the bregma) with tissue adhesive. Focal cerebral ischemia was carried out by using an intraluminal filament technique [130-132]. Firstly, a small midline neck incision was made and then, left common and external carotid arteries were detached and ligated. Internal carotid artery was temporally ligated using microvascular clips (FE691; Aesculap, Germany). A 180-190 µm silicon coated (Xantropen; Bayer Dental, Japan) 8.0 nylon monofilament (Ethilon; Ethicon, Germany) was inserted through a narrow incision into the common carotid artery and forwarded 9 mm distal to the carotid bifurcation for MCAO (Figure 2.9).

Reperfusion was initiated by with-drawl of the thread throughout 72 hours for 30 minutes MCAO and 24 hours for 90 minutes MCAO in order to study acute effects. After that, LDF recordings continued for 30 minutes for control of the reperfusion. Then, anesthesia was ceased, and mice were placed to their cages. After 24 hours (90 minutes MCAO) or 72 hours (30 minutes MCAO) of reperfusion, mice were re-anesthetized and decapitated. Reperfusion

was completed at post-ischemic day 55 after decapitation of animals for experimental set established to study long term effects of Lv-GDNF-GFP treatment. Brains were removed quickly and frozen with dry ice. Thereafter, brains were sectioned with a cryostat (CM1850-UV; Leica, Germany) into 18 µm coronal sections for conventional histologic and immune stainings and 40 µm coronal sections for axonal projection analysis.

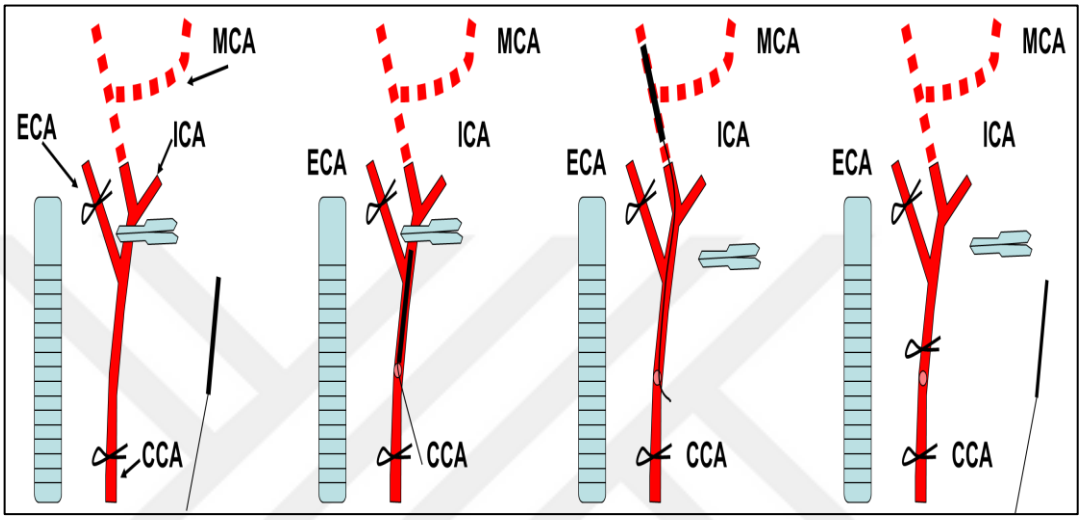

Figure 2.9. Representation of the MCAO.

2.4. NEUROLOGICAL SCORE

In mice submitted to 90 minutes of focal cerebral ischemia, neurological deficits were evaluated 24 hours after MCAO using following scores; 1= flexion of torso and contralateral forelimbs after lifting animals by the tail, 2= rotating to the contralateral side but having normal posture at rest, 3= reclination to the contralateral side at rest, and 4= absence of voluntary motor activity [132].

2.5. FUNCTIONAL NEUROLOGICAL TESTS

2.5.1. Grip Strength Test

Grip strength test consists of a spring balance interconnected with a Newton meter. A triangular steel wire, which is hold by animals instinctively, is attached to the Newton meter. When pulled by tail, animals exert force on the steel wire [130]. Grip strength test was

evaluated at the right forepaws of the animals which were paralyzed because of left-sided ischemia. The left non-paretic forepaws were wrapped with adhesive tape during the test. The test was repeated five times successively and mean values for each measurement were calculated.

2.5.2. Rota Rod Test

Rota Rod is a rotating platform with an accelerating speed from 6 to 40 rpm (model 47600; UgoBasile, Italy) which allows evaluating motor coordination skills [130]. At the end of 245 seconds, maximum speed is reached and time at which animals drop off the drum is evaluated (maximum testing time: 300 seconds) [130]. Measurements were performed five times successively, and mean values for all five measurements were calculated.

2.5.3. Open Field Test

Open field is a round arena with a 150 cm diameter. It is covered with a white plastic floor. A side wall made of white polypropylene at the height of 35 cm high surrounds the platform. It allows to measure spontaneous locomotor activity and exploration behavior. The arena is partitioned into three sections, an outer wall zone, an intermediate transition zone and an inner zone [130]. Each mouse was released near the wall of outer zone and observed for 10 minutes. Paths traveled by animals were tracked and recorded with an electronic imaging system (Anymaze Version 4.99, Stoelting, USA). In order to determine exploratory behavior and anxiety, total resting and progressing times of the animals and the time spent in each of the zones were analyzed.

2.5.4. Light/Dark Transition Test

Light/dark transition test involves a closed box with sizes of 40 cm x 20 cm x 20 cm equally divided into two light and dark chambers by a 4 cm x 4 cm door opening at the floor level. Light/dark transition test serves information about the anxiety like behavior of animals depending on the instinct of abstaining from the illuminated environments [130]. Each mouse was placed in the distant corner of the light chamber and monitored for 10 minutes. Paths traveled by animals were tracked and recorded with an electronic imaging system

(Anymaze Version 4.99, Stoelting, USA). The time spent in the light or dark chamber was assessed.

2.6. ANALYSIS OF SURVIVING NEURONS

For calculating neuronal survival, coronal sections from mice which were exposed to 30 minutes MCAO and followed by 72 hours reperfusion were used. Sections cut in 18 μm width from the level of striatum were immune-stained with anti-NeuN (neuron specific nuclear antigen) antibody in order to mark surviving neurons. Firstly sections were fixed with 4 per cent paraformaldehyde (PFA) for 10 minutes at RT. Next, slides were washed three times with PBS for 5 minutes and immersed in PBS composed of 0.3 per cent Triton X-100 (T8787; Sigma Aldrich, USA) (PBS-T) and 10 per cent normal goat serum (NGS; G9023, Sigma Aldrich, USA) for 1 hour. Sections were incubated at 4°C for overnight with monoclonal antibodies against NeuN (MAB377, Millipore, USA). Next day, sections were incubated with conjugated secondary antibodies at RT for 1 hour which was followed by an incubation with 4'-6-diamidino-2-phenylindole for 10 minutes (DAPI; D9542, Sigma Aldrich, USA). Sections were mounted with fluoromount (F4680, Thermo Scientific) and analyzed with confocal microscopy (LSM 780, Carl Zeiss, Germany). Twelve region of interest, each measuring 62,500 μm^2, were evaluated and mean values were calculated both ischemic (ipsilateral) and non-ischemic (contralateral) areas. Therefore, percentage of surviving neurons was designated.

2.7. CRESYL VIOLET STAINING

18 μm width 4 coronal brain sections, 2 mm apart from each other were selected from mice which were exposed to 90 minutes MCAO following 24 hours of reperfusion and stained with cresyl violet dye (C5042, Sigma Aldrich, USA) according to standard histological staining procedures [131, 132]. Briefly, following fixation with 4 per cent PFA (P6149, Sigma Aldrich, USA), sections were washed with PBS and incubated in cresyl violet dye solution. After alcohol series, sections were mounted with entellan (1079610500, Merck, USA) and the sections were analyzed to outline infarct zones using the ImageJ software (NIH, USA). The area of infarction was assessed by subtracting the non-lesioned area of the ipsilateral hemisphere from that of the contralateral side. The volume of infarction was

calculated by integration of these lesion areas. Edema was calculated as the volume difference between the ischemic and the non-ischemic hemisphere and expressed as percentage of the volume of the non-ischemic hemisphere [132].

2.8. ANALYSIS OF DNA FRAGMENTATION

From animals subjected to 30 minutes MCAO, brain sections from mid-striatum level were stained by terminal transferase fluorescein-dUTP nick end labeling (TUNEL) using a *in situ* cell death detection kit (11684795910; Roche, Switzerland). Firstly, sections were fixed with 4 per cent PFA and washed with PBS. Secondly, antigen retrieval was performed by incubating the sections in citrate buffer. Then brain sections were blocked with a mixture of NGS, bovine serum albumin (BSA; A2058, Sigma Aldrich, USA) and gelatin (48723, Sigma Aldrich, USA). Lastly, they were reacted with TUNEL reaction mixture and counterstained with DAPI. In stained sections, TUNEL positive DNA-fragmented cells were counted from 12 region of interest (ROI) in the striatum via confocal microscopy.

2.9. IMMUNOFLUORESCENCE STAINING

Animals having 30 minutes MCAO and 55 days reperfusion were used for immunofluorescence studies. For conventional immunofluorescence analysis, 18 µm coronal sections from bregma level were used. Immunofluorescence analysis was applied to brain sections of mice treated with BrdU to identify cells in S phase only. Briefly after immersion fixation in 4 per cent PFA, sections were pretreated with citrate buffer for antigen retrieval, rinsed and immersed in PBS-T containing 10 per cent NGS for 1 hour. Sections were then incubated with the monoclonal antibody against BrdU at 4°C for overnight (Ab6326, Abcam, UK). Next day, sections were incubated with Alexa Fluor 488 conjugated goat anti rat secondary antibody (ab150157, Abcam, UK) at RT. Finally, DAPI staining was used for counterstaining of nucleus. Positively stained cells were analyzed using confocal microscopy.

For analysis of neurogenesis and gliogenesis, sections were double stained for BrdU/NeuN, BrdU/GFAP and BrdU/Iba-1 antibodies together. Briefly after immersion fixation in 4 per cent PFA, sections were pretreated with citrate buffer for antigen retrieval, rinsed and

immersed in PBS-T containing 10 per cent NGS for 1 hour. Sections were incubated with monoclonal antibodies against Cy3 conjugated NeuN (MAB377c3, Millipore, USA), Alexa Fluor 555 conjugated GFAP (3656, Cell Signaling, USA) and Iba-1 (019-1974, Wako, Japon) which was detected with Alexa Fluor 555 conjugated goat-anti rabit secondary antibody (A27039; Thermo Fisher Scientific). Next day, sections were treated with 2N HCl at 37°C for 2 hours in order to break strands of DNA and double stain BrdU. Next, sections were incubated with anti-BrdU antibody for 2 hours and then Alexa Fluor 488 conjugated goat anti rat secondary antibody for 1 hour at RT. Finally, sections were incubated with DAPI for 10 minutes.

In the event of anti-GFAP staining for glial scar analysis (3656, Cell Signaling, USA) Alexa Fluor 555 fluorescently conjugated primary antibody was used without need of a secondary antibody incubation step and all the procedure defined above was performed. Positively stained cells from 12 different region of interest (ROI) in the ischemic striatum, each measuring 62,500 μm^2, were evaluated by confocal microscopy.

2.10. ANALYSIS OF AXONAL PROJECTIONS

Brain sections of tracer injected animals that had been perfused transcardiacly with 4 per cent PFA followed by 0.9 per cent NaCl at day 55, were stained for axonal projection analysis. For immunohistochemistry of BDA, fixation was performed using 4 per cent PFA in PBS and then sections were washed three times with PBS. After blocking with 10 per cent NGS for 1 hour at RT sections were washed with PBS and incubated with Alexa Fluor 555 conjugated streptavidin (S21381, Invitrogen) for 2 hours at RT. Sections were mounted with fluoromount (F4680, Thermo Fisher Scientific, USA) and analyzed with confocal microscopy.

Axonal projections were evaluated at the level of the parvocellular red nucleus (bregma -3.0 to -3.5 mm) for corticorubral and facial nucleus (bregma -5,8 to -6,3 mm) for corticobulbar tracts. For analysis, a 1,000 μm long straight line was drawn on section images at the midline to separate the hemispheres. Along that line, fibers crossing into the contralateral hemisphere in direction of the red nucleus and facial nucleus were quantified. In order to eliminate the possibility of variation among animals during tracer injections total numbers of BDA

positive fibers in the pyramidal tract were also counted. Therefore, fibers crossing the section throughout the corticospinal tract were counted in four regions of interest each of which were 2,865 μm^2. For each animal, total number of counted fibers were normalized with the total number of labeled fibers in the pyramidal tract.

2.11. ANALYSIS OF PROTEIN EXPRESSION

Brain tissue samples harvested from the animals subjected to 30 minutes MCAO followed by 55 days reperfusion were separated as ischemic and non-ischemic hemispheres. Tissue samples of the same groups were pooled and homogenized with radioimmunoprecipitaion assay lysis buffer (RIPA; 89900, Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitor cocktail (Bullet Blender, Next Advance). Then, proteins on the supernatant phase were extracted after 15 minutes of centrifugation at 14,000 rpm. Protein concentrations were measured by using BCA protein assay kit (23227; Thermo Fisher Scientific, USA). Briefly, working solution was prepared with mixing copper sulfate reagent with buffer solution including bicinchoninic acid. Protein samples were mixed with working solution inside a 96 well-plate and incubated at 37 ºC for 30 minutes. Then, colorimetric measurement was made at 562 nm wavelengths by a spectrophotometer (51119200; MultiskanTM go microplate reader, Thermo Fischer, USA). After determining concentrations, protein samples were prepared for western blotting by mixing with loading buffer (1610737, Laemli buffer, Biorad Life Sciences Research, USA).

