Evaluating the role of Neuregulin-1β1 in neuroprotection after spinal cord injury

By

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A thesis will be submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Abstract:

Spinal cord injury (SCI) results in significant degeneration of neurons and axons that leads to permanent impairment in motor, sensory and autonomic function. Thus, development of neuroprotective and neuroregenerative strategies are critical to reduce neuronal damage and facilitate axon regeneration in injured neurons. Our laboratory has uncovered that SCI results in acute and persistent downregulation of Neuregulin-1beta 1 (Nrg-1 β1) after SCI. We demonstrated that restoring the declined levels of Nrg-1β1 fosters oligodendrogenesis and promotes an anti-inflammatory, pro-regenerative response in resident glia and infiltrating leukocytes, which culminates in improved recovery of function after SCI. Nrg-1β1 is well-known for its critical roles in the development, maintenance and physiology of neurons and glia in the developing and adult spinal cord. However, despite this pivotal role, Nrg-1β1 specific effects and mechanisms of action on neuronal injury remain largely unknown in SCI.

In this study, we have demonstrated, for the first time, that Nrg-1β1 protects neurons from degeneration in acute and subacute phases of SCI through downregulation of several cell injury pathways implicated in oxidative damage, lipid peroxidation, necrosis and apoptosis. Utilizing a SCI-relevant *in vitro* model of glutamate excitotoxicity, a major mechanism of neuronal injury in SCI, we have provided direct evidence that Nrg-1 protects neurons by blocking Caspase-3 and mitochondrial mediated cell death mechanisms, and modulation of MAPK and Akt signaling pathways. Altogether, our work provides novel insights into the role and mechanisms of Nrg-1β1 in neuronal injury after SCI and introduces its potential as a new neuroprotective target for this debilitating neurological condition.

Acknowledgement:

Firstly, I would like to express my gratitude and appreciation to my supervisor, professor Soheila Karimi -Abdolrezaee, whose guidance, support and encouragement has been invaluable throughout this study. I am extremely grateful for our friendly meetings and your support in my academic endeavors. During this journey, I have practiced and learnt several critical skills such as: being independent, using critical thinking, team working, and writing efficiently and scientifically. Extensive support and mentorship in Dr. Karimi's lab can guarantee the success of students in their future career.

I also wish to thank my advisory committee members, Dr. Michael F. Jackson, Dr. Katinka Stecina and Dr. Jillian Stobart who have been a great source of support, guidance, and constructive feedbacks.

I would like to thank all previous and current lab members: Arsalan Alizadeh, Christopher G. Hart, Seyed Mojtaba Hosseini and Shiva Nemati for being friendly, supportive and collaborative. I would particularly like to single out Dr. Hardeep Kataria for his patience, support, and compassionate academic and personal advice.

In addition, I would like to thank my husband for his wise counsel and sympathetic ears. He was always there for me.

Dedication:

This thesis is dedicated to my loving family.

My parents

Nasrin and Ali-Mohammad Shahsavani

For their unconditional love, forgiveness and endless support.

My beloved husband

Hossein Ataei

For his love and support throughout this challenging journey

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Contributions

The contents of the introduction (Chapter 1) were prepared independently for this dissertation, and portions have been published in a peer-reviewed journal. Components of an article that is published in a peer-reviewed research journal are presented in Chapter 2, and the contributions of the authors are listed below.

Narjes Shahsavani, Hardeep Kataria, Soheila Karimi-Abdolrezaee, (2021). **Mechanisms and repair strategies for white matter degeneration in CNS injury and diseases.** Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 2021. © Elsevier Inc.

My contribution to this work included concept and design, manuscript writing and final approval of manuscript. Manuscript preparation was done in collaboration with Dr. Hardeep Kataria and my supervisor, Dr. Soheila Karimi-Abdolrezaee.

Narjes Shahsavani, Arsalan Alizadeh, Hardeep Kataria, Soheila Karimi-Abdolrezaee, (2021). **Availability of neuregulin-1beta1 protects neurons in spinal cord injury and against glutamate toxicity through caspase dependent and independent mechanisms.** Experimental neurology, 2021in press.

My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. Conceptual and experimental design, data interpretation and manuscript preparation were done in collaboration with my supervisor, Dr. Soheila Karimi-Abdolrezaee. Dr. Arsalan Alizadeh performed all surgical procedures in this study. Dr. Hardeep Kataria provided training for culture and molecular biology techniques.

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AChR, acetylcholine receptor;
AD, Alzheimer's disease;
ADAM, a disintegrin and metalloproteinase;
AIF, apoptosis inducing factor;
ALS, amyotrophic lateral sclerosis;
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;
ARIA, acetylcholine receptor inducing activity;
ATP, adenosine triphosphate;
BACE, β-site of amyloidprecursor protein cleaving enzyme;
BBB, blood brain barrier;
Breg, regulatory B cell;
BSB, blood spinal barrier;
CCL, C-C motif ligand;
CNS, central nervous system;
CRD, cysteine-rich domain;
CSPGs,chondroitin sulfate proteoglycans;
CXCL, C-X-C motif ligand;
DA, dopamine;
DAMP, damage associated molecular pattern;
DAT, dopamine transporter;
DC, dendritic cell;
DRG, dorsal root ganglion;
EAAC, excitatory amino acid carrier;
EAE, experimental autoimmune encephalomyelitis;
ECM, extracellular matrix;
ErbB, erythroblastic leukemia viral oncogene homolog;
EGF, epidermal growth factor;
ErbB, erythroblastic oncogeneB;
ERK, extracellular signal-regulated kinase;
FADD, Fas associated via death domain;
FDA, food and drug administration;
GABA, γ-aminobutyric acid;
GDNF, glial cell line derived neurotrophic factor;
GFAP, glial fibrillary acidic protein;
GGF, glial growth factor;
HMGB1, High mobility group box1;
HNE, Hydroxynonenal;
HSPGs, heparan-sulfate proteoglycans;
IL, interleukin;

