PROTECTIVE MECHANISMS OF GRANULOCYTE-COLONY STIMULATING FACTOR AGAINST EXPERIMENTAL MODELS OF STROKE

by

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Jang-Yen Wu, Department of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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Title: Protective Mechanisms of Granulocyte-Colony Stimulating Factor Against Experimental Models of Stroke

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Ischemic stroke has a multiplicity of pathophysiological mechanisms. Granulocyte-colony stimulating factor (G-CSF) is an endogenous growth factor that exerts a diverse range of neuroprotection against ischemic stroke. Several lines of evidence demonstrated the contribution of endoplasmic reticulum (ER) in apoptotic cell death involving ischemia. Cell culture of undifferentiated PC12 cells were subjected to 10mM glutamate and selected doses of G-CSF (25ng/ml, 50ng/ml, 100ng/ml and 250ng/ml) for 24 hours. Cell viability, expression of the G-CSF receptor and expression level of CHOP were assessed *in vitro*. Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO). Rats were subcutaneously injected with G-CSF (n= 15; 50ug/kg body weight) 24 hours post-MCAO for 4 days. Vehicle treated rats were administered 5% dextrose for 1 day (n=4) or 4 days (n=16). Sham-operated rats (n=9) were not subjected to MCAO. Neurological deficit and infarct volume were measured

while expression levels of pAKT, Bcl2, Bax, Bak, cleaved caspase-3, GRP78, ATF4, ATF6, p-p38MAPK, pJNK, CHOP and HSP27 were analyzed by western blotting. *In vitro* G-CSF receptor was expressed on undifferentiated PC12 cell, and an optimal dose of 50 ng/ml G-CSF significantly protected these cells against glutamate-induced cytotoxicity ($P < 0.05$). G-CSF significantly down-regulated ($P < 0.01$) the ER stress-induced pro-apoptotic marker CHOP *in vitro*. In vivo, G-CSF reduced infarct volume to 50% while significantly improved neurological deficit compared to vehicle rats. G-CSF significantly ($P < 0.05$) up-regulated pro-survival proteins pAKT and Bcl2 while down-regulating pro-apoptotic proteins Bax, Bak and cleaved caspase 3 in the ischemic brain. It also significantly ($P < 0.05$) downregulated the ER intraluminal stress sensor GRP78, proteins of ER stress induced intracellular pathway; ATF4, ATF6, p-p38MAPK, pJNK and the ER stress induced apoptotic marker CHOP, which suggests that ER stress is being ameliorated by G-CSF treatment. G-CSF also reduced the level of HSP27, providing additional evidence of cellular stress reduction. G-CSF treatment increased cell survival by attenuating both general pro-apoptotic proteins and specific effector proteins in the ER stress induced apoptotic pathways. Our data has provided new insight into the anti-apoptotic mechanism of G-CSF, especially as it relates to ER stress induced apoptosis in ischemia.

DEDICATION

This dissertation is dedicated to first and foremost to my Lord and Savior Jesus Christ, whom I know has been my source of strength throughout the preparation of this dissertation. I am also dedicating this dissertation to my parents; my father, Mr. Withel Menzie and my mother, though no longer with me, Mrs. Emlin Menzie. These two people are/ have been the most supportive and caring parents I am/was blessed to have in my life. To my wonderful husband, Pastor David Suderam, who cried with me, prayed with me and gave me unlimited support and encouragement during the latter years of completing this work – love you lots. And last but not least to my present church family at First Alliance International church, who prayed for me constantly, especially when the going was really tough. To God be the Glory, great things He has done.

PROTECTIVE MECHANISMS OF GRANULOCYTE-COLONY STIMULATING FACTOR AGAINST EXPERIMENTAL MODELS OF STROKE

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LIST OF ABBREVIATIONS

AKT/PKB:	Protein kinase B
AMPA:	Amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA:	Analysis of variance
APAF1:	Apoptotic protease activity factor-1
ATF4:	Activating transcription factor 4
ATF6:	Activating transcription factor 6
ATP:	Adenosine 5 triphosphate
BAK:	Bcl-2 antagonist/killer
BAX:	Bcl-2 associated protein X
BCL2:	B cell lymphoma 2
BCL2-xL	B cell lymphoma 2 extra long
BDNF:	Brain-derived neurotrophic factor
BFGF:	Basic fibroblast growth factor
BH3:	Bcl-2 homology domain 3
BH:	Bcl-2 homology

BiP:	Binding immunoglobulin protein
CCA:	Common carotid artery
Caspase:	Cysteine aspartic acid protease
CHOP:	C/EBP homologous protein
ERK1/2/5:	Extracellular signaling regulated kinase 1,2 & 5
DNA:	Deoxyribonucleic acid
EIF2α:	Eukaryotic translation initiation factor 2 alpha
ECA:	External carotid artery
ERAD:	Endoplasmic reticulum associate degradation
GADD135:	Growth arrest and DNA damage-inducible 135
GAPDH:	Glyceraldehayde-3-phosphade dehydrogenase
G-CSF:	Granulocyte-colony stimulating factor
G-CSFR:	Granulocyte-colony stimulating factor receptor
GRP 78:	Glucose-regulated protein 78
GSH:	Reduced glutathione
ICA:	Internal carotid artery
IGF-1:	Insulin-like growth factor
IRE1α:	Inositol-requiring protein-1alpha
IP3R:	Inositol 1,4,5-trisphosphate receptor

JAK:	Janus kinase
JNK:	cJUN NH2-terminal kinase
NMDA:	*N*-methyl-D-aspartate
MAM:	Mitochondria-associated ER membrane
MCA:	Middle cerebral artery
MCAO:	Middle cerebral artery occlusion
OMM:	Outer mitochondrial membrane
PACA:	Proximal anterior cerebral artery
PCA:	Posterior communicating artery
PERK:	Protein kinase RNA (PKR)-like ER kinase
PDK:	Phosphoinositide dependent protein kinase
PPA:	Pterygopalatine artery
PI3K:	Phosphatidylinositol-3-kinase
P38MAPK:	Mitogen-activated protein kinase
PUMA:	p53-upregulated modulator of apoptosis
STAT3:	Signal transducer and activator of transcription
RCBF:	Regional cerebral blood flow
TRAF2:	Tumor necrosis factor-α receptor-associated factors 2
XBP-1:	X-Box-binding protein

1. INTRODUCTION

1.1 Stroke

Stroke is one of the world's leading cause of death and disability (Feigin et al. 2009; Strong et al. 2007). The World Health Organization (WHO) reported that 5.71 million people died of stroke in 2004 with an estimated increase to 7.8 million by 2030 (MacKay, and Mensah 2004). In keeping with this estimated trajectory of stroke, it was also reported that within the next decade there will be a 12% global rise in stroke morbidity (Rosamond et al. 2007) with an estimated 8% -10% possibility of developing stroke during a lifetime (Seshadri et al. 2006). This is due to an increase in unhealthy life style such as smoking, physical inactivity, unhealthy diets which results in obesity, hypertension, hypercholesterolemia and type II diabetes (Garber 2007)

There are two types of stroke, one pertains to vessel occlusion (ischemic stroke), while the other is primarily due to intracerebral bleeding (hemorrhage stroke). Of these two types of strokes, 85% occurs as ischemic stroke, while the remainder as hemorrhage stroke (Beal 2010). Ischemic stroke may result from either a transient or a permanent reduction in cerebral blood flow, and may be global (global ischemia) due to cardiac arrest or focal (focal ischemia) due to a blockage in a specific blood vessel by either an embolus or by thrombosis

The pathophysiology of ischemic stroke involves an insufficient supply of oxygen and glucose which leads to unsustainable cellular homeostasis and initiated cell injury Cellular injury progresses as a result of excitotoxicity, ionic imbalance, oxidative and nitrosative stresses, endoplasmic reticulum (ER) stress and mitochondrial disturbances, ultimately resulting in programmed cell death, apoptosis, and necrosis (Hossmann et al. 1994). The cerebral tissue undergoing an ischemic insult has two regions, (1) an inner central region termed the core and (2) a surrounding perifocal region termed the penumbra (Fig.1)

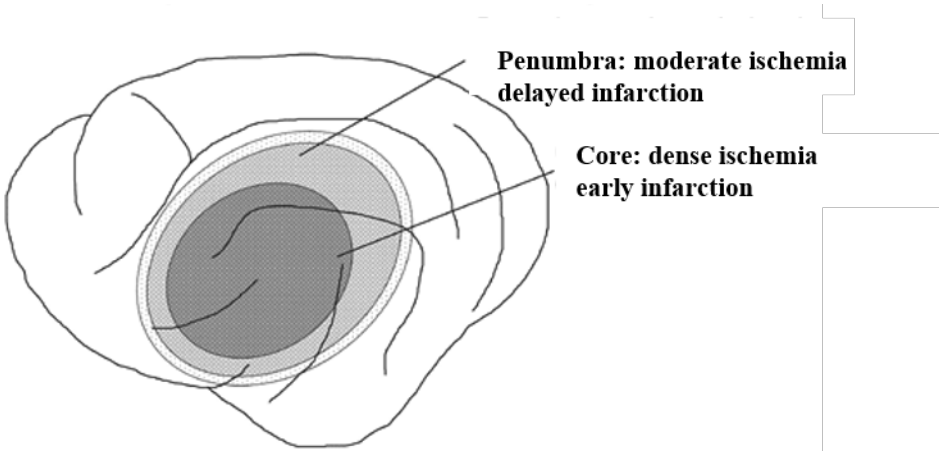


Figure 1. Illustrative diagram showing core and penumbra of ischemic tissue. ***(Adapted from Heiss, 2001)***

The blood flow in the core of the ischemic tissue (which represents the infarct) is severely compromised and both neurons and glial are subjected to apoptosis and necrosis, with necrosis being the predominant type of cell death (Smith 2004). The surrounding penumbra is supplied by collateral blood vessels and is therefore less adversely affected by the ischemia. The cells in the penumbra retain an active metabolic state and hence

are very susceptible to apoptosis, but can be readily rescued by early therapeutic interventions (Astrup, et al. 1981; Hossmann, 1994; Heiss et al., 1998). If the ischemic process is not arrested, the ischemic core will recruit the perifocal penumbra by a process called “spreading depression”. Spreading depression is a recurrent wave of depolarization initiated in the ischemic core that triggers intermittent depolarization in the penumbra resulting in an increase of the infarct volume (Fig. 2).

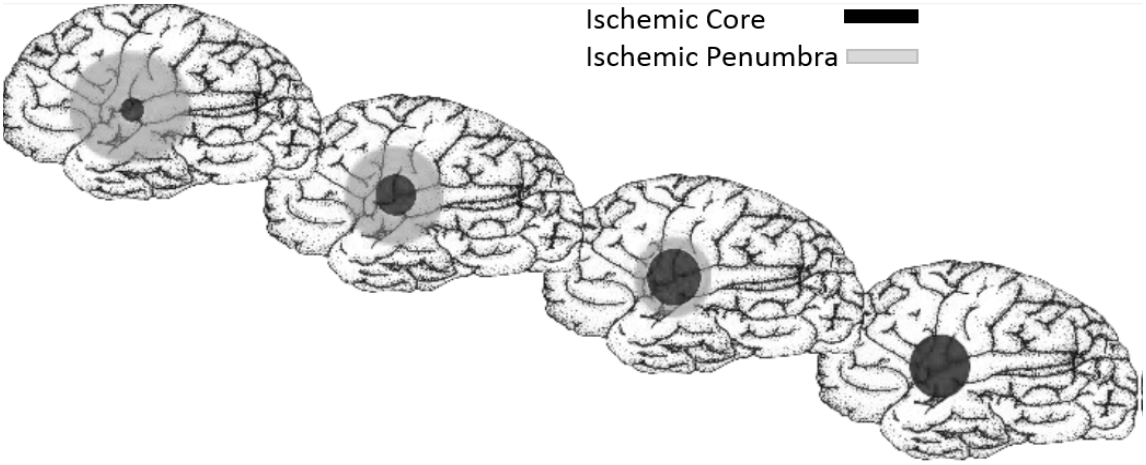


Figure 2. Represented diagram of spreading depression.

Cells within the ischemic core (black circle) can undergo anoxic depolarization without repolarizing, which triggers the cells in the surrounding penumbra (grey circle) to experience intermittent depolarization, resulting in the recuitment of penumbral tissue into the infarctcore. ***(Adapted from Dirnagl et al., 1999)***

1.2. Pathophysiologic mechanisms of ischemic stroke

The blockage of an artery in ischemic stroke initiates a series of multistep pathophysiologic events referred to as the ischemic cascade (Fig. 3). The mechanisms involve in the ischemic cascade are; energy failure, increased level of intracellular calcium ions (Ca^{2+}), excitotoxicity, dysfunction of cellular organelles such as the endoplasmic reticulum and mitochondrion, generation of reactive oxygen species, inflammation, disruption of the blood brain barrier (BBB) and apoptosis (Durukan and

Tatlisumak 2007). Within 10 - 20 sec. of an ischemic insult there is a loss of consciousness and loss of neuronal electrical activity within the ischemic area (Kristian and Siesjo 1998). This initial 20sec. is followed by the failure of energy-dependent pumps; such as the Na^+/K^+-ATPase and Ca^{2+}-ATPase pump, in addition to the Na^{+}-Ca^{2+} transporter being reversed (Reuter & Philipson, 2002; Phan et al.2002).

Due to impairment of the energetics that are required to maintain ionic gradients, ion homeostasis becomes dysfunctional (Martin et al. 1994; Kalogeris et al., 2012) Increased influx of sodium ions ($Na^{+)}$ and reduced efflux of potassium ions (K^+) induce membrane depolarization of neurons and glia, resulting in a subsequent increase of Ca^{2+} influx through voltage gated calcium channels (VGCC) (Katsura et al. 1994; Cross et al., 2010) and the subsequent release of the excitatory amino acid glutamate into the extracellular space from presynaptic neuronal terminals (Phillis & O'Regan 2003). Another source that accounts for the increased accumulation of extracellular glutamate is the reversal of the neuronal and astrocytic glutamate transporter (GLT-1) (Krzyżanowska et al., 2014). The reversal of GLT-1 occurs as a result of an increase in intracellular Na^+ and extracellular K^+ (Szatkowski et al. 1990; Jabaudon, et al., 200; Rossi et al. 2000). In addition to direct neurotoxicity by excessive extracellular glutamate, activation of ionotropic glutamate receptors [NMDA (*N*-methyl-D-aspartate), AMPA (aamino-3-hydroxy-5-methyl-4-isoxazolepropionate), and kainate] and metabotropic glutamate receptors (mGluRs) further augments the increased level of intracellular Ca^{2+} (Meldrum 2000). Interestingly AMPA/Kainate receptors are not normally permeable to Ca^{2+} but ischemia activates a population of AMPA receptors that are Ca^{2+} permeable (Kwak and Weiss 2006). The excess activation of these glutamate receptors results in an overload of

intracellular calcium. Excessive intracellular calcium is also a by-product of acidosis (fall from pH 7.3 to 6.2); a condition that develops due to the production of lactic acid during anaerobic metabolism in the ischemic region. Although acidosis may be a direct cause of cell death by either necrosis or apoptosis, it also augments increased intracellular calcium through acid-sensing ion channels (Ding et al. 2000).

Elevated intracellular calcium $[Ca^{2+}]_i$ initiates cellular events, by activating catabolic enzymes such as proteases (Araújo et al. 2004), phospholipases and endonucleases which initiate the development of cellular injury and cell death. For example, increases in $[Ca^{2+}]_i$ activate phospholipase A2 (PLA_2) which acts on membrane phospholipids, altering membrane structure and rendering it more permeable (Farooqui, et al. 2002; Adibhatla & Hatcher, 2008).

The endoplasmic reticulum (ER) and the mitochondrion are two cellular calcium storage organs that are intricately involved in intracellular calcium signaling (Wulf Paschen and Mengesdorf 2005). Both organelles become dysfunctional in response to high levels of calcium, resulting in cell death by apoptosis and necrosis. Activation of mGluRs by excessive extracellular glutamate results in the mobilization of Ca^{2+} from the endoplasmic reticulum (ER). This action is GTP-binding protein-dependent and results in the binding of inositol, 1,4,5-triphosphate (IP_3) to its receptor (IP_3R) on the membrane of the endoplasmic reticulum. The ryanodine receptor (RyR), located on the ER membrane will also release Ca^{2+} from the ER via a calcium-induced-calcium-release

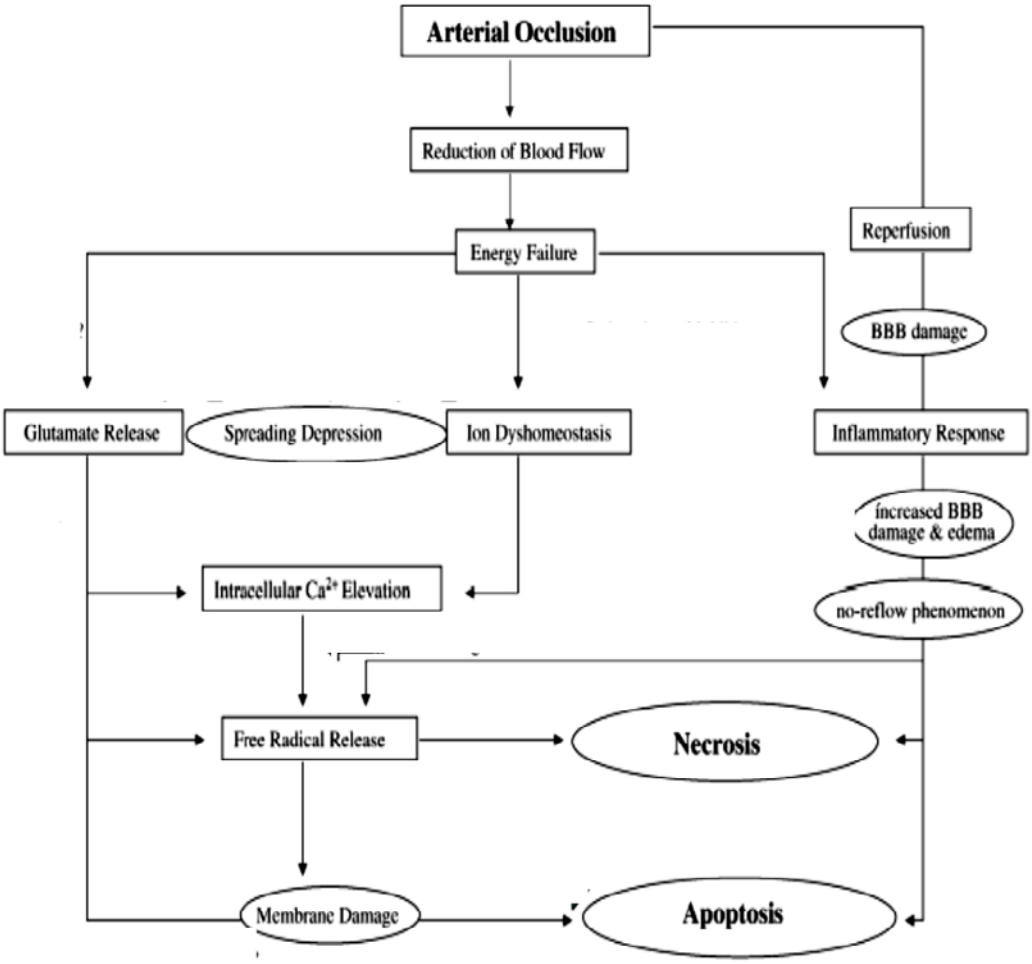


Figure 3. Ischemic Cascade.

The mechanisms in the ischemic cascade are: energy failure, increased level of intracellular calcium ions (Ca^{2+}), excitotoxicity, generation of reactive oxygen species, inflammation, disruption of the blood brain barrier (BBB) and apoptosis ***(Adapted from Durukan & Tatlisumak, 2007)***

mechanism (Berridge, 1995; Sukhareva et al., 2002). A reduction of ER calcium disrupts the function of the ER, such as protein folding, which is calcium dependent. This imbalance in ER calcium homeostasis, if not remedied, propagates ER stress and resultant apoptosis (Paschen & Mengesdorf, 2005).

One important physiological function of the mitochondria is to sequester cytosolic calcium. The mitochondrion becomes dysfunctional in brain ischemia due to

intramitochondrial calcium overload, which may disrupts the electron transfer chain (ETC) resulting in an excessive production of free radicals/reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Duan et al., 2007; Alleyne et al., 2011).

The production of ROS is augmented in the reperfusion phase of ischemia due to the reintroduction of oxygen to the injured site. Un-scavenged excessive ROS causes protein- and lipid-oxidation, interfering with membrane structure and causing DNA damage which inevitably leads to apoptotic or necrotic cell death (Lipton, 1999;Nakka, et al., 2008). Not only are oxygen radicals important signaling molecules that trigger apoptosis but they play an active role in the inflammatory process as well (Dirnagl, et al., 1999).

Ischemic injury also triggers an inflammatory response which may potentiate tissue damage by various mechanisms. For instance an upregulation of gene expression of inflammatory mediators, platelet-activating factor, tumor necrosis factor α, and interleukin 1 β, occurs after the ischemic insult. While the vascular endothelium promotes inflammation through the upregulation of adhesion molecules such as inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin, bind to circulating leukocytes and facilitate their migration into the brain tissue. On entering the brain tissue, leukocytes may initiate the production of the toxic molecules; nitric oxide and superoxide, which increases brain injury. The adverse effect of having leukocytes enter the brain tissue is debatable, since leukocytes such as macrophages can aid in the inflammatory condition by scavenging necrotic debris (Emerich et al. 2002; Danton & Dietrich, 2003) .

The pathogenesis of ischemic stroke also involves a deficiency of inhibitory GABAergic mechanisms due to the activation of the mGlu1 receptor located on GABAergic neurons. Extracellular glutamate activation of presynaptic mGlu1 receptors on GABAergic interneurons suppresses the release of GABA (Pellegrini-Giampietro 2003) resulting in a subsequent imbalance between the excitatory and inhibitory neurotransmitter.

1.3. Endoplasmic reticulum stress in ischemic stroke

The endoplasmic reticulum is an essential organelle responsible for calcium storage and signaling, calcium-dependent processes such as the folding and processing of synthesized proteins and lipid biosynthesis (Kuznetsov & Brostrom, 1992; Verkhratsky & Toescu, 2003; Pizzo & Pozzan, 2007). The implications of endoplasmic reticulum (ER) stress in neurological diseases remain to be fully elucidated. However, in the last decade a growing body of evidence indicates that ER stress/dysfunction leads to apoptosis in ischemic stroke and other neurological diseases such as Alzheimer disease (AD) and Parkinson's disease (PD) (DeGracia & Montie, 2004; Hoozemans et al., 2007; Yang & Paschen, 2009; Pan et al., 2010)

During ischemia excessive excitation of the metabotropic glutamate receptor 1 (mGluR1) by extracellular glutamate causes the associated enzyme phospholipase C to hydrolyze phosphoinositide phospholipids in the plasma membrane resulting in the production of inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol. Inositol 1,4,5-trisphosphate receptor (IP_3R), opens up calcium channel once IP_3 binds to it, and releases calcium from the ER [$[Ca^{2+}]_{ER}$]. This depletes $[Ca^{2+}]_{ER}$ which interrupts the normal function of the ER (Endoh 2004). Another source that contributes to ER dysfunction

during ischemia is the over production of reactive oxygen species (ROS) which is compounded by reperfusion (Murphy et al., 1999; Kalogeris et al., 2012). Both conditions interfere with the protein folding machinery of the ER, resulting in a build up of unfolded or misfolded proteins.

Unfolded/misfolded proteins activate the unfolded protein response (UPR) (Malhotra and Kaufman, 2007) which either improves local protein folding by triggering the expression of genes to control the level of unfolded/misfolded proteins or triggers apoptotic pathways which results in cell death (Patil and Walter 2001). The UPR functions via the activation of three stress sensors on the ER membrane: double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1α (IRE1 α). The sensing mechanism is initiated by the ER chaperone, glucose regulated protein 78 (GRP78), which recognizes unfolded proteins and then dissociates from each of the three sensing molecules, releasing them from their inactive state (Mercado 2013). Each of the three ER stress sensors (PERK, IRE1 α and ATF6), once released from Grp78 will in turn activate cytosolic pathways that will upregulate UPR genes and ER-associated degradation (ERAD) genes that will degrade the unfolded protein in the ER (Pan, 2011) (Fig. 4). Activated PERK phosphorylates eukaryotic initiation factor 2 α (eIF2 α) which induces suppression of global protein synthesis while also increasing translation of selective mRNA such as activating transcription factor 4 (ATF-4) (Mercado 2013). ATF4 induces the expression of genes involved in restoring ER homeostasis as well as expression of genes involved in cell death. Activated IRE1 α signals to regulate the mRNA for the transcription factor X box binding protein (XBP1). XBP1 is a transcription factor responsible for regulating a

specific subset of UPR target genes, involved in restoring protein folding or degrading unfolded proteins (Hetz 2012). A second function of activated IRE1 α is to bind cytosolic proteins, TNF receptor associated factor 2 (TRAF2) and activate signaling pathways known as alarm pathways involving: apoptosis signal regulating kinase 1 (ASK1), downstream kinases including mitogen activated protein kinase (p-38 MAPK), and Jun-N- terminal kinase (JNK) resulting in activation of autophagy and apoptosis (Mercado 2013). The third stress sensor, ATF6, once released from Grp 78, translocates to the golgi apparatus, where it undergoes proteolytic cleavage before translocating to the nucleus to contribute to the induction of protein quality control genes (Paschen & Mengesdorf 2005).

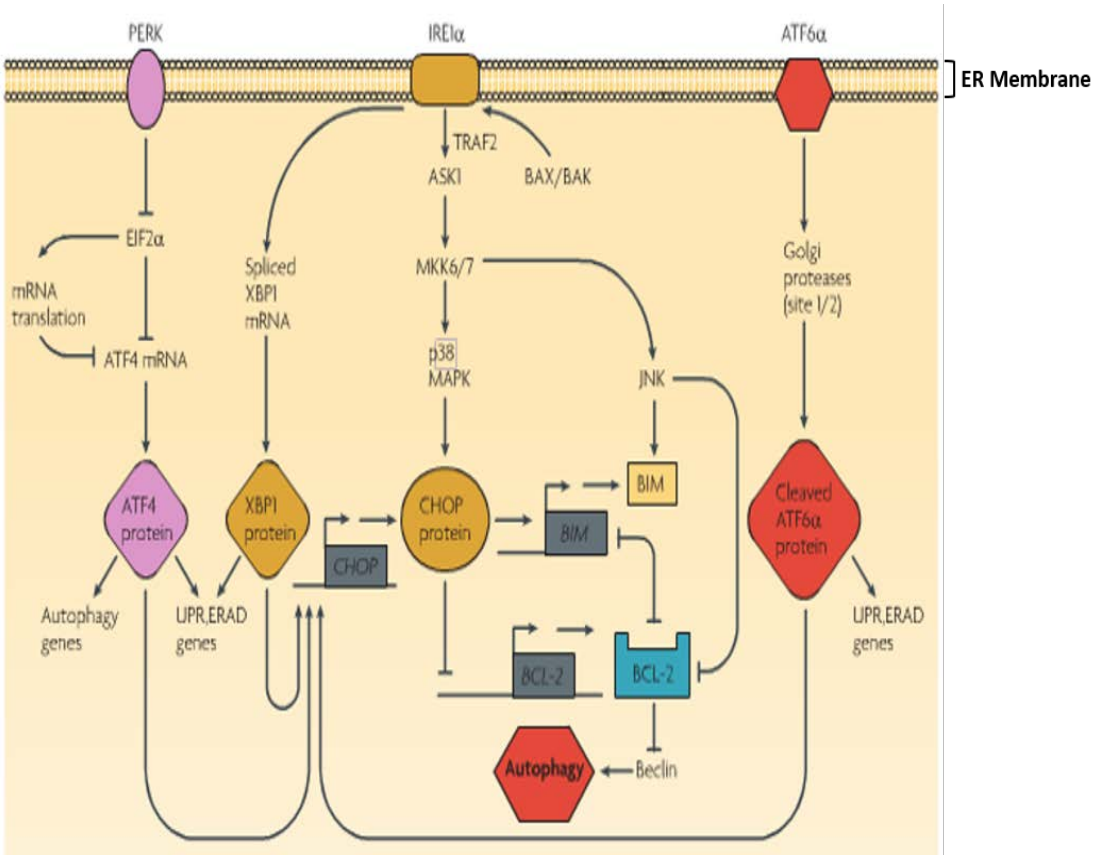


Figure 4. Unfolded protein response and endoplasmic reticulum stress pathway. ***(Adapted from Kim et al., 2008)***

In the case of severe stress as found with ischemic stroke, the regulating effect of the UPR to restore ER homeostasis is dominated by an apoptotic effect of the UPR in which all three stress sensing pathways upregulate the expression of the transcription factor, [CCAAT-enhancer-binding proteins (C/EBP) homologous protein] (CHOP) (Fig.4) also known as growth arrest and DNA damage inducible protein 153 (GADD 153) (Ron and Walter 2007). CHOP is a transcription factor that regulates the balance between pro-apoptotic members and the anti-apoptotic members of the B-cell leukemia/lymphoma 2 (Bcl2) family (Ferri and Kroemer 2001). CHOP suppresses the expression of anti-apoptotic protein Bcl2 while potentiating the expression of the pro-apoptotic protein Bim (a member of the BH3-only protein family).