Equal amounts of protein samples (20 μg) was loaded into 4-20 per cent bis-tris gels, run for 1 hour at 150 V and then transferred to polyvinylidene fluoride (PVDF) membranes (162-0174, Biorad Life Sciences Research, USA) via semi-dry blotting system (1704155, Biorad Life Sciences Research, USA). After transfer, membranes were blocked with 5 per cent non-fat milk powder solved in Tris-buffered saline containing Tween-20 (TBS-T), for 1 hour at RT. Membranes were washed with TBS-T and incubated overnight with primary antibodies GDNF (sc-13147, Santa Cruz), Brevican (610895, BD Biosciences, USA), Versican (ab19345, Abcam, UK), Ephrin-b1 (sc-515264, Santa Cruz, USA) and Ephrin-b2 (sc-398735, Santa Cruz, USA). Next day, membranes were washed with TBS-T and incubated with goat anti rabbit-HRP (horse radish peroxidase) secondary antibody (31460, Thermo Fisher Scientific, USA) or goat anti mouse-HRP secondary antibody (31430, Thermo Fisher

Scientific, USA) for 1 hour at RT. For normalization of protein loading membranes were stripped and reprobed with an anti-β-actin (4970, Cell Signaling) antibody. All of the proteins to be examined were studied as triplicate. Blots were developed via enhanced chemiluminescent substrate (ECL) (K-12043-D10; Western Bright Sirius, Advansta, USA) and visualized by the CCD camera integrated visualization cabinet (Fusion FX7, Vilber, Germany). Protein levels were analyzed densitometrically using the software (Image J; NIH, USA) and corrected with values determined on β-actin blots.

2.12. QUANTITATIVE REAL-TIME PCR (qPCR)

Striatal areas of brains were dissected and each of the samples studied separately; without pooling. Firstly, total RNA was isolated by the same protocol stated in section 2.2.1.1. Approximately 100 mg of tissue was homogenized with 500 µl TRIzol reagent. Chloroform (200 µl) was added onto the homogenate and incubated at RT for 1-2 minutes. After centrifugation at 14,000 rpm for 15 minutes soluble phase at the supernatant was separated and taken to a clean tube. After addition of absolute ethanol, the mixture was loaded onto DNA elimination columns and centrifuged at 14,000 rpm for 3 minutes. After washing steps RNA was eluted with RNase free water. After determining RNA concentration first strand cDNA synthesis reaction was applied with the protocol indicated in section 2.2.1.2. Reaction mixtures were prepared and performed under defined conditions. Forward and reverse primers against GDNF and control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were selected from Harvard primer bank database. Information about primers was given in Table 2.12.

Table 2.12. qPCR primers for GDNF and GAPDH.

	Forward Primer	Reverse Primer
GDNF	5' -TCT TTC GAT ATT GCA GCG GTT- 3'	5' -GTC ACT TGT TAG CCT TCT ACT CC- 3'
GAPDH	5'-AGG TCG GTG TGA ACG GAT TTG- 3'	5' -TGT AGA CCA TGT AGT TGA GGT CA- 3'

qPCR reaction mixture and conditions are demonstrated in Table 2.13 and Table 2.14. For PCR reaction SensiFAST^{TM} SYBR No-ROX kit which includes Sybr green was used as nonspecific interchelating agent for staining DNA (BIO-980005, Bioline, USA) was used. PCR reactions were executed by CFX96 Touch Real-Time PCR (Bio-Rad, USA).

Table 2.13. Components of qPCR reaction.

Reagent	Volume (µl)
cDNA	1.0 µl
Primer forward	0.5 µl
Primer reverse	0.5 µl
Reaction mixture (SYBR green, buffer, dNTPs, DNA polymerase)	15.0 µl
Total	17.0 µl

Table 2.14. qPCR reaction conditions.

Step	Time	Temperature	
Initial denaturation	2 minutes	95 °C	
Denaturation	5 second	95 °C	38 cycle
Annealing	10 seconds	60-65 °C	
Extension	15 seconds	72 °C	
Final	∞	4 °C	

Gene expressions were analyzed by relatively quantifying the threshold cycle (C_T). C_T values were normalized according to endogenous reference which was GAPDH and compared with control group which was Lv-GFP using the C_T formula. Data were analyzed using logarithmic transformation of fold induction ratios according to relative quantification (R_F) formula ($2^{-\Delta\Delta Ct}$) (Formula 2.3).

$$\Delta C_T = C_T\,[\text{GAPDH}] - C_T[\text{GDNF}]$$
$$\Delta\Delta C_T = \Delta C_T\,[\text{Lv-GDNF-GFP}] - \Delta C_T\,[\text{Lv-GFP}] \qquad (2.3)$$

2.13. STATISTICAL ANALYSIS

Statistical data analyses were done with a standard software package (SPSS for Windows; SPSS Inc., USA). Differences between the groups were calculated by independent sample t test. All values were given as mean ± SD and “n” values indicate the number of different animals analyzed p values ≤ 0.05 were considered significant.

3. RESULTS

3.1. CORRELATION OF LENTI-VIRAL GDNF EXPRESSION

Initially, lenti-viral vectors were aimed to be tested for accurate delivery of the viral vector and expression of the desired gene in healthy animals. For that pupose exogenous GDNF production was analysed by western blot analysis on protein level and by real time PCR on RNA level. GDNF protein level was detected to be almost three times higher in the Lv-GDNF-GFP treated animals compared to the control animals which received only GFP carrying vector (p= 0.01) (Figure 3.1 a). In gene expression analysis, GDNF mRNA was found to be expressed significantly higher after Lv-GDNF-GFP treatment (Figure 3.1 b) (p= 0.01). On the basis of these data, lenti-virus mediated delivery of GDNF gene was accepted as successful.

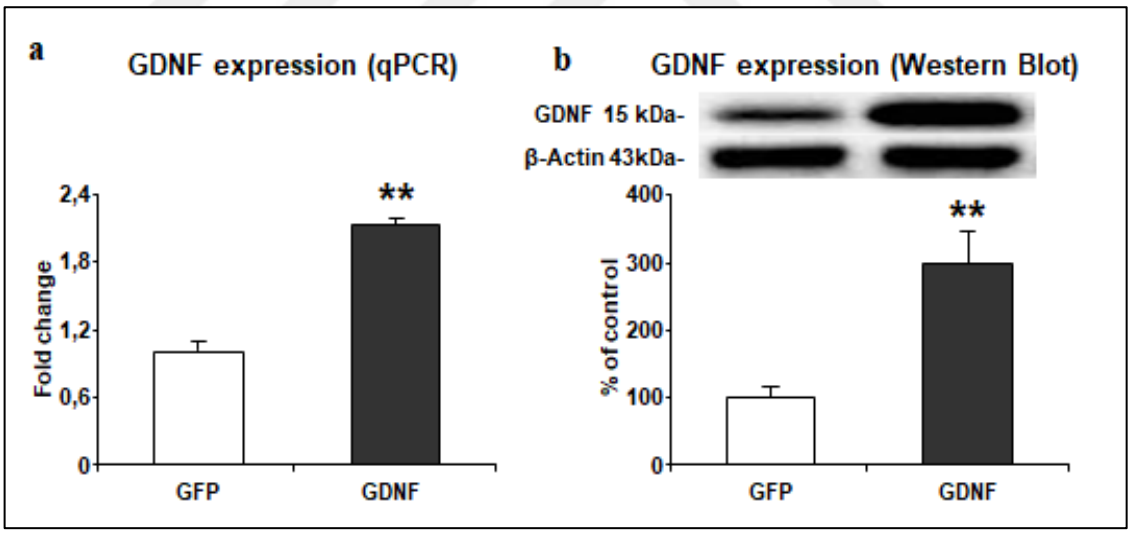

Figure 3.1. Expression of GDNF levels on (a) protein and (b) gene expression after virus injection to healthy brains. Data were indicated as mean and error bars show ± standard deviation values (n=4 mice per group). Significance was denoted as **p≤ 0.01 according to Lv-GFP treated group.

3.2. LASER DOPPLER FLOW DURING MCAO

During occlusion of the MCA throughout the experiments, CBF was controlled by laser doppler flow records. Monitoring the real time blood flow is essential for verifying the experimental procedure. Records were taken from the region (Bregma; -2 mm posterior; 6 mm lateral) where MCA feeds primarily. Blood flow was confirmed to decrease down to 20 per cent in average just after induction of occlusion by the filament technique for both 90 (Figure 3.2 a) and 30 minutes (Figure 3.2 b) ischemia. This stable decrease was observed until the filament was removed at the end of 90 or 30 minutes for each experimental group. After reperfusion, restoration of blood supply to brain within minutes can be seen from Figure 3.2.

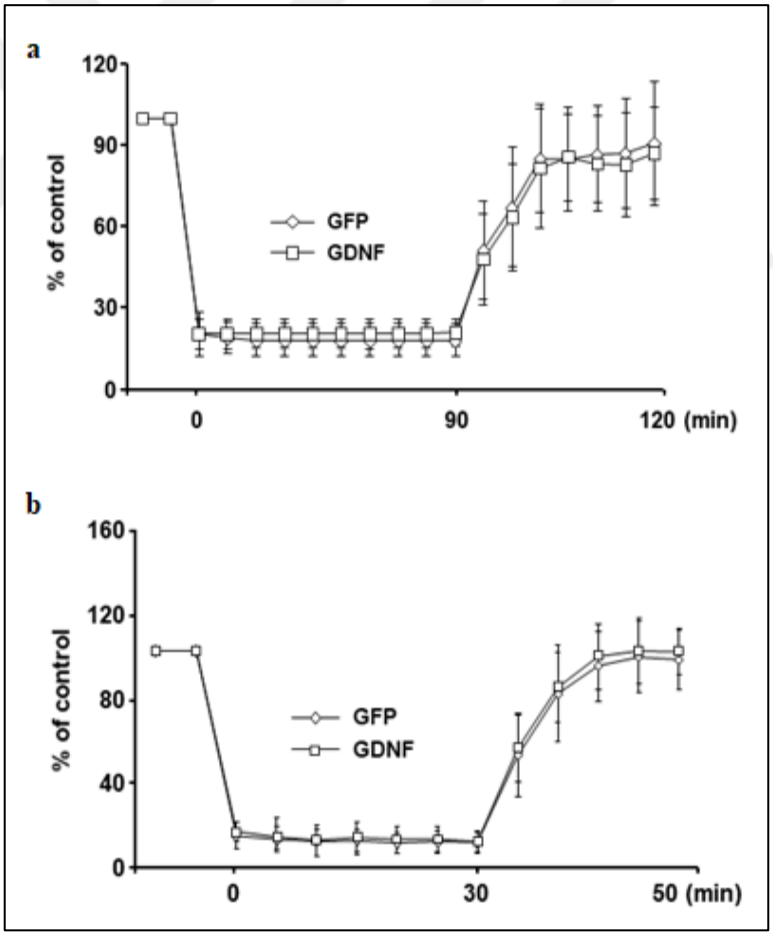

Figure 3.2. CBF, evaluated during ischemia and initial reperfusion by Laser Dopler Flow for (a) 90 minutes and (b) 30 minutes of focal cerebral ischemia. Data were indicated as mean (n=7 mice per group).

3.3. ASSESSMENT OF NEUROLOGICAL RECOVERY

Neurological examination of animals was interpreted on 4 grades according to the severity of their condition. Since neurological scoring is based on observations by directly examining animals it is an important parameter indicating neurological conditions of animals. Scoring analysis showed that neurological statute of animals was significantly better after Lv-GDNF-GFP treatment (p= 0.045) (Figure 3.3).

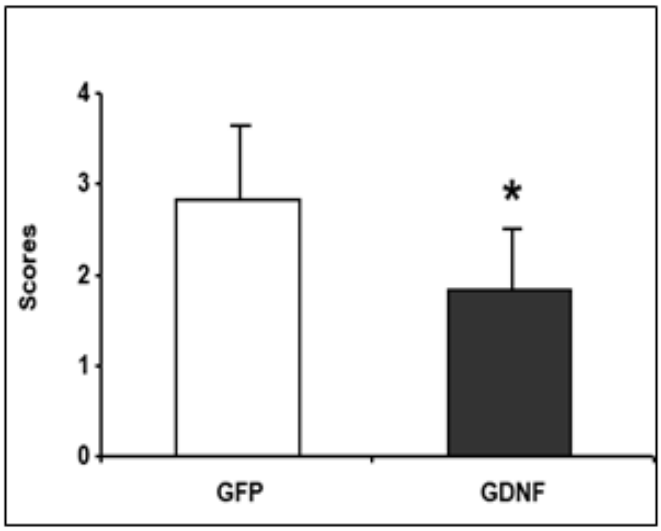

Figure 3.3. Neurological deficit score after 90 minutes of MCAO. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.4. ASSESSMENT OF EDEMA AND INFARCT SIZE

Next step in the study, was analyzing acute effects of Lv-GDNF-GFP treatment on mice. Putting down the animals at time points of 24 hours post-reperfusion in case of 90 minutes MCAO and 72 hours post-reperfusion in case of 30 minutes MCAO was because of investigating acute effects which refer to processes initiated after completion of ischemic damage. As severity of injury increased along with duration of ischemic condition, infarct size and edema were evaluated from brains exposed to 90 minutes MCAO. Mice treated with Lv-GDNF-GFP (n=7) or Lv-GFP (n=7) vectors 10 days before induction of MCAO for 90 minutes were sacrificed after 24 hours of reperfusion. Infarct volume and brain swelling

were analyzed from cresyl violet stained sections which were selected from four equidistant brain levels, 2 mm apart. It was observed that infarct volume and brain swelling were significantly reduced by GDNF gene treatment (p= 0.003 and p= 0.001 respectively) (Figure 3.4).

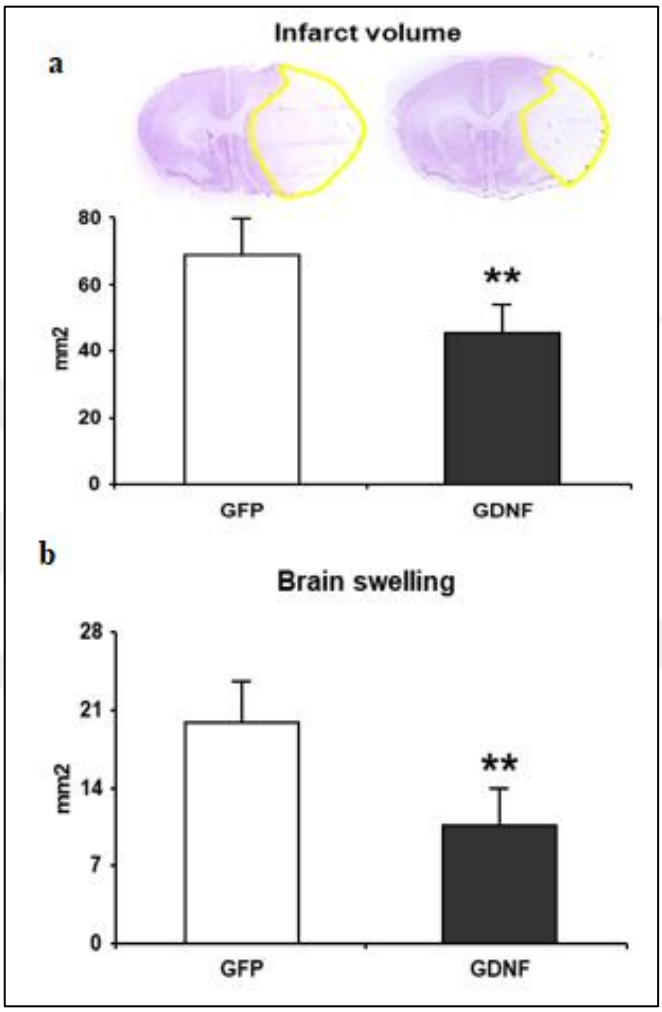

Figure 3.4. Effect of GDNF on (a) infarct volume and (b) brain swelling after focal cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as **p≤ 0.01 according to Lv-GFP treated group.