Ig, immunoglobulin;
ICD, intracellular domain;
IFN-γ, interferon gamma;
iNOS, inducible nitric oxide synthase;
JNK, c-Jun N-terminal kinase;
KA, kainic acid;
LC3, Microtubule-associated protein 1A/1B-light chain 3;
LPC, Lysophosphatidylcholine;
LPS, Lipopolysaccharide;
MAPK, mitogen activated protein kinase;
MEK, MAPK/ERK kinase;
mGluR1, metabotropic glutamate receptor 1;
Mpz, myelin protein zero;
MLKL, mixed lineage kinase domain like pseudokinase;
MMP, matrix metalloprotease;
MPTP, mitochondrial permeability transition pore;
MS, multiple sclerosis;
Myd88, Myeloid differentiation primary response 88;
MuSK, muscle specific kinase;
NCC, neural crest cells;
NF-κ B, nuclear factor kappa-light-chain-enhancer of activated B cells;
NGF, nerve growth factor;
NMJ, neuromuscular junction;
NO, nitric oxide;
Nrg-1, neuregulin-1;
NMDA, N-methyl-D-aspartate;
NPC, neural precursor cells;
NT-3, neurotrophin-3;
OPCs, oligodendrocyte precursor cells;
P75NTR, P75 neurotrophin receptor;
PARP, Poly (ADP-ribose) polymerase;
PD, Parkinson's disease;
PI3K, phosphatidylinositol 3-kinase;
PKC, protein kinase C;
PLC, phospholipase C;
PNS, peripheral nervous system;
PSD-95, postsynaptic density protein of 95 kDa;
RAF, rapidly accelerated fibrosarcoma;
RIPK, receptor-interacting serine/threonine-protein kinase;
RMS, rostral migratory stream;

ROS, reactive oxygen species;
SCI, Spinal cord injury;
SD, Sprague dawly
SOD1, superoxide dismutase 1;
STAT3, Signal transducer and activator of transcription 3;
TACE, Tumor necrosis factor-alpha converting enzyme;
TGF-β, transforming growth factor beta;
TLR, toll-like receptor;
TM, transmembrane domain;
TNF, tumor necrosis factor;
Treg, regulatory T cell;
VDAC, voltage dependent anion channel

Chapter I: Introduction

1.1 Pathophysiology of traumatic spinal cord injury

1.1.1 Epidemiology and clinical impact

Traumatic spinal cord injury (SCI) results in significant tissue degeneration that leads to permanent impairment of motor, sensory and autonomic functions. Based on the National Spinal Cord Injury Statistical Center, there are 12,500 new cases of SCI each year in North America (1). There are approximately 325,000 Canadians and 27 million individuals globally with SCI (2). Over 90% of SCI cases are caused by accidents, violence, sports or falls (3) with a rate of 2:1 in males to females and at different stages of adulthood (3). Based on the level of the injury, patients can experience a range of sensory and motor loss and sympathetic/parasympathetic imbalance. Injuries in lower thoracic region cause paraplegia while injury at cervical level results in quadriplegia (4). Of note, SCI mainly occurs in cervical levels and particularly at C5 (1). Early surgical decompression within 24hrs post injury is shown to improve functional outcomes in patients (5, 6). Advances in surgical procedures, rehabilitation and long-term care have improved the survival rate and recovery of patients; however, there is no clinically approved treatment to significantly improve spinal cord repair processes and regain the impaired sensory and motor functions in SCI patients.

1.1.2 Primary spinal cord injury

Traumatic SCI is classified into two major events: primary and secondary injury. Primary injury is defined as the tissue damage following physical and mechanical impact to the spinal column (7). These forces do not only destroy pathways, but often the cells (neuron or supporting cells), which eventually cause spinal shock, hypotension, ischemia, ionic imbalance and neurotransmitter accumulation (8, 9). All these processes eventually result in neurodegeneration

and formation of a cavity at the lesion site in chronic stages that has been also observed in human SCI. There are several types of experimental SCI models that each have their advantages for studying pathological responses and repair. For example, the complete or dorsal/lateral transection SCI model is suitable for evaluating axonal regeneration and plasticity (9). However, the contusive compressive SCI model is the most clinically relevant as it simulates the most common type of injury observed in human cases (10). Degree of tissue sparing at the lesion site greatly influences the degree of recovery in patients. To study pathophysiological aspects of SCI, rats are beneficial as they are small in size and more economic comparing to the larger animal models and primates (11). Additionally, they share similar pathological and regenerative features with primate and human SCI (12). Mice are also frequently used to study SCI since they can be genetically manipulated however, they exhibit several pathophysiological characteristics that are different from those of rats and humans including inflammatory responses (13, 14). Altogether, these primary events are followed by a cascade of secondary injury mechanisms that lead to progressive degeneration of spinal cord as well as muscle and bone tissue in the periphery. These hallmarks will be discussed in the following sections.