1.4. Animal model of transient focal ischemic stroke

The most commonly used animal in non-transgenic stroke research is the rat. In addition to being acceptable from an ethical perspective when compared to larger animals, the anatomy and physiology of the rat's cerebrovascular is similar to humans (Macrae, 1992). Clinical relevancy of any research model is an important criteria when using animal models to mimic a disease and should take into consideration how close the model is in resembling the human disease. While permanent stroke models allow for the study of stroke without the effect of reperfusion, the transient focal ischemic model patterns the effect of a clot removal after arterial blockage, which is common in most cases of human stroke. The transient focal ischemic model also allows the study of reperfusion injury in the ischemic region (Gürsoy-Ozdemir et al. 2004).

Most experimental focal ischemic models involve the occlusion of the middle cerebral artery (MCAO) because according to clinical studies, 75% of focal ischemia occurs due to a blockage of this artery (Mergenthaler et al. 2004; Lloyd-Jones et al., 2009). The most frequently used model of MCA occlusion is the intraluminal suture model which is less invasive compared to others. A monofilament is inserted into the internal carotid artery (ICA) and advanced until it blocks the blood flow of the MCA (Fig. 5.)

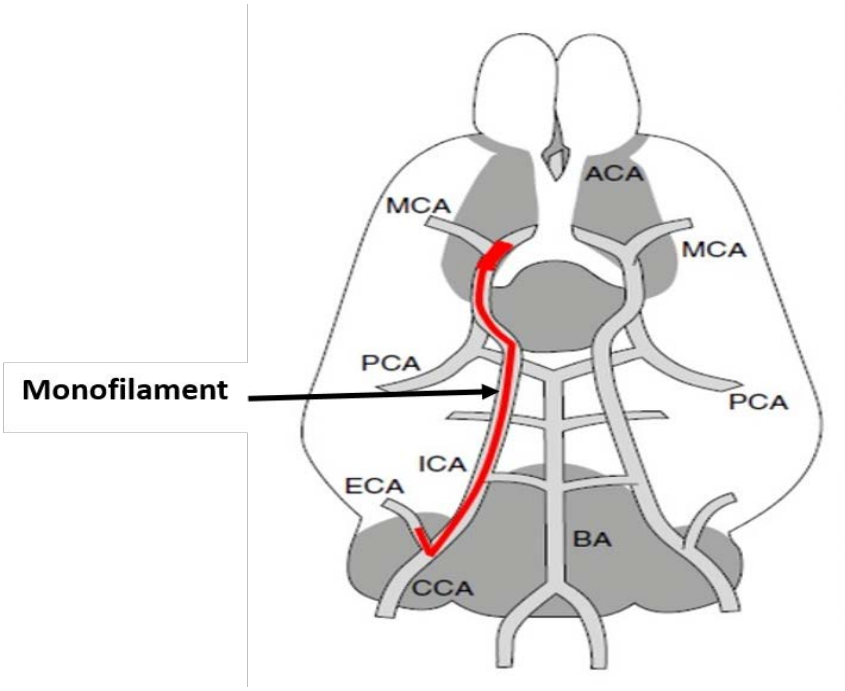


Figure 5. Carotid and cerebral arteries in the rat with the insertion of the intraluminal monofilament (red) on the left side.

Carotid and cerebral arteries of the rat with the insertion of the intraluminal monofilament (red) on the left side. ACA, anterior cerebral artery; BA, basilar artery; CCA common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery. ***(Adapted from O'Neill and Clemens, 2000)***

The model allows good reproducibility of the MCA region infarctions which includes the frontoparietal cortex and lateral caudoputamen (the striatum). If the desired model is that of transient focal ischemia, the MCAO model allows for reperfusion by retracting the intraluminal suture. The MCAO model also provides good ischemic lesion growth and therefore is advantageous for neuroprotective studies. Because of these features of the MCAO model, in addition to the fact that most human stroke are transient, we decided to use this model to investigate transient focal ischemia.

1.5. Stroke treatment

Due to the multiple pathophysiological mechanisms observed in ischemic stroke, current treatments remain challenging and mostly ineffective. Protection of the neurovascular unit may be achieved by enhancing reperfusion, modifying neuronal activity and augmenting neurorepair (del Zoppo et al. 1992). Of these, reperfusion by using thrombolytic therapy is effective. This treatment uses recombinant human thrombolytic tissue plasminogen activators (rt-PA) (the only FDA approved stroke treatment) (NINDS Group, 1995) such as Alteplase (Hacke et al. 2008) to break down blood clots. The disadvantage of this therapy is that it only allows a 3 – 4 ½ hours window for effective treatment so that only a small percentage of all stroke patients undergo thrombolysis. Many neuroprotective agents focusing on ischemic mechanisms such as glutamate toxicity or free radical formation lack efficacy or have intolerable side effects (for review: Fisher & Schaebitz, 2000). Generally a successful treatment agent for stroke should be well tolerated, not interfere with essential brain function and show a multi-approach capability to address several stroke pathophysiological mechanisms in parallel.

1.6. Granulocyte-colony stimulating factor

Granulocyte-colony stimulating factor (G-CSF) is a 19.6 kDa glycoprotein that belongs to the family of cytokine hematopoietic growth factor (Burgess and Metcalf 1980). It was initially identified as a serum activity that induced differentiation of the murine myelomonocytic leukemic cell line (Welte et al. 1985). The G-CSF gene was cloned in 1986 (Nagata et al. 1986) and soon after the recombinant form of the protein was synthesized [Filgrastim: recombinant methionyl human granulocyte colony-

stimulating factor (r-metHu G-CSF)]. G-CSF regulates the generation, proliferation, survival, and maturation of neutrophilic granulocytes and induces their mobilization from bone marrow (BM) to the peripheral blood (Liu et al., 1996; Puthalakath et al., 2007; Bendall & Bradstock, 2014). Due to these properties of G-CSF, it has been in clinical practice to treat neutropenia induced by chemotherapy since it was U.S.A. FDA approved in 1991. It is also clinically used for bone marrow reconstitution and stem cell mobilization (Begley et al., 1986; Metcalf, 1990; Weaver et al., 1993; Welte et al., 1996). It has been given to over 3 million patients with few side effects (Frampton et al., 1994; Crawford, 2003).

Increasing studies have shown G-CSF to be beneficial in a number of neurodegenerative diseases (McCollum et al. 2010) and neurological diseases including ischemic stroke (Schäbitz et al., 2003; Schneider, et al., 2005; Komine-Kobayashi et al., 2006) For instance it has been reported that in several murine stroke models, G-CSF treatment led to an improved functional recovery (Shyu et al., 2004; Schneider et al., 2005; Gibson, et al,. 2005), reduced lesion size (Six et al., 2003; Schäbitz et al., 2003; Sevimli et al., 2009) and enhanced survival rate (Six et al., 2003). The beneficial effects of G-CSF have been attributed to different concurrent mechanisms, including anti-apoptotic activity (Schäbitz et al., 2003; Schneideret al., 2005) immunomodulation (von Aulock et al., 2004), stimulation of neurogenesis (Schneider et al., 2005; Kawada et al., 2006; Diederich et al., 2009), and angiogenesis (Lee et al., 2005). The biological effect of G-CSF is mediated by G-CSF binding to its specific receptor; granulocyte-colony stimulating factor receptor (G-CSFR) (Solaroglu et al., 2006). The receptor is expressed on both hematopoietic cells as well as on non-hematopoietic cells, such as neurons,

endothelial cells and glial cells (Bussolino et al., 1989; Hanazono et al., 1990; Schäbitz et al., 2003; Schneider et al., 2005)

The Granulocyte colony-stimulating factor receptor, was characterized by Nicola and colleagues (Nicola et al., 1988). It homo-oligomerizes once G-CSF binds to it and activates associated intracellular signaling pathways such as the JAK/STAT (Janus kinase /signal transducer and activator of transcription, Ras/ERK 1, 2 &5 (Ras/extracellular signaling regulated kinase 1,2 & 5) and PI3K/AKT [phosphatidylinositol 3-kinase/protein kinase B (also known as Akt)] signaling pathways (Nicholson et al., 1995; Dong & Larner, 2000). It has been reported that the phosphoinositide-3-kinase pathway hinders neuronal cell death after stroke (Chan 2004; Zhao et al., 2006) and *in vitro* studies have provided evidence that G-CSF exerts receptor-mediated neuroprotection through the PI3-AKT pathway (Schneider et al., 2005).

1.7. Research aim.

Because stroke is such a burdensome disease to society with a high prevalence rate, it is important to find efficacious stroke treatment apart from rt-PA. Establishing effective neuroprotective agents could increase the window for stroke treatment. Such agents should have the potential to address the diverse pathophysiologic conditions observed in ischemic stroke. G-CSF has shown this potential from reported evidence of its anti-apoptotic activity (Schäbitz et al., 2003; Schneider et al., 2005) immunomodulation (von Aulock et al., 2004), stimulation of neurogenesis (Schneider et al., 2005; Kawada et al., 2006; Diederich et al., 2009), and angiogenic capabilities (Lee et al., 2005). While both the mitochondrion and the endoplasmic reticulum are two main organelles known to be involved in neuronal cell death due to ischemia, very little is

known about the role of ER stress induced apoptosis in ischemia. Therefore the aim of this study is to investigate the protective effect of G-CSF against ER stress apoptosis in (1) a stroke-like cell culture model involving glutamate-induced cell death and (2) a rat model of transient focal ischemia.

2. GRANULOCYTE-COLONY STIMULATING FACTOR PROTECTS AGAINST GLUTAMATE-INDUCED CYTOTOXICITY IN UNDIFFERENTIATED PHEOCHROMOCYTOMA RAT CEL

This *in vitro* study was designed to determine the efficacy of G-CSF to exert a protective function against cell death. Glutamate was used to simulate glutamate-induced toxicity in the rat pheochromocytoma (PC12 cell) line and then the effect of G-CSF against glutamate-induce toxicity was determined by measuring cell viability.

While both the mitochondrion and endoplasmic reticulum are major suborganelles that contributes to cellular death during an ischemic insult (mitochondrial dysfunction and endoplasmic reticulum stress), only a few studies have investigated ER stress resulting from glutamate (Kitao et al., 2001; Sokka et al., 2007; Choe et al., 2011). The use of an *in vitro* model allowed a relatively quick investigation into the effect of glutamate toxicity-induced ER stress and to also perform experiments which analyzed protective effects by G-CSF against this toxic condition.

I hypothesized that in glutamate-induced ER stress PC12 cells, G-CSF will downregulate the ER stress apoptotic marker CHOP. Since the effect of G-CSF is receptor mediated, I predict that the G-CSF receptor will be located on the plasma membrane of PC12 cell and that an attenuation of CHOP by G-CSF will increase the survival of PC12 cells against glutamate-induced ER stress apoptosis

2.1. MATERIALS AND METHODS

2.1.1. Cell culture

Rat pheochromocytoma cell (PC 12 cells) (Greene and Tischler 1976) were purchased from American Type Culture Collection (ATCC, catalog no. CRL-1721). Cells were prepared and treated as previously described (Seyfried et al. 2000) with slight modifications. Cells were grown in suspension in 25 cm^2 tissue culture flasks in growth medium of RPMI 1640 (Invitrogen; Carlsbad, CA, USA) which was supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum and penicillin (50U/ml)/streptomycin (50ug/ml) (all from Invitrogen; Carlsbad, CA, USA). Growth maintenance occurred in a humidified incubator at 37^oC, containing 95% air, 5% CO_2 and cells were fed three times per week by centrifuging at 225 x g for 8 -10 mins., 26^oC, removing the supernatant and then re-suspended in fresh growth medium. The cells were passed every 7 days or when there were at 80% - 90% confluence, after which their population were either sub divided and plated for growth in a new 25 cm^2 tissue culture flasks with a new passage number or plated on poly-D-lysine-coated (10ug /ml) (Sigma Aldrich; St. Louis, MO, USA) plates or cover slips for experimental purposes. Cells between passages 2 – 7 were used in experimental protocols. Cell density was determined by cell counting using a hemocytometer and a tissue culture microscope. After cell density was determined, 96-well plates, 6 – well plates were plated with 1.5 x 10^4 cells per well and 5 x 10^5 cells per well for Adenosine 5'-triphosphate (ATP) assay and Western blot analysis, respectively. Coverslips were seeded with 1.5 x 10^5 cells for immunofluorescence analysis.

2.1.2. Glutamate cytotoxicity

Undifferentiated PC 12 cells were seeded on 96-well plates coated with poly-D-lysine at a density of 1.5×10^4. Twenty – four hours after initial seeding the medium was renewed with fresh culture medium containing various final concentrations (0.05, 0.25, 0.5, 5, 10, 20 and 40 mM) of glutamate (Sigma Aldrich; St. Louis, MO, USA), as described previously (S. Ma et al. 2012) with some slight modifications. Cell viability analysis was made after 8, 12 or 24 hours of glutamate incubation.

2.1.3. Treatment groups of undifferentiated PC 12

Undifferentiated PC12 cells were divided into 7 groups: (1) control group, (2) glutamate group: cells were treated with 10mM glutamate (Sigma Aldrich; St. Louis, MO, USA) (3) G-CSF group: cells were treated with a final concentration of 25ng/ml, 50ng/ml, 100 ng/ml, 250ng/ml G-CSF (Filgrastim, Akron; Boca Raton, FL, USA); (4) glutamate and G-CSF group: cells were treated with a final concentration of 25ng/ml, 50ng/ml, 100 ng/ml, 250ng/ml G-CSF and 10mM glutamate respectively, (5) anti-G-CSF receptor group: cells were preincubated with 1:100 dilution of G-CSF receptor antibody for 60 minutes, after which the medium with G-CSF receptor antibody (anti-G-CSFR) (Santa Cruz Biotechnology; Santa Cruz, CA, USA) was removed and replaced with fresh medium without the anti-G-CSFR, (6) G-CSF and anti-G-CSF receptor group: cells were preincubated with 1:100 dilution of G-CSF receptor antibody for 60 minutes after which the medium was removed and replaced with either 50ng/ml or 100ng/ml or 250 ng/ml G-CSF in fresh media (7) glutamate, anti-G-CSF receptor and G-CSF: preincubated with 1:100 dilution of G-CSF receptor antibody for 60 minutes after which the medium was removed and replaced with either 50ng/ml or 100ng/ml or 250 ng/ml G-CSF in fresh

media, simultaneously adding 10mM glutamate respectively. Dilution of the anti-G-CSF receptor and solutions of glutamate and G-CSF were made with growth medium.

Table 1: Treatment groups of undifferentiated PC12 cell.

Group	**Treatment**
1. Control	-
2. Glutamate	10mM Glutamate (Froissard, et al., 1997; Seyfried et al., 2000)
3. G-CSF	25ng/ml, 50ng/ml, 100ng/ml or 250ng/ml G-CSF
4. G-CSF + Glutamate	25ng/ml or 50ng/ml or 100ng/ml, or 250ng/ml G-CSF + 10mM glutamate
5. Anti-G-CSF Receptor	1:100 diluted Anti-G-CSF Receptor
6. Anti-G-CSF Receptor + G-CSF	1:100 diluted (60 minutes preincubated) Anti-G-CSF Receptor + 50ng/ml or 100ng/ml or 250ng/ml G-CSF
7.Anti-G-CSF Receptor + G-CSF + Glutamate	1:100 diluted (60 minutes preincubated) Anti-G-CSF Receptor + 50ng/ml or 100ng/ml or 250ng/ml G-CSF + 10mM Glutamate respectively

All treatments were administered to PC12 cells after cells were seeded for 24 hours at 37^{O}C and 5% CO_2 incubation in 96-well plates. Twenty-four hours following treatment regimes, analyses of cell viability were performed.

2.1.4. Adenosine 5'-triphosphate (ATP) viability assay

Adenosine 5'-triphosphate (ATP) quantification is a widely accepted method for detecting cell viability because healthy cells exhibit ATP as biomarker while non-viable cells both lose their ability to synthesize ATP, as well as produce endogenous ATPase that quickly depletes the existing ATP (Lundin et al. 1986; Crouch et al. 1993). The ATP is quantified by a luciferase chemical reaction which generates a luminescence signal that is proportional to the number of viable cells (Maehara et al., 1987). The ATP assay is one of the most sensitive and rapid tests of cell viability (Niles et al. 2009) detecting as few as 10 cells in 10 mins.

The assessment of the number of cells that survived after each treatment was measured with a Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega Corporation; Madison, WI, USA) which quantified the amount of ATP generated from cells that are metabolically active. The procedure was performed according to the manufacturer's protocol. Briefly, for the glutamate cytotoxicity; experiments were carried out in 96-well plates with undifferentiated PC12 cells incubated with various glutamate concentration (0.05, 0.25, 0.5, 5, 10, 20 and 40 mM) for either 8 or 12 or 24 hours. After each respective time point the 96-well plates were removed from the incubator and allowed to equilibrate at room temperature for 30 mins. Cells were then incubated in the ATP Kit's lysis buffer for 10 – 20 mins which released any ATP synthesized from viable cells. The ATP was then detected by a luciferase reaction which produced a luminescence signal. The luminescence signal was detected at an absorbance of 550nm by a microplate reader (Spectra Max, Molecular Devices) after transferring

100ul of the lysate to a standard opaque walled 96-well plate. The background luminescence of the culture medium was subtracted. Cell viability for other experiments were performed in a similar manner as that of the glutamate cytotoxicity experiment, section 2.1.2, except that treatment conditions involving glutamate used only 10mM glutamate and 24 hour was the selected time point for all experiments preceding the glutamate cytotoxicity experiment.

2.1.5. Cell morphology

To assess morphologic cellular changes the method used was previously described by Cao et al. 2006 with some slight modifications. Briefly, undifferentiated PC12 cells were seeded on 24-well plates (1.5×10^5) and subjected to four experimental conditions: (1) control, (2) G-CSF (50ng/ml), (3) glutamate (10mM), and (4) glutamate (10mM) + G-CSF (50ng/ml) for 24 hours incubation time respectively. After the 24 hours incubation, cells were observed under phase-contrast imaging for morphologic changes with a Trinocular Fluorescence Inverted microscope (Motic; Carlsbad, CA, USA)

2.1.6. Immunofluorescence

Undifferentiated PC12 cell samples (control group; cells without any treatment) for immunofluorescence were plated (1.5×10^5) on sterile poly-D- lysine (Sigma Aldrich; St. Louis, MO, USA) coated glass coverslips in a 24-well plate for 24 hours. After which cells were washed briefly with phosphate buffered saline (PBS; pH 7.4), fixed with 4% paraformaldehyde for 5 mins. at room temperature, then washed with PBS for 5 mins, 3 times. Cells were blocked in blocking buffer (10% normal goat serum, 1% BSA, in PBS) for 60 mins. at room temperature, then subsequently incubated overnight at 4^0C with

primary polyclonal rabbit antibody against G-CSFR (1:1000) (Santa Cruz Biotechnology; Santa Cruz, CA, USA) in diluent (1% normal goat serum, 1% BSA, in PBS). Following overnight incubation in primary polyclonal rabbit anti-G-CSFR, cells were washed for 5 mins, 3 times in PBS, then incubated with secondary goat anti-rabbit IgG conjugated to Alexa Fluor 594 (red) (1:3000) (Invitrogen; Carlsbad, CA, USA) for 2 hours at RT in diluent (1% normal goat serum, 1% BSA, in PBS). After immunostaining, the coverslips were mounted onto silicone-coated microscope slides (Fisher Scientific; USA) with Prolong Gold anti-fade mounting reagent containing DAPI (Invitrogen; Carlsbad, CA, USA), a nuclear counterstain. Fluorescent signals were detected using a Zeiss confocal laser scanning microscope. For negative controls, cells underwent similar procedure as described above except that primary anti-G-CSFR was omitted.

2.1.7. Western blotting

Undifferentiated PC12 cells were seeded in 6-well plates at a density of 5 $x10^5$ cells per well. Twenty-four hours after seeding, cells were subjected to either glutamate (10mM), or glutamate (10mM) added simultaneously with G-CSF (50ng/ml) or 60 minutes preincubated anti-G-CSFR followed by simultaneous addition of glutamate (10mM) with G-CSF (50ng/ml), for an additional 24 hours incubation. Cells were then harvested and western blot analysis performed according to Pan et al. 2012, with slight modifications. Briefly, cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1% (v/v) mammalian protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail from Sigma Aldrich, USA). Whole cell lysate was obtained and protein concentration was determined by Bradford assay (Bradford 1976). Protein samples (50 -

60 ug) were suspended in laemmli SDS sample buffer (125 mM Tris–Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% bromophenol blue), boiled for 7 mins., and resolved by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers (Biorad, USA) were loaded simultaneously with protein sample on the gel for protein band identification. Resolved protein samples were electro-blotted from the gel to nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane with blocking buffer, [TBST: (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk)] for 1 hour at room temperature (RT). Membranes were probed with polyclonal rabbit anti-CHOP (1:100) (Santa Cruz Biotechnology; Santa Cruz, CA, USA) polyclonal rabbit anti-GAPDH (1:3000) (for normalization) and left overnight at 4^0C. After overnight incubation, antibody was removed and membrane washed in TBST, three times for 5 mins respectively. This was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit (1: 3000) (Cell Signaling Technology). Signals were detected by enhanced chemiluminescence and analyzed with NIH image J.

Western blot analysis of the G-CSF receptor was performed as described in the above paragraph with some exceptions; only PC12 cells of the control group (no treatment was given to this group) was harvested and once protein samples were suspended in laemmli SDS sample buffer (125 mM Tris–Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% bromophenol blue), they were heated at 37^oC for 10 mins before resolved on 12 % SDS-PAGE. Protein denaturation was done at this temperature and time period because higher temperature or longer incubation leads to the aggregation of membrane proteins (Kaur and Bachhawat 2009). Primary polyclonal

rabbit anti-G-CSFR (1:1000) (Santa Cruz Biotechnology; Santa Cruz, CA, USA) was left to incubate overnight at 4^0C. Followed by overnight incubation at 4^oC with fluorescence-conjugated secondary goat anti-rabbit (1: 10,000) (LI-COR; 800). Detection of protein expression was performed using LI-COR Odyssey Fc system.

2.1.8. Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). Statistical significance was determined by either one-way, two-way or three-way ANOVA followed by Tukey post hoc test. Differences in p-values were considered significant if $p < 0.05$. Data are represented as the mean ± S.E.M. At least three independent replicates were performed for each experiments

2.2. RESULTS

2.2.1. A cell culture model of stroke-related condition

Firstly**,** we wanted to simulate a stroke-related condition in cell culture. It is well known that subsequent to a cerebral ischemic insult the level of extracellular glutamate increases, resulting in excitotoxicity. The clonal cell line of the rat pheochromocytoma, PC12 cells, has long proven to be a well-established cell line for the investigation of neuronal injury (Greene and Tischler 1976) and has been shown to be susceptible to glutamate toxicity (Froissard & Duval, 1994) The addition of high level of glutamate to PC12 cells creates a stroke-related condition whereby cells die from cytotoxicity (Tyurin et al. 1998; Hong et al. 2004; Penuogonda et al. 2006; Pan et al. 2010). Based on these previous findings, we subjected undifferentiated PC12 cells to a range of glutamate concentrations in order to create stroke-related conditions in PC12 cell culture and also to identify the ideal glutamate concentration for eliciting PC12 cell death.

2.2.2. Glutamate cytotoxicity in undifferentiated PC12 cell

Glutamate toxicity was evaluated by the quantification of ATP in cells after incubating PC12 cells for 8 or 12 or 24 hours with glutamate (glutamate concentration ranges from 0.05– 40 mM). Cell viability was then expressed as a percentage (%) of control. As shown in Fig. 6, there was a significant decrease in cell survival to glutamate which concentration- and time-dependent [$F(22, 326) = 6.293$; $P < 0.0001$].

There was a significant reduction of cell viability when cells were incubated for 24hrs at 5, 10, 20 and 40 mM glutamate exposure (71.5± 5.9%, 51.2± 6.1%, 31±2.3%, and 25±2.6% respectively when compared with control value; **#**$P < 0.0001$).

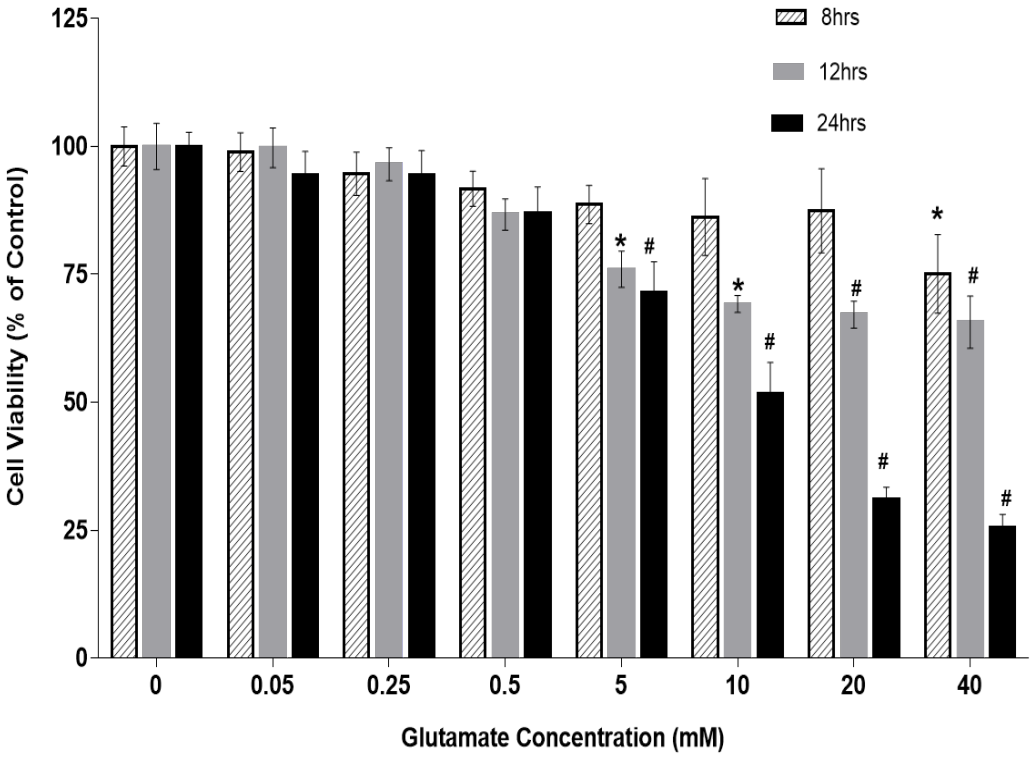


Figure 6. Glutamate-induced cytotoxicity in PC12 cells.

Cells were incubated with different concentrations of glutamate, 0.05–40 mM for 8, 12 and 24 hours respectively at 37^0C. Cell viability was measured with ATP assay as a percentage (%) of control. Data expressed as the percentage (%) of control values, are the mean ± SEM for triplicate determinations of 4 different experiments. #P<0.0001; *P<0.001; significantly different compared with control conditions. Two-way ANOVA, Post hoc Tukey.

A similar significant reduction of cell viability was seen for cells treated with glutamate (20 and 40 mM glutamate) for 12 hrs. (67±2.6%, 65±5.1%, compare with control values; #P < 0.0001) and also when cells were incubated for 40 mM glutamate (75±7.7%, compared with control values; #P < 0.0001) for 8 hours. The drastic decrease in cell viability was first observed when cells were treated 5 mM glutamate for 24 hours. The same drastic increase in cell death that was observed at 5 mM glutamate for 24 hours

was also observed at shorter incubation time (12 and 8 hours) but at higher glutamate concentration (20 mM and 40 mM respectively). Although there was a drastic increase in cell death with 5 mM glutamate for 24 hours; this same concentration induced a lesser significant cell death when cells were incubated for 12 hours, which was significantly similar to cell death with 10 mM glutamate for the same 12 hour incubation (76 ±3.6%, 69.2±1.7% respectively when compared to control values; $*P<0.001$). There was no significant difference between control value and glutamate concentration less than 5 mM at any time point. Incubation in 10mM glutamate concentration for 24 hours gave approximately 50% loss of cell viability (51.2± 6.1%) compared to control. This concentration was representative of the half maximal effective concentration (the concentration where the response is reduced by a half) for glutamate in our toxicity experiment with undifferentiated PC12 cells. Based on this observation we decided to use 10 mM glutamate for 24 hours in all subsequent *in vitro* experiments.

2.2.3. The expression of G-CSF receptor on undifferentiated PC12 cell.

After establishing the glutamate-induced toxicity model (stroke-related condition) in undifferentiated PC12 cells, our next step was to investigate the potential protective effect of G-CSF against glutamate-induced cytotoxicity in this model.