3.5. ANALYSIS OF NEURONAL SURVIVAL

After determining positive roles of GDNF on infarct size and edema, neuronal survival in lesioned striatum was investigated by staining neuronal cells. Neurons were marked with

NeuN which is a neuron specific nuclear antigen and NeuN positive cells were counted from 12 different areas. According to data, lenti-virus mediated GDNF treatment enhanced neuronal survival in the ischemic striatum significantly, compared to the control group (p= 0.044) (Figure 3.5).

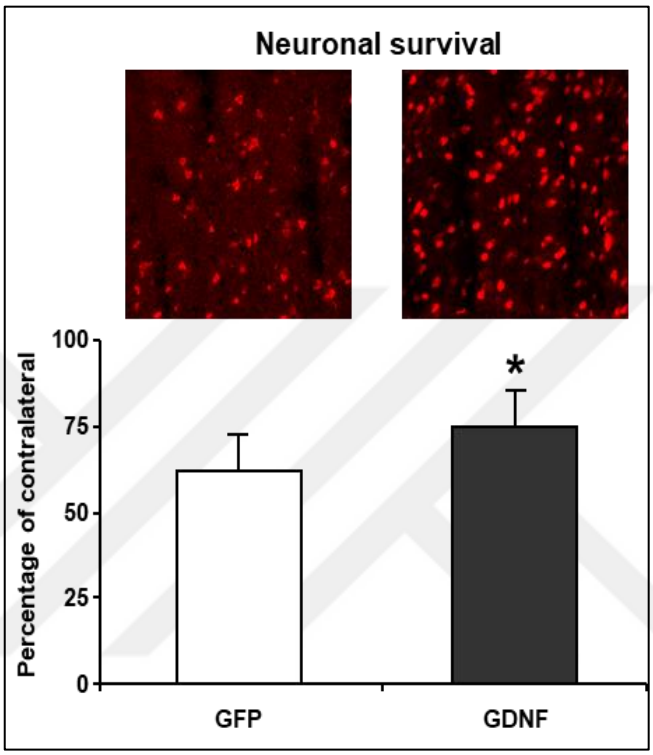

Figure 3.5. Neuronal survival after cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.6. ANALYSIS OF DNA FRAGMENTATION

One important indicator giving hint about post-ischemic recovery mechanism is reduction of cell death in lesioned area. For this purpose TUNEL assay was applied in order to label 3' OH sites of fragmented DNA in apoptotic cells. The amount of positively stained cells which were accepted as apoptotic cells was found to reduce in Lv-GDNF-GFP treated group after focal cerebral ischemia (p= 0.034) (Figure 3.6).

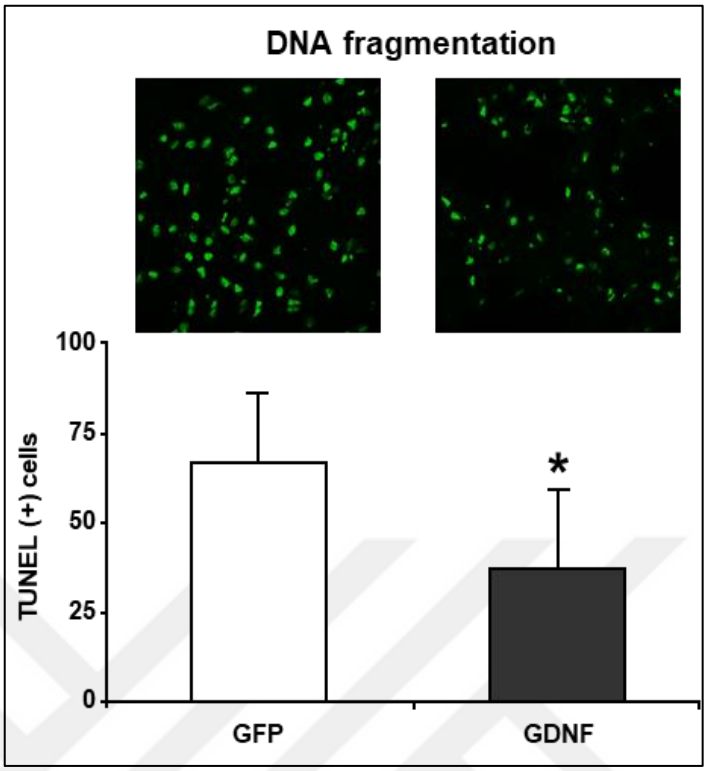

Figure 3.6. The amount of DNA fragmanted cells after focal cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.7. ASSESSMENT OF FUNCTIONAL RECOVERY

3.7.1. Grip Strength Test

Behavioral tests answered notable questions about functional outcomes of Lv-GDNF-GFP treatment. Especially in clinic, difficulty of molecular studies makes functional studies advantageous because of its practicability. In this study, behavioral data were placed on the ground of neuroplasticity studies. Grip strength and rota rod tests were performed for evaluating spontaneous motor activity while open field and light-dark transition tests enabled analysis of both voluntary movements and anxiety like behaviors such as; fear, apprehension and depression. The data belonging to grip strength test is shown in Figure 3.7. According to data, GDNF treatment improved functional deficit at the right paretic forepaw at the end of day 42, significantly (p= 0.026). There were no significant differences at previous days (Figure 3.7).

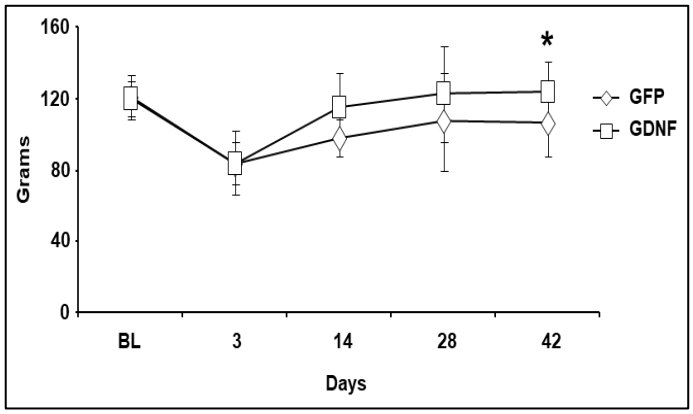

Figure 3.7. Grip strength of animals after focal cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=12 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group. BL refers to baseline which shows the pre-ischemic condition.

3.7.2. Rota Rod Test

According to the graph showing measurements recorded in the course of rota rod test indicated that, motor coordination skills of animals started to develop after day 3. At day 42, there was a significant increase in Lv-GDNF-GFP group relative to Lv-GFP group (p= 0.045) (Figure 3.8).

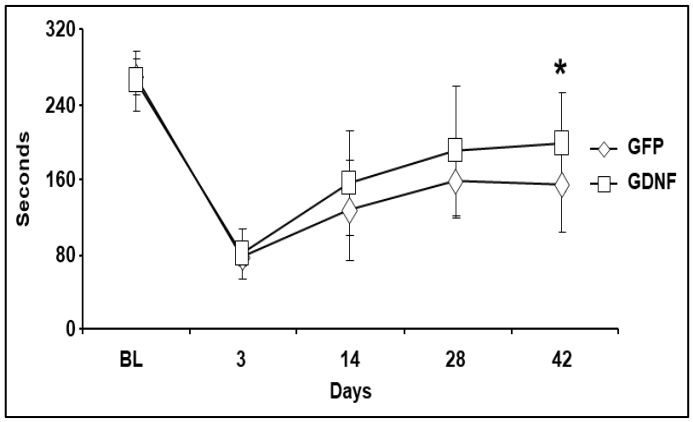

Figure 3.8. Motor coordination of animals after focal cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=12 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group. BL refers to baseline which shows the pre-ischemic condition.

3.7.3. Open Field Test

Mobility can be correlated with anxiety together with motor ability. Open field test revealed that, at day 42, Lv-GDNF-GFP treated mice showed a higher mobility in accordance with Lv-GFP treated mice (p= 0.047 for mobile time and p= 0.047 for immobile time) (Figure 3.9).

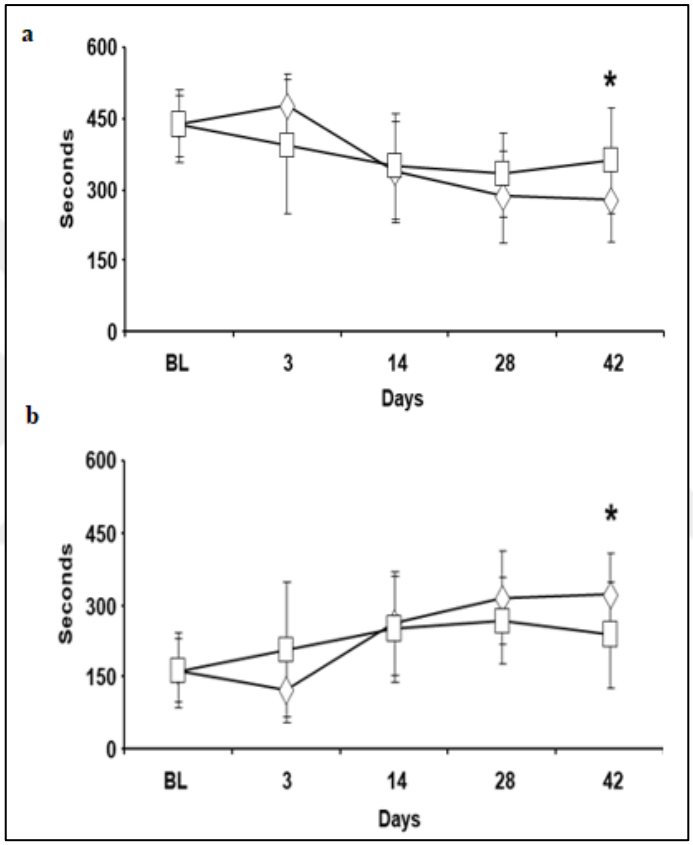

Figure 3.9. (a) Mobile and (b) immobile time spent by animals during open field test. Data were indicated as mean and error bars show ± standard deviation values (n=12 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group. BL refers to baseline which shows the pre-ischemic condition.

3.7.4. Light/Dark Transition Test

In Figure 3.10, it is possible to see the graphs showing regular increasing of the time spent in the light room (a), and on the contrary regular decreasing of the time spent in dark room (b) throughout the trial days depending on GDNF treatment. At day 42, this increase and decrease, respectively, reached the point of significance for GDNF treated animals (p= 0.045 for light room and p= 0.045 for dark room).

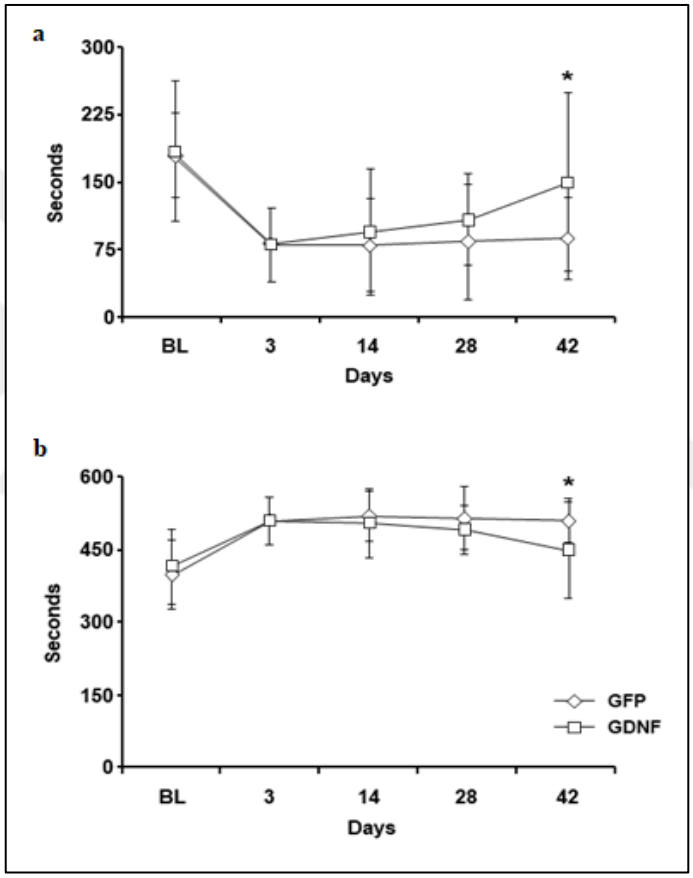

Figure 3.10. Time spent in (a) light and (b) dark zones by animals after focal cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=12 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group. BL refers to baseline which shows the pre-ischemic condition.

3.8. ASSESSMENT OF CELL PROLIFERATION IN THE ISCHEMIC STRIATUM

BrdU is a nucleoside analog which incorporates in DNA during building of new strands. Thus, it is a well established S phase indicator for proliferation assays [133]. BrdU immunostaining was applied for identification of total numbers of proliferating cells. This number gives an idea about the renewal and restoration of cells in the damaged area. Lv-GDNF-GFP treatment induced proliferation in a significant portion of cells in striatum (p= 0.04) (Figure 3.11).