1.1.3 Secondary injury mechanisms

Secondary injury mechanisms are cellular and molecular processes that begin within minutes following the primary injury and continues for weeks, months or years and eventually lead to tissue degeneration and scar formation (15). Secondary injury mechanisms are temporally categorized into three phases of acute, sub-acute and chronic. The acute phase, which immediately starts following primary injury includes vascular damage, ionic imbalance, neurotransmitter accumulation (excitotoxicity), free radical formation, calcium influx, lipid peroxidation,

inflammation, edema, and necrotic cell death (16, 17). With progression of injury, the sub-acute phase begins that involves apoptosis, demyelination of surviving axons, Wallerian degeneration, axonal dieback, matrix remodeling, and formation of a glial scar around the injury site. Further changes in the chronic phase of injury include the formation of a cystic cavity, progressive axonal die-back, and maturation of the glial scar (9). I will review the key relevant components of the secondary injury mechanisms in the following sections.

1.1.3.1 Neuroinflammation

Early after primary injury, there is a wave of stressor release from damaged cells to the extracellular space (e.g., glutamate and HMGB1). These stressors activate resident microglia and stimulate infiltration of peripheral immune cells (18, 19). Activation and recruitment of immune cells stimulate inflammatory responses that include release of pro-inflammatory cytokines, chemokines and free radicals at the site of injury (20, 21). Originally, inflammatory responses were thought to be detrimental. However, it is recognized now that inflammation can have both beneficial and detrimental effects. The dual role of inflammation will be discussed further in this section.

Neuroinflammation following SCI involves both the innate and the adaptive immune responses. Reactive astrocytes, resident microglia, monocyte derived macrophages and neutrophils are the main players of the innate immune response and lymphocytes orchestrate the adaptive immune response (9). The first wave of inflammation is characterized by acute recruitment of resident microglia and astrocytes and neutrophils to the injury site (22). Three days following injury, the second wave of inflammation is organized by recruitment of macrophages, B and T lymphocytes (23-25). Presentation of antigen by macrophages and microglia to T lymphocytes

activates these cells. Activated T cells produce cytokines that stimulate B cells to produce antibodies to further activate phagocytes (26). Inflammatory responses are more prominent in the acute stages of SCI; however, they persist throughout the sub-acute and chronic stages. The role of each immune cell population in the pathophysiology of SCI will be discussed below.

Microglia/ Macrophages: Microglia are specialized population of macrophage-like cells in the CNS with the capability to orchestrate the inflammatory response (27). In contrast, macrophages are derived from monocytes that infiltrate to the CNS following injury (20). In the healthy CNS, quiescent ramified microglia are implicated in synaptic organization, neuronal support, phagocytosis of apoptotic cells, myelin turnover, control of neuronal excitability and phagocytic debris removal (27, 28). Upon reaching their final destination during development, in CNS parenchyma, they differentiate into mature microglia and acquire a ramified morphology (29-31). Following injury, in response to environmental stressors (NO, ATP or glutamate) reactive microglia transition from ramified to amoeboid morphology and migrate to the site of injury (32, 33). The phenotype of activated microglia can be classified into two different categories: proinflammatory (M1) phenotype and anti-inflammatory (M2) phenotype (34). M1 microglia/macrophages release a number of pro-inflammatory factors, free radicals and matrix metalloproteases (MMPs) (35-37), while M2 microglia help in promoting regeneration by phagocytosis of inhibitory myelin debris or secreting anti-inflammatory cytokines such as IL-10 or TGFβ (38-40). The proportion of M1/M2 phenotype is instructed by the environmental factors following injury. As injury progresses, the pro-inflammatory population becomes more prominent (41).

Astrocytes play a critical role in maintaining CNS homeostasis by contributing to function of the blood brain barrier (BBB), providing nutrients to neurons and removal of excess ions and

neurotransmitters such as glutamate from synaptic regions (26). They also contribute to CNS pathology by regulating immune cell activity and trafficking (42). Astrocytes react acutely to CNS injury by increasing cytokine and chemokine production through an IL-1R1-Myd88 pathway (43). A few minutes following injury, expression of IL-1β is significantly elevated in astrocytes and microglia (37). Additionally, other chemokines such as monocyte chemoattractant protein (MCP)-1, chemokine C-C motif ligand 2 (CCL2), C-X-C motif ligand 1 (CXCL1), and CXCL2 are produced by astrocytes, and enhance the recruitment of neutrophils and pro-inflammatory macrophages following injury (42, 43). Due to production of TNF-α, IL-12, and IFN-γ by astrocytes, they promote pro-inflammatory M1-like phenotype in microglia/macrophages in the injured spinal cord (44, 45). Growing evidence shows astrocytes also play anti-inflammatory roles as they produce anti-inflammatory cytokines such as TGF-β and IL-10, which can promote a pro-regenerative M2-like phenotype in microglia/macrophages (46). TGF-β signaling in astrocytes has been implicated in modulation of neuroinflammation through inhibition of NF-κB activity and nuclear translocation (47). STAT3 is another key signaling pathway in astrocytes with beneficial effects on neuroinflammation as increase in STAT3 phosphorylation promotes astrocytic scar formation and restricts the expansion of inflammatory cells in mouse SCI that is associated with improved functional recovery (48).

Neutrophils are attracted to the site of injury by pro-inflammatory chemokines that are released by reactive astrocytes and microglia including C-C motif ligand-2 (CCL2) and C-XC motif chemokine ligand-1 and -2 (CXCL1/2) (43). Neutrophils promote inflammatory cell recruitment and activation by releasing IL-8, and matrix metalloprotease (MMPs) to degrade the extracellular matrix (ECM) (49-51). Moreover, they release TNFα and reactive oxygen species (ROS) that lead to cell death indirectly (51, 52).