It has been reported that the effect of G-CSF is receptor mediated (Solaroglu et al., 2006) and that the receptor is expressed on both hematopoietic cells as well as on

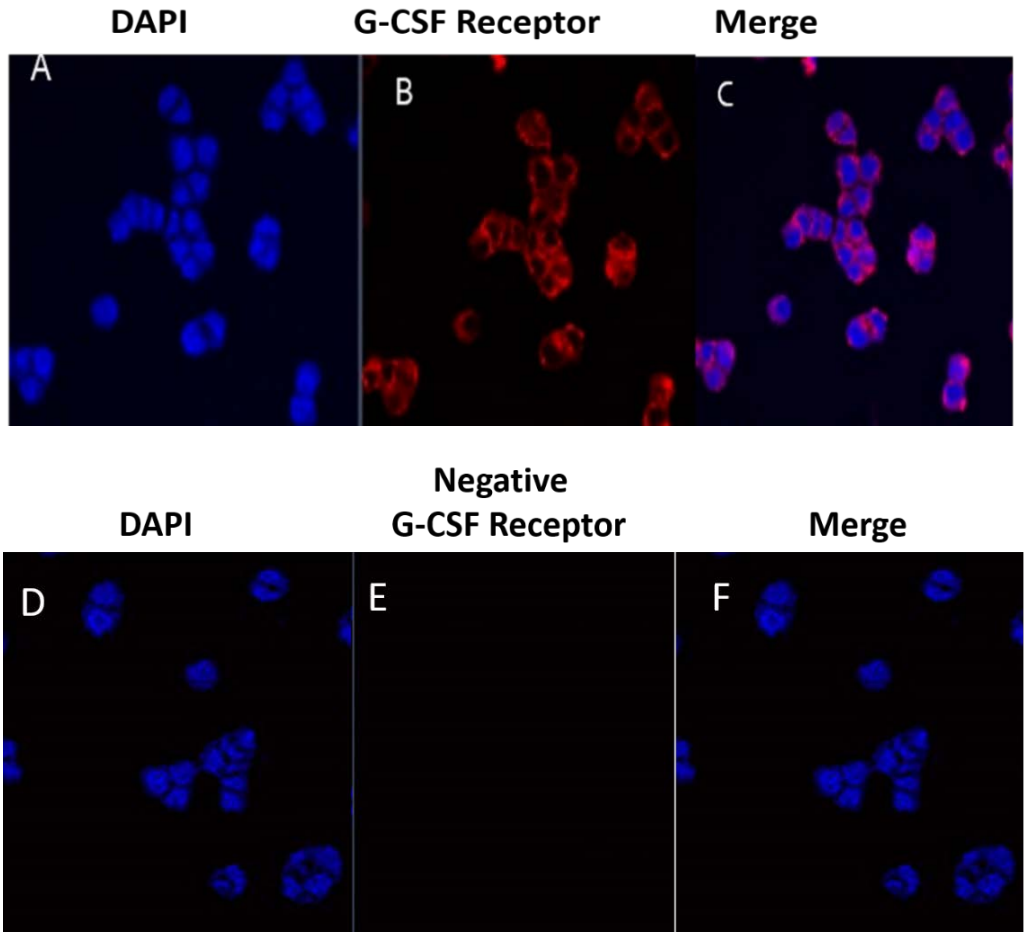
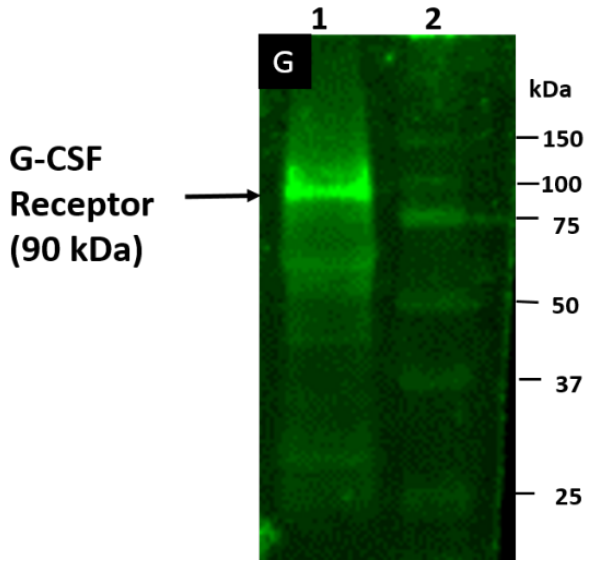


Figure 7. G-CSFR expression on undifferentiated PC12 cells.

(A). DAPI fluorescence blue and stains the cell nucleus. **(B**). Red fluorescence is for G-CSFR, expressed on the plasma membrane of the cells. **(C).** A merger of both DAPI and G-CSFR on the plasma membrane of the cells**. (D-F)** Negative control, without primary anti-G-CSFR **(G).** Western blotting showing G-CSFR expression; undifferentiated PC12 protein sample (without any treatment condition in lane 1) and standard protein ladder (lane 2).



non-hematopoietic cells, such as neurons, endothelial cells and glial cells (Bussolino et al., 1989; Hanazono et al., 1990; Schäbitz et al., 2003; Schneider et al., 2005). I wanted to be certain that the G-CSF receptor was also expressed on undifferentiated PC12 cells before examining the protective effect of G-CSF in this cell line.

To detect the G-CSF receptor (G-CSFR), immunofluorescence staining as well as western blotting were performed on undifferentiated PC12 cells. As shown in Fig.7, the G-CSFR was expressed in undifferentiated PC12 cells (Fig. 7G); specifically on the plasma membrane of the cells (Figs. 7B and 7C). The control experiment without the addition of the primary anti-G-CSF receptor antibody was immunonegative for the G-CSFR (Fig. 7D-F).

2.2.4. Granulocyte colony-stimulating factor protects against glutamate-induced cytotoxicity in undifferentiated PC12 cells.

To examine protection by G-CSF against glutamate-induced cell death, increasing doses of G-CSF (25ng/ml or 50ng/ml or 100ng/ml or 250ng/ml) were given to cultured PC12 cells simultaneously with 10mM glutamate, for 24 hours incubation. PC12 cells also received glutamate (10mM) only and G-CSF (25ng/ml or 50ng/ml or 100ng/ml, 250ng/ml) only. Cell viability was measured as the release of ATP from viable cells. The resulting data were expressed as a percentage (%) of control cells (glutamate and G-CSF free cells). The results showed that G-CSF significantly decreased glutamate-induced cell death in a dose-dependent manner [$F(4, 53) = 8.975$; $P < 0.0001$)] (Fig 8). Cells exposed to 10mM glutamate for 24 hrs. had a cell viability of 42.4 ± 2.8 % of control value, while the viability of cells treated simultaneously with 10 mM glutamate and different doses of G-CSF (50, 100 and 250 ng/ml) significantly increased to 80.5 ±

8.3%, and 84.5 ± 8.1% to 89.2 ± 2.8% of the control value respectively.

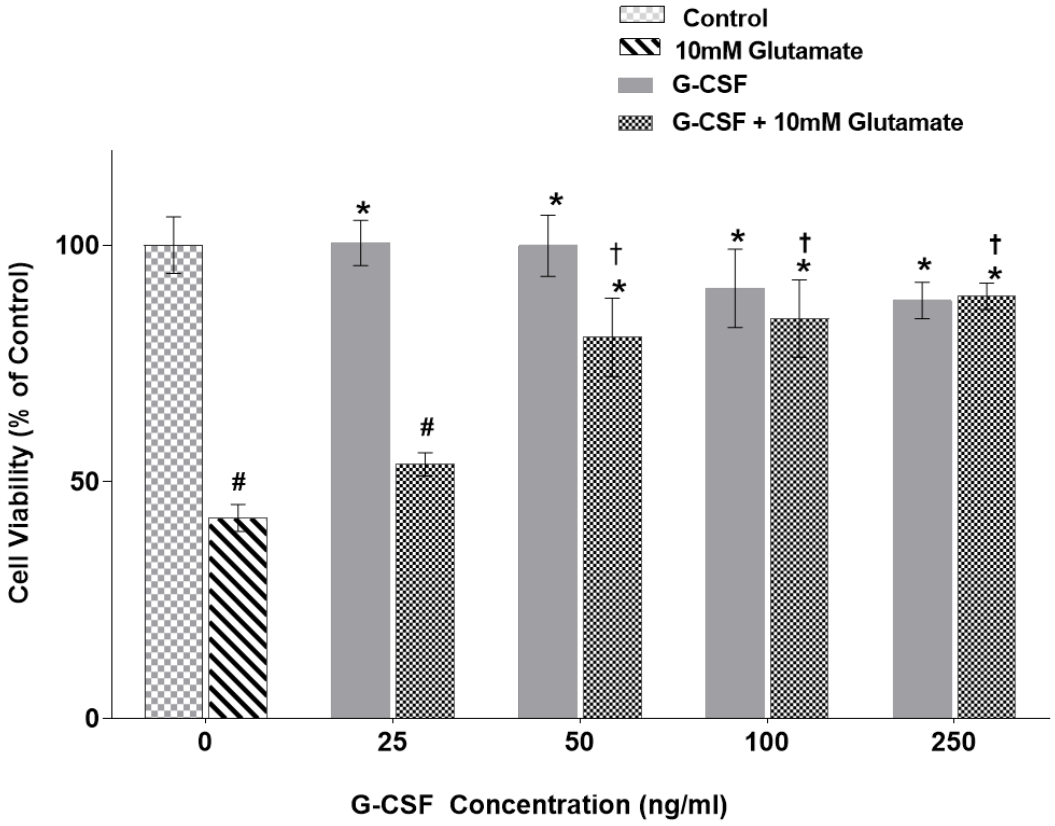


Figure 8. Granulocyte colony-stimulating factor (G-CSF) attenuates glutamate-induced undifferentiated PC12 cell death.

PC12 cells were exposed simultaneously to glutamate (10mM) and G-CSF (25, 50, 100, 250 and 500 ng.ml) for 24 hours in 96 well plate. Viability of PC12 cell was evaluated by ATP assay and expressed as a percentage (%) of control (cells without any treatment). Data expressed as the mean ± SEM for triplicate determinations of 4 different experiments. #$P < 0.0001$, significantly different compared with control conditions (cells without any glutamate or G-CSF added); *$P < 0.05$, significantly different compared with 10mM glutamate-treated cells; † $P < 0.05$, significance compared to 25ng/ml G-CSF + 10 mM glutamate treatment. Two-way ANOVA, Post hoc Tukey.

The optimal protective dose for G-CSF against glutamate induced-toxicity was 50ng/ml since a lower dose of 25ng/ml, showed no significant protection (53.7 ± 2.5%) and higher doses (100 and 250 ng/ml) were not significantly different from 50ng/ml (80.5 ± 8.3%) nor from each other (84.5 ± 8.1% and 89.2 ± 2.8% respectively). This was

an interesting observation and indicated that the G-CSFR was saturated by doses of G-CSF that were higher than 50ng/ml. Our results also showed that G-CSF was not toxic to PC12 cells, since increasing doses (25, 50, 100, and 250 ng/ml) of G-CSF administered alone to PC12 cells showed no significant effect (100 ± 4.8%, 99.9 ± 6.4%, 90.9 ± 8.3% and 88.3 ± 3.8% respectively) on cell viability of control values.

2.2.5. Effect of G-CSF on glutamate-induced changes in undifferentiated PC12 cell morphology- A qualitative description

Generally cells either die by apoptosis or necrosis. Apoptosis is active programmed cell death while necrosis is passive cell death by default. Both types of death are distinguishable by morphological changes that can be observed under a phase contrastm microscope. Cells undergoing apoptosis exhibit shrinkage, numerous plasma membrane deformations (blebbing) and chromatin condensation (Rello et al. 2005). Those undergoing necrosis show signs of swelling, nuclear and plasma membrane disintegration and eventually become a shadow of a cell, "ghost cell" with only the presence of attached cellular debris that remained on the substrate (Rello et al. 2005).

Subsequent to exposing PC12 cells to four conditions of; (1) control (untreated group) (2) glutamate (10mM), (3.) G-CSF (50ng/ml) and (4) glutamate (10mM) + G-CSF (50ng/ml) for 24 hours incubation time respectively, we observed cellular (not nuclear) changes in cell morphology with a phase contrast microscope (Fig. 9). We observed that cells treated with only G-CSF (50ng/ml) (Fig. 9a) exhibited the same healthy round morphology as control cells (Fig. 9b) without any sign of cellular changes. Cells exposed to only glutamate (10mM) (Fig. 9c) showed both signs of apoptosis (black arrows), indicative of cell shrinkage and cell deformity, as well as some necrosis (white

arrow with white circle), indicative of disintegrated plasma membrane with the presence of attached cell debris.

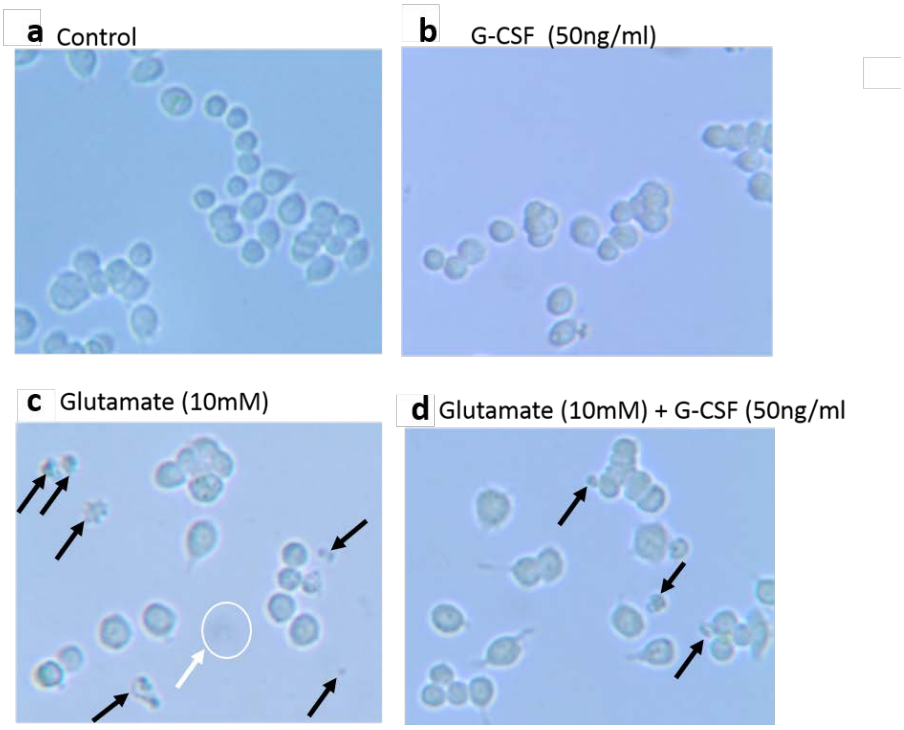


Figure 9. Effect of G-CSF on glutamate-induced change cell morphology of PC12 cell.

Photomicrographs (a-d) shows cell morphology exposed to glutamate and recusing effect of G-CSF. (a) Control, (b) G-CSF (50ng/ml), (c) 10 mM Glutamate, (d) 10mM Glutamate + G-CSF (50ng/ml). Cells were incubated for 24 hours for respective treatment conditions. Following treatments, cells were visualized using phase/contrast microscopy (x200) and photographs were taken. Black arrows indicates apoptosis, white arrow with white circle indicates necrosis. Images depict a representative field obtained from each treatment condition**.**

The cells exposed to glutamate (10mM) + G-CSF (50ng/ml) (Fig. 9d) showed a representative reduction of cell number with cellular (not nuclear) changes compared to cells only exposed to glutamate (10mM). Based on this qualitative description, G-CSF was able to rescue undifferentiated PC12 cells from glutamate-induced apoptotic and necrotic cellular morphological changes.

2.2.6. Receptor-mediated protection by Granulocyte colony-stimulating factor against glutamate-induced toxicity in undifferentiated PC12 cells.

In order to validate the protective effect by G-CSF against glutamate-induced toxicity (Fig. 8), G-CSF receptor (G-CSFR) activation was blocked by the addition of an antibody against (anti-G-CSFR) the receptor. The anti-G-CSFR antibody (1:100 dilution) was incubated 60 minutes prior to the addition of any other treatment. After 60 minutes incubation with the anti-G-CSFR antibody, cell culture medium was removed and replaced with fresh medium containing either glutamate (10mM) or G-CSF or glutamate (10mM) + G-CSF for a further 24 hours incubation. There was no interaction between the inhibitor and the various G-CSF concentrations on ATP release (ATP release is an indicator of cell survival) [$F(3, 96) = 2.41$, $P=0.07$], and when compared to the control group (group without any form of treatment) there was no significant effect by either the inhibitor alone or G-CSF alone on ATP release ($P > 0.05$). An interactive effect was observed between G-CSF concentrations and 10 mM glutamate [$F(3, 96) = 3.01$, $P=0.03$] on the release of ATP, whereby G-CSF concentrations significantly rescued (1811.65± 144.19, 1737.99± 96.04, 1933.72± 259.48: 50 ng/ml, 100 ng/ml and 250 ng/ml respectively, $P<0.05$) the PC12 cells from 10 mM glutamate (898.83±29.28). The interaction between G-CSFR antibody (the inhibitor), G-CSF concentrations and 10 mM [$F(3, 96) = 6.992$, $P=0.0003$] was clearly observed when G-CSFR antibody abolished the protective effect of G-CSF against 10 mM glutamate-induced cytotoxicity in the presence of G-CSF (Fig. 10). The effect of the anti-G-CSFR was clearly shown since there was no significant difference in ATP release between the groups treated with

anti-G-CSFR + glutamate + G-SCF (1042.26±76.01, 883.53±41.65, 852.99±64.90; P<0.05) versus 10 mM glutamate only (898.83±29.28).

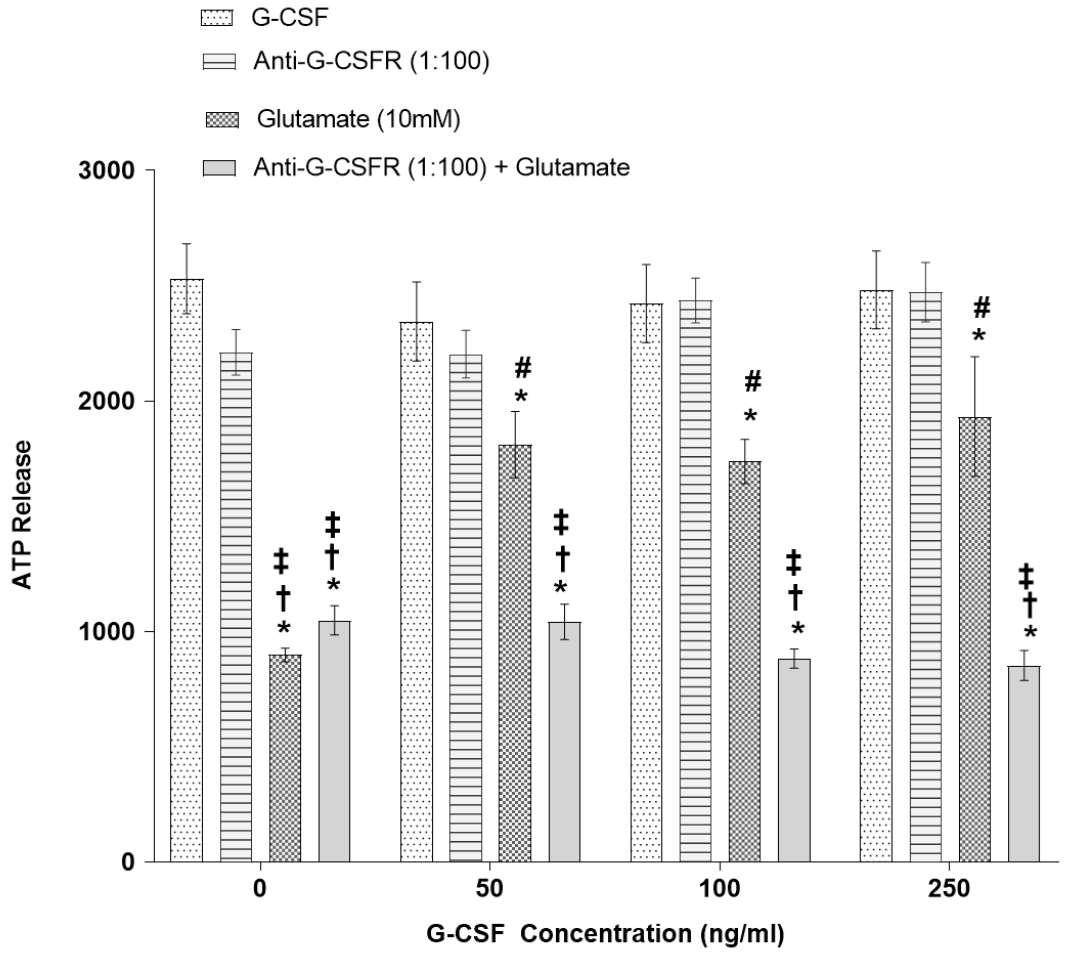


Figure 10. Effect of anti G-CSF receptor on G-CSF protection against glutamate-induced-toxicity in undifferentiated PC12 cell.

Data expressed as the mean ± SEM for triplicate determinations of 4 different experiments. (a) #*P* < 0.05, significantly different compared with anti G-CSFR treated 60 mins prior to added G-CSF and glutamate (10mM); *P < 0.05, significantly different compared with control group (no treatment); † P < 0.05, significance compared to G-CSF treatment alone; ‡ P < 0.05, significantly different compared to anti-G-CSFR + G-CSF. Three-way ANOVA, Post hoc Tukey

We also observed that a 1:100 anti-G-CSFR dilution at 60 minutes preincubation was an ideal condition for the anti-G-CSFR antibody to be effective, since increasing doses of G-

CSF (50, 100 and 250 ng/ml) were unable to rescue the cells from glutamate-induced toxicity after 60 minutes of preincubated anti-G-CSFR. Indeed, Schneider and colleagues (2005) showed that a 1:100 dilution of anti-G-CSFR was effective in abolishing the neuroprotective effect of G-CSF (50 ng/ml) in the human neuroblastoma (SHSY-5Y) cell line

2.2.7. Granulocyte colony-stimulating factor downregulates CHOP

The endoplasmic reticulum is one of the subcellular organelles that becomes stressed during an ischemic insult. Generally subsequent to the ER being stressed, the three ER stress membrane sensors, PERK, IRE1 α and ATF6 activate intracellular pathways that lead to the upregulation of [CCAAT-enhancer-binding proteins (C/EBP) homologous protein] (CHOP) (an ER stress marker) (Ron & Walter, 2007). In order to investigate the effect of glutamate toxicity on the endoplasmic reticulum and to determine the effect of G-CSF against endoplasmic reticulum stress, using western blotting, I monitored the expression level of CHOP under different treatments conditions; (1) Control, (2) 10mM glutamate treatment, (3) 10mM glutamate simultaneously treated with 50ng/ml G-CSF and (4) 10mM glutamate treated with 50ng/ml G-CSF after 60mins pre-incubation with anti-G-CSFR. Expressed results were normalized to GAPDH and presented as a percentage of the control group.

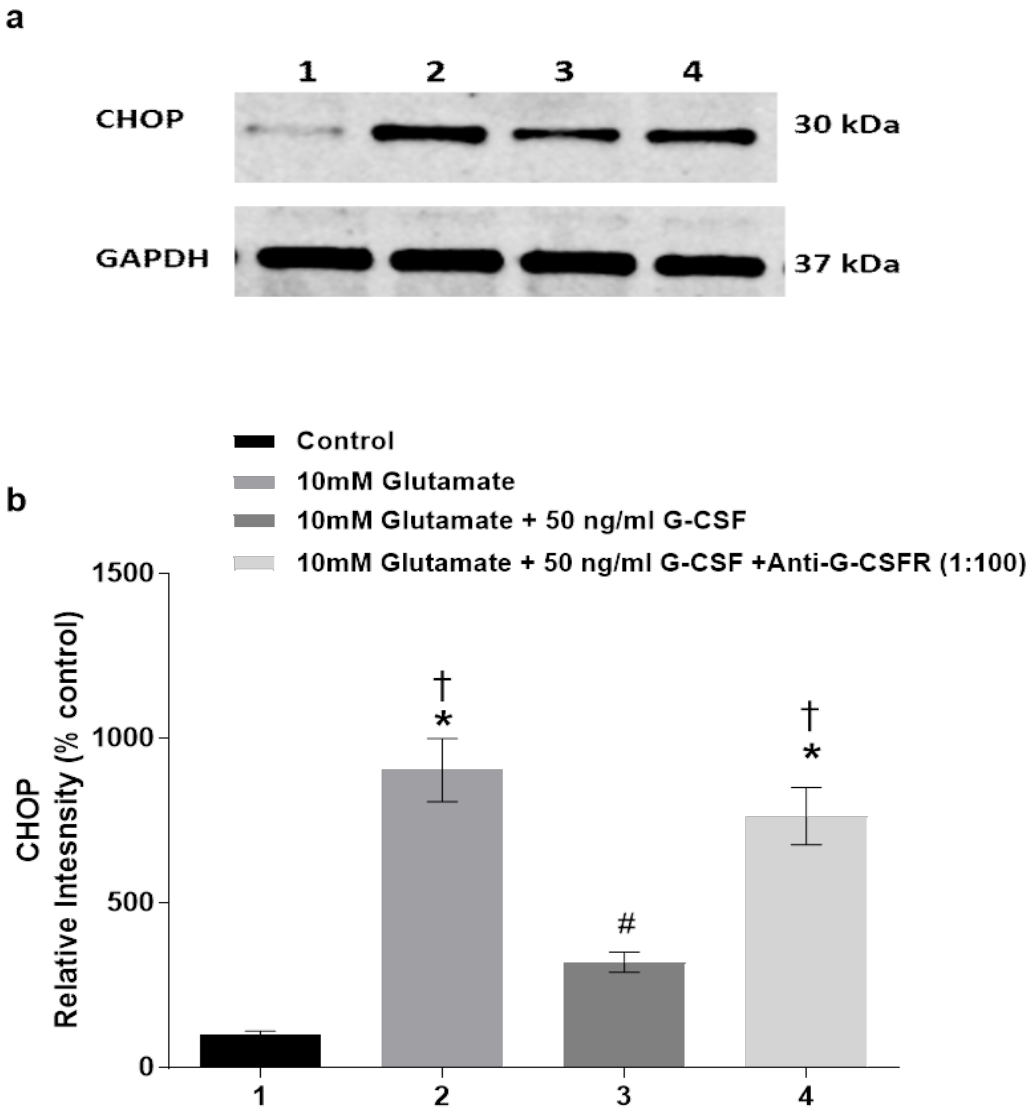


Figure 11. Effect of G-CSF on CHOP expression in undifferentiated PC12 cells

(a.) Western blot: 1. control, 2. 10mM glutamate treatment, 3. 10mM glutamate simultaneously treated with 50ng/ml G-CSF, 4. 10mM glutamate simultaneously treated with 50ng/ml G-CSF after 60mins preincubation with anti-G-CSFR. (b.) Densitometry of bands from CHOP Western blot: 1. control, 2. 10mM glutamate treatment, 3. 10mM glutamate simultaneously treated with 50ng/ml G-CSF, 4. 10mM glutamate simultaneously treated with 50ng/ml G-CSF after 60mins preincubation with anti-G-CSFR. Values were obtained from three independent experiments with duplicated blotting respectively, normalized to GAPDH and express as percentage control. Values are expressed as mean ± SEM. *$P < 0.01$, statistical significance compared with group 1 †$P < 0.01$, statistical significance compared with group, #$P < 0.05$, compared to group 1. One-way ANOVA, Tukey post hoc.

Our results showed that there was significant differences amongst the groups [$F(3, 20) = 29.79$, $P < 0.01$] with a clear demonstration that glutamate induced ER stress (Fig.11), since the expression level of CHOP was greatly upregulated in the glutamate (10mM) treated group by 9 times the level in the control group (904.5 ± 95.90% vs 100 ± 12.45%; $P < 0.01$). G-CSF was able to ameliorate the glutamate-induced level of CHOP (247.85 ± 30.68% in G-CSF treated group compared to 904.5 ± 95.90% in 10mM glutamate treated group, $P < 0.05$); consequently protecting the PC12 cells from glutamate toxicity-induced ER stress.

The receptor-mediated effect of G-CSF was validated, because G-CSF in the presence of the antibody against its receptor, anti-G-CSFR, was unable to significantly ($P = 0.47$) attenuate the level of CHOP produced by 10mM glutamate (765.20 ± 87.22 compared to 904.5 ± 95.90%; respectively).

2.3. DISCUSSION

This phase of our study focused on the use of glutamate-induced toxicity in undifferentiated PC12 cells to simulate an *in vitro* model of stroke-like condition. Using this model we investigated the protective effect of granulocyte colony-stimulating factor (G-CGF) against glutamate induced-toxicity; specifically glutamate toxicity involving endoplasmic reticulum stress. Our study has provided evidence that (1) high extracellualar glutamate concentration induced toxicity resulting in cell death, (2) G-CSF was able to protect undifferentiated PC 12 cells from glutamate-induced cell death, (3) the protective action of G-CSF was receptor mediated, and (4) G-CSF protected undifferentiated PC12 cells against glutamate-induced endoplamsic recticulum stress by attenuating the ER stress apoptotic protein, CHOP.