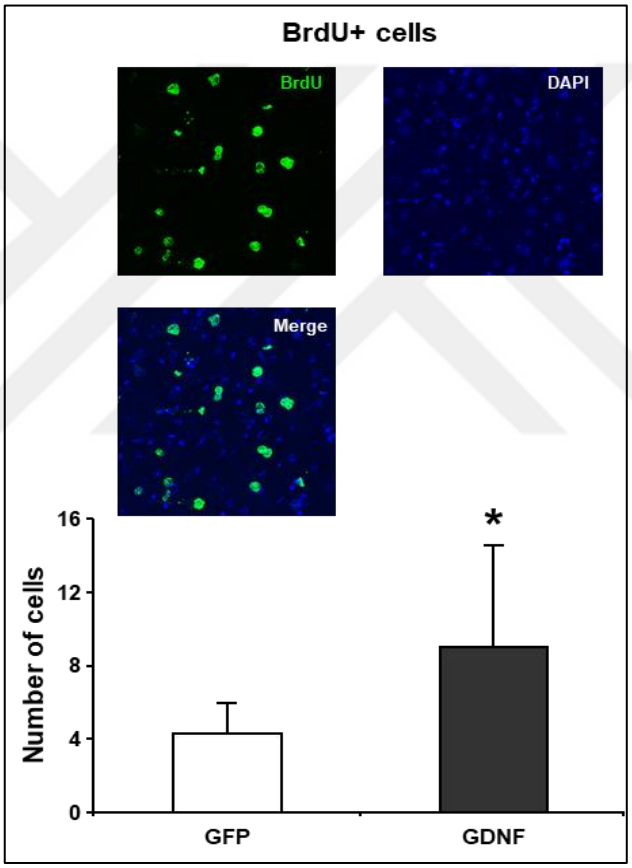

Figure 3.11. Post-ischemic cell proliferation in the striatum. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.9. ANALYSIS OF NEUROGENESIS

For detailed information, another experiment to show the extent of proliferation of neurons, astrocytes and microglia was carried out by double stainings of BrdU with NeuN (Figure 3.12), Iba-1 (Figure 3.13 a) and GFAP (Figure 3.13 b). These data provided information about both neurogenesis profile and rate of glial activation.

GDNF treatment was found to increase significantly neuronal cell proliferation in ischemic striatum (p= 0.025) (Figure 3.12). Double staining with mature neuronal nuclei marker NeuN, and BrdU antibodies were merged on the software (Zen Black, Carl Zeiss) and number of overlapped cells were counted from 12 different ROIs.

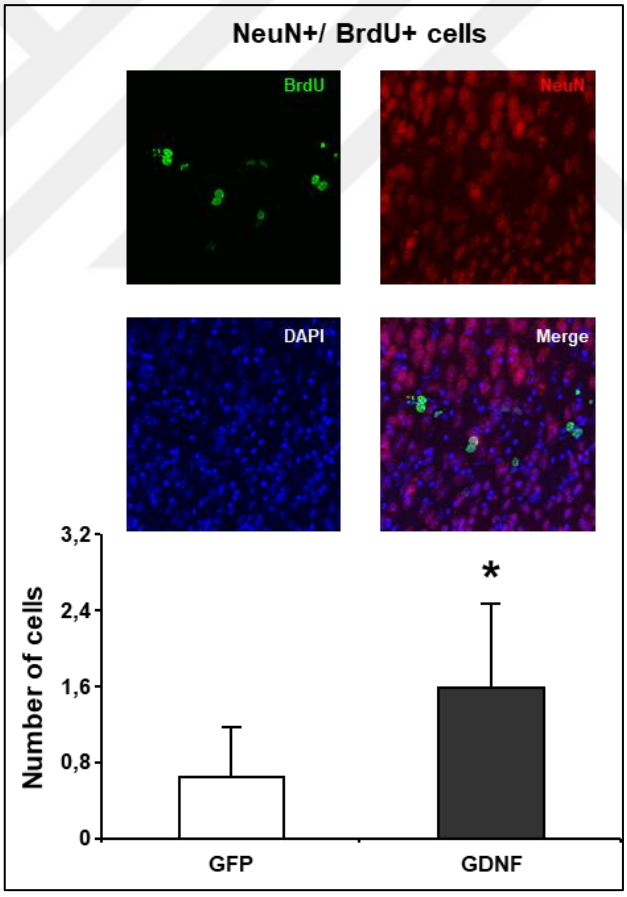

Figure 3.12. Co-expression of BrdU and NeuN in animals exposed to 30 minutes MCAO followed by 55 days reperfusion. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p ≤ 0.05 according to Lv-GFP treated group.

3.10. GLIAL ACTIVATION PROFILE

Similar with neurogenesis, glial activation was determined by co-screening of BrdU with, Iba-1 for microglia and GFAP for astrocytes, separately. Microglial activation (double immunostaining with Iba1 and BrdU) was not significantly but slightly increased in Lv-GDNF-GFP treated group (Figure 3.13 a). In addition, number of proliferating astrocytes (double immunostaining with GFAP and BrdU) was significantly decreased in Lv-GDNF-GFP treated group (p= 0.012) (Figure 3.13 b).

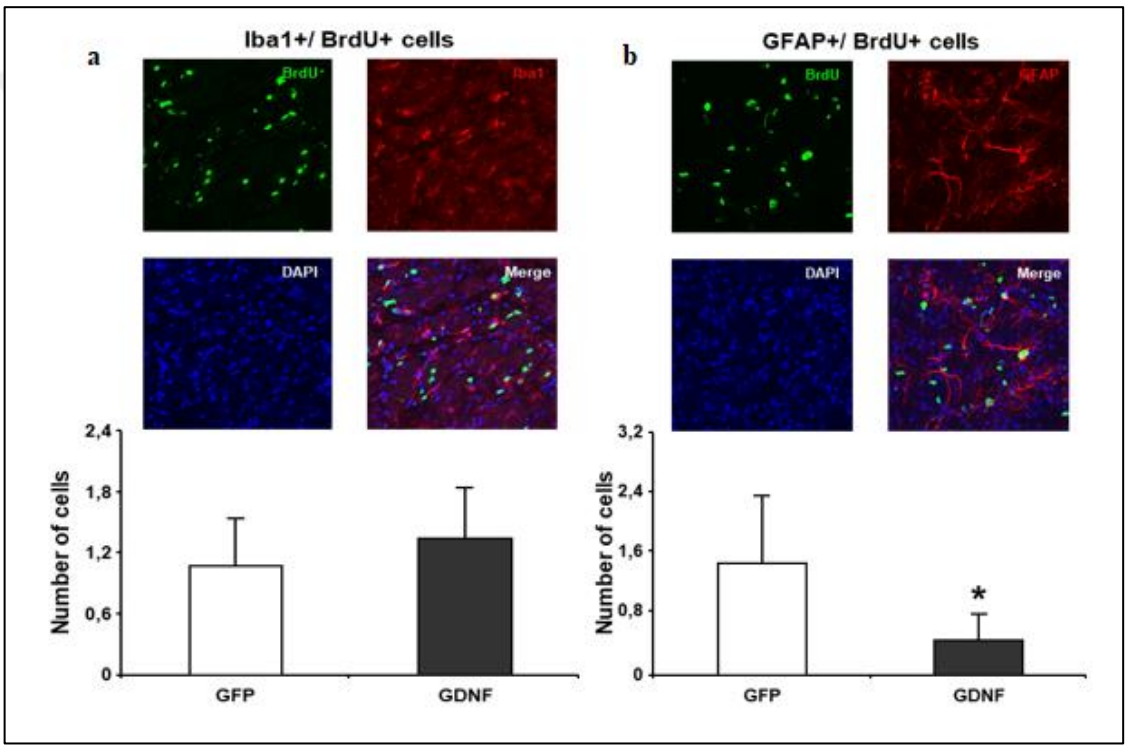

Figure 3.13. Co-expression of (a) BrdU and Iba1 representing microglial activation and (b) BrdU and GFAP representing astrocytic activation was evaluated in ischemic striatum focal after cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.11. GLIAL SCAR FORMATION

Area of glial scar was measured in GFAP-stained sections in animals submitted to 30 minutes focal cerebral ischemia followed by 55 days reperfusion. Glial scar formation was analyzed from GFAP stained sections of 55 days lived animals. Briefly, distribution of GFAP positive signals throughout the brain was measured via ZenPro software for each brain sections (Zeiss, Germany). Glial scar formation was significantly decreased in Lv-GNDF-GFP treated group (p= 0.001) (Figure 3.14).

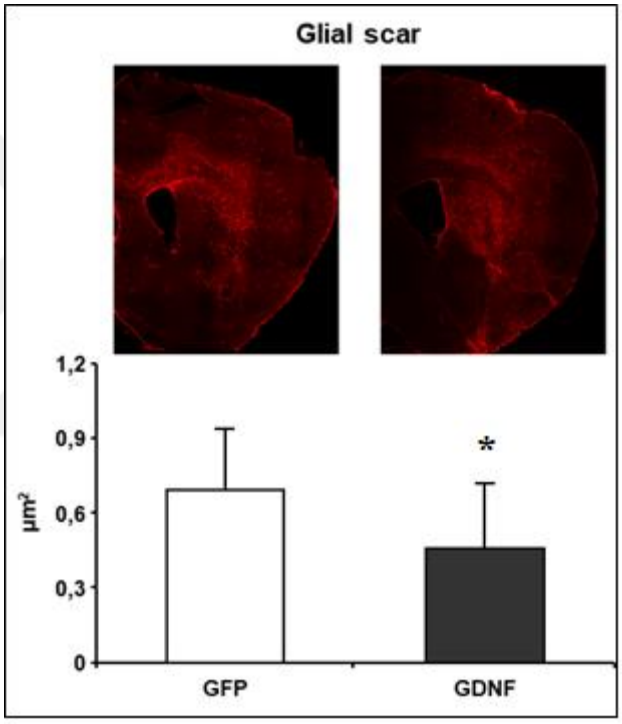

Figure 3.14. Glial scar formation after cerebral ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.12. ANALYSIS OF AXONAL PROJECTIONS

Extent of axonal growth and branching is directly related to neuroplasticity. Because, the term of neuroplasticity is not only addition of newborn neurons to existing circuits but also improvement of neuronal contacts. For this purpose, Biotinylated Dextran Amine (BDA) was used as a tract tracer. It was injected into the contralesional motor cortex to determine axonal projections from contralateral to ischemic hemisphere. BDA administration was performed at post-ischemic day 45 and let it to be transported through axonal projections trailing from cortex to spinal cord. Number of tracer stained fibers in the pyramidal tract at the level of the parvocellular red nucleus and facial nucleus was evaluated. Induction of cerebral ischemia significantly increased axonal plasticity contralateral to stroke both at the level of red nucleus and facial nucleus (p= 0.023 and p= 0.045 respectively) (Figure 3.15).

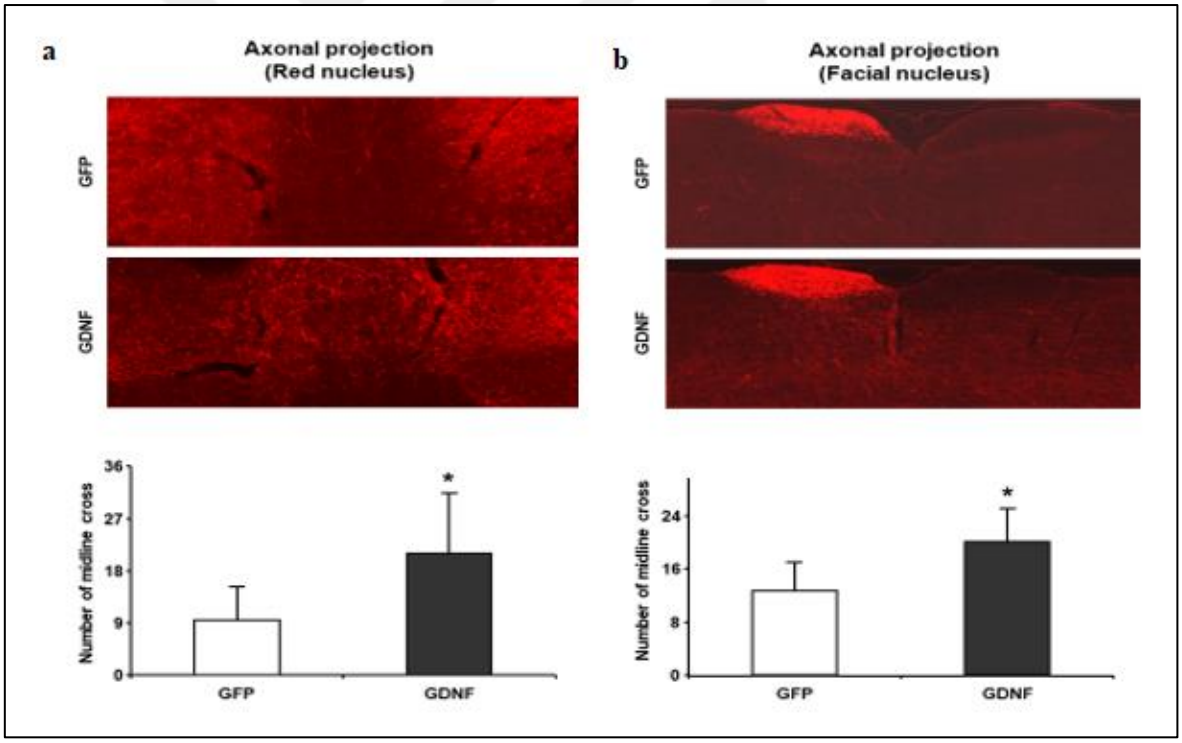

Figure 3.15. Tract tracer analysis of (a) corticorubral and (b) corticobulbar projections. Midline crossing axon fibers in (a) red nuclei and (b) facial nuclei were traced by BDA administration after ischemia. Data were indicated as mean and error bars show ± standard deviation values (n=12 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.13. ANALYSIS OF PROTEIN EXPRESSION

3.13.1. Brevican Expression

Lastly, expressions of proteins known to have an inhibitory role during axonal growth were analyzed as part of neuroplasticity related studies. To analyze the effect of GDNF on protein Brevican, tissue from ipsilateral and contralateral striatum were analyzed with western blot technique. A slight decrease in the level of Brevican protein was observed upon Lv-GDNF-GFP administration in ischemic striatum. In addition, GDNF treatment significantly decreased Brevican protein expression in contralateral striatum after MCAO (p= 0.05) (Figure 3.16).