1.1.3.2 Oxidative stress and lipid peroxidation

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of ROS in cells and the ability to neutralize these reactive products (53). Superoxide radicals (O2•−), hydrogen peroxide (H2O2), hydroxyl radicals (•OH), and singlet oxygen (1O2) are considered as ROS (53). They are produced in mitochondria as by-product of ATP via the electron transport chain or by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (53). All biological processes including protein phosphorylation, activation of transcriptional factors, apoptosis and differentiation are dependent on a proper ROS production inside the cell and at a low level (54). However, excessive levels of ROS is attributed to harmful effects on macromolecules through protein oxidation and lipid peroxidation, which can eventually cause cellular degeneration (54). Neurons, in particular, are susceptible to ROS-mediated cell death due to rapid depletion of their antioxidant NAD^+ levels (55). Another reason for susceptibility of neurons to ROS is that neurons need to utilize high levels of ATP to maintain ionic gradient and support synaptic activity (56, 57). It is estimated that in synaptic activity, 1.64×10^5 ATP per second per neurotransmitter vesicle is consumed (57, 58). Therefore, mitochondria and specifically, synaptic mitochondria play a key role to meet neuronal ATP demand (59). Altogether, since neuronal function is highly dependent on mitochondria, ROS imbalance results in mitochondria permeabilization, glutamate, and glucose uptake, and autophagic degradation of damaged proteins and organelles (60-62).

In addition to neurons, oligodendrocytes are sensitive to oxidative stress because of high metabolic demand and their lipid and iron content (63-65). Moreover, oligodendrocytes have lower antioxidants agents such as glutathione peroxidase with half of the glutathione reductase activity compared to astrocytes (66).

SCI results in a significant increase in oxidative stress that overwhelms the antioxidant defense system during the first two weeks after injury (67, 68). Mitochondria metabolic activity is significantly reduced as early as 1hr post SCI in rats and ROS formation is dramatically increased at 4 and 24hrs post SCI (69). Due to release of inflammatory factors, reactive microglia, astrocytes, neutrophils and macrophages produce high levels of ROS after SCI (70-72). Following SCI one of the major forms of radical-induced oxidative damage involves oxidative attack on polyunsaturated fatty acids of cell membrane, which triggers lipid peroxidation that ultimately generates 4-hydroxynonenal (4-HNE), acrolein or 3-nitrotyrosine as by-products (73). These peroxidized phospholipids promote apoptotic cell death by accumulating in mitochondrial membranes and impairing cellular respiration and calcium buffering, and by inducing protein misfolding (68, 74, 75). Administering antioxidants can alleviate oxidative stress and attenuate tissue degeneration, as reviewed earlier (76), suggesting targeting oxidative stress is a viable therapeutic strategy for SCI.

1.1.3.3 Reactive astrogliosis and glial scar formation

Spinal cord injury leads to formation of a glial scar that surrounds the injury epicenter (77). Reactive astrocytes, NG2+ oligodendrocyte precursor cells (OPCs) and microglia are the main contributors to the glial scar by secreting ECM components such as chondroitin sulfate proteoglycans (CSPGs) (78). CSPGs are the major component of the glial scar and play a central role in inhibition of axon regeneration following SCI. Astrocytes play a central role in glia scar formation (79). Upon activation, they increase the expression level of intermediate filaments, glial fibrillary acidic protein (GFAP), nestin, vimentin and more importantly CSPG as early as 24hrs post injury that persists in chronic stages (80). Then, they proliferate and migrate to the site of injury to generate a mesh like structure around the injury epicenter (81, 82). Glial scar plays a dual

role in SCI pathology as it is inhibitory to axon regeneration while it serves as a physical barrier to prevent spread of inflammation to the healthy adjacent tissue (81, 82).

In addition to resident glia, infiltrating immune cells also play an important role in glial scar formation by producing pro-inflammatory cytokines (IL-1β and IL-6), chemokines and enzymes. Release of these factors leads to disruption of blood spinal barrier (BSB) and facilitates astrocyte activation (79). Activated microglia and macrophages produce proteolytic enzymes such as MMPs that exacerbates infiltration of peripheral cells by increasing vascular permeability causing further disruption of BSB (83). Inhibition of MMPs has been implicated in neuroprotection and functional recovery following SCI (83, 84).

Fibroblasts, pericytes and ependymal cells also contribute to glial scar formation (79). Fibroblasts secret ECM components including collagen, laminin, fibronectin and a number of inhibitory molecules for axon regeneration such as semaphorins (85). SCI also activates quiescent stem cell pool in the spinal cord that mainly differentiate to the astrocytes and NG2+ OPCs which further contribute to glial scar formation (81, 86, 87). Structurally, activated microglia/ macrophages locate in the inner portion of the glial scar, while reactive astrocytes occupy the outer compartment (79).

As mentioned above, although the glial scar plays beneficial roles in restricting the inflammatory zone, in the long term it also poses a barrier to axon regeneration (88, 89). CSPGs are the major component of the glial scar that are shown to inhibit axon regeneration (90-95), sprouting (96, 97), conduction (98, 99) and remyelination (100-104). In normal condition, CSPGs are expressed in low levels and are mainly implicated in neuronal guidance and synapse stabilization (105, 106). Mechanistically, CSPGs inhibit microtubule organization, which leads to formation of dystrophic endbulb and axon retraction (107, 108). Extracellularly, CSPGs can induce

their inhibitory effects through different mechanisms. They can bind and activate several receptor proteins, including PTPσ, LAR, NgR1 and NgR3 (109). They can convert Sema5A from an attractive form to an inhibitory cue. CSPGs can also suppress the function of growth-promoting factors such as laminin (109). Intracellularly, CSPGs activate RhoA-Rho kinase signaling while inactivate survival pathways such as Akt and Erk. GSK-3β and mTOR are among the identified downstream signaling pathways that mediate the inhibitory effects of CSPGs.