2.3.1. High extracellular glutamate concentration induces undiffereniated PC 12 cell death.

One of the major conditions related to stroke pathophysiology is excitotoxicity, which is mediated by the excitatory neurotransmitter; glutamate (Hazell 2007). Under normal physiological conditions extracellular glutamate concentration is maintained at a micro molar range (2 μM) (Benveniste et al., 1984; Baker et al., 2003), where it activates postsynaptic action potential through its ionotropic [NMDA (*N*-methyl-D-aspartate), AMPA (aamino-3-hydroxy-5-methyl-4-isoxazolepropionate), and kainate] and metabotropic receptors (Meldrum et al. 2000). Once bound to its receptors, calcium influx is initiated through the glutamate receptors. Subsequent to an ischemic insult there is an excessive increase in the concentration of extracellular glutamate (Globus et al., 1988; Hillered et al., 1989; Budd, 1998) due to one or all of the following: increased

exocytotic vesicular release, reduced glutamate uptake via EAATs (excitatory amino acid transporters), and non-vesicular glutamate release via reversal of EAAT-mediated glutamate uptake or opening of astrocytic volume-sensitive organic anion channels (Seki et al., 1999; Jabaudon, et al., 2000;). This excessive extracellular glutamate leads to the over-activation of glutamate receptors, intracellular calcium overload, dysfunction of cellular metabolism and ultimate cell death by apoptosis and/or necrosis.

Another type of glutamate toxicity that contributes to stroke pathophysiological conditions is oxidative glutamate toxicity which is non-receptor mediated (Murphy et al., 1989). This condition is initiated by high concentrations of extracellular glutamate that prevent cystine (a critical component for glutathione synthesis) uptake into the cells due to the reversal of the cystine/glutamate antiporter (Xc^-). This results in a depletion of the major cellular antioxidant, glutathione (GSH) (Chen et al. 2011). With a diminishing supply of GSH, there is an accumulation of excessive amounts of ROS which cause protein- and lipid-oxidation, interfere with membrane structure, causing DNA damage and eventual apopototic and necrotic cell death (Penugonda et al., 2006).

Although different cell lines respond differently to excessive glutamate exposure, the PC12 cell line, a cell line derived from the rat adrenal medulla pheochromocytoma, demonstrates sensitivity to glutamate injury. In this study we showed that glutamate significantly decreased undifferentiated PC12 cell viability. We found that the glutamate-induced cell injury in undifferentiated PC12 cells was both concentration – and time- dependent (Fig.6). These results are consistent with those from other studies, (Naito et al., 1995; Pereira et al., 1998). While it has been reported that the glutamate toxic concentration in PC 12 cell varies between 0.01 and 10mM, for incubation periods

between 3, 12, 24 or 48 hours (Pereira et al., 1998; Pourzitaki et al., 2008; Lu et al., 2011), we found that in our study with undifferentiated PC12 cell, 10mM glutamate concentration for 24 hour exposure provided the desired toxicity of 50% cell death (Fig. 6); a finding that was further supported by Froissard and others (1994, 1997). It is obvious from our data that the concentration (10 mM glutamate) used to achieved optimal glutamate toxicity is much higher than those (50 – 500 μM glutamate) necessary for glutamate-induce toxicity in primary neuronal cultures (Lysko et al., 1989; Leon et al., 2009) and *in vivo* ischemia (16 μM) (Benveniste et al. 1984). This discrepancy could be due to the differences between cell culture systems and also differences between in *vivo* systems. There is a lot of controversy in the literature as to whether glutamate-induced toxicity in PC12 cells is NMDA receptor-mediated, since some reports have shown that even though there were trace amounts of the NMDA receptor, no functional NMDA-operated channels were found in both differentiated and undifferentiated PC12 cells (Sucher et al. 1993). While using the same experimental approach, Casado, et al. (1996) detected functional activity of the NMDA receptor. Murphy and others (1990) showed that glutamate exerted cytotoxic action at high extracellular concentrations (5 – 10 mM) in immature cortical neurons and that it was NMDA receptor-independent which involved glutamate oxidative toxicity via the inhibition of cystine uptake. Several reports have demonstrated glutamate oxidative toxicity in PC12 cell at a glutamate concentration of 10mM (Pereira and Oliveira, 1997; Tyurin, et al., 1998; Penugonda, et al., 2005). Interestingly, it was recently demonstrated in PC 12 cells that 10 mM glutamate-induced toxicity was both NMDA receptor mediated, involving intracellular calcium overload, and non NMDA receptor mediated involving oxidative glutamate toxicity via inhibited

cystine uptake (Ma et al., 2012) It is therefore reasonable to infer from our study that glutamate-induced toxicity in undifferentiated PC12 cells is both NMDA- and non NMDA- mediated since we observed toxicity at high (10mM) glutamate concentration.

Our use of high glutamate concentration to simulate a stroke-like condition in undifferentiated PC12 cells, mimics the ischemic pathology of the presence of excessive extracellular glutamate (an eight-fold increase from 2uM to 16uM, Benveniste et al., 1984) in the extracellular space.

2.3.2. Protective effect of G-CSF against glutamate-induce toxicity in undifferentiated PC12 cell is receptor-mediated.

The protective effect of G-CSF is receptor mediated, activating associated intracellular signaling pathways (Nicholson et al., 1995; Dong & Larner, 2000). The granulocyte colony-stimulating factor receptor (G-CSFR) is found on a variety of cells (Bussolino et al., 1989; Hanazono et al., 1990; Schäbitz et al., 2003; Schneider et al., 2005). While it has been reported that the mRNA (Meuer et al., 2006; Kook et al., 2011) and protein expression (Kook et al., 2011) for the G-CSFR is found in PC12 cells, there has been no report on the specific location in PC12 cells. By using western blotting and immunofluorescence, we confirmed that undifferentiated PC12 cells expressed the G-CSF receptor protein and that this expression was located on the plasma membrane of the cells (Fig. 7). To our knowledge our data showing this specific location of the G-CSFR on the plasma membrane of undifferentiated PC12 cells is a novel finding.

Granulocyte colony-stimulating factor (G-CSF) has been reported to physiologically regulate survival, differentiation, and proliferation of the neutrophil leukocyte lineage (Hartung, 1998; Sakamoto et al., 2003; Mangan & Reddy, 2005), partly

because of its antiapoptotic activity (Solaroglu et al., 2006). It has been shown to be protective against several toxic compounds in a variety of cell lines. For instance it protects against: MPP^+ (1-methyl-4-phenylpyridinium) in primary neuronal midbrain and PC12 cell cultures model of Parkinson Disease (Kook et al., 2011); and glutamate-induced toxicity in cerebellar granule cell culture (Schäbitz, et al., 2003). We have demonstrated that G-CSF potently attenuated glutamate-induced toxicity in undifferentiated PC12 cells in a dose dependent manner with an optimal dose of 50ng/ml (Fig.8). This optimal protective dose (50ng/ml) of G-CSF against glutamate-induced toxicity was also reported to be effective in human neuroblastoma (SHSY-5Y) cell model (Schneider et al., 2005). We then investigated if the protective effect observed by G-CSF would be reversed once its receptor was inhibited. We have demonstrated that an antibody against the G-CSFR reversed the protective effect observed by G-CSF (Fig. 10). Based on our data we have provided evidence that the G-CSFR located on the plasma membrane of undifferentiated PC12 cells were functional and we have also confirmed evidence from the literature that the effect of G-CSF is receptor mediated.

2.3.3. The effect of G-CSF against glutamate-induced endoplasmic reticulum stress.

Endoplasmic reticulum (ER) stress has been implicated in several neurodegenerative diseases, including Alzheimer's disease (Sherman and Goldberg 2001) Parkinson's disease, and neuronal damage by ischemia (Paschen & Frandsen, 2001) (DeGracia et al., 2002). ER stress can be induced by pathophysiological conditions, such as neurotrophic factor deprivation, hypoxia and oxidative stress (Feldman et al., 2005) and we have previously shown that glutamate induced endoplasmic reticulum stress in primary neuronal cell culture (Pan et al., 2010; 2012). One of the major indicative

markers of ER stress is CHOP (Wang et al., 1996; Lu et al., 2014) . CHOP is a transcription factor and has been shown to play a critical role in the pathological process of ER stress. For instance, cells overexpressing CHOP showed an increasing susceptibility to apoptosis induced by ER stress (McCullough et al., 2001).

In this study we have shown that 10mM glutamate induced ER stress in undifferentiated PC12 cells. This was observed by the significant upregulation of CHOP in the glutamate-treated group (Fig. 11). Several studies had reported that the transcriptional property of CHOP induced apoptosis (Wang et al., 1998; Sok et al., 1999; McCullough et al., 2001). Using a phase/contrast microscope we observed apoptotic morphological changes in undifferentiated PC12 cells treated with 10mM glutamate (Fig. 9c). This observation may be partly due to upregulated CHOP which is transcription factor that enhances the transcription of apoptotic protein, Bim, and inhibits the anti-apoptotic protein, Bcl-2. Growth factors such as insulin-like growth factor -1 (IGF-1), have being reported to be protective against ER stress-induced apoptosis in differentiated PC12 cells (Zou et al. 2009). In our present study, we demonstrated that G-CSF, another type of growth factor, protected undifferentiated PC12 cells against glutamate-induced ER stress by downregulating CHOP.

3. GRANULOCYTE COLONY-STIMULATING FACTOR PROTECTS AGAINST APOPTOSIS IN A RAT MODEL OF TRANSIENT FOCAL ISCHEMIA

While biological experiments carried out *in vitro* provide relatively quick data that are both important and critical in understanding a specific area of research, they tend to be less exact than experiments done *in vivo* (experiments carried out inside a living organism) because they do not exhibit the actual physiological and/or pathophysiological conditions of a living organism (Emelda, 2011). For instance, studies in animal models of stroke enables the control over the severity, duration, location and cause of the ischemia and also allows for the monitoring of physiological parameters, such as body temperature, (such physiological parameter influences the magnitude of the ischemic lesion) (Macrae, 1992); all of which presents a more similar scenario to human conditions. Hence data obtained from *in vivo* studies are more easily extrapolated to human conditions than those obtained from *in vitro* studies.

This *in vivo* study was designed to determine the efficacy of G-CSF to exert protection against transient focal ischemia in a rat model. The following hypotheses would be tested: (1) G-CSF would exert protection against ER stress induced apoptosis in the transient focal ischemic brain. (2) G-CSF would reestablish ER homeostasis in the transient focal ischemic brain. (3) G-CSF would reduced the general stress level of the brain due to the ischemic injury. Subsequent to prolonged ER stress (the accumulation of unfolded proteins in the ER), the three ER stress transmembrane sensors; PERK, IRE1α

and ATF6 are activated, leading to the consequential initiation of intracellular pathways that upregulate the ER stress proapoptotic transcription factor, CHOP. CHOP contributes to apoptosis by inhibiting the transcription of the antiapoptotic molecule, Bcl-2 while poteniating the transcription of the proapoptotic protein, Bim. In addressing the first hypothesis, I predict that G-CSF will negatively modulate the PERK, IRE1α and ATF6 pathways. I also predict that G-CSF will downregulate or attenuate CHOP, thereby protecting the ischemic brain against ER stress apopotsis.

One criteria for maintaining ER homeostasis is the concentration level of calcium (Verkhratsky et al. 2003). Calcium in the ER is important for the proper folding of proteins, since the enzymes (chaperone molecules) responsible for proper protein folding are Ca^{2+} dependent. Ischemia leads to a reduction of the calcium level in ER, resulting in the disruption of the ER's homeostasis and the consequential accumulation of unfolded/missed folded proteins. Increased levels of unfolded protein results in the upregulation of protien chaperons, such as GRP78. I predict that G-CSF treatment will downregulate GRP78 in the transient ischemic brain, which would be a reflection of the reestablishment of the ER homeostasis.

The overall stress level of a cell is reflected in the expression level of salient biomakers, such as members of the heat shock family of proteins (Tiwari et al. 2015). I predict that G-CSF will not only reestablish the ER homeostasis, but will also reduce the overall stress level of the cell, reflected by the attenuation of HSP27 (a very popular heat shock protein) in the transient focal ischemic brain that is treated with G-CSF.

3.1. MATERIAL AND METHOD

3.1.1. Animal preparation

Animals were prepared accordingly to our previous studies (Gharibani et al., 2013; Modi et al., 2014). Briefly, adult male Sprague–Dawley rats (weighing 270–300 g, Harlem Chicago, IL, USA) were place on overnight fasting before surgery to ensure a stable blood glucose level. All rats were weighed and anesthetized by intra-peritoneal (IP) injection with a cocktail of ketamine hydrochloride (80 mg/kg body weight; Putney) and xylazine hydrochloride (20 mg/kg body weight; Vedco). If necessary, animals were injected with the cocktail, (50 mg/kg body weight) to maintain anesthesia. During surgery body temperature was maintained at 37 °C ± 0.5 °C by a thermostatically controlled heating pad regulated by a rectal temperature probe (CMA 450). After surgery the animals were allowed to recover from anesthesia and were given food and water ad libitum. All animal procedures were carried out in accordance with the guidelines for Animal care and use and were institutionally approved by the Institutional Animal Care and Use Committee (IACUC) of the Florida Atlantic University (FAU), Boca Raton.

3.1.2. Induction of transient focal ischemia by occlusion of the proximal middle cerebral artery.

The induction of cerebral ischemia by the transient proximal occlusion of the middle cerebral artery (MCAO) was carried out as described in detailed, Longa et al., 1989) and recently published, Gharibani et al., 2013; Modi et al., 2014. Briefly, a midline cervical incision was made ventrally. Using an operating microscope (Leica Wild M3C) the left common carotid artery (CCA) was exposed and carefully dissected

away from the vagus nerve. The left CCA was dissected until its bifurcation into the external carotid artery (ECA) and internal carotid artery (ICA) was observed. Two branches of the left ECA (occipital and superior thyroid artery) were cut between double ligatures using 6-0 silk suture. The left ECA was double ligated as far distally as possible while its proximal end was loosely held with a 5-0 silk suture. The left ICA was dissected away from the surrounding fascia and vagus nerve until the pterygopalatine artery (PPA) of the ICA was observed and tied (5-0 silk suture). Both the CCA and the ICA were clamped with micro vessels clips while the ECA was cut between the two distal ligatures. A microincision was made in the ECA close to the cut end and a 4–0 monofilament nylon suture with a silicon coated tip (Doccol Co., Albuquerque, NM, USA) was inserted into the ECA through the microincision. The silicon coated tip 4-0 monofilament (intraluminal suture) was gently advanced into the ICA, from the ECA, until it abutted the clamped end of the ICA. The micro vessel clip of the ICA was removed and the intraluminal suture was advanced 18–22 mm from the carotid artery bifurcation into the internal carotid artery until there was slight resistance (O'Neill and Clemens 2001). The microincision in the ECA was clamped with a micro vessel clip while the clip at the CCA was removed. In this position the tip of the intraluminal suture occluded the origin of the MCA (Fig. 5) which was confirmed by a 20 – 50 % drop of baseline blood flow monitored by a Laser Doppler Flowmeter (LDF) (Periflux 4001 Master, Perimed Inc, Cleveland, OH, USA) (Fig. 12). The intraluminal suture remained in position for 90 mins. after which it was gently withdrawn and the ECA was tied (5-0 silk suture) just below the microincision. The removal of the intraluminal suture post ischemia facilitated reperfusion.

Animals with a reduced regional cerebral blood flow (RCBF) greater than 50% were removed from the study as well as animals with an increased core body temperature greater than 39 °C (Li et al., 1999; O'Donnell et al., 2006). Sham-operated animals underwent the same experimental procedures, but the silicon coated monofilament was not inserted beyond the bifurcation of the CCA; thus preventing an ischemic injury.

3.1.3. Measurement of regional cerebral blood flow

At the beginning of surgery, a small incision was made in the skin overlying the parietal bone of the animal's cranium. A 0.7 mm flexible laser Doppler probe (model P10) was placed 6mm lateral and 2mm posterior from bregma on the superior aspect of the left temporal bone and secured with glue (Loctite) (Popp et al.,2009). This positioning was over the area supplied by the middle cerebral artery (MCA) of the left cerebral hemisphere. Recorded value of regional cerebral blood flow (RCBF) was presented as a percentage of baseline value.

3.1.4. Experimental animal group and drug treatment

Rats were assigned to three experimental groups. Rats that were subjected to MCAO were randomly assigned into vehicle-treated (n = 20); administered 0.3ml of 5% dextrose) group or G-CSF-treated group (n =15). Sham operated control group (n = 9; in 0.3ml of 5% dextrose) were rats subjected to surgery but without the MCAO being occluded. The G-CSF-treated group received recombinant human G-CSF (Filgrastim,: Akron, FL, USA) dissolved in 0.3ml of 5% dextrose, injected subcutaneously (50μg/kg body weight) (Crouch et al., 2003;Solaroglu et al. 2006). Initial administration of G-CSF was 24 hrs. after MCAO, followed by the administration of the same dose (50μg/kg body weight) daily for an additional 3 days. Sham and vehicle-treated groups underwent

the same experimental protocol except that they received only 0.3ml of 5% dextrose. Rats were euthanized either 1 day (vehicle-treated, n = 4) or 4 days (vehicle-treated, n = 16; sham, n = 9; and G-CSF- treated n = 15) after MCAO.

3.1.5. Neurological evaluation

Rats were carefully evaluated for motor and behavioral deficits according to Menzies et al., (1992) with slight modifications. Briefly, neurological evaluations were made 24 hrs. after surgery and every consecutive day after that for 4 days. A six-point neuro-score grading scale of 0 to 5 was used as assessment. The tests described below were conducted sequentially; if a rat exhibited a particular behavior at one step but not at the subsequent step, it was graded as the former. The grading score was as follows: rats were held gently by the tail, suspended 30 cm above the floor, and observed for forelimb posture. Grade 5 = normal rats that extended both forelimbs toward the floor and that

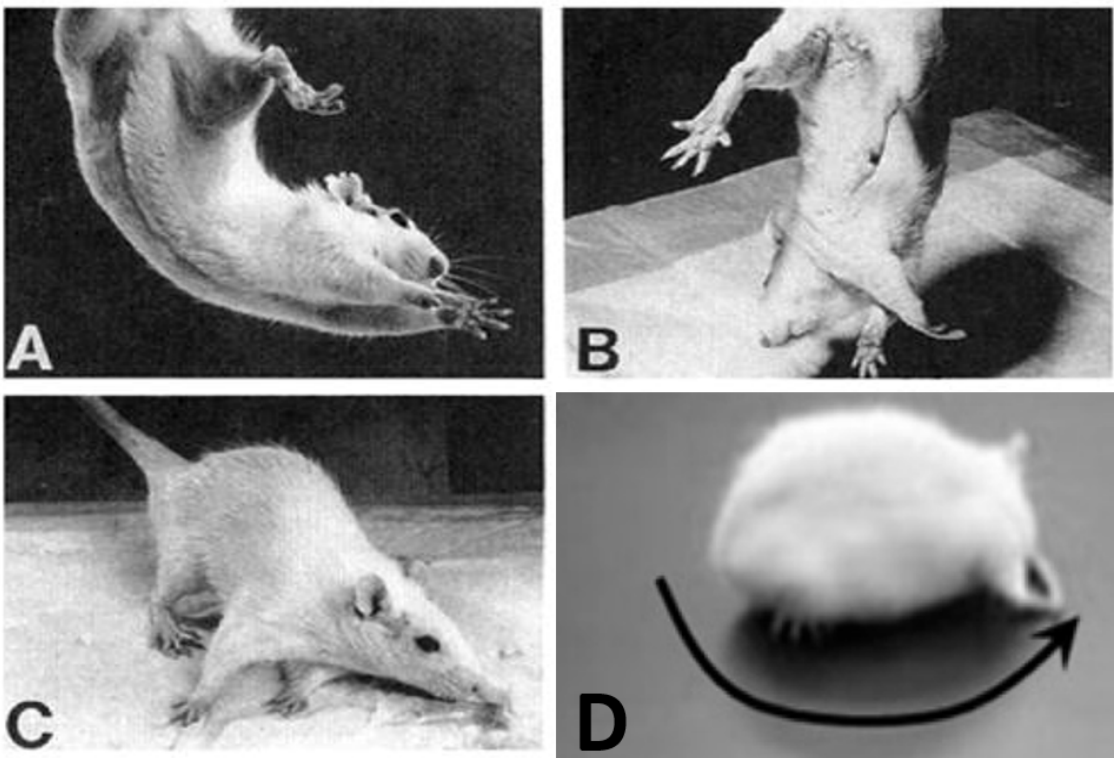


Figure 12. Representation of neurological scoring scheme (for scores 2 – 5).

Rats were suspended from tail approximately 30 cm above the floor and observed for forelimb posture. (A) Score 5 = normal rats extended both forelimbs. (B) Score 4 = rats with contralateral forelimb flexion. (C) Score 3 = with the tail held by hand, a gentle pull on the tail produced an apparent decrease in grip in the contralateral forelimb. (D) Score 2 = rats circled towards the paretic side if pulled by the tail ***(Adapted from Menzies et al., 1992).***

had no other neurological deficit (Fig. 12A). Grade 4 = rats that consistently flexed the forelimb contralateral to the injured hemisphere during suspension and there were no other abnormalities (Fig. 12B). Grade 3 = rats that were placed on a large sheet of soft, plastic coated paper that could be gripped firmly by their claws. With the tail held by hand, a gentle pull on the tail produced an apparent decrease in grip in the contralateral forelimb. The maneuver was repeated a few times. Normal rats resisted being pulled by showing a firm grip in both forelimbs. Severely dysfunctional rats had consistently reduced resistance to tail pull in forelimb on the paretic side (Fig 12C). Thereafter rats were allowed to move freely and were observed for circling behavior. Grade 2 = rats circled towards the paretic side if pulled by the tail (Fig. 12D). Grade 1 = rats that consistently circled spontaneously towards the paretic side. Grade 0 = rats that exhibited "no spontaneous" movement. Forelimb flexion was always observed in rats with decreased resistance to lateral push; both forelimb flexion and decreased resistance to lateral push were always observed in rats that displayed circling behavior. While all scores were noted, the lowest score observed was the score assigned as the rat's behavior for that day.

3.1.6. Histochemical assessment of infarct volume

Animals were deeply anesthetized by isoflurane (Phoenix) and decapitated, after which their brains were rapidly dissected for sectioning. Using an adult rat brain slicer

(Matrix, Zivic Instruments), animal brains were cut in coronal sections of six 2-mm coronal slices (2, 4, 6, 8, 10, and 12 mm) (Fig. 13a), starting from the frontal pole. Brain slices were then immersed in a 2 % (w/v) solution of 2, 3, 5-triphenyltetrazolium chloride (TTC; J.T. Baker, India) at 37 °C in the dark, for 10 minutes. Slices were flipped at the 5 minute time point for consistent staining of anterior and posterior surfaces. TTC, a water soluble salt, is reduced by mitochondrial dehydrogenases to formazan, which turns normal tissue deep red, leaving damaged tissue colorless (Lundy et al., 1986; Rich et al., 2001). Therefore a reduction in TTC stain indicates regions of diminished mitochondrial function in ischemic tissue. In assessing the lesion volume, the TTC-stained slices were scanned, on both anterior and posterior sides, using an HP ScanJet 5530 and analyzed by Image J analysis software [public domain software developed at NIH. The average infarct area (mm^2) in each section was calculated by the following formula: (infarct area on the anterior surface + infarct area on the posterior surface)/2. Infarct volumes (mm^3) were calculated by the sum of all the section areas and multiplying by the slice thickness (2mm) (Cisneros-mejorado et al. 2015) and determined as the percent of the total ipsilateral hemispheric volume. Brain edema was corrected as previously described (Swanson et al., 1990; Schäbitz et AL., 2000; O'Donnell et al. 2006). Briefly, the corrected infarct volume was calculated using the formula:

$$[(VC - VL)/ VC] \times 100$$

in which VC is the volume of control (right) hemisphere and VL is the volume of non-infarcted tissue in the lesioned (left) hemisphere. The TTC stain validated the occurrence of an ischemic insult and hence only those animals that showed evidence of ischemia was used in the study.

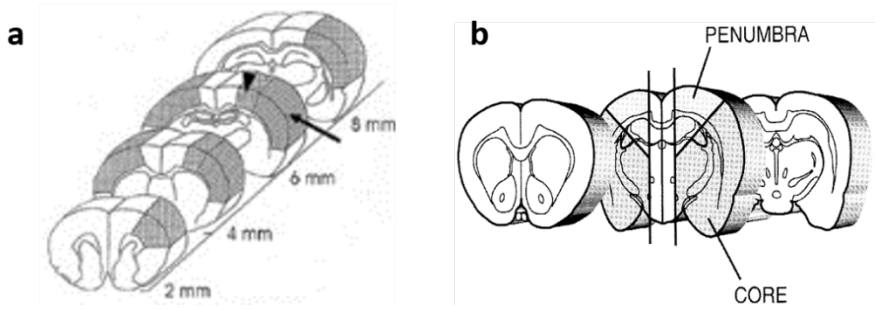


Figure 13. Schematic representation.

(a) Representative sections of rat brain cut into 2mm sections from frontal pole to occipital pole. (b) Anatomical distribution of penumbra and core. Longitudinal cut made on both hemisphere approximately 2 mm from the midline in order to avoid mesial hemispheric structures, which are supplied primarily by the anterior cerebral artery. A transverse diagonal cut at approximately the "2 o'clock" position was made to separate the core from penumbra. ***(Adapted from Sun et al., 2008)***

3.1.7. Collecting ischemic core and penumbra

After TTC staining core and penumbra regions were quickly dissected while the respective brain sections were on ice. Briefly, as previously described by (Ashwal et al. 1998) a midline cut was made to separate the right and left hemisphere. After which a longitudinal cut (from top to bottom) was made on the separated hemispheres, approximately 2 mm from the midline. This was done to avoid mesial hemispheric structures, which are supplied primarily by the anterior cerebral artery. Then a transverse diagonal cut at approximately the "2 o'clock" position (Fig. 13b) was made to separate the core (i.e., striatum and overlying cortex) from the penumbra (adjacent cortex). (Folbergrová et al., 1992; Sun et al., 2008). Brain sample of ischemic core and penumbra of vehicle treated and G-CSF treated were stored at -80^{o}C until needed for further experimental assay. Corresponding areas (Fig. 13b) that represented "core" and "penumbra" in sham-operated rats were also collected and stored at -80^{0}C.

3.1.8. Western blotting

Rat brain samples (core and penumbra) were collected (Fig. 13b) and lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) (Thermo Scientific, Rockford, IL, USA) containing 1%(v/v) mammalian protease inhibitor cocktail and 2 % (v/v) phosphatase inhibitor cocktail from Sigma and Thermo Scientific, respectively. The samples were them homogenized in RIPA buffer and centrifuged at 20,000 g for 15 min at 4°C. Whole cell lysate was obtained and protein concentration was determined by Bradford assay (Bradford 1976). Protein samples (50 -60 ug) were suspended in laemmli SDS sample buffer (125 mM Tris–Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% bromophenol blue), boiled for 7 mins., and resolved by 12 % SDS-PAGE. Molecular weight markers (Biorad, USA) were loaded simultaneously with protein sample on the gel for protein band identification. Resolved protein samples were electro-blotted from the gel to nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the nitrocellulose membrane with blocking buffer, [TBST: (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk)] for 1 hour at room temperature (RT). Membranes were then probed with one of the following primary antibodies overnight at 4^{o}C; abcam: GRP78 (1:1000), Hsp27 (1:1000), ATF4, (1:1000), caspase-12 (1:1000), p-JNK (1:1000); Cell Signaling: GAPDH (1:3000), Bax (1:1000), Bcl-2 (1:1000), Bak (1:500), cleaved caspase-3 (1:1000), AKT (1:1000), pAKT (1:1000), p38MAPK (1:500), p-p38MAPK) (1:500); Santa Cruz: CHOP/GADD153 (1:100); Imgenex: ATF6 (1:1000) and Sigma Aldrich: JNK (1:10,000).

After overnight incubation, antibodies were removed and membranes washed in TBST, three times for 5 mins respectively. This was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit (1:3000; Cell Signaling Technology) or goat anti-mouse (1:3000; abcam). GAPDH was used as internal loading control. Signals were detected by enhanced chemiluminescence and quantitative western blot results were obtained by densitometry analysis by NIH Image J. Sham-operated "core" and "penumbra" samples served as baseline for samples of the left ischemic hemisphere. Data were presented as percentage (%) sham-operated value.