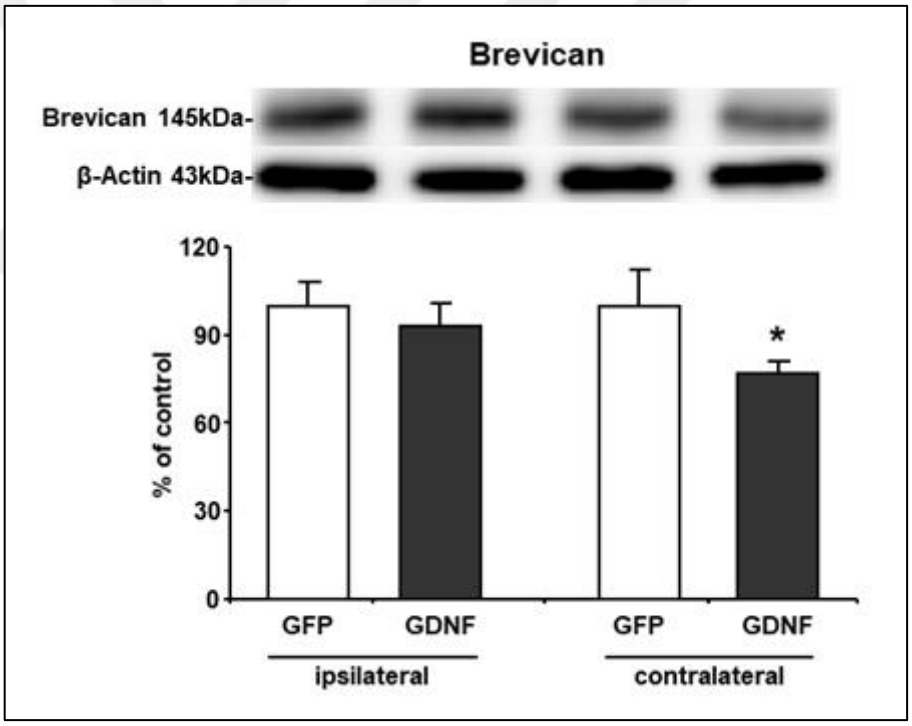

Figure 3.16. Brevican protein expression after 30 minutes MCAO followed by 55 days reperfusion. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.13.2. Versican Expression

To analyze the effect of GDNF on extracellular matrix proteoglycan, Versican, tissue samples harvested from ischemic and non-ischemic striatum were analyzed using Western blot. Results showed that administration of GDNF significantly reduced Versican protein expression in ischemic striatum (p= 0.017). In addition to this, there was slighty decreased Versican expression in contralateral striatum (Figure 3.17).

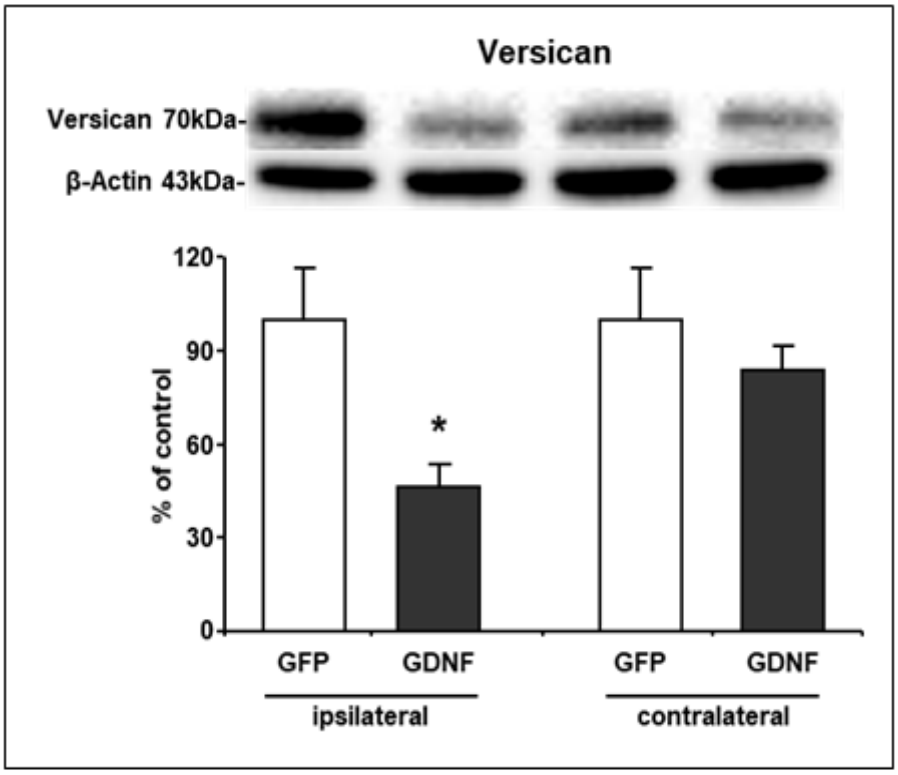

Figure 3.17. Versican protein expression after 30 minutes MCAO followed by 55 days reperfusion. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

3.12.3. Ephrin b1 and Ephrin b2 Expression

To analyze the effect of GDNF on axonal guidance molecule Ephrin b1/b2, tissue samples harvested from ischemic and non-ischemic striatum were analyzed using Western blot. It was observed that GDNF significantly reduced Ephrin b1 protein expression in non-ischemic

striatum after focal cerebral ischemia (p= 0.021) (Figure 3.18 a). In addition, GDNF treatment also significantly increased Ephrin B2 protein expression in contralateral striatum (p= 0.040) (Figure 3.18 b). However, there were no statistically significant differences observed in Ephrin b1 and Ephrin b2 expressions in ischemic striatum.

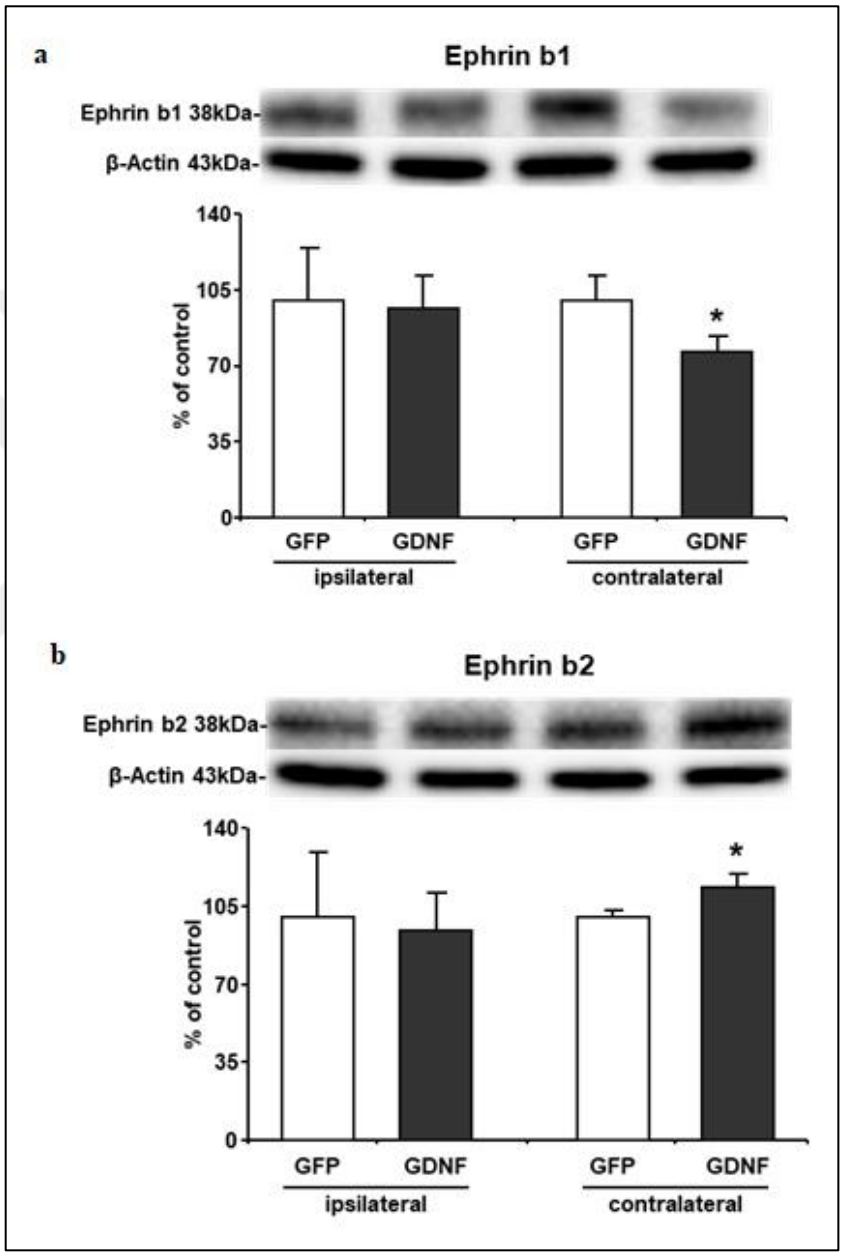

Figure 3.18. (a) Ephrin b1 and (b) Ephrin b2 protein expressions after 30 minutes MCAO followed by 55 days reperfusion. Data were indicated as mean and error bars show ± standard deviation values (n=7 mice per group). Significance was denoted as *p≤ 0.05 according to Lv-GFP treated group.

4. DISCUSSION

Stroke influences millions of people's lives adversely every year. It is responsible for most of the *de novo* disabilities which can vary in accordance with the dimension of damage. Many of the survivors remain disabled or lose their motor functions permanently while some lucky ones can maintain their lives with minor injuries such as partial deficiencies in vision, speaking, thinking and other cognitive functions. Together with becoming the second leading cause of death, economic and social burden of the disease make stroke a challenging issue. Millions of patients are on welfare from the government sanitary budgets for their care and needs.

Tissue plasminogen activator (tPA) is the unique treatment being applied to patients within first 4 hours after initiation of an attack. This may provide unclogging of the vessels in ischemic stroke cases and prevents progression of damage. However, reversal of the functional and physiological integrity which is lost as a result of oxygen and nutrition deprivation cannot be obtained. Middle cerebral artery occlusion (MCAO) is responsible for most of the cerebral ischemia cases. Primary sensory and motor cortexes are mainly affected areas upon MCAO [11].

Retrieval in the long term is primarily related with regenerative mechanisms in the brain. Researchers basically aim to trigger a regeneration process that ends up with formation of new neuronal circuits and functional recovery. Within this context, neuronal plasticity is an essential phenomenon that should be examined. Brain has been accepted as a non-regenerative organ for decades until discovery of adult neural stem cells which have the characteristics of self-renewal and multi-potency, giving rise to 3 different cell lines; neurons, astrocytes and oligodendrocytes [134]. Regenerative medicine for stroke has been examined with an accelerating potential, particularly after findings revealing the renewal power of the brain. At this point, neurotrophic factors gain an importance due to their roles as rescuers in nervous system in the course of any damage. GDNF can be set apart from other neurotrophic factors for its distinguishing roles on dopaminergic neurons.

GDNF is a type of TNF-α growth factors and famous with its beneficial actions specifically on dopaminergic neurons. Dopaminergic neurons are found in the region of substantia nigra and ventral tegmental area of midbrain. Although they are not abundant in numbers, they are important regulators of various motor and cognitive functions such as voluntary movements, learning, mood, reward, addiction, stress, etc. [135]. Dopaminergic neurons send axonal projections to dorsal striatum that is one of the main input areas for the basal ganglia. These afferent neurons known as nigrostriatal pathway play an important role in movement. Dopaminergic neurons in the nigrostriatal pathway synapse onto GABAergic neurons in the basal ganglia and in part make up the basal ganglia loop. This pathway is primarily responsible for voluntary movements [136, 137].

Innervation of dopaminergic neurons of the striatum by GDNF is considered to be higher during development, as GDNF expression is much higher in developing striatum than adult striatum [138]. Interestingly, striatal GDNF levels are higher when compared to other brain regions [139]. Midbrain dopaminergic neurons are identified to be differentially vulnerable against 6-OHDA injection with a same parallelism to GDNF expression in the striatum. GDNF expression in ventral striatum was demonstrated to be significantly higher than dorsal striatum and dopaminergic neurons in ventral tegmental area that project to ventral striatum were found to be more resistant than those in substantia nigra that project to dorsal striatum [140]. It shows the possible neuroprotective role of striatal GDNF expression on dopaminergic neurons.

GDNF is necessary for the survival of the dopamine neurons apparently but it targets other types of neurons residing in the striatum as well. We hypothesized a regenerative mechanism induced by GDNF that could proceed primarily on dopaminergic neurons together with other types of neurons. Suggesting transport of GDNF from striatum through different brain structures, we examined neuronal plasticity and functional recovery after cerebral ischemia in the long term. In literature, neuroprotective and therapeutic effects of GDNF on cerebral ischemia have been demonstrated by many researchers. We adopted a differential approach from the literature and designed experiments around neuroplasticity. For this purpose, animals were followed for 55 days, a relatively long period of time after induction of MCAO. One other point putting this study advanced from the others is succeeding continuous expression of GDNF in the brain via a lenti-viral vector system. Previous works were mainly

utilized adeno-viral [128, 141, 142] or adeno-associated viral vectors [127] as a gene transfer vehicle while there was no lenti-viral mediated GDNF therapy as therapeutic purposes.

Firstly, confirmation of Lv-GDNF vectors was made before administration in MCAO model of animals. Expression of GDNF was shown to increase up to 3-fold on protein level and 2,5 fold on RNA level when Lv-GDNF applied 10 days before sacrification (Figure 3.1). This time point was designated since the most efficient production of the desired gene could be managed within this period of time. These 2 to 3 fold expressions were accepted as sufficient for following procedures from previous experiences [128]. Secondly, neuroprotective influences of GDNF in short term were analyzed on two different models of MCAO for 30 minutes and 90 minutes.