CSPGs also play a beneficial role in restricting tissue damage in CNS injuries (110). Removal of the glial scar (48) or limiting its formation (111) leads to enlarged lesion area, increased inflammatory responses and impaired functional recovery. Glial scar is also protective chronically, as removal of the chronic glial scar and adjacent reactive astrocytes causes more extended lesions and tissue degeneration (111). Interestingly, Rolls and colleagues have demonstrated that removal of CSPGs in acute stages of SCI by xyloside exacerbates tissue degeneration and impairs functional recovery (112), while its inhibition in chronic stages results in enhanced functional recovery in mice with SCI. These findings suggest CSPGs play a dual protective and inhibitory role in SCI.

1.1.3.4 Cell death

In multicellular organisms, cell death is a critical and active process that maintains tissue homeostasis and eliminates potentially harmful cells. However, following SCI, the primary insult initiates a complex secondary injury cascade that eventually leads to the death of neurons and glial cells (113). During the acute phase of SCI (< 48hrs), the mechanical disruption and dislocation of vertebral column causes compression or transection of the cord that leads to immediate death of neurons and oligodendrocytes at the lesion through necrosis (9). As injury progresses, in the acute to sub-acute stages (48hrs- < 7days), ischemia and excitotoxicity play a key role in ionic imbalance

that leads to cell death in both neurons and glia (9). Necrosis of neurons and glia results in release of ATP, potassium and DNA that activates microglia. Activated microglia in addition to other inflammatory cells contribute to apoptosis in neurons and oligodendrocytes in penumbra regions surrounding the injury (114). Major forms of cell death in SCI include necrosis, apoptosis, autophagy and necroptosis. All these types of cell death are morphologically distinct from each other and can occur through distinct or overlapping signaling pathways in response to injurious stimuli (115).

1.1.3.4.1 Necrosis

Necrosis is a hallmark of primary injury following SCI in neurons, oligodendrocytes and endothelial cells (116). It is defined as a passive and accidental cell death in which the cellular content is released to the extracellular space (117). There are several morphological changes in the cells that undergo necrosis such as increased cell volume, swelling of organelles, and early ruptures of plasma membrane (118). High Mobility Group Box 1 protein (HMGB1) is a ubiquitous nuclear protein that is passively released by cells that are damaged in a traumatic and non-programmed manner (necrosis). Upon binding of HMGB1 to its receptors they infiltrate leukocytes to from the blood into the injured tissue to trigger adaptive immunity (119). Most SCI studies have focused on apoptosis and autophagy as necrosis is essentially irreversible period (120, 121). However, some therapeutic approaches have been implemented to reduce tissue damage such as the use of necrostatin-1 this approach mainly targets programmed necrosis (necroptosis) that will be discussed later (119). Necrosis is commonly described as an accidental and unregulated type of cell death however, recently evidence suggest that there is a caspase-independent process that resembles necrosis and is called necroptosis. This will be discussed in following sections.

1.1.3.4.2 Apoptosis

The term of apoptosis (a-po-toe-sis) was first used by Kerr, Wyllie, and Currie in 1972 to introduce a morphologically different type of cell death; although some aspects of apoptosis had been introduced earlier (122-124). The knowledge about apoptosis mechanisms was obtained from exploration around programmed cell death in *Caenorhabditis elegans* (125). This organism contains 1090 cells in which 131 cells undergo programmed cell death at specific points during the development, which is conserved among worms, indicating the accuracy and control in this system (126). Apoptosis is characterized as a cell death in which a sequence of programmed events leads to elimination of the cell without the release of intracellular components to the outside environment (127). Apoptosis is a normal function of the body during development and aging to maintain homeostasis in an organism or tissue. It can also occur as a defense approach in immune reactions or in response to disease condition (128). Following apoptosis, cells exhibit specific morphological features, which distinguishes them from necrotic cells. In apoptosis, the cell membrane is intact, the cytoplasm is restrained in apoptotic bodies and does not trigger inflammation. In contrast, in necrotic cells, cell swelling, and disruption of membrane is observed. Moreover, during necrosis the cytoplasm and its components are released to the extracellular space and usually inflammation is present (129). In addition to morphological changes, several biochemical changes occur in apoptotic cells such as: protein cleavage, protein cross linking, DNA breakdown and phagocytic recognitions (130). One of the prominent features of apoptosis is involvement and activation of caspases, which are proteases that are ubiquitously expressed in different cells. They are mainly expressed as inactive proenzymes that can cleave proteins at aspartic acid residues upon activation. The mechanisms of apoptosis are sophisticated including

several energy consuming events. To date, there are two major apoptotic pathways: extrinsic or death receptor dependent and intrinsic or mitochondrial pathway (Figure 1).

The extrinsic pathway is mediated by death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (131). These receptors share similar cyteine-rich extracellular domains and a cytoplasmic domain of 80 amino acids called the "death domain". This death domain plays the central role in signal transduction (132). Currently, the common death signal/ and receptors are FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (132-135). In response to stimulus, after ligand binding, a cytoplasmic adaptor protein is added that contains the death domain which is already bound to the receptor. For example, binding of Fas ligand to Fas receptor, recruits FADD adaptor while binding of TNF ligand leads to recruitment of TRADD adaptor following by FADD (136, 137). In next step, procaspase-8 will be added and a death-inducing signaling complex (DISC) will be formed, leading to the auto-catalytic activation of procaspase-8 (138). Upon activation of caspase-8 the execution step will be initiated that is discussed in following sections.