3.1.9 Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). Regional cerebral blood flow and behavioral measurements were analyzed using the two- way ANOVA repeat measures. The 2-tailed Student's *t* test or one-way ANOVA was used to determine significant difference between infarct volumes. Western blotting (performed in duplicates) densitometries were assessed by one-way ANOVA. Tukey post hoc test was used for ANOVAs and $P < 0.05$ was considered statistically significant. Data are represented as the mean ± S.E.M

3.2. RESULTS

The occlusion of the middle cerebral artery (MCAO), remains one of the most relevant stroke models, simply because the majority of human strokes result form an occlusion of this artery (Mohr et al., 1986). The middle cerebral artery may be transient or permanently occluded. Permanent occlusion models the problem of long term vessel blockade that sometimes occurs in humans while transient occlusion mimics the problem of both ischemia and reperfusion. We decided on using the transient occlusion model which allowed us to mimic both ischemia and reperfusion conditions of stroke.

3.2.1. Induction of transient focal ischemia

We used the intraluminal technique to induce transient focal ischemia by occluding the middle cerebral artery (MCA) (for description see section 3.1.2). Although there exist several methods to induce transient focal ischemia via the MCA (Sharkey et al., 1993; Kanemitsu et al., 2002; Bacigaluppi et al., 2010; Boyko et al., 2010; Soylu et al., 2012; Morancho et al., 2012) the intraluminal suture method has been widely used and was identified as the method employed in more than 40% of 2,582 neuroprotection stoke experiments (Howells et al., 2010). By using this technique we had successfully induced transient focal ischemia with reperfusion as monitored by Laser Doppler Flowmeter (Fig 14a). During the occlusion period (90 mins) rats were randomly selected to receive G-CSF or vehicle treatment. All rats subjected to MCAO exhibited a significant reduction in regional cerebral blood flow (RCBF) [$F(2, 21) = 199.7$; $P < 0.01$] to at least 50% pre-MCAO values (G-CSF assigned group: 45 ± 2.35 % and 50 ± 2.35% for vehicle assigned group) at the beginning of the MCAO period.

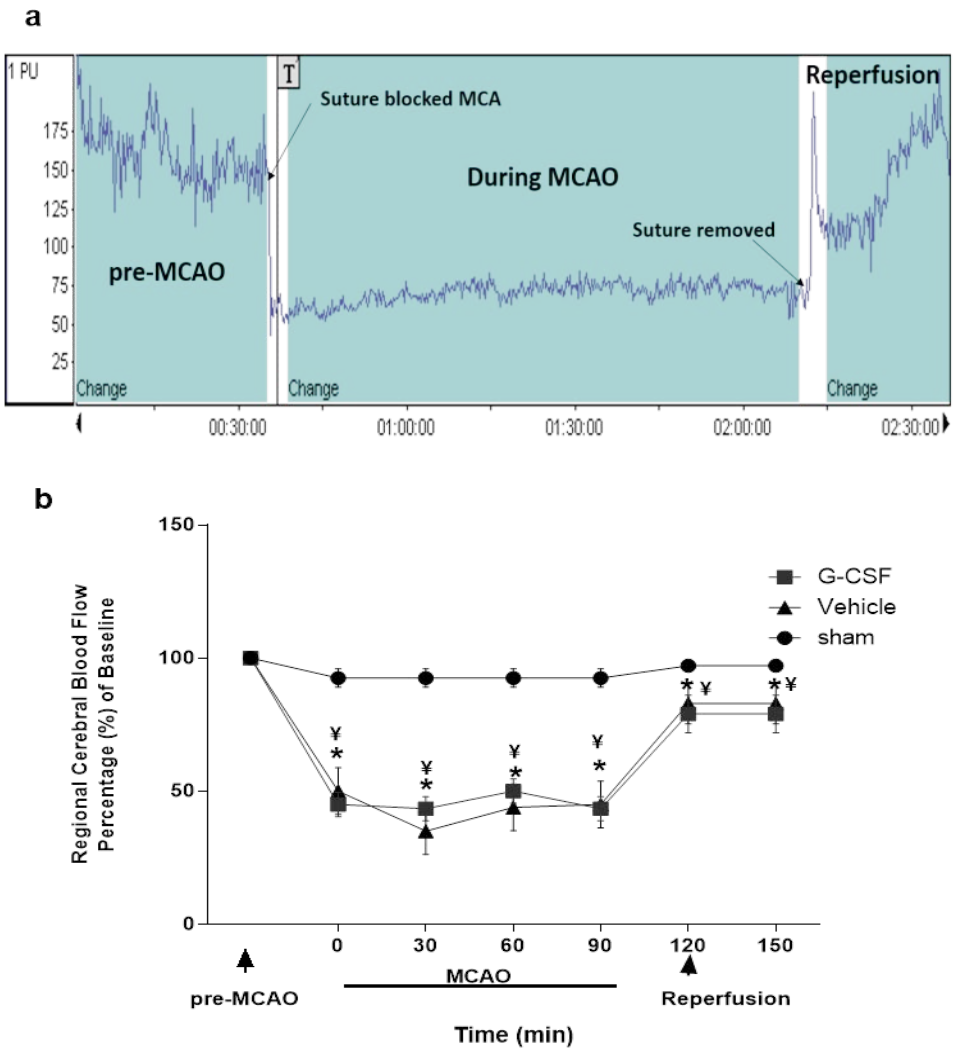


Figure 14. Regional cerebral blood flow (RCBF).

(a) A Laser Doppler Flowmeter (LDF) recording of RCBF: pre- MCAO (baseline flow 161.78 PU), during 90mins MCAO (70.69 PU, 43.7% drop from baseline) and reprefusion (144.12 PU, 89.1% of baseline) PU : perfusion uit. (b) At the start of MCAO, rats were randomly assigned to be treated with either G-CSF (n = 15) or vechile (n=20). Sham operated rats (n=9) were not subjected to MCAO. Data were presented as percentage (%) value of baseline RCBF. Values are mean ± SEM. * $P < 0.01$ vs baseline values and ¥ $P < 0.01$ vs sham operated rats. Two-way ANOVA repeat measure, post hoc Tukey.

We allowed the intraluminal suture to remain in position for 90 mins. Doppler data showed no differences in RCBF between rats that received subsequent G-CSF (43.43 ± 1.22 %) or vehicle treatment (45 ± 1.22 %) at the end of the 90 mins. occluded

time. The intraluminal suture was withdrawn after 90 mins MCAO and RCBF had to rise to at least 70% of pre-MCAO values for the rats to be included in the study. After the removal of the intraluminal suture (for at least the first 30 min.) there were no significant differences in the increase of RCBF between the rats that were to receive G-CSF (79 ± 1.89%) and those to receive vehicle (83 ± 2.05%) (Fig 14b). Sham operated rats experienced the same surgical procedure except that the intraluminal suture was not advanced far enough to occlude the MCA. This was confirmed since sham operated rats showed no significant decrease (92.5 ± 1.24%) in RCBF from baseline values (Fig. 14b). This initial RCBF decreased (92.5 ± 1.24%) in sham operated rats was maintained throughout the 90 mins (30, 60 and 90 mins respectively) occlusion tine and was significantly different from rats subjected to MCAO [(G-CSF assigned; 45 ± 1.22%, 43.43± 1.22 % , 50± 1.22% and vehicle assigned; 50± 2.35%, 35 ±2.35%, 43.93± 2.35%) $F(10, 105) = 66.27$, $P < 0.01$]. Rats that showed signs of hyperthermia (body temperature $\geq 39^{o}C$) or hypothermia (body temperature $\leq 33^{o}C$) were removed from the study since such temperatures above and/or below body temperature affected lesion size (Li et al., 1999; O'Donnell et al., 2006; Krieger et al., 2001).

3.2.2. Behavioral assessment – Neuro-score

Impaired motor function, sensorimotor integration and cognitive functions are neurological deficits of ischemic stroke. Several studies have shown behavioral changes, such as impaired motor coordination, hemiplegia and abnormal posture in rats subjected to middle cerebral artery occlusion (Yamamoto et al., 1988; Markgraf et al., 1992; Menzies et al., 1992). We assessed the effect of G-CSF (50μg /kg. body weight) on neurological behavior based on a six-point neuro-scoring scheme as previously described

in section 3.1.5. The neurological scores were evaluated 24, 48, 72 and 96 hours after surgery in the G-CSF treated group, vehicle treated group and sham-operated group as shown in Fig 15.

Sham-operated rats showed an unimpaired motor deficit and normal behavior that remained constant over the 4 day neurological evaluation and were therefore omitted from statistical analysis. The overall neurological behavior of the G-CSF treated rats showed consistent improvement over a time period of 96 hours, compared to the vehicle treated rats, the neurological behavior of the G-CSF treated rats did not reach significance [$F(4, 56) = 1.81$, $P=0.14$]. This was because treatment conditions had no effect on neurological behavior [$F(1, 14) = 0.6819$, $P=0.43$] but time did [$F(4, 56) = 117.4$, $P<0.01$]. The lack of a significant effect by G-CSF could be because the six point neuro-scoring scheme may not have been sensitive enough for a four day observation, or it could be that the behavioral time point of 4 days was too short so observe the effect of G-CSF on behavior. It would have interesting to observe the effect of G-CSF over a longer time period of 1 – 2 weeks, since several reports provided evidence of neurological improvement by G-CSF over 7 days (Solaroglu et al., 2006; Sevimli et al., 2009).

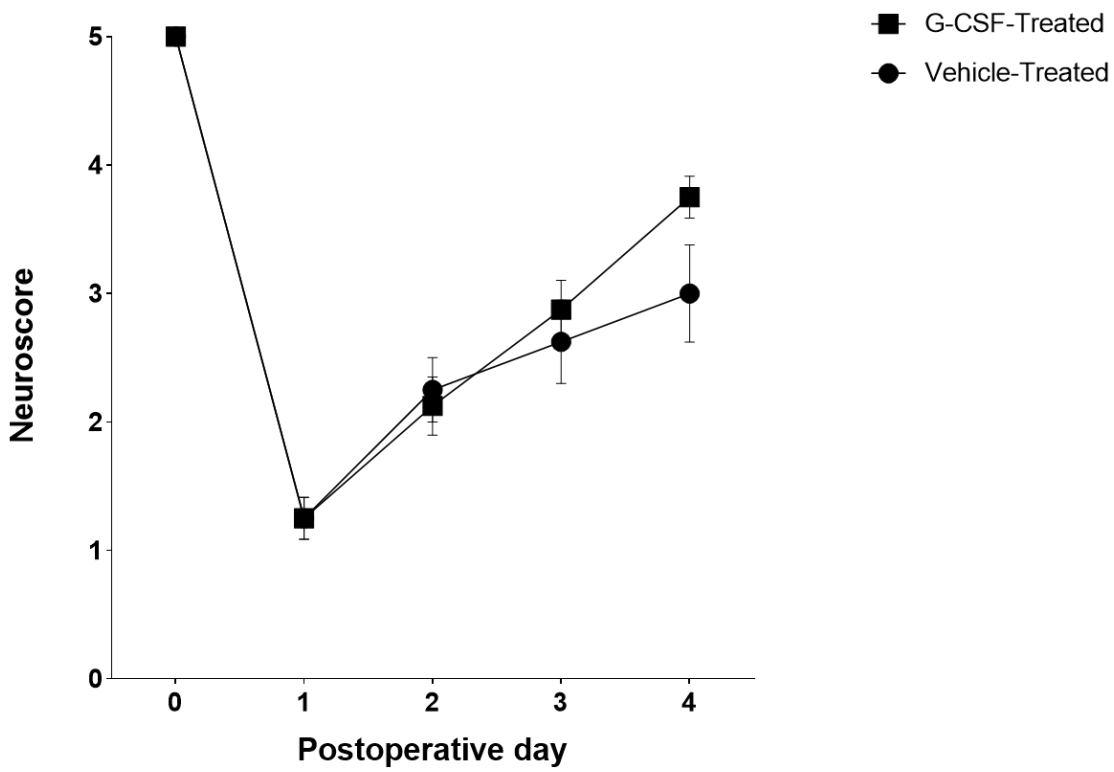
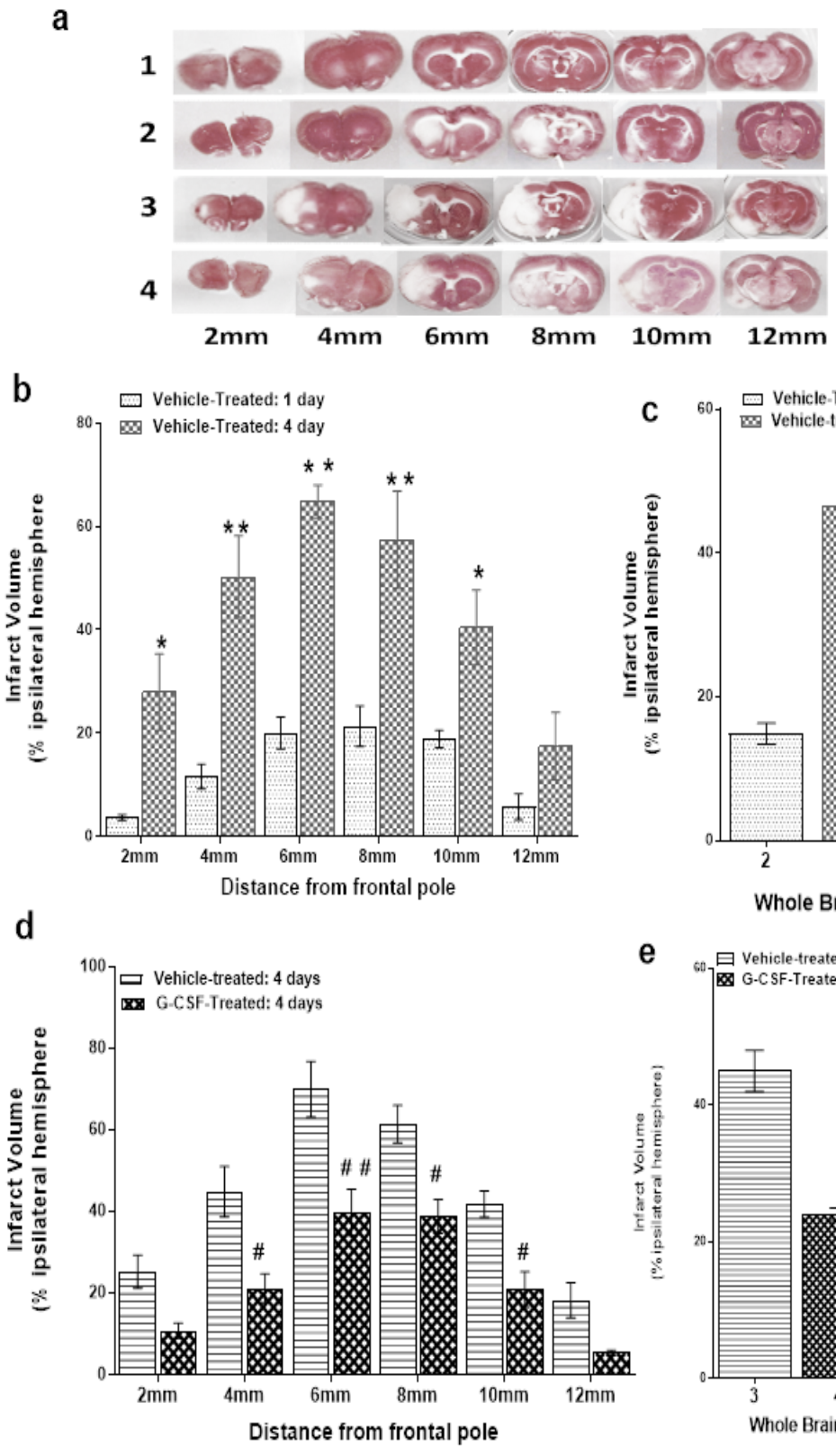


Figure 15. Neuro-score.

Neurological recovery during a 4-day examination postoperative in sham-operated (n=8), vehicle treated (n=8) and G-CSF treated rats (n=8). All sham-operated rats exhibited normal behavior. Both G-CSF and vehicle treated rats showed improved neurological score with time. The G-CSF animal showed greater and better sustained improvement in neurological score, but this did not reach significance [F (4, 56) = 1.81, P=0.14]. Two way ANOVA, repeat measure. Tukey post hoc.

3.2.3 Infarct volume analysis

Analysis of infarct volume is one of the major morphological assessment of successfully inducing ischemia in stroke research. Infarct volumes were quantified as a percentage of the contralateral hemisphere in order to correct for edema in the ipsilateral hemisphere (Swanson et al., 1990; Schäbitz et al., 2000; O'Donnell et al. 2006). Using 2% TTC [TTC a water soluble salt is reduced by mitochondrial dehydrogenases to formazan, which turns normal tissue deep red, leaving damaged tissue colorless (Lundy et al., 1986; Rich et al., 2001)], we examined the brains of sham-operated rats and MCAO rats for physical signs of infarctions. Sham-operated rats (n=9) showed no sign of an infarct (Fig. 16a-1) and were therefore excluded from infarct volume quantification. As shown in Fig 16a-2-4, the MCAO rats showed varying degree of physical infarction based on their treatment conditions. By analyzing the infarct size at 2, 4, 6, 8, 10, and 12 mm from the frontal pole we observed that the extent of the ischemic volume was greatly increased [$F(5, 36) = 2.63$ $P < 0.01$)] from 1 day vehicle-treated rats (n =4) to 4 days vehicle-treated rats (n=4) in brain slices that were 2, 4, 6, 8 and 10 mm from the frontal pole (Fig. 16b) (3.69 ± 0.52 %, 11.61 ± 2.42%, 20.05 ± 3.06%, 21.34 ± 3.91% and 18.86 ± 1.68% versus 27.96 ± 7.39%, 50.29 ± 7.91%, 64.87 ± 3.16%, 57.41 ± 9.43% and 40.46 ± 7.23%, $P< 0.01$).


a
1
2
3
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2mm
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b
Vehicle-Treated: 1 day
Vehicle-Treated: 4 day
Infarct Volume
(% ipsilateral hemisphere
Distance from frontal pole
c
Vehicle-Treated: 1 day
Vehicle-treated: 4 days
Infarct Volume
(% ipsilateral hemisphere)
Whole Brain
d
Vehicle-treated: 4 days
G-CSF-Treated: 4 days
Infarct Volume
(% ipsilateral hemisphere
Distance from frontal pole
e
Vehicle-treated: 4 days
G-CSF-Treated: 4 days
Infarct Volume
(% ipsilateral hemisphere)
Whole Brain

Figure 16. Representation of infarct volume.

(a) Coronal brain sections (2, 4, 6, 8, 10, and 12 mm from the frontal pole) stained with 2% TTC (White areas reflect a lack of staining and hence a loss of viable tissue). 1- Sham-operated rats (n =9); MCAO animals that were either 2- vehicle-treated (1 day reperfusion, n= 4) or 3 – vehicle-treated (4 days reperfusion, n=16) or 4 – G-CSF-treated (4 days reperfusion, n= 15). (b) Quantitative analysis of infarct volume on each coronal brain sections (2, 4, 6, 8, 10, and 12 mm) for 1 day vehicle-treated (n=4) and 4 days vehicle-treated (n=4) (mean ± SEM * * P< 0.01 and * P< 0.05 versus 1 day vehicle-treated for corresponding brain sections, two way ANOVA, Tukey post hoc test) (c) Quantitative analysis of infarct volume on whole brain for 1 day vehicle-treated (n=4) and 4 days vehicle-treated (n=4), (mean ± SEM * P< 0.05 versus 1 day vehicle- treated, Student's t-test, two tail). (d) Quantitative analysis of infarct volume on each coronal brain sections (2, 4, 6, 8, 10, and 12 mm) for 4 day vehicle-treated (n=12) and 4 days G-CSF-treated (n=15) (mean ± SEM # # P< 0.01 and # P< 0.05 versus 4 days vehicle-treated for corresponding brain sections, two way ANOVA, Tukey post hoc test). (e) Quantitative analysis of infarct volume on whole brain for 4 days vehicle-treated (n=8) and 4 days G-CSF-treated (n=14) (mean ± SEM # P< 0.05 versus 4 days vehicle- treated, Student's t-test, two tail**.**

Overall, there was a 31% increase in infarct volume of the whole brain of MCAO rats (46.48 ± 5.86%) at 4 days compared to those at 1 day (14.88 ± 1.47 %) ($P < 0.05$) (Fig. 16c). This observation confirmed the phenomenon of "spreading depression", a characteristic condition of focal ischemia whereby the ischemic core (if not arrested) will recruit the perifocal penumbra via waves of electrophysiological hyperactivity, resulting in infarct expansion (Dirnagl et al. 1999). The administration of G-CSF (50μg/kg body weight, s.c.) to MCAO rats for 4 days (n=15), markedly reduced the infarct volume (Fig 16a-4), especially in brain sections, 4, 6, 8 and 10 mm from the frontal pole compared to MCAO rats (n=12) without G-CSF also for 4 days (Fig. 16d) (21.20 ± 20%, 39.80 ± 5.65%, 38.80 ± 4.18% and 20.90 ± 4.34% compared to 44.87 ± 6.18%, 69.96 ± 6.86%, 61.41 ± 4.58% and 41.83 ± 3.21%). This reflected an overall reduction of 53% ($P < 0.05$) infarct volume of the whole brain of rats given G-CSF (50μg/kg body weight, s.c.)

(45± 3.00 %) for 4 days compared to MCAO rats without G-CSF (24.05 ± 0.94 %) also at 4 days (Fig. 16e).

3.2.4. Effect of granulocyte-colony stimulating factor on apoptosis.

Apoptosis (programmed cell death) is an energy-dependent process that initates intracellular death pathways (Kerr et al., 1972). There are extensive documentation of both morphological and biochemical evidences of apoptosis in experimental animal models of focal ischemia (Li et al., 1995;Charriaut-Marlangue et al., 1996; Du et al., 1996; Unal-Cevik et al., 2004; Sugawara et al., 2004; Durukan & Tatlisumak, 2007). Many molecular markers exist that are indicators of apoptosis. Because it was reported that one of the protective mechanisms of G-CSF was anti-apoptotic (Schäbitz et al., 2003) we therefore investigated the effect of G-CSF on some of these apoptotic markers.

3.2.4.1. Bcl-2 family members

Some of the major players that regulate intracellular apoptotic signal transduction are members of the Bcl-2 family. Bcl-2 family members such as Bax and Bak are major pro-apopotic proteins (Deckwerth et al. 1996) while on the other hand Bcl-2 and Bcl-xL are major anti-apoptoic proteins (Michaelidis et al. 1996). We investigated the effect of G-CSF on the expression level of anti-apoptotic protein Bcl-2 and the pro-apoptotic proteins Bax and Bak. Brain samples of sham (n = 6), vehicle-treated (n =8) and G-CSF-treated (n = 8) rats 4 days after MCAO were harvested and western blotting was performed on the core and penumbra of the ischemic region (core and penmubral tissue were collected as previously decribed in section 3.1.7.)

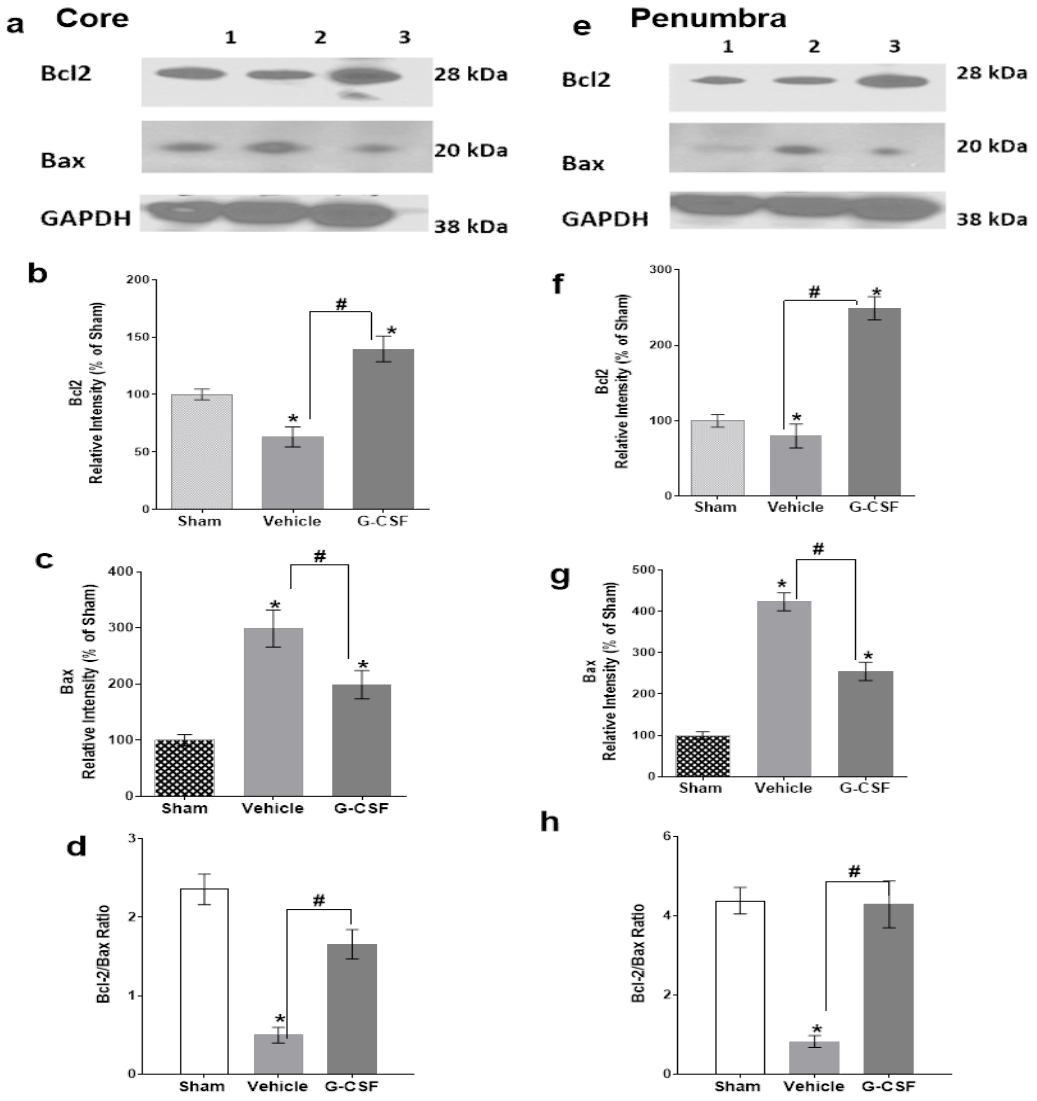


Figure 17. Effect of G-CSF on the levels of Bcl-2 and Bax.

Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against Bcl-2 and Bax in core (a) and penmumbra (e); 1 – Sham-operated rats, 2- vehicle treated and 3-G-CSF treated rats. The *bar* graphs reflected the densitometric data from the experiment of Bcl-2 and Bax Western blot in the core (b –c) and penumbra (f – g). The results were expressed as percentage of the levels in sham-operated rats. *Bar* graphs also reflected the ratio of Bcl-2/Bax in the core (d) and the penumbra (h). All data were expressed as mean ± SEM, *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

We observed that vehicle treatment and G-CSF treatment affected the expression level of Bax, Bak and Bcl2 in both the core [$F(2, 12) = 10.73$, $P = 0.002$], [$F(2, 9) = 89.15$, $P < 0.001$] and [$F(2, 16) = 18.37$, $P < 0.001$] and the penumbra [$F(2, 9) = 17.59$, $P = 0.008$], [$F(2, 9) = 67.69$, $P < 0.001$] and [$F(2, 22) = 38.67$, $P < 0.001$], respectively. We demonstrated that the level of Bax in both the core and penumbra of vehicle treated rats were significantly increased compared to sham-operated rats (298.81 ± 32.98 %, 423.23 ± 21.97% vs 100 ± 10.05%, 100 ± 8.95%; respectively, *P < 0.05) as shown in Fig 17a, e, c and g. A similar significant increase was shown for the level of Bak in both core and penumbra (339.47 ± 16.23%, 419.51 ± 23.65% vs 100 ±10.25%, 100 ± 10.18%; respectively, *P < 0.05) as shown in Fig. 18a – d. In contrast, the levels of Bcl-2 in vehicle treated rats of both the core and penumbra were markedly reduced versus sham-operated rats (63.21 ±8.81 %, 79.84 ± 15.93 % vs 100 ± 4.69 %, 100 ± 8.42% respectively, *P < 0.05) as exhibited in Fig 17a, e, b and f. Ischemia-induced changes of Bcl-2 and Bax levels resulted in a significant reduction in the ratio of Bcl-2:Bax in both the core and the penumbra compared to sham-operated rats (0.49 ± 0.10, 0.83 ± 0.15 vs 2.35 ± 0.20, 4.384 ± 0.335 respectively, *P < 0.05).

Compared with vehicle-treated rats, treatment with G-CSF (50 ug/kg body weight s.c) significantly reduced Bax level (198.81 ± 25.23%, 254.54 ± 22.05% vs 298.81 ± 32.98 %, 423.23 ± 21.97%; #P<0.05) while increased Bcl-2 levels (139.75 ± 11.10%, 249.08 ± 15.36% vs 63.21 ±8.81 %, 79.84 ± 15.93 % , # P < 0.05) in both core and penumbra, which also significantly enhanced the ratio of Bcl-2:Bax (1.657 ± 0.187, 4.29 ± 0.59, vs 0.49 ± 0.10, 0.83 ± 0.15; respectively #P < 0.05).