For conceiving rapid effects of Lv-GDNF therapy, 90 minutes MCAO animal model was used. Animals were evaluated for their neurological condition as it was offered in data based on observations. Neurologic status of animals can be considered as the earliest response concerning whether GDNF showed a positive impact or not. According to scoring, neurological functions of animals were apparently better after Lv-GDNF treatment but not Lv-GDNF-GFP treatment (Figure 3.3). Even it is too early to mention about a recovery mechanism yet, it can be said that GDNF may provide such an escape hatch to the brain in order to tolerate ischemic injury as early as possible. The extent of infarct size and swelling induced just after ischemia are two prominent indicators and they give information about the severity of ischemic event. Size of infarction, the mass of death tissue was conspicuously smaller. Edema is the result of broken ionic balance and gets shape during acute phase of ischemia. Decrease in both infarct size and swelling depending on Lv-GDNF treatment shows neuroprotective capacity of GDNF on macro scale (Figure 3.4). Despite this is not a novel finding in the scientific world, it was an evidence of being on the right way for our hypothesis about GDNF. Previously, data about the reducing role of GDNF on ischemic lesions were shared by several studies as stated previously. When looking at the molecular perspective and going into particulars, analysis of neuronal survival was the next stage of the experiments. MCAO model with 30 minutes was used for molecular analysis. It is known that cells localized in the injury site undergo necrosis just after ischemic onset. However, during acute phase of ischemia apoptosis is liable for most of the cell deaths [11]. Increase in the percentage of surviving neurons in the ipsilateral hemisphere to contralateral

hemisphere was an expected outcome based on the surviving promoting actions of GDNF (Figure 3.5). From this view, together with the rise in numbers of neurons, it was suggested that apoptosis could be downregulated (Fig 3.6).The results of DNA fragmentation analysis showed a correlation with data of neuronal survival analysis. There was an inverse proportion between neuronal survival and cell death as estimated. Lv-GDNF treatment lowered TUNEL positive cell numbers in the ischemic striatum. Here, despite the fact that types of cells were not clearly shown, it is known from previous works that neuronal subtypes compose most of the apoptotic cells in the striatum after ischemia [143]. Findings related to cell death and neuronal survival are the consequence of activated PI3K/Akt and ERK1/2/MAPK pathways which were regulated by GDNF signaling. GDNF is already known to activate survival kinases and inhibits apoptotic pathways [71].

In the next part, long term action of GDNF on neuronal plasticity was identified. Behavioral tasks can be considered as the ground of the hypothesis about GDNF's potential restorative role on injured neuronal system. By observing and following animals for 42 days, retrieval of disabilities in motor movements and mood which were caused by ischemic damage during such a relatively longer period of time was designated. This information is so valuable that there was no data shared before about long term functional recovery examined in detail. Four sets of behavioral tests; grip strength, rota-rod, open field and light-dark transition, performed within the scope of this thesis filled a gap in the literature by exhibiting neuroplasticity related functional recovery induced by GDNF not only at early periods but also at late periods of chronic phase post-ischemia. As a matter of fact, recovery was observable at post-ischemic day 42 for each of the tests. Grip strength of animals at post-ischemic day 42 reached almost the same level as the one in pre-ischemic conditions (Figure 3.7). Similarly, rota-rod test revealed that Lv-GDNF treated animals were able to continue walking on the rotating rod up to 200 seconds with a better performance when compared to control (Figure 3.8). Open field test, another task applied for evaluating motor activity showed increasing mobility and physical activity of animals at post-ischemic day 42 depending on recovery process carried out by Lv-GDNF treatment (Figure 3.9).

In a previous study where GDNF was administered via fusing with PEP-1 peptide, limb use was scored and found to be significantly better at post-ischemic day 21 and day 28 [144]. In another study, behavioral recovery was determined with grasping power and rota rod tests

at post-ischemic day 7, day 14, day 21 and day 28 in animals treated with GDNF-fibrin glue mixture [120]. In the stated studies, Lv-GDNF treatment was not used before for the induction of ischemia model as it was in this thesis. Continuous expression of GDNF maintained by lenti-viral vector system created the differences among the other works. As lenti-virus is a retro-virus and integrates into a genome, attaining the sufficient GDNF expression takes longer period of time when compared to other vectors.

Because of alterations in temper triggered by stroke, light-dark transition test was used to measure anxiety of animals. In light-dark transition test, level of anxiety correlates with the time spent in dark zone. As expected, time spent in light zone began to increase gradually from the post-ischemic day 3, but significance was found only in post-ischemic day 42 (Figure 3.10). Importance of this piece of experiment is its uniqueness in the literature by showing the neuroprotective effect of GDNF on mood.

The next stage was detailed molecular analysis to see the mechanism behind of these beneficial outcomes. In this thesis, elaborating the phenomenon of neuroplasticity was intended. For analyzing cell proliferation capacity in the striatum, BrdU stainings were applied by using both single and double immune-labeling. Overall proliferation was evaluated by counting BrdU positive cells. At post-ischemic day 55, cells in S phase that can be suggested as undergoing cell division were detected to be higher due to Lv-GDNF treatment (Figure 3.11). In order to look at the picture closer and identify proliferating cell types, double stainings with NeuN, Iba-1 and GFAP were applied. This gave an idea that neurogenesis was promoted by a mechanism activated by GDNF (Figure 3.12). Although increased cell proliferation and neurogenesis in striatum upon GDNF treatment is not a novel argument, it was conducted for the first time for a long term application [121, 122, 144]. Neurogenesis is accomplished by adult neural stem cells after ischemic injury but it is limited within a time window [44]. Therapeutic approaches against stroke emphasize this issue and aim to increase neuronal cell populations in longer periods. Here, it was thought to be managed by continuous expression of GDNF. In addition, proliferation status of glial cells were found to be different from neurons. It should be noted that glial cells become activated quickly due to inflammatory response triggered by ischemic event and keep on their position during recovery process. Although astrocytes and micro-glia have roles such as buffering of extracellular environment and helping production of neurotransmitters and anti-oxidants,

lowering in the number, glial cells are known to be associated with the degree of improvement [11, 29, 30]. On this basis, different strategies aim to decrease glia-dependent inflammation as a therapeutic approach for stroke [15]. In our study, the significant decrease in number of newborn astrocytes at post-ischemic day 55 can be interpreted as inhibitory role of GDNF on inflammatory mechanisms (Figure 3.13).

Another important mechanism should be examined within the context of inflammation that is formation of glial scar which is a barrier preventing regeneration by inhibiting axonal growth. Glial scar is composed of activated astrocytes and proteoglycans such as chondroitin and keratan sulfate. When regenerating axons encounter the territory of the glial scar, growth cones fail to protrude [145]. Based on this piece of information, Lv-GDNF treatment significantly reduced the distribution of scar (Figure 3.14). Probably, scar formation in control animals was approximated to GDNF treated ones towards post-ischemic day 55 as it is evaluated mostly as an acute finding.

Lastly and most importantly, neuroplasticity which is the hinge of the thesis was correlated with analyzing the numbers of axonal fibers projecting the ischemic hemisphere from contralateral. Corticorubral tract which refers descending neuronal fibers from cortex towards red nucleus of midbrain and corticoburbal tract which refers to descending neuronal fibers from cortex toward facial nucleus of medulla oblongata were traced with BDA staining in our study. Both of corticorubral and corticobulbar tracts descend through the spinal cord and are primarily involved in carrying the motor function [146]. BDA was transported anterogradely by axonal termini of neurons in cortex through red nucleus and facial nucleus in which they were terminated. Lv-GDNF treatment was demonstrated to induce both contralesional corticorubral and corticobulbar sprouting (Figure 3.15). The corticorubral and corticobulbar fibers that were observed to cross the midline and reached the red nucleus and facial nucleus from the contralateral cerebral cortex were significantly higher due to Lv-GDNF treatment. These results reflected a behavioral correlation of the neural plasticity on a cellular basement.

Expression analysis on protein level was the final touch for completing the overall hypothesis aiming to show the potential role of continuous GDFN treatment on neuroplasticity. Apart from regenerative capacity, the growth inhibitory attributes of CNS

after an injury is a barrier against axonal growth and sprouting and must be overcome. Those inhibitory factors can be categorized into three general classes; members of canonical axon guidance molecules (semaphorins, ephrins, netrins), prototypic myelin inhibitors (Nogo, MAG, and OMgp) and chondroitin sulfate proteoglycans (lecticans, NG2). Brevican and Versican are two major proteoglycans in adult brain [147, 148]. As glial scar is mainly composed of proteoglycans which are the inhibitory factors limiting neuronal outgrowth, expression levels of Brevican and Versican give an important deduction about the molecular mechanism of post-ischemic plasticity. Although there is very limited information in the literature clarifying the role of Brevican and Versican in the process of recovery, it was apparently displayed that deposition of them is associated with lesional progression [149, 150]. In this study, despite being a tendency in decrement of expression levels in both hemispheres, Brevican was significantly lower in contralateral hemisphere whereas Versican was significantly lower in ipsilateral hemisphere (Figure 3.17). In a study, aiming to characterize regeneration induced by proteoglycans after spinal cord injury showed that Versican was primarily produced by oligodendrocytes and fibroblasts. On the other hand, Brevican production was mainly carried out by astrocytes [150]. This piece of information may offer a logical explanation for crosswise expressions. Number of oligodendrocytes in the ipsilateral hemisphere and number of astrocytes in the contralateral hemisphere may be regulated in this way. Another proteins, examined within the perspective of neuroplasticity were Ephrin-b1 and Ephrin-b2. Ephrin ligands which signal through Ephrin receptor tyrosine kinases play essential roles in neuronal targeting. They modulate axonal guidance and targeting, synaptic plasticity and neurotrophic factor mediated neuroprotection [151]. Ephrin-b ligands (Ephrin-b1-b3) are transmembrane ligands which can signal towards forward direction as well as reverse direction which refers the transduction through intracellular phosphotyrosines. Ephrin-b ligands are important regulators of pre and post synaptic development [152]. During development, Ephrin-b ligands have missions as attractant and repellent molecules according to the site where they act. However, in case of adult brain, the major role of Ephrin-b ligands in regeneration is their repulsive functions [153]. From this perspective, the downregulation of Ephrin-b1 in the contralesional hemisphere that was similar to Brevican and Versican may be the result of building a suitable environment in order to pave the way for generation of new neuronal networks (Figure 3.18 a). Importantly, regulation of neuroplasticity by guidance molecules should be commented as a dynamic process. Due to the combination of both attractive and repulsive signals, overall

neuronal structures were built up beyond assembly of neuronal networks. This may explain the upregulation of Ephrin-b2 in contralesional hemisphere (Figure 3. 18 b).

5. CONCLUSION

Numerous different therapeutic factors including neuroprotective, neuromodulator, neurotrophic agents, growth factors, hormones, cell-based systems, scaffolds, etc. have been identified till today for stroke treatment. All of these approaches were shown to provide partial recovery. Rather than administration of recombinant proteins directly, modified biological systems (viral vectors, cell-based implants) are much more advantegous due to their maintenance. From this perspective and behind the findings of structural and molecular improvements by previous studies, it was hypothesized that, GDNF which is an essential neurotrophic factor by means of its positive impacts on especially dopaminergic neurons, could possibly increase post-ischemic neuroplasticity. Lenti-viral vector system was chosen as the delivery method in order to gain a continuous GDNF expression.

For this purpose, addition to acute effects, which were analysed by measuring infarct size, brain swelling, neuronal survival and cell death, action of GDNF in a long period of time were also investigated by focusing on neuroplasticity. Therefore, functional behavioral tests, axonal projection analysis and expression analysis of plasticity related proteins were designed.

In conclusion, it was the first time documented in this thesis that, lenti-viral mediated GDNF gene therapy provided post-ischemic recovery in early stages and initiated several regenerative mechanisms in the progressing periods. It was also demonstrated that exogenous GDNF expression contributed to functional recovery which was the indicator of increased neuronal plasticity. This inference was accompanied with molecular studies which showed increased axonal projections in corticorubral and corticobulbar tract. Also, axonal growth inhibitory molecules were identified to decrease as a result of neuroprotective impacts of GDNF. Data obtained within the scope of this thesis demonstrated that, GDNF can be a promising candidate for stroke which causes permanent devastations.

6. FUTURE PROSPECTS

As brain is protected by the rigid skull and because of the impermeable nature of BBB, it is difficult to apply therapeutic agents to the site of injury. Therefore, most probable way of giving such molecules which cannot pass through BBB is direct injections into the brain. For ensuring a stable long term application vector directed delivery of GDNF gene appears as a potential strategy. Viral vectors especially lenti virus derived systems are applicable due to their capability to provide sustained expressions. Adeno-virus and adeno-associated virus are two other commonly used vectors for virus mediated gene transfer. Among these 3 vector systems, each of them possess different advantegous and disadvantages. GDNF gene therapy mediated by viral vectors for treatment of various disease is under clinical researches. GDNF gene therapy by using adeno-viral vectors were investigated in Parkinson's disease patients with clinical studies and they can be also tried in stroke. Although, there is no clinical trial subjecting lenti-viral mediated GDNF delivery, integration ability of lenti-viral genome to the host genome after transduction, makes lenti-viral vectors attractive and useful vehicles for gene therapy. So that, when considering the protective role of GDNF it is a promising way of gene therapy for stroke by inducing long term neuroplasticity. However, further clinical studies are needed for observing potential side effects caused by the surgical intervention for intracerebral administration. Also despite being fully safe, immunogenic reactions triggered by virus particles are the main problems to be overcome.

Eventhouhg GDNF is a powerful biosubstance and an apparent therapeutic candidate for cerebral ischemia in order to increase efficiency related to syptomatic recovery, additional strategies should be taken into account. In modern medicine, combinational therapies appear as a more plausible way of medications. As the mechanism underlying the pathophysiology of stroke is much complicated, expectations relating to complete recovery can be potentially met by combination of some other neurotrophic factors, neuroprotective agents, antioxidants, hormones, etc.

REFERENCES

1. Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al. Heart disease and stroke statistics-2018 update: A report from the American Heart Association. *Circulation.* 2018;137(12):67-492.

2. Katan M, Luft A. Global burden of stroke. *Seminars in Neurology.* 2018;38(2):208-11.

3. Correction to: Heart disease and stroke statistics-2017 update: A report from the American Heart Association. *Circulation.* 2017;136(10):196.

4. Kunz A, Dirnagl U, Mergenthaler P. Acute pathophysiological processes after ischaemic and traumatic brain injury. *Best Practice & Research Clinical Anaesthesiology.* 2010;24(4):495-509.

5. Roth JM. Recombinant tissue plasminogen activator for the treatment of acute ischemic stroke. *Proceedings.* 2011;24(3):257-9.

6. Lackland DT, Carey RM, Conforto AB, Rosendorff C, Whelton PK, Gorelick PB. Implications of recent clinical trials and hypertension guidelines on stroke and future cerebrovascular research. *Stroke.* 2018;49(3):772-9.

7. Mink JW, Blumenschine RJ, Adams DB. Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. *The American Journal of Physiology.* 1981;241(3):203-12.

8. Harris JJ, Jolivet R, Attwell D. Synaptic energy use and supply. *Neuron.* 2012;75(5):762-77.

9. Nortley R, Attwell D. Control of brain energy supply by astrocytes. *Current Opinion in Neurobiology.* 2017;47:80-5.

10. Klehmet J, Harms H, Richter M, Prass K, Volk HD, Dirnagl U, et al. Stroke-induced immunodepression and post-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. *Neuroscience.* 2009;158(3):1184-93.

11. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences.* 1999;22(9):391-7.

12. Zechariah A, ElAli A, Doeppner TR, Jin F, Hasan MR, Helfrich I, et al. Vascular endothelial growth factor promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. *Stroke.* 2013;44(6):1690-7.

13. Yao H, Takasawa R, Fukuda K, Shiokawa D, Sadanaga-Akiyoshi F, Ibayashi S, et al. DNA fragmentation in ischemic core and penumbra in focal cerebral ischemia in rats. *Brain Research Molecular Brain Research.* 2001;91(1-2):112-8.

14. Astrup J, Siesjo BK, Symon L. Thresholds in cerebral ischemia - the ischemic penumbra. *Stroke.* 1981;12(6):723-5.

15. Lakhan SE, Kirchgessner A, Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *Journal of Translational Medicine.* 2009;7:97.

16. Caraballo JC, Yshii C, Butti ML, Westphal W, Borcherding JA, Allamargot C, et al. Hypoxia increases transepithelial electrical conductance and reduces occludin at the plasma membrane in alveolar epithelial cells via PKC-zeta and PP2A pathway. *American Journal of Physiology Lung Cellular and Molecular Physiology.* 2011;300(4):569-78.

17. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiology of Disease.* 2008;32(2):200-19.

18. Martin RL, Lloyd HG, Cowan AI. The early events of oxygen and glucose deprivation: setting the scene for neuronal death? *Trends in Neurosciences.* 1994;17(6):251-7.

19. Katsura K, Kristian T, Siesjo BK. Energy metabolism, ion homeostasis, and cell damage in the brain. *Biochemical Society Transactions.* 1994;22(4):991-6.

20. Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Research Reviews.* 2007;54(1):34-66.

21. Mies G, Iijima T, Hossmann KA. Correlation between peri-infarct DC shifts and ischaemic neuronal damage in rat. *Neuroreport.* 1993;4(6):709-11.

22. Hossmann KA. Periinfarct depolarizations. *Cerebrovascular and Brain Metabolism Reviews.* 1996;8(3):195-208.

23. Noshita N, Sugawara T, Fujimura M, Morita-Fujimura Y, Chan PH. Manganese superoxide dismutase affects cytochrome c release and caspase-9 activation after transient focal Cerebral Ischemia in Mice. *Journal of Cerebral Blood Flow and Metabolism.* 2001;21(5):557-67.

24. Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR. Significance of melatonin in antioxidative defense system: reactions and products. *Biological Signals and Receptors.* 2000;9(3-4):137-59.

25. Wong CH, Crack PJ. Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. *Current Medicinal Chemistry.* 2008;15(1):1-14.

26. Yilmaz G, Granger DN. Cell adhesion molecules and ischemic stroke. *Neurological Research.* 2008;30(8):783-93.

27. Letechipia-Vallejo G, Gonzalez-Burgos I, Cervantes M. Neuroprotective effect of melatonin on brain damage induced by acute global cerebral ischemia in cats. *Archives of Medical Research.* 2001;32(3):186-92.

28. Yamasaki Y, Matsuo Y, Matsuura N, Onodera H, Itoyama Y, Kogure K. Transient increase of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family, in ischemic brain areas after focal ischemia in rats. *Stroke.* 1995;26(2):318-22.

29. Lucas SM, Rothwell NJ, Gibson RM. The role of inflammation in CNS injury and disease. *British Journal of Pharmacology.* 2006;147(1):232-40.

30. Trendelenburg G, Dirnagl U. Neuroprotective role of astrocytes in cerebral ischemia: focus on ischemic preconditioning. *Glia.* 2005;50(4):307-20.

31. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science.* 2004;305(5684):626-9.

32. Bowden MG, Woodbury ML, Duncan PW. Promoting neuroplasticity and recovery after stroke: future directions for rehabilitation clinical trials. *Current Opinion in Neurology.* 2013;26(1):37-42.

33. Carmichael ST. Emergent properties of neural repair: elemental biology to therapeutic concepts. *Annals of Neurology*. 2016;79(6):895-906.

34. Di Pino G, Pellegrino G, Assenza G, Capone F, Ferreri F, Formica D, et al. Modulation of brain plasticity in stroke: a novel model for neurorehabilitation. *Nature Reviews Neurology.* 2014;10(10):597-608.

35. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nature Medicine.* 2002;8(9):963-70.

36. Hallett M. Plasticity of the human motor cortex and recovery from stroke. *Brain Research Brain Research Reviews.* 2001;36(2-3):169-74.

37. Lee E, Son H. Adult hippocampal neurogenesis and related neurotrophic factors. *Biochemistry and Molecular Biology Reports.* 2009;42(5):239-44.

38. Sharma N, Classen J, Cohen LG. Neural plasticity and its contribution to functional recovery. *Handbook of Clinical Neurology.* 2013;110:3-12.

39. Hara Y. Brain plasticity and rehabilitation in stroke patients. *Journal of Nippon Medical School.* 2015;82(1):4-13.

40. Li S. Spasticity, Motor Recovery, and Neural Plasticity after Stroke. *Frontiers in Neurology.* 2017;8:120.

41. Netz J, Lammers T, Homberg V. Reorganization of motor output in the non-affected hemisphere after stroke. *Brain: A Journal of Neurology.* 1997;120:1579-86.

42. Cramer SC. Brain repair after stroke. *The New England Journal of Medicine.* 2010;362(19):1827-9.

43. Meves JM, Zheng B. Extrinsic inhibitors in axon sprouting and functional recovery after spinal cord injury. *Neural Regeneration Research.* 2014;9(5):460-1.

44. Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. *Science.* 1999;286(5439):548-52.

45. Gage FH. Mammalian neural stem cells. *Science.* 2000;287(5457):1433-8.

46. Ibanez CF. Neurotrophic factors: versatile signals for cell-cell communication in the nervous system. *Results and Problems in Cell Differentiation.* 2000;30:163-88.

47. Althaus HH. Remyelination in multiple sclerosis: a new role for neurotrophins? *Progress in Brain Research.* 2004;146:415-32.

48. Kalinowska-Lyszczarz A, Losy J. The role of neurotrophins in multiple sclerosis-pathological and clinical implications. *International Journal of Molecular Sciences.* 2012;13(10):13713-25.

49. Kolb B. Overview of cortical plasticity and recovery from brain injury. *Physical Medicine and Rehabilitation Clinics of North America.* 2003;14(1):7-25.

50. Markus A, Patel TD, Snider WD. Neurotrophic factors and axonal growth. *Current Opinion in Neurobiology.* 2002;12(5):523-31.

51. Xiao N, Le QT. Neurotrophic factors and their potential applications in tissue regeneration. *Archivum Immunologiae et Therapiae Experimentalis.* 2016;64(2):89-99.

52. Ibanez CF. Neurotrophic factors: from structure-function studies to designing effective therapeutics. *Trends in Biotechnology.* 1995;13(6):217-27.

53. Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nature Medicine.* 2003;9(5):589-95.

54. Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature.* 1987;329(6134):65-8.

55. Fischer W, Bjorklund A, Chen K, Gage FH. NGF improves spatial memory in aged rodents as a function of age. *The Journal of Neuroscience.* 1991;11(7):1889-906.

56. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science.* 2000;290(5492):767-73.

57. Marx J. Neurodegeneration. Huntington's research points to possible new therapies. *Science.* 2005;310(5745):43-5.

58. Ikeda K, Wong V, Holmlund TH, Greene T, Cedarbaum JM, Lindsay RM, et al. Histometric effects of ciliary neurotrophic factor in wobbler mouse motor neuron disease. *Annals of Neurology.* 1995;37(1):47-54.

59. Haase G, Pettmann B, Vigne E, Castelnau-Ptakhine L, Schmalbruch H, Kahn A. Adenovirus-mediated transfer of the neurotrophin-3 gene into skeletal muscle of pmn mice: therapeutic effects and mechanisms of action. J*ournal of the Neurological Sciences.* 1998;160(1):97-105.

60. Beck M, Flachenecker P, Magnus T, Giess R, Reiners K, Toyka KV, et al. Autonomic dysfunction in ALS: a preliminary study on the effects of intrathecal BDNF. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders.* 2005;6(2):100-3.

61. DiStefano PS. Neurotrophic factors in the treatment of motor neuron disease and trauma. *Experimental Neurology.* 1993;124(1):56-9.

62. Yu SJ, Tseng KY, Shen H, Harvey BK, Airavaara M, Wang Y. Local administration of AAV-BDNF to subventricular zone induces functional recovery in stroke rats. *PloS One.* 2013;8(12):81750.

63. Zhang Y, Gao F, Wu D, Moshayedi P, Zhang X, Ellamushi H, et al. Lentiviral mediated expression of a NGF-soluble Nogo receptor 1 fusion protein promotes axonal regeneration. *Neurobiology of Disease.* 2013;58:270-80.

64. Levi-Montalcini R, Angeletti PU. Growth control of the sympathetic system by a specific protein factor. *The Quarterly Review of Biology.* 1961;36:99-108.

65. Lindholm P, Saarma M. Novel CDNF/MANF family of neurotrophic factors. *Developmental Neurobiology*. 2010;70(5):360-71.

66. Abe K. Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury. *Journal of Cerebral Blood Flow and Metabolism.* 2000;20(10):1393-408.

67. Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annual Review of Neuroscience.* 2001;24:677-736.

68. Herpin A, Lelong C, Favrel P. Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. *Developmental and Comparative Immunology.* 2004;28(5):461-85.

69. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science.* 1993;260(5111):1130-

70. Massague J. The transforming growth factor-beta family. *Annual Review of Cell Biology.* 1990;6:597-641.

71. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nature Reviews Neuroscience.* 2002;3(5):383-94.

72. Trupp M, Raynoschek C, Belluardo N, Ibanez CF. Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. *Molecular and Cellular Neurosciences.* 1998;11(1-2):47-63.

73. Walker MJ, Xu XM. History of glial cell line-derived neurotrophic factor (GDNF) and its use for spinal cord injury repair. *Brain Sciences.* 2018;8(6):109-19.

74. Baloh RH, Enomoto H, Johnson EM, Jr., Milbrandt J. The GDNF family ligands and receptors - implications for neural development. *Current Opinion in Neurobiology.* 2000;10(1):103-10.

75. Wang X. Structural studies of GDNF family ligands with their receptors-Insights into ligand recognition and activation of receptor tyrosine kinase RET. *Biochimica et Biophysica Acta.* 2013;1834(10):2205-12.

76. Ibanez CF. Beyond the cell surface: new mechanisms of receptor function. *Biochemical and Biophysical Research Communications.* 2010;396(1):24-7.

77. Springer JE, Seeburger JL, He J, Gabrea A, Blankenhorn EP, Bergman LW. cDNA sequence and differential mRNA regulation of two forms of glial cell line-derived

neurotrophic factor in Schwann cells and rat skeletal muscle. *Experimental Neurology.* 1995;131(1):47-52.

78. Cristina N, Chatellard-Causse C, Manier M, Feuerstein C. GDNF: existence of a second transcript in the brain. *Brain Research Molecular Brain Research.* 1995;32(2):354-7.

79. Schindelhauer D, Schuffenhauer S, Gasser T, Steinkasserer A, Meitinger T. The gene coding for glial cell line derived neurotrophic factor (GDNF) maps to chromosome 5p12-p13.1. *Genomics.* 1995;28(3):605-7.

80. Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK. GDNF, NGF and BDNF as therapeutic options for neurodegeneration. *Pharmacology & Therapeutics.* 2013;138(2):155-75.

81. Saarma M, Sariola H. Other neurotrophic factors: glial cell line-derived neurotrophic factor (GDNF). *Microscopy Research and Technique.* 1999;45(4-5):292-302.

82. Lin LF, Zhang TJ, Collins F, Armes LG. Purification and initial characterization of rat B49 glial cell line-derived neurotrophic factor. *Journal of Neurochemistry.* 1994;63(2):758-68.

83. Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, et al. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(18):8274-8.

84. Schaar DG, Sieber BA, Dreyfus CF, Black IB. Regional and cell-specific expression of GDNF in rat brain. *Experimental Neurology.* 1993;124(2):368-71.

85. Trupp M, Belluardo N, Funakoshi H, Ibanez CF. Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *The Journal of Neuroscience.* 1997;17(10):3554-67.

86. Bohn MC. A commentary on glial cell line-derived neurotrophic factor (GDNF). From a glial secreted molecule to gene therapy. *Biochemical Pharmacology.* 1999;57(2):135-42.

87. Suter-Crazzolara C, Unsicker K. GDNF is expressed in two forms in many tissues outside the CNS. *Neuroreport.* 1994;5(18):2486-8.

88. Oo TF, Ries V, Cho J, Kholodilov N, Burke RE. Anatomical basis of glial cell line-derived neurotrophic factor expression in the striatum and related basal ganglia during postnatal development of the rat. *The Journal of Comparative Neurology.* 2005;484(1):57-67.

89. Hidalgo-Figueroa M, Bonilla S, Gutierrez F, Pascual A, Lopez-Barneo J. GDNF is predominantly expressed in the PV+ neostriatal interneuronal ensemble in normal mouse and after injury of the nigrostriatal pathway. *The Journal of Neuroscience.* 2012;32(3):864-72.

90. d'Anglemont de Tassigny X, Pascual A, Lopez-Barneo J. GDNF-based therapies, GDNF-producing interneurons, and trophic support of the dopaminergic nigrostriatal pathway: Implications for Parkinson's disease. *Frontiers in Neuroanatomy.* 2015;9:10.

91. Eigenbrot C, Gerber N. X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. *Nature Structural Biology.* 1997;4(6):435-8.

92. Parkash V, Goldman A. Comparison of GFL-GFRalpha complexes: further evidence relating GFL bend angle to RET signalling. *Acta Crystallographica Section F, Structural Biology and Crystallization Communications.* 2009;65(6):551-8.

93. Baloh RH, Tansey MG, Johnson EM, Jr., Milbrandt J. Functional mapping of receptor specificity domains of glial cell line-derived neurotrophic factor (GDNF) family ligands and production of GFRalpha1 RET-specific agonists. *The Journal of Biological Chemistry.* 2000;275(5):3412-20.

94. Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, et al. Renal and neuronal abnormalities in mice lacking GDNF. *Nature.* 1996;382(6586):76-9.