The intrinsic pathway is not mediated by receptors and its stimuli are within the cells and target mitochondrial dependent events. These stimuli affect the inner mitochondrial membrane and result in opening of mitochondrial permeability transition (MPT) pore, loss of mitochondrial membrane potential and release of two different groups of pro-apoptotic proteins into the cytosol.

The first group of proapoptotic proteins contain cytochrome C, which triggers caspase dependent mitochondrial apoptosis (139). Following release of cytochrome C to cytosol, it binds to and activates Apaf-1 as well as procaspase-9 to form "apoptosome" (140, 141). The second group of proapoptotic proteins include apoptosis inducing factor (AIF) that triggers caspase-independent mitochondrial apoptosis. Upon release of AIF from mitochondria to cytosol, it will

then translocate to the nucleus and cause DNA fragmentation into ~50–300 kb pieces and then condensation of peripheral nuclear chromatin (142). Regulation of mitochondrial pathways is related to Bcl-2 family of proteins (143). Currently, 25 genes have been identified to be associated with Bcl-2 proteins. Some of the members of this family have anti-apoptotic roles (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG) and some have pro-apoptotic functions (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk). Regardless, their function converges in regulation of cytochrome C release from mitochondria by modifying mitochondrial membrane permeability (144). Bad's phosphorylation is a key to determine the fate of a cell. Upon phosphorylation, Bad is sequestered from the cytosol but when it is not phosphorylated it will move to mitochondria and contribute to cytochrome C release (145). Moreover, Bad can heterodimerize with Bcl-Xl or Bcl-2 and neutralize their protective effect and promote cell death (146). When both Bcl-2 and Bcl-Xl are not trapped by Bad, they inhibit the release of cytochrome C from the mitochondria (147). Puma is another member of the Bcl2 with pro-apoptotic activity. Puma plays an important role in *p53*-mediated apoptosis. Overexpression of Puma is accompanied by increased Bax expression, Bax conformational change, translocation to the mitochondria, cytochrome *c* release and reduction in the mitochondrial membrane potential and finally cell death (148).

Both extrinsic and intrinsic pathways converge in execution stage. At this point, caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases that cleave several substrates including cytokeratins, poly ADP-ribose polymerase (PARP), the plasma membrane cytoskeletal protein, the nuclear protein NuMA, among others, that ultimately cause the morphological and biochemical changes in apoptotic cells (149). Caspase-3 is the main executioner caspase as it can get activated by any of the initiator caspases (150). It is important to mention that extrinsic apoptosis usually occurs in inflammatory conditions. Inflammatory cells

secrete death receptor ligands, which bind to receptors on neurons, oligodendroglial and other receptor-expressing cells and causes cell death while intrinsic pathway usually becomes activated in response to internal stressors such as radiation, hypoxia, ER and metabolic stress.

Studies in rat SCI revealed that apoptosis begins as early as 4hrs following injury (151). Activation of caspases is observed in neurons located at the injury site within a few hours post SCI indicating that in addition to necrosis, apoptosis is active in neurons at the injury epicenter (151). Apoptosis is also the major cell death mechanism in oligodendrocyte at the epicenter and the penumbra of lesion. Of note, apoptosis also leads to loss of neurons and glia that are in distant regions and unaffected by initial injury (151).

Several factors following SCI can trigger apoptosis. SCI results in increase in expression of death receptors and their ligands as well as activation of caspases and calpain (151). As mentioned previously, SCI dramatically enhances ROS levels, which leads to mitochondrial damage through cell membrane lipid/protein/carbonylation peroxidation and eventually cell death (152). SCI also leads to extensive activation of PARP-1 which induces apoptosis through NAD consumption and release of AIF (153). Mitochondrial mediated apoptosis plays a pivotal role in cell death following SCI. Morphological analyses reveals that alteration in morphology of mitochondria initiates 2hrs post SCI and continues for at least 24hrs (154). At 3–6 hrs post-SCI, mitochondria become larger in size and fewer in number. It is also reported that the SCI leads to increase in expression level of mitofusin (Mfn)1 and Mfn2 while it decreases the expression of mitochondrial fission 1 and dynamin-related protein 1 (Drp1) (155). Therefore, SCI promotes mitochondrial fission shortly after the injury leading to apoptosis and cell death.