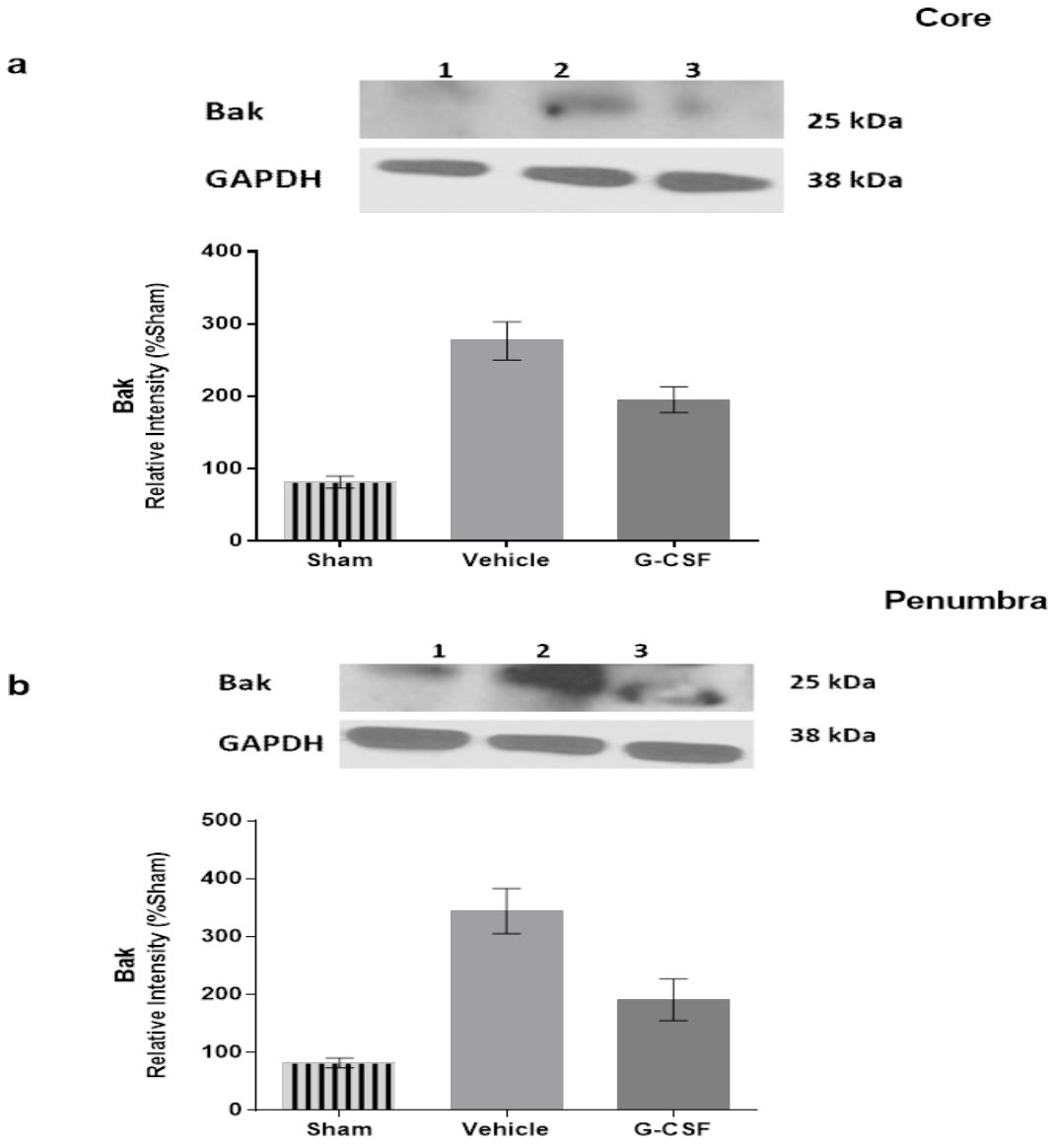


Figure 18. Effect of G-CSF on Bak expression.

Rtreated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starating 24hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against Bak in core (a) and penmumbra (c); 1 – Sham-operated rats, 2-vehicle treated and 3-G-CSF treated rats. The *bar* graphs reflected the densitometric data from the experiment of Bak Western blot in the core (b) and penumbra (d). The results were expressed as percentage of the levels in sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

It is interesting to note that the increased level of Bcl-2 and reduced level of Bax by G-CSF was so markedly significant that the ratio of Bcl-2:Bax was not significantly different from the Bcl-2:Bax ratio in sham-operated rats. A similar significant reduction by G-CSF was shown for the level of Bak compared to vehicle treated rats (239.47 ± 10.94%, 232.93 ± 21.85% vs 339.47 ± 16.23%, 419.51 ± 23.65% respectively, # $P < 0.05$) (Fig. 18a-d). This demonstrated that G-CSF could reverse the levels of pro-apoptotic proteins in the ischemic region.

3.2.4.2. Caspase 3

Another group of proteins that are crucial regulators of apopoptic pathways are caspases (cysteine aspartic acid proteases) (Shi 2002). To date there are 14 known mamalian capases, some are initiators for apoptosis (eg., caspase 8, caspase 9, caspase 10 and caspase 12) while others are executers of apoptosis (eg., caspase 3 and caspase 7).

Generally activated initiator caspases cleave inactive forms of executioner caspases which cleave other protein substrates that contribute to apoptosis (Fischer et al., 2003; Bredesen et al., 2006). Caspase 3 is a frequently activated death protease and catalyzes specific cleavage of key cellular proteins. It is also required for some typical characteristics of apoptosis such as apoptotic chromatin condensation and DNA fragmentation in all cell types (for review : Porter & Jänicke, 1999).

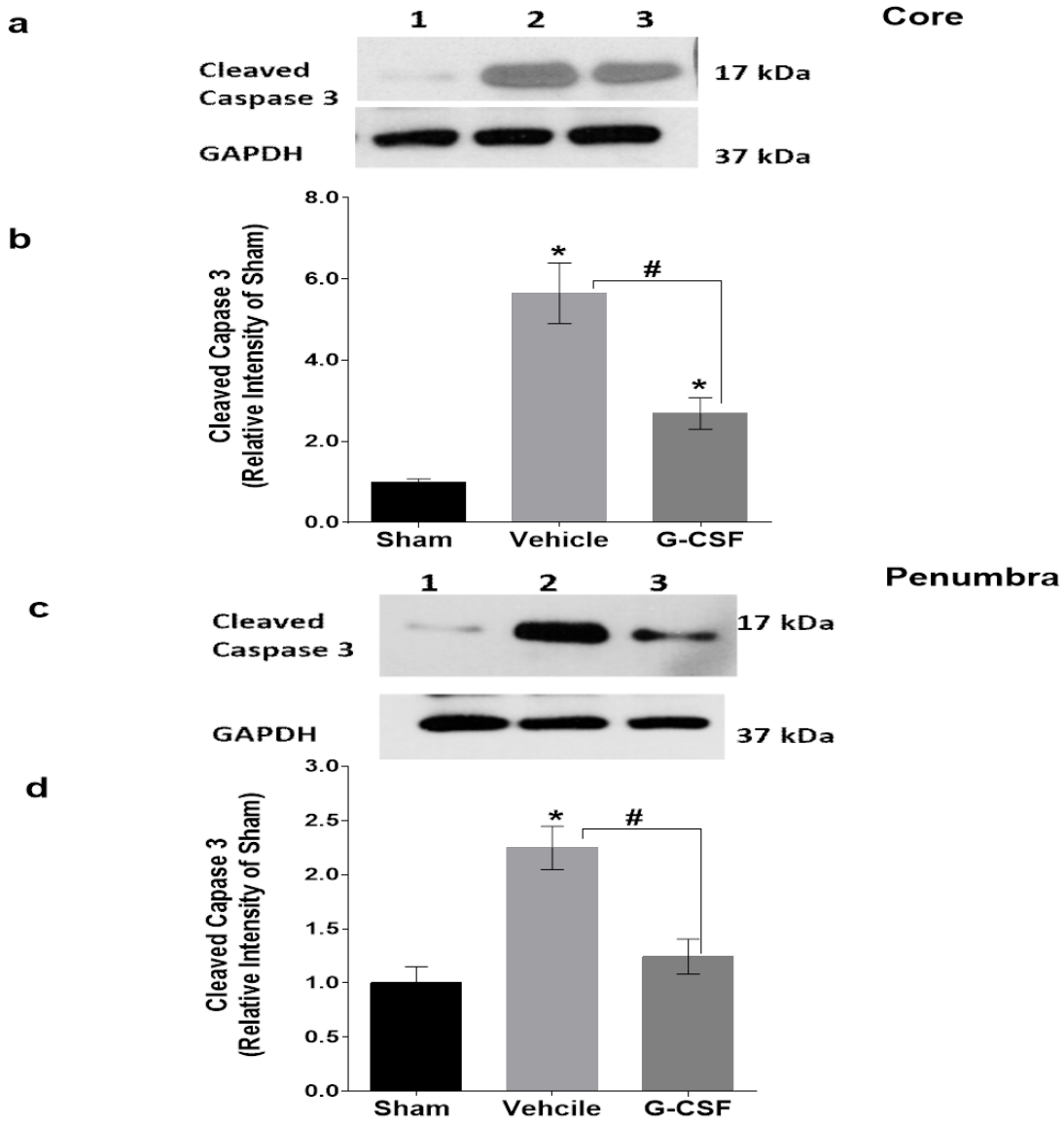


Figure 19. Effect of G-CSF on the level of cleaved caspase 3.

Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against cleaved caspase 3 in core (a) and penmumbra (c); 1 – Sham-operated rats (n=4) , 2- vehicle treated (n=6) and 3-G-CSF treated rats (n =6). The *bar* graphs reflected the densitometric data from the experiment of cleaved caspase 3 Western blot in the core (b) and penumbra (d). The results were expressed as relative intensity of the levels in sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

Several lines of evidence have shown that cerebral ischemia causes the activation of caspase 3; for instance, upregulation and activation of caspase 3 have been found to precede neuronal cell death in animal models of focal cerebral ischemia (Chen et al., 1998; Niwa et al., 2001) and increased level of procaspase 3 has been observed in strokes resulting from permanent arterial occlusion in humans studies (Love et al., 2000) .

Using western blotting we decided to investigate the effect of vehicle treatment and G-CSF treatment had on on the level of activated caspase 3 (cleaved caspase 3) in both the core and penumbra. We observed a significant effect of ischemia and G-CSF on activated caspase 3 level in both the core [$F(2, 13) = 21.54$, $P < 0.0001$] and penumbra [$F(2, 13) = 9.820$, $P = 0.0025$]. We confirmed previous reported evidence of an increased level of activated caspase 3 after focal ischemia (Niwa et al., 2001) (Fig. 19a & c). This significant increase of ischemia-induced cleaved caspase 3 was observed in both the core and penumbra of vehicle treated rats (n= 6) compared to sham-operated rats (n = 4) (5.65 ± 0.74, 2.25 ± 0.20 vs 1.00 ± 0.078, 1.00 ± 0.15 respectively $P < 0.05$). Treatment with G-CSF significantly reduced the level of cleaved casapase 3 in both core and penumbra compare to vehicle treated rats (n = 6; 2.69 ± 0.39, 1.24 ± 0.16 vs. 5.65 ± 0.74, 2.25 ± 0.20; n= 6 respectively, $P < 0.05$) (Fig. 19b& d). There was no significant difference of the level of cleaved caspase 3 in the penumbra of G-CSF treated rats compared to sham-operated rats ($P = 0.76$) indicating that G-CSF treatment had returned the expression level of cleaved caspase 3 approximately to baseline in the penumbra.

3.2.5. Effect of granulocyte-colony stimulating factor on endoplasmic reticulum stress induced apoptosis

The function of the endoplasmic reticulum (ER)is to synthesize and fold proteins destined for secretion, the cell membrane, golgi apparatus or lysosomes. Any condition, such as ischemia, that interferes with the normal folding of protiens in the ER results in ER stress (Kaufman 1999). In order to overcome the initial ER stress signal, the ER elicits a response called the Unfolded Protein Response (UPR) which is excuted by the three ER transmembrane stress sensors: IRE1α (inositol-requiring protein-1α), PERK (protein kinase RNA (PKR)-like ER kinase), and ATF6 (activating transcription factor 6). These three ER sensors will in turn activate respective intracellular pathways in order to alleviate ER stress (Malhotra & Kaufman, 2007). However if the cell is subjected to prolonged ER stress which it is unable to alleviate, CHOP, a pro-apoptotic protein is induced by the three ER stress sensors (Oyadomari & Mori, 2004). CHOP also known as GADD135 (growth arrest and DNA damage-inducible 135) is a transcription factor and has been reported as the most important mediator of ER stress induced apoptosis protein (Oyadomari & Mori, 2004) It modulates the transcription of Bcl-2 family members by inhibiting transcription of the anti-apoptitic protein, Bcl-2 (McCullough et al., 2001) while enhancing the transcription of the pro-apoptotic protein, Bim (Puthalakath, O'Reilly, Gunn, Lee, Kelly, Huntington, et al. 2007).

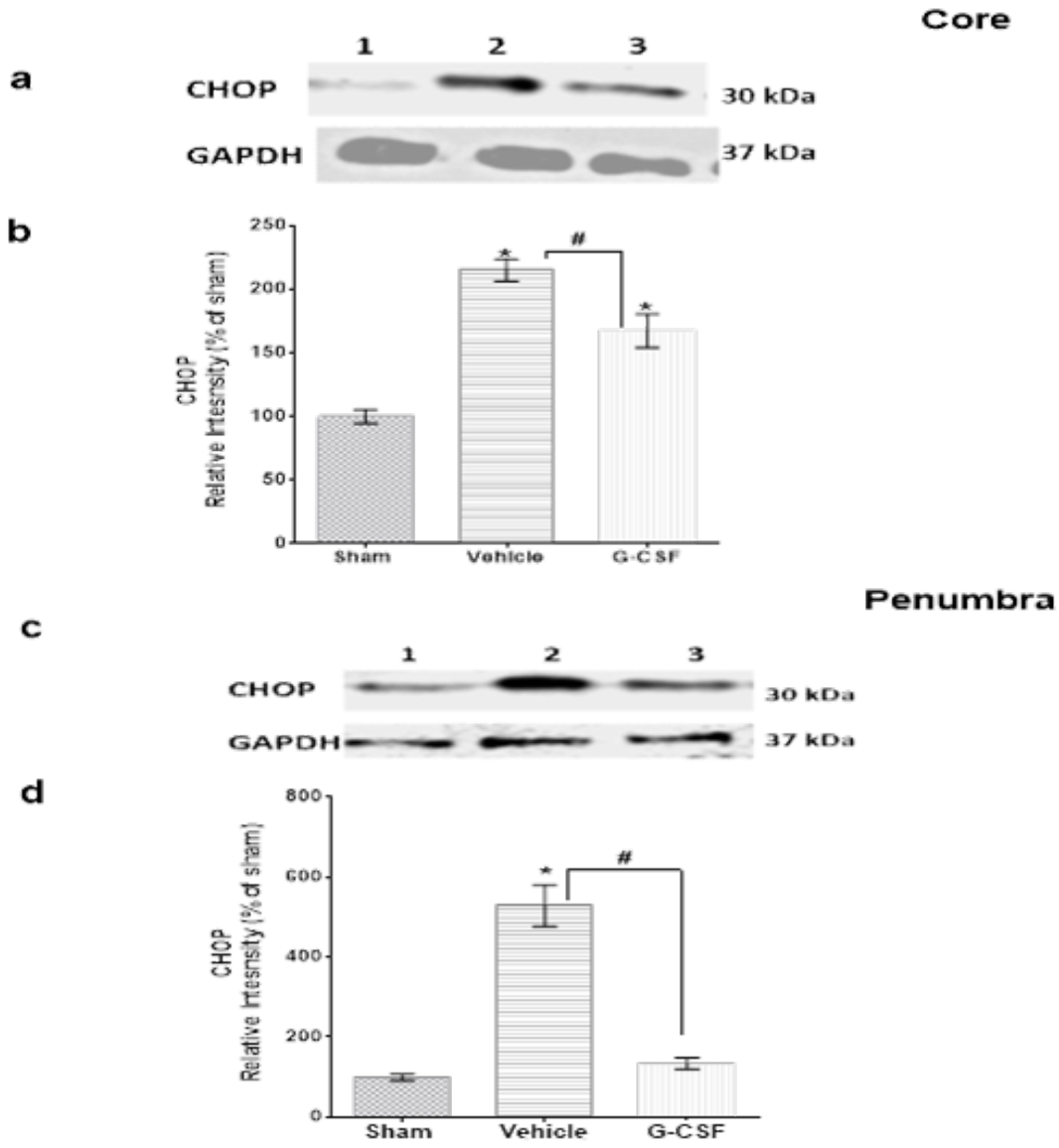


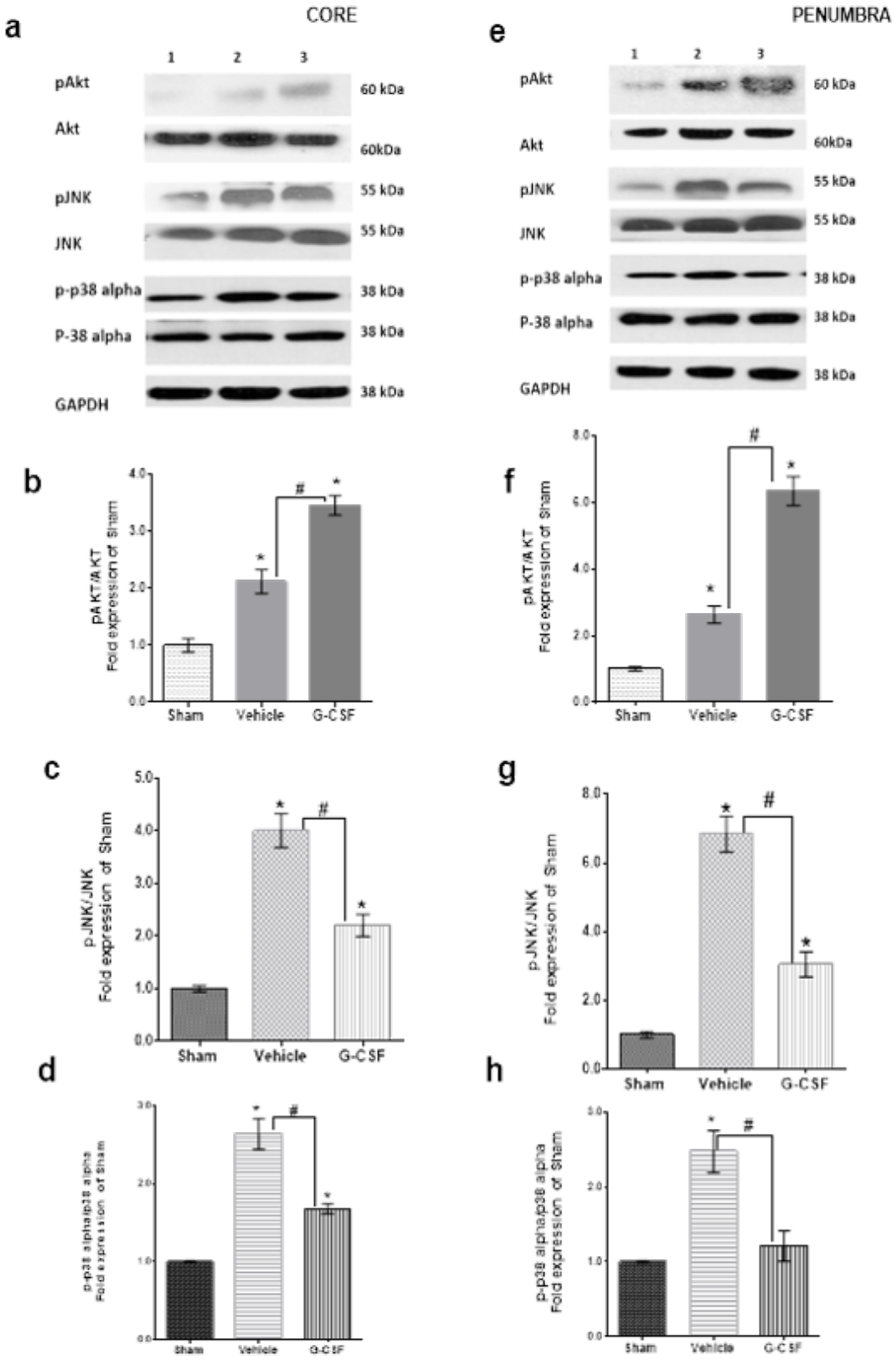
Figure 20. Effect of G-CSF on CHOP expression.

Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against CHOP in core (a) and penmumbra (c); 1 – Sham-operated rats (n=6) , 2- vehicle treated (n=8) and 3-G-CSF treated rats (n =8). The *bar* graphs reflected the densitometric data from the experiment of CHOP Western blot in the core (b) and penumbra (d). The results were expressed as relative intensity of the levels in sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

Based on the importance of CHOP in ER stress-induced apoptosis (Oyadomari & Mori, 2004), we decided to investigate the effect of G-CSF on CHOP expression in our rat model of transient focal ischemia. We observed that ischemia and G-CSF treatment affected the level of CHOP in the core [$F(2, 19) = 29.12$, $P < 0.0001$] and in the penumbra [$F(2, 20) = 55.98$, $P < 0.0001$]. As shown in Fig. 20 the level of ischemia-induced CHOP in the vehicle treated rats ($n = 8$) was significantly higher compared to sham- operated rats ($n = 6$) in both the core ($215.06 \pm 8.6\%$ vs. $100 \pm 5.38\%$ respectively, $^{*}P < 0.05$) and penumbra ($528.79 \pm 52.43\%$ vs. $100 \pm 7.97\%$ respectively, $^{*}P < 0.05$) This increased level of CHOP was significantly reduced by the administration of G-CSF (50ug/kg body weight, s.c) in both the core and the penumbra compared to the vehicle treated rats (core: $167.47 \pm 13.23\%$ vs. $215.06 \pm 8.6\%$; penumbra: $134.44 \pm 14.30\%$ vs. $528.79 \pm 52.43\%$ $^{\#}P < 0.05$). The reduction of CHOP expression by G-CSF in the core of G-CSF treated rats ($n = 8$) was significantly different compared to sham-operated rats ($167.47 \pm 13.23\%$ vs. $100 \pm 5.38\%$ respectively, $^{*}P < 0.05$) unlike in the penumbra where CHOP expression was not significantly different from sham-operated rats ($134.44 \pm 14.30\%$ vs. $100 \pm 7.97\%$ respectively, $^{*}P < 0.05$). This indicated that G-CSF was able to return the level of CHOP approximately to baseline (level present in sham-operate rats) in the penumbra of ischemic rats. This also indicated G-CSF could possibly protect the cell from CHOP-apopotosis in both the core and the penumbra of ischemic rats, with a more potent protection in the penumbra.

Since CHOP is the downstream effector of the three ER stress senor pathways, we next investigated which of the three pathways G-CSF modulated in order to achive the reduced expression of CHOP observed in Fig. 20. Firstly we investigated the IRE1α

pathway. Activated IRE1α binds TNF receptor-associated factor 2 (TRAF2), activating apoptosis signal-regulating kinase 1 (ASK1) which in turn activates downstream kinases that activate mitogen activated protein kinase (p-38 MAPK), and Jun-N-terminal kinase (JNK) (Ron & Hubbard, 2008; Mercado, 2013). Activated p-38MAPK phosphorylates and activates CHOP post-translationally, thus constituting the IRE1α-TRAF2-ASK1-p38MAPK-CHOP pathway. We demonstrated that the expression of activated p38MAPK (p-p38MAPK) increased markedly in both the core and the penumbra of vehicle treated rats compared (n = 8) to sham-operated rats (n = 5) (core: 2.47 ± 0.28 fold vs. 1.0 ± 0.02 fold; penumbra: 2.64 ± 0.19 fold vs. 1.00 ± 0.01 fold respectively, *P < 0.05) which was greatly reduced in G-CSF treated rats (n = 8) compared to vehicle treated rats (core: 1.68 ± 0.07 fold vs. 2.47 ± 0.28 fold; penumbra: 1.21 ± 0.20 fold vs. 2.64 ± 0.19 fold respectively, #P < 0.05) (Fig. 21a, d, e & h). The decrease of p-p38MAPK in the penumbra of the G-CSF rats was substantial, since the level of p-p38MAPK was only 0.21 fold difference from baseline which is 20% of basal p-p38MAPK.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38 kDa
e
PENUMBRA
b
pAKT/AKT
Fold expression of Sham
Sham
Vehicle
G-CSF
f
c
pJNK/JNK
Fold expression of Sham
g
d
p-p38 alpha/p38 alpha
Fold expression of Sham
h

Figure 21. Effect of G-CSF on downstream players in the IRE1α pathway.

Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against pAKT/AKT, pJNK/JNK and p-p38MAPK/p38MAPK in core (a) and penmumbra (e); 1 – Sham-operated rats (n=5) , 2- vehicle treated (n=8) and 3-G-CSF treated rats (n =8). The *bar* graphs reflected the densitometric data from the respective experiments of pAKT/AKT, pJNK/JNK and p-p38MAPK/p38MAPK western blot in the core (b-d) and penumbra (f-h). The results were expressed as fold expression of sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

The IRE1α-TRAF2-ASK1 pathway has another branch that does not directly modulate CHOP expression but nevertheless contributes to ER stress aopoptosis due to the apoptotic protein, JNK, a downstream effector of the pathway (Ron & Hubbard, 2008; Mercado, 2013). Activated JNK (pJNK) inhibites Bcl-2 (anit-apoptotic protein) and activates Bim (a pro-apoptotic protein) by phosphorylation (Lei and Davis 2003). We established a similar trend in expression level of pJNK as p-p38MAPK in vehicle treated and G-CSF treated rats (Fig. 21 a, c, e & g). There was a significant increase of pJNK expression in both the core and the penumbra of vechicle treated rats compared to sham-operated rats (core: 4.00 ±0.32 fold vs. 1.00 ± 0.06; penumbra: 6.83 ± 0.52 fold vs. 1.00 ± 0.09 fold respectively, *P < 0.05) which was markedly reduced, in both areas, by G-CSF administration when comapared to vehicle treated rats (core: 2.20 ± 0.21 fold vs 4.00 ±0.32 fold; penumbra: 3.04 ± 0.36 fold vs. 6.83 ± 0.52 fold, respectively, #P < 0.05). Overall our data suggested that the decrease in expression of p-p38MAPK in the core and the penumbra of G-CSF treated rats (Fig. 21a, d, e & h) could decrease the activity of CHOP in these areas. The reduction of pJNK expression in the core and

penumbra of G-CSF treated rats provided protection against apoptosis (Fig. 21 a, c, e & g).

Next, we investigated the mode by which G-CSF effectively downregulates the expression of both activated p38MAPK and JNK. We decided to examine the receptor mediated effect of G-CSF because several lines of evidence showed G-CSF to exert an antiapoptotic effect on neurons both *in vivo* and *in vitro* (Komine-Kobayshi et al., 2005; Park et al., 2005) via its specific receptor (Schabitz et al., 2005; Schneider et al., 2005), G-CSFR, a finding we also confirmed from our *in vitro* study in chapter 2, section 2.2.6. The G-CSFR activates three intracellular signaling pathways once G-CSF binds to it. These pathways are: JAK2/STAT3, RAS/ERK1/2/5 and the PI3K/AKT pathways, of which Schneider and colleagues reported that the PI3K/AKT pathway had the most potent protective effect against apoptosis in cortical neurons *in vitro* (Schneider et al., 2005). The PI3K/AKT pathway is a survival pathway and upon activation, PI3K phosphorylates AKT thereby activating it. Activated AKT (pAKT) inhibits apoptosis by suppressing the activity of its pro-apopotic substrates including ASK 1 (Kim et al., 2001). ASK 1 is upstream to p-38MAPK and JNK where it activates these apoptotic proteins via phosphorylation, therefore inhibiting ASK1 which should reduce the expression of phosphorylated p-38 MAPK (p-p38MAPK) and phoshorylated JNK (pJNK) . Using western blotting, we assessed the expression level of pAKT (activated AKT).

Interestingly we demonstrated that the expression of pAKT was significantly increased in the core and penumbra of both vehicle treated rats (n = 8) compared to sham-operated rats (n= 5) (core: 2.12 ± 0.21 fold vs. 1.00 ± 0.12 fold; penumbra: 2.63 ± 0.26

fold vs. 1.00 ± 0.06 fold, respectively, *P < 0.05) as well as in G-CSF treated rats (n = 8) compared to sham-operated rats (core: 3.46 ± 0.17 fold vs 1.00 ± 0.12; penumbra: 6.34 ± 0.43 fold vs. 1.00 ± 0.06 fold, respectively, *P < 0.05) (Fig. 21 a,e,b &f). The increased pAKT was significantly enhanced in both the core and penumbra but especially the penumbra of G-CSF treated rats compared to vehicle treated rats (core: 3.46 ± 0.17 fold vs. 2.12 ± 0.21 fold; penumbra: 6.34 ± 0.43 fold vs. 2.63 ± 0.26, repectively, #P < 0.05). It is reasonable to deduce that the overexpressed level of pAKT in the core and penumbra of G-CSF treated rats indicated that the downregulation of apoptotic pJNK and p-p38MAPK in the G-CSF treated rats (Fig. 21) could be partially due to the G-CSFR-mediated upregulation of pAKT. This confirmed previous reports of the protective effect of G-CSF being partially mediated through the PI3K/AKT pathway (Schneider et al., 2005).