95. Kholodilov N, Yarygina O, Oo TF, Zhang H, Sulzer D, Dauer W, et al. Regulation of the development of mesencephalic dopaminergic systems by the selective expression of glial cell line-derived neurotrophic factor in their targets. *The Journal of Neuroscience.* 2004;24(12):3136-46.

96. Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature.* 1995;373(6512):335-9.

97. Kearns CM, Gash DM. GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. *Brain Research.* 1995;672(1-2):104-11.

98. Oppenheim RW, Houenou LJ, Johnson JE, Lin LF, Li L, Lo AC, et al. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature.* 1995;373(6512):344-6.

99. Beck KD, Valverde J, Alexi T, Poulsen K, Moffat B, Vandlen RA, et al. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature.* 1995;373(6512):339-41.

100. Wang CY, Yang F, He X, Chow A, Du J, Russell JT, et al. Ca(2+) binding protein frequenin mediates GDNF-induced potentiation of Ca(2+) channels and transmitter release. *Neuron.* 2001;32(1):99-112.

101. Ibanez CF. Structure and physiology of the RET receptor tyrosine kinase. *Cold Spring Harbor Perspectives in Biology.* 2013;5(2).

102. Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, et al. Characterization of a multicomponent receptor for GDNF. *Nature.* 1996;382(6586):80-3.

103. Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, et al. Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature.* 1996;381(6585):785-9.

104. Airaksinen MS, Titievsky A, Saarma M. GDNF family neurotrophic factor signaling: four masters, one servant? *Molecular and Cellular Neurosciences.* 1999;13(5):313-25.

105. Ugarte SD, Lin E, Klann E, Zigmond MJ, Perez RG. Effects of GDNF on 6-OHDA-induced death in a dopaminergic cell line: modulation by inhibitors of PI3 kinase and MEK. *Journal of Neuroscience Research.* 2003;73(1):105-12.

106. Encinas M, Tansey MG, Tsui-Pierchala BA, Comella JX, Milbrandt J, Johnson EM, Jr. c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. *The Journal of Neuroscience.* 2001;21(5):1464-72.

107. Paratcha G, Ledda F, Ibanez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell.* 2003;113(7):867-79.

108. Kramer ER, Liss B. GDNF-Ret signaling in midbrain dopaminergic neurons and its implication for Parkinson disease. *FEBS Letters.* 2015;589(24):3760-72.

109. Sawada H, Ibi M, Kihara T, Urushitani M, Nakanishi M, Akaike A, et al. Neuroprotective mechanism of glial cell line-derived neurotrophic factor in mesencephalic neurons. *Journal of Neurochemistry.* 2000;74(3):1175-84.

110. Li X, Peng C, Li L, Ming M, Yang D, Le W. Glial cell-derived neurotrophic factor protects against proteasome inhibition-induced dopamine neuron degeneration by suppression of endoplasmic reticulum stress and caspase-3 activation. *The Journals of Gerontology Series A, Biological Sciences and Medical Sciences.* 2007;62(9):943-50.

111. Abe K, Hayashi T. Expression of the glial cell line-derived neurotrophic factor gene in rat brain after transient MCA occlusion. *Brain Research.* 1997;776(1-2):230-4.

112. Kitagawa H, Sasaki C, Zhang WR, Sakai K, Shiro Y, Warita H, et al. Induction of glial cell line-derived neurotrophic factor receptor proteins in cerebral cortex and striatum after permanent middle cerebral artery occlusion in rats. *Brain Research.* 1999;834(1-2):190-5.

113. Sarabi A, Chang CF, Wang Y, Hoffer BJ, Morales M. Time course study of GFRalpha-1 expression in an animal model of stroke. *Experimental Neurology.* 2001;170(2):283-9.

114. Curcio M, Salazar IL, Inacio AR, Duarte EP, Canzoniero LM, Duarte CB. Brain ischemia downregulates the neuroprotective GDNF-Ret signaling by a calpain-dependent mechanism in cultured hippocampal neurons. *Cell Death & Disease.* 2015;6:1645.

115. Duarte EP, Curcio M, Canzoniero LM, Duarte CB. Neuroprotection by GDNF in the ischemic brain. *Growth Factors.* 2012;30(4):242-57.

116. Wang Y, Lin SZ, Chiou AL, Williams LR, Hoffer BJ. Glial cell line-derived neurotrophic factor protects against ischemia-induced injury in the cerebral cortex. The *Journal of Neuroscience.* 1997;17(11):4341-8.

117. Kitagawa H, Hayashi T, Mitsumoto Y, Koga N, Itoyama Y, Abe K. Reduction of ischemic brain injury by topical application of glial cell line-derived neurotrophic factor after permanent middle cerebral artery occlusion in rats. *Stroke.* 1998;29(7):1417-22.

118. Zhang WR, Hayashi T, Iwai M, Nagano I, Sato K, Manabe Y, et al. Time dependent amelioration against ischemic brain damage by glial cell line-derived neurotrophic factor after transient middle cerebral artery occlusion in rat. *Brain Research.* 2001;903(1-2):253-6.

119. Abe K, Hayashi T, Itoyama Y. Amelioration of brain edema by topical application of glial cell line-derived neurotrophic factor in reperfused rat brain. *Neuroscience Letters.* 1997;231(1):37-40.

120. Cheng H, Huang SS, Lin SM, Lin MJ, Chu YC, Chih CL, et al. The neuroprotective effect of glial cell line-derived neurotrophic factor in fibrin glue against chronic focal cerebral ischemia in conscious rats. *Brain Research.* 2005;1033(1):28-33.

121. Shang J, Deguchi K, Ohta Y, Liu N, Zhang X, Tian F, et al. Strong neurogenesis, angiogenesis, synaptogenesis, and antifibrosis of hepatocyte growth factor in rats brain after transient middle cerebral artery occlusion. *Journal of Neuroscience Research.* 2011;89(1):86-95.

122. Kobayashi T, Ahlenius H, Thored P, Kobayashi R, Kokaia Z, Lindvall O. Intracerebral infusion of glial cell line-derived neurotrophic factor promotes striatal neurogenesis after stroke in adult rats. *Stroke; A Journal of Cerebral Circulation.* 2006;37(9):2361-7.

123. Kilic U, Kilic E, Dietz GP, Bahr M. Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice. *Stroke; a Journal of Cerebral Circulation.* 2003;34(5):1304-10.

124. Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *Journal of the Neurological Sciences.* 2001;189(1-2):49-57.

125. Ou Y, Yu S, Kaneko Y, Tajiri N, Bae EC, Chheda SH, et al. Intravenous infusion of GDNF gene-modified human umbilical cord blood CD34+ cells protects against cerebral ischemic injury in spontaneously hypertensive rats. *Brain Research.* 2010;1366:217-25.

126. Yang C, Zhou L, Gao X, Chen B, Tu J, Sun H, et al. Neuroprotective effects of bone marrow stem cells overexpressing glial cell line-derived neurotrophic factor on rats with intracerebral hemorrhage and neurons exposed to hypoxia/reoxygenation. *Neurosurgery.* 2011;68(3):691-704.

127. Tsai TH, Chen SL, Chiang YH, Lin SZ, Ma HI, Kuo SW, et al. Recombinant adeno-associated virus vector expressing glial cell line-derived neurotrophic factor reduces ischemia-induced damage. *Experimental Neurology.* 2000;166(2):266-75.

128. Hermann DM, Kilic E, Kugler S, Isenmann S, Bahr M. Adenovirus-mediated GDNF and CNTF pretreatment protects against striatal injury following transient middle cerebral artery occlusion in mice. *Neurobiology of Disease.* 2001 (4):655-66.

129. pLenti-CMV-GFP-2A-Puro vector details: ABM Goods; [cited 2018 12 November]. Available from: https://www.abmgood.com/vectors/vectorDisplay.php?vec=pLenti-CMV-GFP-2A-Puro&page=prop.

130. Kilic E, Reitmeir R, Kilic U, Caglayan AB, Beker MC, Kelestemur T, et al. HMG-CoA reductase inhibition promotes neurological recovery, peri-lesional tissue remodeling, and contralesional pyramidal tract plasticity after focal cerebral ischemia. *Frontiers in Cellular Neuroscience.* 2014;8:422.

131. Reitmeir R, Kilic E, Reinboth BS, Guo Z, ElAli A, Zechariah A, et al. Vascular endothelial growth factor induces contralesional corticobulbar plasticity and functional neurological recovery in the ischemic brain. *Acta Neuropathologica.* 2012;123(2):273-84.

132. Beker MC, Caglayan AB, Kelestemur T, Caglayan B, Yalcin E, Yulug B, et al. Effects of normobaric oxygen and melatonin on reperfusion injury: role of cerebral microcirculation. *Oncotarget.* 2015;6(31):30604-14.

133. Gratzner HG. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science.* 1982;218(4571):474-5.

134. Bond AM, Ming GL, Song H. Adult mammalian neural stem cells and neurogenesis: Five decades later. *Cell Stem Cell.* 2015;17(4):385-95.

135. Chinta SJ, Andersen JK. Dopaminergic neurons. *The International Journal of Biochemistry & Cell Biology.* 2005;37(5):942-6.

136. Tritsch NX, Ding JB, Sabatini BL. Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. *Nature.* 2012;490(7419):262.

137. Seamans JK, Yang CR. The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Progress in Neurobiology.* 2004;74(1):1-57.

138. Stromberg I, Bjorklund L, Johansson M, Tomac A, Collins F, Olson L, et al. Glial-Cell Line-Derived Neurotrophic Factor Is Expressed in the Developing but Not Adult Striatum and Stimulates Developing Dopamine Neurons in-Vivo. *Experimental Neurology.* 1993;124(2):401-12.

139. Springer JE, Mu X, Bergmann LW, Trojanowski JQ. Expression of GDNF mRNA in rat and human nervous tissue. *Experimental Neurology.* 1994;127(2):167-70.

140. Barroso-Chinea P, Cruz-Muros I, Aymerich MS, Rodriguez-Diaz M, Afonso-Oramas D, Lanciego JL, et al. Striatal expression of GDNF and differential vulnerability of midbrain dopaminergic cells. *European Journal of Neuroscience.* 2005;21(7):1815-27.

141. Zhang WR, Sato K, Iwai M, Nagano I, Manabe Y, Abe K. Therapeutic time window of adenovirus-mediated GDNF gene transfer after transient middle cerebral artery occlusion in rat. *Brain Research.* 2002;947(1):140-5.

142. Kitagawa H, Sasaki C, Sakai K, Mori A, Mitsumoto Y, Mori T, et al. Adenovirus-mediated gene transfer of glial cell line-derived neurotrophic factor prevents ischemic brain injury after transient middle cerebral artery occlusion in rats. *Journal of Cerebral Blood Flow and Metabolism.* 1999;19(12):1336-44.

143. Kokaia Z, Thored P, Arvidsson A, Lindvall O. Regulation of stroke-induced neurogenesis in adult brain--recent scientific progress. *Cerebral Cortex.* 2006;16(1):162-7.

144. Liu Y, Wang S, Luo S, Li Z, Liang F, Zhu Y, et al. Intravenous PEP-1-GDNF is protective after focal cerebral ischemia in rats. *Neuroscience Letters.* 2016;617:150-5.

145. Silver J, Miller JH. Regeneration beyond the glial scar. *Nature Reviews Neuroscience.* 2004;5(2):146-56.

146. Rosen HJ, Viskontas IV. Cortical neuroanatomy and cognition. *Handbook of Clinical Neurology*. 2008;88:41-60.

147. Yamaguchi Y. Brevican: a major proteoglycan in adult brain. *Perspectives on Developmental Neurobiology.* 1996;3(4):307-17.

148. Margolis RU, Margolis RK. Aggrecan-versican-neurocan family proteoglycans. *Methods in Enzymology*. 1994;245:105-26.

149. Leonardo CC, Eakin AK, Ajmo JM, Gottschall PE. Versican and brevican are expressed with distinct pathology in neonatal hypoxic-ischemic injury. *Journal of Neuroscience Research.* 2008;86(5):1106-14.

150. Jones LL, Sajed D, Tuszynski MH. Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: A balance of permissiveness and inhibition. *Journal of Neuroscience.* 2003;23(28):9276-88.

151. Batista CM, Bianqui LLT, Zanon BB, Ivo MMAA, de Oliveira GP, Maximino JR, et al. Behavioral improvement and regulation of molecules related to neuroplasticity in ischemic rat spinal cord treated with PEDF. *Neural Plasticity.* 2014; 2014: 451639.

152. McClelland AC, Sheffler-Collins SI, Kayser MS, Dalva MB. Ephrin-B1 and ephrin-B2 mediate EphB-dependent presynaptic development via syntenin-1. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(48):20487-92.

153. Giger RJ, Hollis ER, Tuszynski MH. Guidance molecules in axon regeneration. *Cold Spring Harbor Perspectives in Biology*. 2010;2(7):001867.

APPENDIX A: ETHICAL APPROVAL FORM

BEZMİÂLEM
VAKIF ÜNİVERSİTESİ

T.C.
BEZMİÂLEM VAKIF ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
KARAR METNİ

SAYI: 2016/252 26.10.2016
KONU: Sn.Ar.Gör.Merve BEKER

Sayın, Ar.Gör.Merve BEKER

"Farede geçici fokal serebral iskemi sonrası lenti-viral vektör aracılı GDNF ekspresyonunun fonksiyonel iyileşme, beyin plastisitesi ve nörogenez üzerine etkilerinin incelenmesi" başlıklı projenize ait başvurunuz 26.10.2016 tarihinde yapılan Yerel Etik Kurul toplantısında değerlendirilmiş ve onanmıştır.

Doç. Dr. Fahri AKBAŞ
Etik Kurul Başkanı

Prof. Dr. Ahmet BELCE
Üye

Prof. Dr. Erhan AYŞAN
Üye

Prof. Dr. İsmail MERAL
Üye

Yrd. Doç.Dr.Fatemeh BAHADORİ
Üye

Yrd.Doç.Dr. Ömer UYSAL
Üye
KATILMADI

Vet. Hek. Mert ÇELİKTEN
Üye

Harun SARIKAMIŞ
Üye

Şerife GÖNCÜ
Üye

- Etik kurulumuzdan onam alan her proje için, çalışma başlamadan üç ay önce çalışılacak hayvan rezervinin uygunluğunu (tür, yaş, cinsiyet) belirlemek amacıyla Deney Hayvanları Laboratuvarına başvurulmalıdır.