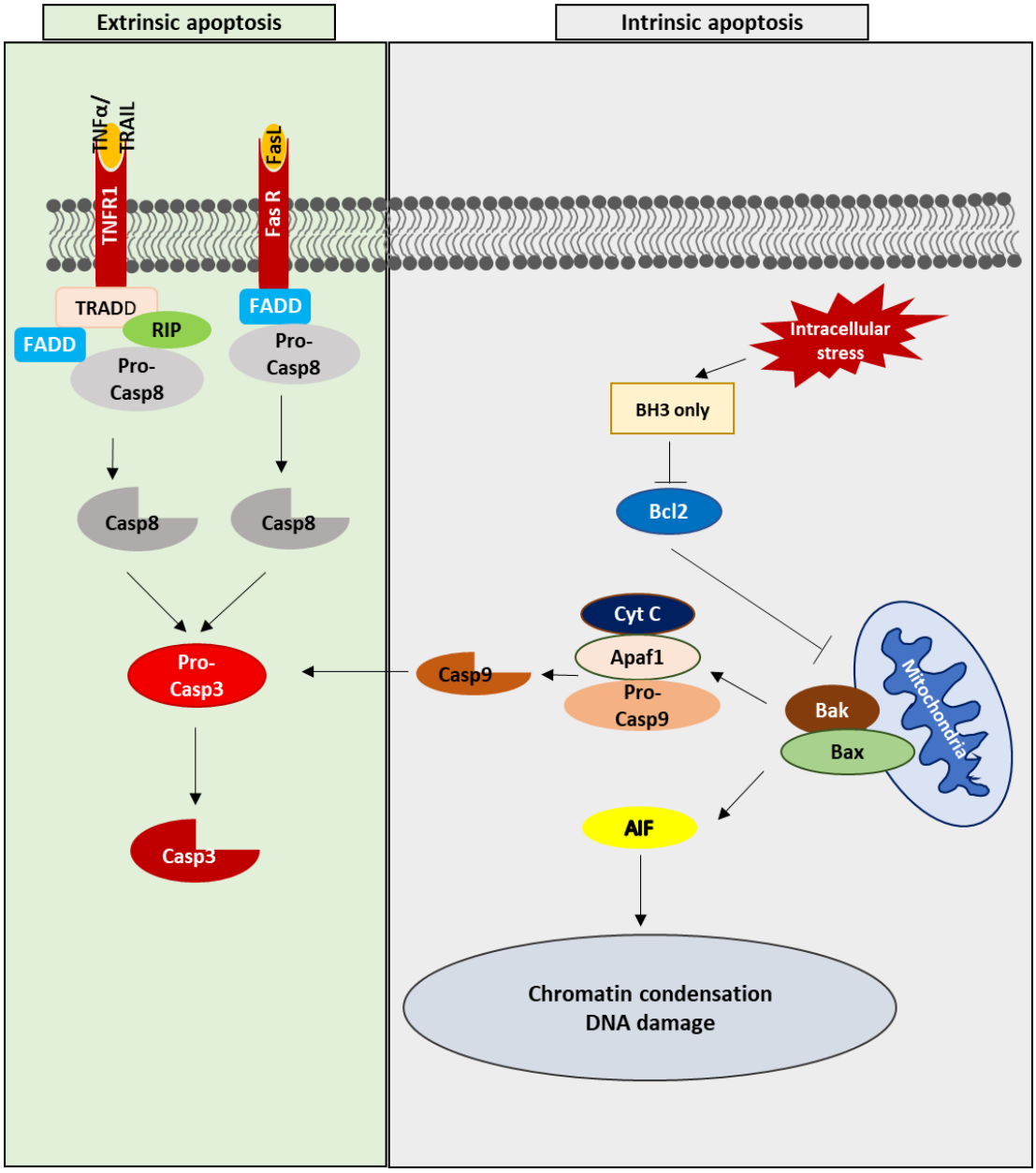

Figure 1: Apoptosis signaling pathways. Apoptosis pathways can be initiated through different entry sites, including the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as Fas or TNF-related apoptosis-inducing ligand (TRAIL) receptors by CD95 ligand (CD95-L) or TRAIL results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase-8 activates and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by stress signals through the release of apoptotic factors such as cytochrome c and apoptosis inducing factor (AIF). The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. The receptor and the mitochondrial pathway can be interconnected at

different levels, for example, by Bid, a BH3 domain-containing protein of the Bcl-2 family which assumes cytochrome-c-releasing activity upon cleavage by caspase-8.

1.1.3.4.3 Necroptosis

A few decades ago, a new type of cell death was recognized where apoptotic pathways were inhibited by necrostatin-1, and morphological features were resembling both apoptosis and necrosis. Therefore, this type of cell death was termed as necroptosis (119). Necroptosis is a regulated type of necrosis that is mediated by death receptors such as TNF superfamily receptors, toll-like receptors (TLR3 and TLR4), and interferon receptors which induces cell death through activation of caspase-8 (119). Evidence suggests that inhibition of caspase-8 shifts the type of cell death from extrinsic apoptosis to necrosis mode via activation of RIPK3 and MLKL (156-159). Therefore, there are two basic factors that must be present in a cell to assure that necroptosis can occur: cell must express RIPK3 and caspas-8 must be inhibited. TNFα-mediated necroptosis is known as extrinsic necroptosis, which binds to other receptors and forms a complex containing TRADD (an adaptor molecule that recruit RIPK1 to TNFR1), FADD, RIPK1, TRAF2/TRAF5, and cIAP1/cIAP2 (160, 161). Following activation of this complex, RIPK1 is ubiquitinated resulting in formation of stable complex that induces survival pathways including NF-κB- and MAPK-mediated pathway (162). NF-κB signaling plays a key role in counteracting the cytotoxic effect of TNFα, and pro-survival effect of NF-κB that is mediated by cIAP1/2 and cFLIPL (cellular FLICE-like inhibitory protein) (163, 164). Therefore, the complex plays the key role in determining the fate of the cell for survival or death (165). Removal of ubiquitin chain from RIPK1 results in its interaction with FADD, TRADD, RIPK3, and caspase-8, which then leads to formation of another complex termed as necrosome that further initiates the downstream signaling resulting in necroptosis (166). Upon activation of RIPK3, it further activates MLKL that plays a

central role in necroptosis. MLKL function is either through recruitment of Na^+ ion or Ca^{2+} channels to the plasma membrane or formation of pore structures in the plasma membrane in relation to phosphotidyl inositol phosphate (PIP) (167, 168). Pore formation eventually leads to the release of chemokines, cytokines and damage associated molecular patters (DAMPs) that causes cell death.