Next we examined the effect of G-CSF on the remaining two ER stress sensor pathways; PERK and ATF6 pathways. The downstream effector of the PERK pathway is activating transcription factor 4 (ATF4) and that of the ATF6 pathway is the cleaved form of ATF6 (activated form). Both ATF4 and cleaved ATF6 converge on the promoter of the gene encoding the transcription factor CHOP (Ma et al., 2002; Kim et al., 2008). We observed that both ischemia and G-CSF treatment affected the expression level of ATF4 in both the core [F (2, 15) = 158.1, P < 0.0001] and the penumbra [F (2, 15) = 140.1, P < 0.0001]. We demonstrated (Fig. 22a,b e & f) that there was a significant increase in the expression level of ATF4 in both the core and penumbra of the vehicle treated rats (n = 7) compared to the sham-operated rats (n = 5) (core: 271.78 ±

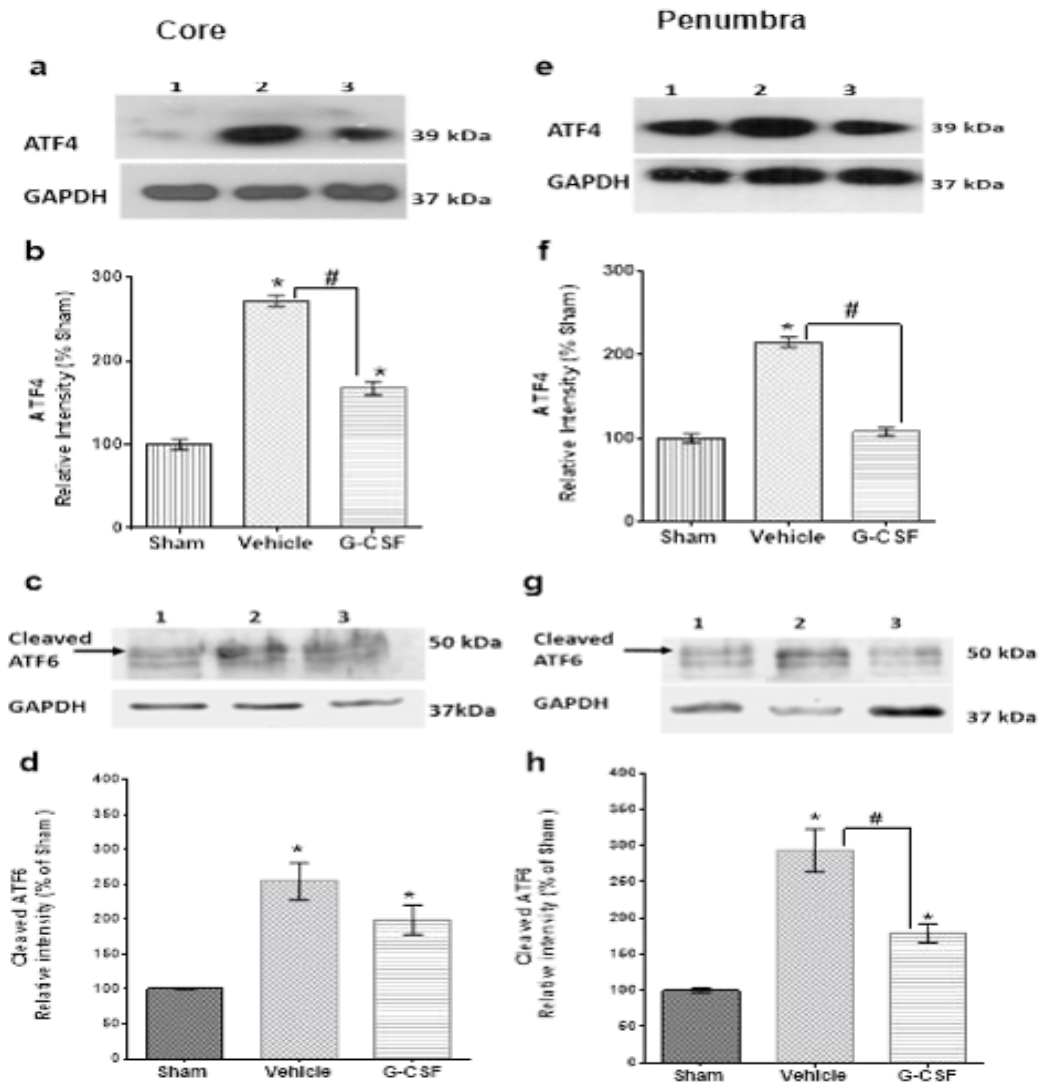

Figure 22. Effect of G-CSF on ATF4 and cleaved ATF6.

ATF4 and cleaved ATF6 are the downstream effectors in the PERK and ATF6 pathways respectively. Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against ATF4 and cleaved ATF6 in core (a & c) and penmumbra (e & g); 1 – Sham-operated rats (n=5) , 2- vehicle treated (n=7) and 3-G-CSF treated rats (n =7). The *bar* graphs reflected the densitometric data from the experiment of ATF4 and cleaved ATF6 Western blot in the core (b & d) and penumbra (f & h). The results were expressed as relative intensity of the levels in sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

16.30 % vs. 100 ± 14.59 %; penumbra: 215.1 ± 14.47% vs. 100 ± 13.44%, respectively) and that this increase was significantly reduced in both the core and penumbra by G-CSF administration compared to the level observed in the vehicle treated rats (core: 167.40 ± 19.35 % vs. 271.78 ± 16.30 %; penumbra: 107.72 ± 11.94 vs. 215.1 ± 14.47% respectively). The level of ATF6 was affect by ischemia and G-CSF treatment in the core [$F(2, 15) = 15.88$, $P = 0.0002$] and penumbra [$F(2, 15) = 26.97$, $P < 0.0001$]. A significant reduction by G-CSF was demonstrated for the ischemia induced expression of cleaved ATF6 in the penumbra of G-CSF treated rats (n= 7) compared to vehicle treated rats (Fig 22c & d) (293.46 ± 29.53% vs. 178.74 ± 13 %).

There was no significant change in ischemia-induced expression of cleaved ATF6 in the core of G-CSF treated rats compared to vehicle treated rats ($P = 0.1470$). Overall these data suggested that G-CSF downregulated CHOP expression in core and penumbra by reducing the expression of ATF4 in the core and penumbra and reducing the expression of cleaved ATF6 in the penumbra.

3.2.6. Effect of Granulocyte-colony stimulating factor on GRP78

It was intriguing that G-CSF was able to indirectly modulate the PERK and ATF6 pathways by downregulating the downstream effectors, ATF4 (in the core and penumbra) and cleaved ATF6 (in the penumbra) respectively (Fig. 22). The observed decrease in ATF4 and cleaved ATF6 expression by the administration of G-CSF could possibility be due to the ER returning to a normal homeostasis as a result of a possible reduction of ER stress (the accumulation of unfolded proteins). Under conditions of ER homeostasis, GRP78 (glucose regulated protein 78) constitutively binds to and maintains the three ER stress transmembrane sensors, ATF6, PERK, and IRE1α in an inactive form (Lee, 2005).

GRP78 is also a molecular chaperone that assists in protein folding and transport in the ER. Subsequent to ER stress, GRP78 goes to aid in the proper folding of the accumulated unfolded proteins, thereby releasing the three ER stress sensors (Li et al., 1993; Lee, 2005).

Our next step was to examine the expression level of GRP78 in the experimental animals. We observed that ischemia and G-CSF treatment affected the level of GRP78 in both the core [$F(2, 19) = 23.21$, $P < 0.0001$] and the penumbra [$F(2, 13) = 27.53$ $P < 0.0001$]. As shown in Fig. 23 there was a significant increase in the level of GRP78 in both the core and penumbra of vehicle treated (core: 233.98 ± 16.43% vs. 100 ± 9.96%; penumbra: 308.20 ± 25.34% vs. 100 ± 17.89% respectively, *$P < 0.05$) and G-CSF treated rats (core: 176.15 ± 17.19% vs. 100 ± 9.96%; penumbra: 212.44 ± 16.93% vs. 100 ± 17.89% respectively, *$P < 0.05$) compared to sham-operated rats, an indication of ER stress. G-CSF treatment significantly reduced GRP78 expression in both core and penumbra of G-CSF treated rats compared to vehicle treated rats (Fig. 23) (core: 176.15 ± 17.19% vs. 233.98 ± 16.43%; penumbra: 212.44 ± 16.93% vs. 308.20 ± 25.34% respectively, #$P < 0.05$). The reduction of GRP78 expression in the G-CSF treated rats is a reasonable indication that G-CSF might be facilitating the restoration of ER homeostasis, thereby protecting neurons and potentially glia from ER stress apoptosis.

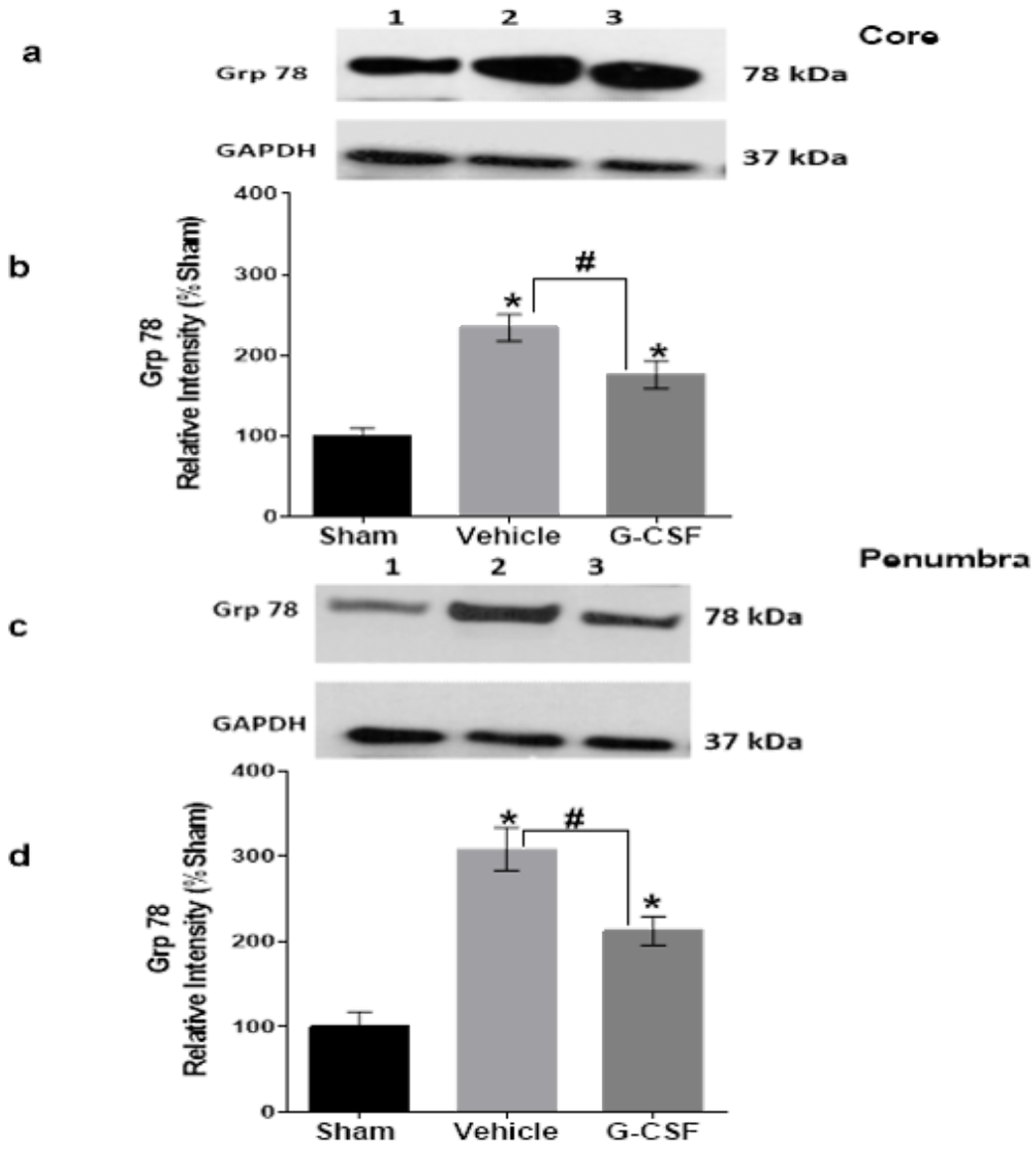

Figure 23. Effect of G-CSF on GRP78 expression.

Rat brains were divided into core and penumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against GRP78 in core (a) and penmumbra (c); 1 – Sham-operated rats (n=4) , 2- vehicle treated (n=8) and 3-G-CSF treated rats (n =8). The *bar* graphs reflected the densitometric data from the experiment of GRP78 Western blot in the core (b) and penumbra (d). The results were expressed as relative intensity of the levels in sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc.

3.2.7. Effect of Granulocyte-colony stimulating factor on HSP27

The 27 kDa heat shock protein (HSP27) belongs to the family of heat shock proteins that are considered as biomarkers of severe cellular stressors of the central nervous system (CNS: that is brain and spinal cord) including ischemia. The level of HSP27 is low in the normal brain but is rapidly induced by ischemia (Li et al., 2004). We next examined the expression level of HSP27 and observed that both ischemia and G-CSF treatment affected the level of HSP27 in both the core [$F(2, 12) = 51.63$, $P < 0.0001$] and the penumbra [$F(2, 14) = 63.82$, $P < 0.0001$]. The expression level was significantly up-regulated in the core and penumbra of both vehicle treated (core: 8.47 ± 0.62 fold vs. 1.00 ± 0.19 fold; penumbra: 8.42 ±0.80 fold vs. 1.00 ± 0.16 fold respectively, *$P < 0.05$) and G-CSF treated rats (core: 3.31 ± 0.54 fold vs. 1.00 ± 0.19 fold; penumbra: 1.65 ± 0.38 fold vs. 1.00 ±0.16 fold respectively, *$P < 0.05$) compared to sham-operated rats (Fig. 24). The up-regulation of HSP27 in the MCAO animals (both vehicle treated and G-CSF treated) indicated ischemic stress, a finding that is similar to previously reported evidence of HSP27 expression in ischemia (Li et al., 2004). The administration of G-CSF greatly reduced HSP27 level in both the core and penumbra of the G-CSF treated rats compared to the vehicle treated rats (core: 3.31 ± 0.54 fold vs. 8.47 ± 0.62 fold; penumbra: 1.65 ± 0.38 fold vs. 8.42 ± 0.80 fold, respectively, #$P < 0.05$). The reduction of HSP27 is therefore another biomarker that indicates G-CSF treatment reduces stress after ischemia/reperfusion injury.

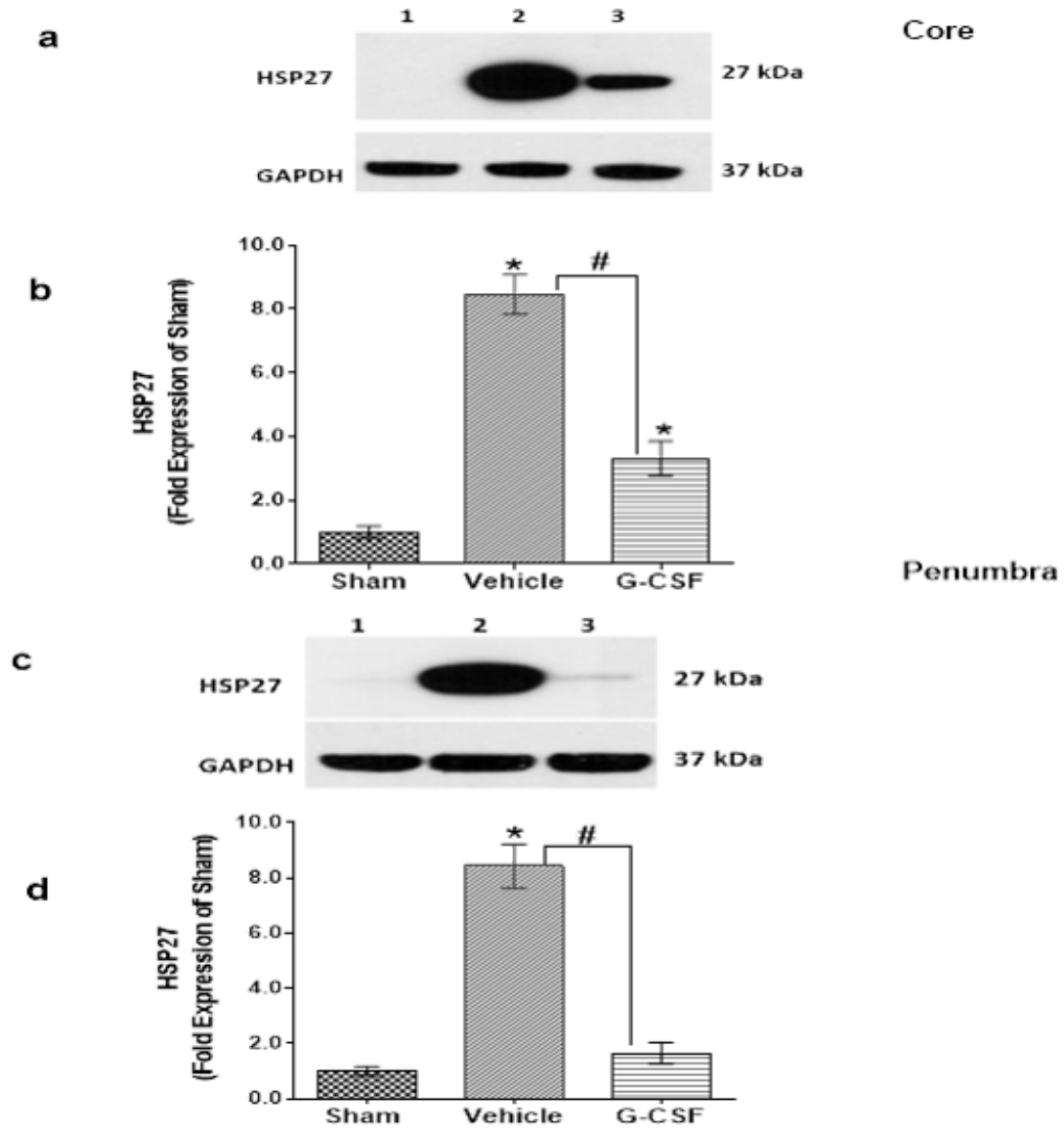

Figure 24. Effect of G-CSF on HSP27 expression.

Rat brains were divided into core and penmumbra, 4 days after MCAO surgery. Vehicle treated rats were given 5% dextrose, while G-CSF treated rats received 50ug/kg body weight, s.c of G-CSF for 4 days starting 24 hrs. after MCAO. Brains of sham-operated rats were divided in representative areas of core and penumbra. Western blot analysis using antibody against HSP27 in core (a) and penmumbra (c); 1 – Sham-operated rats (n=4) , 2- vehicle treated (n=6) and 3-G-CSF treated rats (n =7). The *bar* graphs reflected the densitometric data from the experiment of HSP27 Western blot in the core (b) and penumbra (d). The results were expressed as fold expresion level of sham-operated rats and mean values were expressed as mean ± SEM. *P < 0.05 vs. sham-operated, #P < 0.05 vs vehicle treated, one way ANOVA, Tukey post hoc

3.3. DISCUSSION

In this phase of our study we successfully induced transient focal ischemia/ reperfusion injury in male Sprague Dawley rats by temporarily occluding the proximal aspect of the middle cerebral artery (MCAO). Using this model we demonstrated the neuroprotective effect of exogenous G-CSF against ischemia. The chosen dose for G-CSF (50ug/kg body weight. s.c.) used in this study is consistent with previously reported studies on the beneficial effect of G-CSF against ischemic injury (Gibson et al., 2005; Schneider et al., 2005; Yata et al., 2007; Li et al., 2013). In order to investigate the efficacy of G-CSF beyond the usual 4 hour window of treatment for stroke, the initial dose of G-CSF was administered 24 hours post-MCAO. This initial dose was then followed by a single application (50ug/kg body weight. s.c.) another 3 days resulting in a total of 4 days of G-CSF administration. Our experimental paradigm was supported by Six and colleagues (2003) who subjected mice to 60 mins. MCAO with the initial administration of G-CSF (50ug/kg body weight. s.c.) 24 hours post-MCAO. They also administered G-CSF for a total of 4 days and reported on the beneficial effect of G-CSF to reduce infarct volume (Six et al., 2003).

In this study we provided evidence that G-CSF reduced infarct volume, improved neurological score, protected against apoptosis by modulating the expression of Bcl2 family members via upregulating levels of anti-apoptotic Bcl2 and downregulating pro-apoptotic Bax and Bak expression level. G-CSF also reduced the expression level of cleaved caspase 3, a cell death executioner caspase. In addition we demonstrated that G-CSF was protective against ER stress apoptosis by downregulating ER stress-induced apoptotic CHOP and also downregulating the expression levels of ATF4, p-p38MAPK /

pJNK and cleaved ATF6, which are respective downstream targets in the ER stress sensor pathways: PERK, IRE1 and ATF6, respectively. The protective effect of G-CSF was also evident from the attenuation of molecular chaperones/stress markers such as GRP78 (ER intraluminal stress sensor) and HSP27 (a general stress marker). The effect of G-CSF on the above mentioned proteins was significant in both the core and penumbra of the ischemic brain, expect for its effect on ATF6 (effect was only significant in the penumbra). Cells in the ischemic penumbra experience death by apoptosis (programmed cell death which is ATP-dependent) because of collateral or residual blood supply which make the penumbra more susceptible to G-CSF modulating proteins involved in the apoptotic machinery. In contrast in the core, the apoptotic pathway initiated in the early stages of ischemia may be blocked due to the depletion of ATP, causing a shift from apoptosis towards secondary necrosis. Thus cells in the ischemic core experience a continuum between apoptosis and necrosis (Nicotera et al., 1998; Nakka et al., 2008;Martin, et al., 2010) G-CSF is still able to have an effect on the ischemic core because dying cells may show the characteristic of apoptosis and necrosis.

3.3.1. Induction of ischemia/reperfusion by the intraluminal suture method

We used the intraluminal suture method to occlude the proximal aspect of the middle cerebral artery. Subsequent to the first description of the intraluminal suture MCAO model by Koizumi and colleagues (1986), it has become one of the most frequently and widely used models by stroke researchers (Durukan and Tatlisumak 2007). By using this model we were able to withdraw the intraluminal suture from the occluded MCA, which allowed us to study ischemic/reperfusion injury (a common human stroke condition). Our study maintained a 90 mins ischemia which provided

adequate reduction in regional blood flow ranging between 50% and 60 % drops in regional cerebral blood flow (Fig. 14). This also produced reproducible infarction which is in line with previous reports that a minimum 60 to 90 mins of ischemia is required for reproducible infarct volume (Grotta, 1998). After 90 mins ischemia, removal of the suture resulted in immediate reperfusion with approximately 80% return of regional cerebral blood flow (Fig. 14). During our surgical procedure to occlude the MCA, we utilized the intraluminal silicon-coated suture (versus using uncoated suture). This provided an added advantage since silicon-coated suture reduced the problem of subarachnoid hemorrhage (a common problem in this model) thereby reducing mortality rate (Schmid-Elsaesser et al., 1998). Animals experiencing extremes in body temperatures were removed from our study since hyperthermia (body temperature $\geq$ 39^{o}C) could increase infarct volume up to three fold (Kim, et al., 1996; O'Donnell et al., 2006), while hypothermia (body temperature $\leq$ 33^{o}C) can be profoundly neuroprotective (Won et al. 2006).

3.3.2. G-CSF arrested infarct volume and improved neurological deficit

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that mobilizes stem cells from the bone marrow into the peripheral circulation (Lu and Xiao, 2006). It stimulates the proliferation, survival and maturation of the neutrophilic granulocyte lineage (Xiao et al., 2007) and is therefore commonly used after the treatment of hematologic diseases and for counteracting chemotherapy-induced neutropenia in cancer patients when leukocyte numbers tend to be low (Metcalf, 1990). G-CSF is mainly secreted from hematopoietic cells such granulocytes, a subset of monocytes and macrophages and its physiological action is via a specific G-CSF

receptor located on these cells (Nicola & Metcalf, 1985; Metcalf, 2013). Although initially thought to be functionally restricted to granulocytopoiesis, recent studies have shown the presence of both G-CSF and its receptor on neurons (Meeuwsen et al., 2003; Schäbitz et al., 2003; Schneider et al., 2005) and glial cells [astrocytes (Schäbitz et al., 2003) and microglia (Komine-Kobayashi et al., 2006)] in the central nervous system (CNS) in addition, several lines of evidence have shown its neuroprotective effect after cerebral ischemia (Aapro et al., 2003; Gibson et al. 2005a; Gibson et al., 2005b; Komine-Kobayashi et al., 2006; Ihsan Solaroglu et al., 2006; Kong et al., 2009; Strecker et al., 2010; Hong et al., 2012).

One pathophysiological hallmark of ischemia is a condition called "spreading depression" which involves a wave of depolarization that starts in the ischemic core and extends outwards to surrounding tissue, resulting in an increase of the infarct volume (Hartings et al., 2003; Dohmen et al., 2008). Several studies reported the effect of G-CSF to reduce infarct volume after ischemia (Six et al., 2003; Gibson et al., 2005). Schäbitz and colleagues showed a 47% infarct volume reduction after 90 mins of transient focal ischemia in rats (Schäbitz et al., 2003). Our results showed a significant reduction of 53% infarct volume after subcuatneous administration of exogenous G-CSF (50ug/kg body weight) for 4 days in comparison to vehicle treated rats. This percentage of infarct reduction by G-CSF is comparable to that of other growth factors, such as brain derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1**)** when administered systemically (Schabitz et al., 2000, 2001). Because G-CSF reduced the infarct volume by such a significant percentage it is reasonable to deduce that G-CSF arrested

"spreading depression", which suggests that G-CSF is preventing the ischemic region from expanding.

The MCA supplies blood to brain regions including the frontoparietal cortex and subcortical structures (lateral caudoputamen / the striatum; (Scremin 1995). These cortical and subcortical structures are involved in the processing of sensorimotor information and an infarction in these brain regions produce neurological deficits which are manifested in motor paresis and abnormal neurological behaviors (Andersen et al., 1991; Rogers et al., 1997). Many studies assessed functional impairment of rats after MCAO using a basic neurological evaluation scoring scheme, originally developed by Bederson and colleagues (Bederson et al. 1986). Menzies and colleagues, modified the original scoring scheme into a six-point scheme, in which the highest score, 5, was assigned to normal behavior and the lowest score, zero, was assigned to rats showing no spontaneous motion (Menzies et al., 1992) while other behavior such as forelimb flexion, weakened contralateral forelimb grip, contralateral circling when pulled by tail, and spontaneous contralateral circling were assigned scores of 4, 3, 2 and 1 respectively (Menzies et al., 1992). Using the six-point scoring scheme, there was no significant effect of G-CSF on neurological behavior (Fig. 15). The lack of G-CSF effect on neurological behavior could have due to the short time period, 4 day, used in our study.

3.3.3. G-CSF and the PI3K/Akt pathway

How is G-CSF able to mediate its beneficial effect of reducing infarct volume and improving neurological deficit reflected in our study? It is well established that the effect of G-CSF is via a specific G-CSF receptor located on various cells including neurons and glia (Meeuwsen et al., 2003; Schäbitz et al., 2003; Schneider et al., 2005; Komine-

Kobayashi et al., 2006). The G-CSF receptor is a transmembrane receptor with its membrane region coupled to Janus family kinases (JAKs). On binding to its specific receptor G-CSF activates, through JAK signaling, three independent pathways: the signal transducer and activation of transcription 3 (STAT-3), the extracellular-signal-regulate kinase 1/2/5 (ERK 1/2/5) and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway (Shimoda et al., 1997; Hunter & Avalos, 1998; Dong & Larner, 2000; Schneider et al., 2005). These three intracellular pathways are survival / anti-apoptotic pathways. Several lines of evidence have shown the anti-apoptotic effects of G-CSF mediated via these pathways. For instance; ischemic neurons exposed to G-CSF has been reported to result in rapid phosphorylation of STAT-3 (Schneider et al., 2005; Solaroglu et al., 2006) with an increase in the expression level of anti-apoptotic STAT-3 target, Bcl-X_L protein (Schäbitz et al., 2003). While members of the ERK family have been shown to become activated by G-CSF, ERK 1/2 showed a transient and weak activation but ERK5 kinase was strongly activated by G-CSF in cultured neurons from rat cortex (Schneider et al., 2005). Schneider and colleagues (2005) also reported that the anti-apoptotic effect of G-CSF on neurons was partially mediated by the PI3K-Akt pathway. They also showed that of the three anti-apoptotic pathways (STAT-3, ERK1,2 &5, PI3K-AkT), the PI3K-Akt pathway was the most potent in regulating apoptosis. This was evident by the magnitude of anti-apoptotic Bcl-X_L level induced by the PI3K-Akt pathway compared to the other two pathways (Schneider et al., 2005). In addition it is well established that the PI3K-Akt pathway supports cell suvival, including protecting neurons from apoptosis after ischemia (Franke et al., 2003; Chan, 2004; Zhao et al., 2006). Hence, our attention was directed to the PI3K-Akt pathway.

Akt is activated via PI3K and 3′-phosphoinositide dependent protein kinase (PDK) and the amount of active Akt is based on phosphorylation of serine 437 (Ser-437) (Yang et al., 2002). We therefore measured the expression level of phosphorylated Akt by normalizing the densities of pAkt (Ser-473) against the density of total Akt. Our results showed a significant upregulation of the pAkt expression level in both the core and penumbra of ischemic rats treated with G-CSF compared to ischemic rats without G-CSF treatment. This suggests an increased sensitivity to G-CSF in both the core and penumbra of G-CSF treated rats. Our data also showed a greater increase of pAkt expression in the penumbra than in the core of G-CSF treated rats (Fig. 21a,b,e &f), indicating a greater sensitivity to G-CSF in the penumbra of these rats. This result is consistent with several previous reports that showed the cells in the penumbra of the ischemic tissue experience apoptosis due to collateral circulation and are therefore more susceptible to protective measures from neuroprotective agents, unlike cells in the ischemic core that primarily experience necrosis (Charriaut-Marlangue et al., 1996; Kaufmann et al., 1999; Paciaroni et al., 2009; Ramos-Cabrer et al., 2011). Intriguingly, our results also demonstrated that pAkt expression was also significantly upregulated in both the core and penumbra of the ischemic vehicle treated rats compared to sham-operated rats.

Since pAkt is a substrate of one of the G-CSF receptor mediated pathway (PI3K-Akt), the upregulated pAkt in the ischemic vehicle treated rats could be due to an upregulation of endogenous G-CSF and its receptor which then activate the PI3K-Akt pathway. This results supports previous reports of cerebral ischemia upregulating the expression of endogenous G-CSF (and the G-CSF receptor) (Kleinschnitz et al., 2004;

Hasselblatt et al., 2007; Yu et al., 2012) which enables an endogenous brain protective mechanism after cerebral ischemia. This school of thought has been consolidated by Sevimli and colleagues who showed that G-CSF deficent mice (G-CSF $^{-/-}$) displayed enlarged infarct volume and impared functional recovery after transient focal ischemia. Futhermore they showed that G-CSF substitution in G-CSF $^{-/-}$ mice prevented the observed consequences of G-CSF deficiency (Sevimli et al. 2009). Several lines of evidence have demonstrated an anti-apoptotic effect of G-CSF in experimental cerebral ischemia, but inspite of these findings the mechanism is not fully elucidated.

3.3.4. The endoplasmic recticulum stress pathways.

Both the mitochondrion and endoplasmic recticulum (ER) are orgnelles that induce apopotosis subequent to conditions that perturb their normal functions. While a plethora of studies have been carried out on mitochondrion induced apoptosis, studies on endoplasmic recticulum induced apoptosis have been few. Our study focused on endoplasmic recticulum induced apoptosis. The ER is responsible for folding and assembly of membrane and secreted proteins, synthesis of lipids and sterols, and storage of free calcium (Gething & Sambrook, 1992; Kaufman, 1999). Recently several studies have revealed that ER stress is an essential signaling event for neuronal injury in ischemia/reperfusion (Ma & Hendershot, 2004; Anelli & Sitia, 2008; Osada et al., 2010). Certain conditions such as ischemia, glucose deprivation, hypoxia and alterations in calcium homeostasis tiggers the accumulation of unfolded proteins in the lumen of the ER, resulting in ER stress. The initial response by the ER to stressful stimuli is to elicit the unfolded protein response (UPR), a self-protective signal tranduction pathway (Malhotra & Kaufman, 2007). The UPR attempts to restore ER function by activating

three ER membrane sensors: PERK, IRE1α and ATF6 (Kim et al., 2008). If the initial phase of the UPR fails to facilitate the recovery from ER stress or if the ER stress is prolonged, the UPR will induce apoptosis, via the PERK, IRE1α and ATF6 intracellular pathways, in order to elimate the stressed cell (Rasheva and Domingos 2009).

3.3.4.1 G-CSF attenuates the transcription factor CHOP.

All three ER stress sensor pathways have downstream players that converge on the promoter of the gene encoding the transcription factor CHOP (Ma et al., 2002; Kim et al., 2008; Yiming et al., 2014) also known as growth-arrest- and DNA-damage inducible gene 153 (GADD153). Several lines of evidence have reported the involvement of CHOP in ischemic cell death; for instance CHOP knock out mice were more resistant to hypoxia/reoxygenation neuronal cell death than wild type mice after bilateral common carotid artery occlusion, and ischemic preconditioning protected the brain from ischemic/reperfusion injury by decreasing the activation of CHOP at 24 hours reperfusion (Kim et al., 2007; Yuan et al., 2011). Our data showed that CHOP was significantly upregulated in both the core and penumbra of ischemic vehicle treated rats compared to sham-operated rats after 4 days reperfusion. This result confirms that the ER is experiencing stress, since an upregulated level of CHOP serves as a hallmark of ER stress (Tajiri et al., 2004; Morimoto et al., 2007; Srinivasan & Sharma, 2011).

CHOP, which is a transcription factor, has been identified to promote apoptosis under ER stress by suppressing the expression of anti-apoptotic protein Bcl2 while potentiating the expression of the pro-apoptotic protein Bim (McCullough et al., 2001; Puthalakath et al., 2007). CHOP also mediates cell death in ER stress by inhibiting the PERK translational block (this translational block is a mechanism employed by cells

undergoing ER stress to reduce the amount of protein being translated and hence reduce the ER protein load) (Oyadomari and Mori 2004). We showed that administration of G-CSF (24hrs. post- MCAO) to ischemic rats markedly reduced CHOP expression in both the core and the penumbra after 4 days reperfusion. Our results reveal that G-CSF protects the ischemic/reperfusion brain from ER stress-induced apoptosis by down regulating CHOP. To our knowledege this finding is novel, since we are the first to report the anti-apoptotic mechanism of G-CSF to attenuate CHOP expression in ischemia/reperfusion-induced ER stress.

3.3.4.2. The effect of G-CSF on the PERK pathway.

Protein kinase RNA (PKR)-like ER kinase (PERK) is activated through dimerization and trans-autophosphorylation. Activated PERK is responsible for attenuating the translation of mRNAs during ER stress, thus preventing an increased influx of newly synthesized proteins into the stressed ER. After activation p-PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2α) (Kumar et al., 2001) which inhibites the recycling of eIF2α to its active form (the active from of eIF2α is needed for the first phase of polypeptide chain sysnthesis), thus leading to the inhibition of gobal protein translation (Harding et al., 1999). However this attenuation is not absolute and therefore does not apply to certain proteins, such as ATF4. AT4 is a stress response transcription factor that is upregulated in ER stress. Following ATF4's translation it tranlocates to the nucleus where it transcribes ER stress survival genes such as GRP78. However if ER stress is prolonged, ATF4 will transcribe the pro-apoptotic gene, CHOP (Kumar et al., 2001). Since ATF4 is a downstream protein in the PERK-eIF2α pathway of ER stress, we measured the expression level of ATF4 in order to

determine if G-CSF has an effect on the PERK pathway in transient focal ischemia after 4 days reperfusion. Our results showed that cerebral ischemia/reperfusion induced the upregulation of ATF4 in both the core and the penumbra, which is consistent with our previous study (Mohammad-Gharibani et al. 2014), and that the elevated ATF4 is strongly suppressed by G-CSF treatment in both the core and the penumbra. Our data suggest that the downregulation of CHOP by G-CSF in both the core and the penumbra may be due partially to G-CSF downregulating ATF 4; which is one of the transcriptional regulation for CHOP mRNA synthesis.. This provides new and added insight into the anti-apoptotic mechanism of G-CSF as it relates to alleviating ER stress is ischemia/reperfusion injury.

3.3.4.3. The effect of G-CSF on the ATF6 pathway.

ATF6, another ER transmembrane sensor, translocates to the Golgi during ER stress, where it is cleaved by serine protease site-1 protease (S1P) and metalloprotease site-2 protease (S2P) (Chen et al., 2002) resulting in an active transcription factor (Yoshida et al. 2000). The cleaved ATF6 then translocates to the nucleus where it induces ER stress genes including ER chaperone proteins; GRP 78 and GRP94, protein disulphide isomerase, ER degradation-enhancing a-mannosidase-like protein 1 (EDEM1) (Adachi et al. 2008) the transcription factors X box-binding protein 1 (XBP1) and CHOP (Yoshida et al. 2000). Indeed, cleaved ATF6 has been demonstrated to upregulate levels of CHOP during sustained ER stress (Okada et al., 2002). Since cleaved ATF6 is the effector of the ATF6 pathway that transcribes CHOP, we thought it appropriate to assess the expression of cleaved ATF6 in order to unmask the effect of G-CSF on the ATF6 pathway. Our data revealed that the increased level of cleaved ATF6 expression in

ischemia/4 days reperfusion injury was significantly reduced in the penumbra by G-CSF treatment. Although G-CSF treatment reduced the cleaved ATF6 level in the core, the reduction was not significance. This suggests that the G-CSF reduction of CHOP is partly due to G-CSF downregulating cleaved ATF6, at least in the penumbra.

3.3.4.4. The effect of G-CSF on the IRE1α pathway.

The third ER transmembrane stress sensor is IRE1α. IRE1α contains a kinase and an endoribonuclease (RNase) domain (Wang et al., 1998) and is activated via oligomerization and auto-transphosphorylation (Shamu and Walter 1996). Once activated the endoribonuclease activity splices XBP1 mRNA resulting in the translation of a transcription factor, spliced XBP1 (XBP1 s; Yoshida et al., 2001) which targets ER stress adaptive genes as well as pro-apoptotic genes involve in ER stress (Wu et al., 1990; Lin et al., 2007). In addition to splicing XBP1, IRE1α recruits TNF receptor associated factor 2 (TRAF2) and this complex (that is the IRE1α-TRAF2 complex) then triggers the activation of Apoptotic-Signaling Kinase-1 (ASK1), which causes activation of downstream stress mitogen activated protein kinases (MAPKs); c-Jun-N-terminal kinase (JNK) and p38 MAPK (Urano et al., 2000; Hetz & Glimcher, 2009). It has been reported that ASK1 is activated in ER stress. In addition neurons from $ASK1^{-/-}$ mice demonstrated resistance to ER stress-induced apoptosis (Nishitoh et al. 2002) therefore showing the importance of ASK1 in ER stress. The IRE1α-TRAF2-ASK1-JNK pathway in ER stress contributes to apoptosis by JNK regulating Bcl2 family members. For instance phosphorylation of Bcl2 by JNK inhibits the anti-apoptotic activity of Bcl2 while JNK phosphorylation of BH3 (Bcl-2 homology domain 3)-only member such as Bim enhances the pro-apoptotic potential of Bim (Deng et al., 2001; Lei & Davis, 2003).

While the other arm of IRE1α stress signaling pathway IRE1α-TRAF2-ASK1-p38MAPK phosphorylates and activates CHOP which causes increased expression of the pro-apoptotic Bim and reduced expression of anti-apoptotic Bcl2 (McCullough et al., 2001; Puthalakath et al., 2007). We measured the expression level of both activated JNK (pJNK) and activated p38MAPK (p-p38MAPK) in order to establish the effect of G-CSF on the IRE1α-TRAF2-ASK1-JNK pathway and on the IRE1α-TRAF2-ASK1-p38MAPK pathway. Our data revealed a significant increase in both pJNK and p-p38MAPK levels in ischemia/4 days reperfusion brain injury in both the core and the penumbra. This increase was markedly reduced, in both the core and the penumbra, by the administration of 50ug of G-CSF over the 4 days of reperfusion. The protective mechanism of G-CSF reducing the levels of both pJNK and p-p38MAPK could be the direct activation of the G-CSFR- PI3K/AKT pathway by G-CSF. This is a reasonable deduction since our present study confirmed several previous reports that G-CSF mediated effect is partially via PI3K/AKT (Schäbitz et al., 2003; Schneider et al., 2005; Solaroglu et al., 2006), suggested by G-CSF upregulating pAKT in this study (Fig. 21a, b, e &f). One of the substrates for pAKT is ASK1 and Kim and colleagues reported that pAKT phosphorylation of ASK1 at serine 83 (Ser-83) decreased the kinase activity of activated ASK1 (Kim et al., 2001). This would be reflected in the reduced activation of JNK and p38MAPK (both downstream targets of ASK1) as our data has shown (Fig. 21a, b, c, d, g & h). Interestingly, Li and colleagues recently reported that hypodermic injection of 50ug/kg G-CSF down-regulated pJNK in cerebral ischemia/reperfusion (Li et al., 2013), thus supporting our result. While Li and colleagues reported the effect of G-CSF on p

JNK in ischemia, there are no known reports about the effect of G-CSF on p-p38MAPK, making our finding a novel one.

3.3.5. G-CSF attenuates one of the intraluminal sensor of ER stress – GRP78.

Because our data have shown that G-CSF has a positive effect on the three ER stress-induced intracellular pathways (PERK-, IRE1α- and ATF6 – pathways), our attention was drawn to the intraluminal environment of the ER. In unstressed cells, the three ER transmembrane sensors are kept in an inactive state through binding to the ER chaperone GRP78. GRP78, also known as BiP (Binding immunoglobulin protein) is localized in the ER lumen and is involved in many cellular process of the ER including translocating the newly synthesized polypeptides across the ER membrane, facilitating the folding and assembly of proteins, targeting misfolded proteins for ER-associated degradation (ERAD), and regulating calcium homeostasis; consequently serving as an ER luminal stress sensor (Hendershot, 2004; Lee, 2005; Li & Lee, 2006).

Increased levels of unfolded proteins are manifested in the ER when a stimulus, such as ischemia, perturbs factors (availability of cellular energy levels, the redox state or calcium concentration) needed for proper disulphide-bond formation of polypeptide the in the ER (Gaut & Hendershot, 1993; Braakman & Bulleid, 2011). This subsequently induces the upregulation of GRP78 which increase binding to unfolded protein in order to establish proper protein folding and reduce the unfolded protein load. Due to the increased need for GRP78 during ER stress, it dissociates from the three ER transmembrane sensors, releasing them from their inactivated state (Mercado 2013).

Since GRP78 is an ER intraluminal stress sensor (Li & Lee, 2006), we investigated the effect of G-CSF on the expression level of brain GRP78 in ischemia and

4 days of reperfusion injury. Our data demonstrated that GRP78 was significantly upregulated in the both the core and penumbra (more so in the penumbra than the core) of ischemic-vehicle treated rats in comparison to sham-operated rats. This result is consistent with previous reports that ischemia induced GRP78 upregulation during ER stress (Kitao et al. 2001). Our data further demonstrated that the administration of 50ug/kg G-CSF markedly diminished the increased level of GRP78 (in both the core and the penumbra) that was observed in the vehicle treated rats. Because GRP78 is an ER stress sensor whose activity is correlated with the level of unfolded protein load in the ER (Li & Lee, 2006; Chambers et al., 2012) our result demonstrating that the G-CSF attenuated levels of GRP78, suggests that G-CSF is decreasing the the unfolded protein load in the ER, resulting in reduced ER stress and is potentially reestablishing normal homeostasis in the ER.

3.3.6. G-CSF modulates the Bcl2 family members.

The Bcl2 family members play a critical role in regulating the intracellular apoptotic signal transduction dynamics. The members of the family are all related by containing one or more Bcl2 homology (BH) domains (Borner et al. 1994). The family consists of both anti-apoptotic proteins such as Bcl2, Bcl-xL, Bcl-w, Bcl2A1 (Bcl-2-related protein A1) and Mcl-1(myeloid cell leukemia sequence 1), as well as pro-apoptotic protein such as Bax, Bak, Bad Bim and Puma (Reed 1997). Several lines of evidence have shown them to be crucial players in the struggle between cell survival and cell death in ischemia. For instance, Bax expression level increased while Bcl2 and Bcl-w expressions were reduced in both the ischemic core and penumbra of the infarct (Ferrer and Planas 2003). An over expression of Bcl-2 in transgenic mice is associated

with reduction of the infarct size following focal cerebral ischemia (Martinou et al. 1994). Similarly, over expression of human Bcl-2 with herpes simplex virus vectors limit neuronal death in focal cerebral ischemia (Linnik et al., 1995).

We investigated the effect of G-CSF on the expression level of three members of the Bcl2 family; anti-apoptotic Bcl2 (which is one of the major anti-apoptotic members) and the pro-apopotic proteins Bax and Bak, for which the presence of both seems to be essential for apoptosis in most cells (Wei et al. 2001). Our data revealed that ischemia downregulated Bcl2 while up-regulated Bax and Bak in both the core and the penumbra and that this condition was reversed with the adminstration of 50 ug/kg G-CSF. G-CSF significantly reduced the levels of both Bax and Bak in the core and penumbra but markedly upregulated Bcl2 expression in the core and penumbra. Our data showing the upregulationg of Bcl2 by G-CSF is well supported by other studies (Schneider et al., 2005; Solaroglu et al., 2006). However, because the anti-apoptotic/pro-apoptotic pendulum may swing in favor of either cell survival or death based on the degree to which the level of anti-apoptotic Bcl2 family members are expressed compared to the level of pro-apoptotic family members, we examined the extent to which Bcl2 was upregulated by G-CSF compared to the G-CSF-downregulated Bax. Our result showed that in the core, G-CSF increased the ratio of Bcl2:Bax at least twice the ratio shown in the ischemic vehicle treated rats while in the penumbra, the Bcl2:Bax ratio quadrupled compared to ischemic vehicle treated rats. This result indicates G-CSF swings the anti-apoptotic/pro-apoptotic pendulum in favor of cell survival.

How could the upregulated ratio of Bcl2:Bax by G-CSF treatment protect the cell from ER stress induced apoptosis? Until the last decade it was thought that the Bcl2

proteins exclusively regulated the mitochondrial-mediated apoptotic pathway. There is a growing body of evidence of the Bcl2 family members' association with the ER (Zong et al. 2003). Several lines of evidence have reported the location of Bcl2, Bax and Bak at the membrane of the ER (Szegezdi et al., 2009; Heath-Engel et al., 2012) and of their role in ER stress apoptosis. For instance it has been reported that overexpression of Bcl2, or deficiency of Bax and Bak, was protective against lethal ER stress (Distelhorst & McCormick, 1996; Wei et al., 2001). In addition expression of ER-targeted Bak (Bak/cb5) in Bax/Bak double-knockout cells induced apoptosis (Zong et al., 2003). Hence we postulate that the G-CSF-upregulated Bcl2 could potentially protect the ischemic brain against ER stress-induced apoptosis by: **(1)** Neutralizing the apoptotic effect of Bax/ Bak at IRE1α. Bax and Bak were reported as upstream regulators of the ER stress sensor IRE1α. During prolonged ER stress Bax and Bak bind to the cytosolic region of IRE1α, resulting in the pathological activation of downstream targets such as JNK, p38MAPK and XBP-1. This action of Bax/Bak is independent of their proapoptotic function at the mitochondria (Hetz et al., 2006). Bcl2 executes its anti-apoptotic activity by sequestering and forming heterodimers with the Bcl2 pro-apoptotic family members (Zha et al.,1996). Therefore the upregulation of Bcl2 by G-CSF in our present study could antagonize the pro-apoptotic effect of Bax/Bak by forming a heterodimer with Bax or Bak, neutralizing their effect on IRE1α. **(2)** Increasing the calcium concentration in the ER. One of the resultant conditions of ischemia is the efflux of intraluminal ER calcium through the inositol 1,4,5-trisphosphate receptor (IP_3R) located at the ER membrane (Ruiz et al., 2009). This depletion of ER calcium reduces the activity of calcium-dependent ER molecular chaperons, such as GRP78, which inevitably affect

proper protein folding (Michalak et al., 2002; Burdakov et al., 2005). It has been reported that both Bax and Bak increase the release of calcium from the ER during prolonged ER stress via IP_3R. Sacorrano and colleagues (2003) demonstrated that cells with double knockout (DKO) Bax and Bak had reduced Ca^{2+} release from the ER upon stimulation with IP3R by its ligand, inositol 1,4,5-trisphosphate (IP_3) (Scorrano et al. 2003). On the other hand pro-apoptotic Bcl-2 reverses the effect of Bax at IP_3R, reducing the release of ER calcium (Pinton et al. 2001). Hence the upregulation of Bcl2 by G-CSF could potentially reduce the efflux of ER calcium through IP_3R, ensuring the reestablishment of the ER homeostasis. The reestablishing of normal ER homeostasis can be deduced from the reduced level of the intraluminal ER stress sensor, GRP78, which we demonstrated in this study (Fig.23).

3.3.7. G-CSF attenuates cleaved caspase 3.

Caspases are another family of proteins that play an integral role in apoptosis (Shi 2002). Several lines of evidence showed the activation of caspase 3 in cerebral ischemia (Chen et al., 1998; Le et al., 2002; Li et al., 2010). Since caspase 3 is one of the critical effectors of cell death, we examined the effect of G-CSF on the level of activated caspase 3. We demonstrated that G-CSF treatment significantly reduced the expression of activated caspase 3 (cleaved caspase 3) in both the core and penumbra of the ischemic brain. This reduction of caspase 3 by G-CSF could be attributed to the increased ratio of Bcl2:Bax shown in Fig. 17 d & h.

One way that caspase 3 may become activated is by cross talk between the endoplasmic recticulum and the mitochondria. There exists a physical and functional interaction between the ER and the mitochondria. Their physical interaction is mediated

by the mitochondria-associated ER membrane (MAM) which is a specialized area of the ER that is in direct contact with the mitochondria. MAM presents a physical platform on which molecules from the ER travels to the outer mitochondrial membrane (OMM) (Hayashi et al., 2009). Functional interaction between the two organelles is mediated by Ca^{2+}. During ischemia, Ca^{2+} is released from the ER through the IP3R and ryanodine receptor (RyR) channels (Luciani et al., 2009; Ruiz et al., 2009). The trafficking of the ER Ca^{2+} to the mitochondria during ischemia, compounds the concentration of excessive mitochondria Ca^{2+} leading to the translocation of Bax/Bak to the mitochondria and the opening of mitochondria permeability transition pore (MPTP). The opened MPTP enables the release of the intramitochondrial protein, cytochrome C, which indirectly activates the executioner cleaved caspase 3, thereby leading to cell death (Li et al., 1997;. Celsi et al., 2009). Since Bcl-2 antagonizes Bax and Bak by forming heterodimers, the upregulated Bcl-2 by G-CSF could neutralize the pro-apopotic effect of Bax and Bak at the mitochondria by binding to them and therefore indirectly inhibiting cleaved caspase 3.

Another way in which caspase 3 maybe activated is via the ER associated caspase 12. During ischemia caspase 12, which is located at the ER membrane, is cleaved into its active form. The activated caspase 12 then cleaves caspase 3 into it's active form (Martinez et al. 2010). Since G-CSF may potentially reestablish ER homeostasis, made evident in this study by the effect of G-CSF on ER stress proteins, G-CSF could inhibit the activation of the ER caspase 12 and hence reduce the expression of the caspase 12 substrate, caspase 3.

3.3.8. G-CSF attenuates HSP27.

Under physiological conditions, heat shock proteins (HSPs) assist in the folding of newly synthesized proteins, as well as being involved in intra-organelle protein trafficking. The 27 kilodalton heat shock protein (HSP 27) is one of the major inducible heat shock proteins and is upregulated in neuronal culture and brain tissue exposed to stress. It is therefore one of the key markers of cells undergoing stress in general (Feder and Hofmann 1999). We investigated the effect of G-CSF on the HSP27 expression and demonstrated that G-CSF treatment attenuated the increased level of HSP27 in ischemic vehicle treated rats. This result provided added credence that G-CSF administration reduces cellular stress after ischemic/reperfusion injury.

4. CONCLUSION

Cerebral ischemic injury presents a wide and diverse set of pathophysiological mechanisms. It is therefore imperative that optimal neuroprotective approaches should include either combination treatment or if using a single compound, that compound should have the capacity to affect more than one of the underlying mechanisms of cerebral ischemic damage. Granulocyte-colony stimulating factor has exhibited this potential; for instance, there are reported evidences of its anti-apoptotic activity (Schäbitz et al., 2003; Schneider et al., 2005) its immunomodulatory action (von Aulock et al., 2004), it is a stimulator of neurogenesis (Schneider et al., 2005; Kawada et al., 2006; Diederich et al., 2009), and it has angiogenic capabilities (Lee et al., 2005). Although there exist several reports of its anti-apoptotic activity in cerebral ischemia, this mechanism is still not fully elucidated.

In this study we have provided new insight into the anti-apoptotic mechanism of G-CSF as it pertains to ER stress-induced apoptosis as well as confirmed previous studies on its general anti-apoptotic activity against cerebral ischemia. We have shown that G-CSF modulates all three ER stress sensor pathways, the PERK, IRE1α and the ATF6 pathway by downregulating their respective downstream targeted proteins; ATF4, (p-p 38MAPK and p JNK), in both the core and the penumbra and cleaved ATF6 in the penumbra. While ATF4 and cleaved ATF6 affect the transcription of the ER stress-induced apoptotic transcription factor CHOP, p-p38MAPK activates it post-translationally, thereby increasing its activity. The transcription and activation of CHOP

in turn leads to the reduced expression of Bcl-2 and increased expression of pro-apoptotic gene such as Bim. Our study has revealed that G-CSF downregulated CHOP in both the core and the penumbra, which could be a reflection of G-CSF-reduced expression of the upstream protein to CHOP:- ATF4, cleaved ATF6 and p-p38MAPK.

The downregulation of CHOP by G-CSF implies that Bcl-2 expression should increase, since CHOP inhibition of Bcl-2 transcription would be reduced. We have shown a drastic increase of Bcl-2 in both the core and the penumbra of ischemic animals treated with G-CSF which could be partly due to decreased in CHOP expression. The expression level of pJNK was also down regulated by G-CSF in the core and the penumbra in our study. Bcl-2, being a substrate of pJNK, is inhibited by pJNK-phosphorylation. Since the level of pJNK was down regulated by G-CSF treatment, it is a reasonable deduction that the anti-apoptotic activity of Bcl2 is uninhibited. These results indicate that G-CSF treatment swings the balance of cell death/cell survival in favor of cell survival. Additionally, G-CSF reduced the expression level of HSP27, a general stress marker for cell stress, thereby providing credence of its protecting cells undergoing ischemic stress.

G-CSF decreased the expression of pro-apoptotic Bak, with an overall increase in the ratio of Bcl-2 to Bax. All three proteins are associated with the ER and the mitochondria. We postulate that the increased expression of Bcl-2 by G-CSF could reduce ER stress by (1) neutralizing the apoptotic effect of Bax/Bak at IRE1α and (2) by reducing calcium efflux from the ER through the IP3R. Both situations would result in the reestablishment of normal ER homeostasis, evident by a reduction in the expression level of the intraluminal ER stress sensor, GRP78. We demonstrated a reduction in the

expression level of GRP78 by G-CSF, which makes our postulations feasible. G-CSF also reduced the expression of executioner cleaved caspase 3, an executioner caspase of cell death in both the core and penumbra of the ischemic tissue. This reduction could be due to the heterodimerization of Bcl2 to Bax or Bak, thereby inhibiting their action (Bax and Bak) at the mitochondria, resulting in reduced release of cytochrome C and subsequent reduction of cleaved caspase 3. However, since we used whole cell lysate in our western analysis it is difficult to make a definitive conclusion on the action of Bcl2 at either the ER or mitochondria. Nevertheless our study has provided new and valued evidence on the anti-apoptotic mechanism of G-CSF against ER stress induced apoptosis in ischemia evident by the positive modulation of the PERK, IRE1α and ATF6 pathway and the downregulation of CHOP and GRP78.

Most growth factors such as, brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF-1) and basic fibroblast growth factor (bFGF), exhibit endogenous neuroprotective and neurotrophic effects with several reports providing evidence of neuroprotection by these growth factors after exogenous administration against stroke (Mattson & Scheff, 1994; Larsson et al., 1999; Semkova & Krieglstein, 1999; Schäbitz et al., 2000; Schäbitz et al., 2001; Li & Stephenson, 2002; Markham et al., 2012). However, such factors have yet to translate into clinical practice. For example, clinical trials of bFGF were discontinued in phase III because of dose related adverse side effects that were not anticipated from preclinical studies (Bogousslavsky et al. 2002). Granulocyte-colony stimulating factor is one of the few growth factor currently approved for clinical use and routinely prescribed to treat neutropenia (Metcalf, 1990), making it an ideal agent for clinical trial for treatment of cerebral ischemia.

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