Evidence suggests that there is a robust increase in RIPK3 and phosphorylated MLKL levels after SCI (169, 170). It is shown that reactive astrocytes undergo elimination via M1 microglia-induced necroptosis after SCI (170). It is also revealed that inhibition of necroptosis by necrostatin-1 can induce neuroprotection and improve functional recovery following SCI in rats (171).

1.1.3.4.4 Autophagy

Autophagy is an essential process in which cytoplasmic constituents are delivered to the lysosomes for removal and degradation (172). Autophagy is categorized into three different groups including: macroautophagy, microautophagy and chaperone mediated autophagy that is specific to mammalian cells (173-175). The most distinguishable feature of macroautophagy is formation of double-membrane structure, autophagosome, which is formed following expansion of phagophore and sequestering the damaged organelle. The autophagosome further fuses with the vacuole membrane and releases the autophagic body into the vacuole, which will be degraded by hydrolyses. In contrast to microautophagy, cargos are taken up by the invagination of the vacuole membrane, followed by scission, and subsequent lysis (176). Members of the autophagic machinery are encoded by autophagy related (ATG) genes that includes close to 19 key Atg proteins which orchestrate autophagy processes (177). In yeast, this machinery is categorized into

5 different groups: the Atg8–phosphatidylethanolamine (PE), the Atg5–Atg12 conjugation systems, the Atg1 kinase complex, the class III phosphoinositide 3-kinase (PI3K) complex I, the Atg2–Atg18 complex; and vesicles containing the integral membrane protein Atg9 (178, 179). Alteration in autophagy flux has been implicated in many pathologies such as neurodegenerative and inflammatory disorders, hence, its modulation has become a potential therapeutic approach (180, 181). Autophagy flux can be measured and monitored either by direct observation of autophagy related structure (e.g., electron microscopy) or by quantification of autophagy related markers. To this end, two markers have been considered for quantification, polyubiquitin-binding protein p62/SQSTM1 that plays an important role in autophagy by binding to ubiquitin-tagged proteins and preparing them for degradation (182). Accumulation and aggregation of p62/SQSTM1 is observed during development of neurodegenerative diseases, which is an indication of autophagy blockade (182). The other protein is microtubule-associated protein 1A/1B-light chain 3 (LC3) that is a soluble protein distributed ubiquitously in mammalian tissues and cultured cells (183). In autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. In normal condition, following fusion of autophagosome with lysosome, LC3-II is also degraded in lysosomal lumen (183). In addition to neurodegenerative diseases, alteration in autophagy is one of the hallmarks of traumatic injuries such as TBI and SCI (184). Lysosomal membrane damage due to neurotrauma leads to autophagosome accumulation and eventually blockade of autophagy flux is a common feature of SCI (185-188). Therefore, induction of autophagy by administration of rapamycin (mTOR inhibitor) or Tat-beclin1, reduces neuronal loss, stabilizes microtubules, promotes axon regeneration, and improves functional recovery (185-188).

1.1.3.5 Axon degeneration

This part of the thesis is extracted from "*Shahsavani N, Kataria H, Karimi-Abdolrezaee S. Mechanisms and repair strategies for white matter degeneration in CNS injury and diseases. Biochim Biophys Acta Mol Basis Dis. 2021;1867(6):166117*".

Due to specific morphology of neurons (being polarized and containing processes), mechanisms that are involved in axon degeneration exhibit similarities with cell death mechanisms that normally occurs in the soma but have fundamental differences (65). Before discussing mechanisms, I will go through different types of axonal injury (Figure 2).

In traumatic CNS injury, acute axonal degeneration (AAD) occurs at the site of lesion both proximal and distal parts of axons (189). Extracellular calcium influx occurs after injury and induces activation of calcium dependent proteases such as calpains leading to condensation of neurofilaments and microtubule fragmentation (190, 191). Cytoskeleton breakdown disrupts axonal transport causing accumulation of organelles and formation of retraction bulbs in axons (192). Another prominent feature of AAD is activation of autophagy due to increase in the number of autophagosome and LC3 II (193). Inhibition of either calcium channels or autophagy pathway significantly attenuates time course of AAD; however, blockade of voltage gated calcium channels seems to be more efficient as this approach has shown to reduce the level of autophagy in an optic nerve injury model (190, 194). The distal part of the injured axon undergoes Wallerian degeneration. WD is a genetically encoded program of subcellular self-destruction (195). Microglia play a pivotal role in WD by phagocytosis of Wallerian debris, which is a prerequisite for recovery and regeneration process (196). Axonal die-back or retrograde degeneration is a process by which the proximal part of injured axons retract from the site of injury and fail to regenerate mainly due to formation of retraction bulbs (197). Axonal die back is mediated by infiltrating macrophages during the neuroinflammatory response. At this stage axons usually are about 600-900µm away from the injury site (198-200). Macrophage-dependent axonal die-back is

mediated by MMPs that disrupt the molecular interactions of axons with the matrix CSPGs (199-201). Targeting MMPs or CSPG removal results in inhibition of macrophage mediated die-back (198, 202).

Axon degeneration is one of the hallmarks of neurodegenerative disease and CNS trauma that can occur due to the disruption of axonal transport (203), lack of neurotrophic factors (204), excitotoxicity, oxidative stress and calcium overload are among the leading mechanisms underpinning axon degeneration (205, 206). In this section I will summarize mechanisms of axon degeneration.

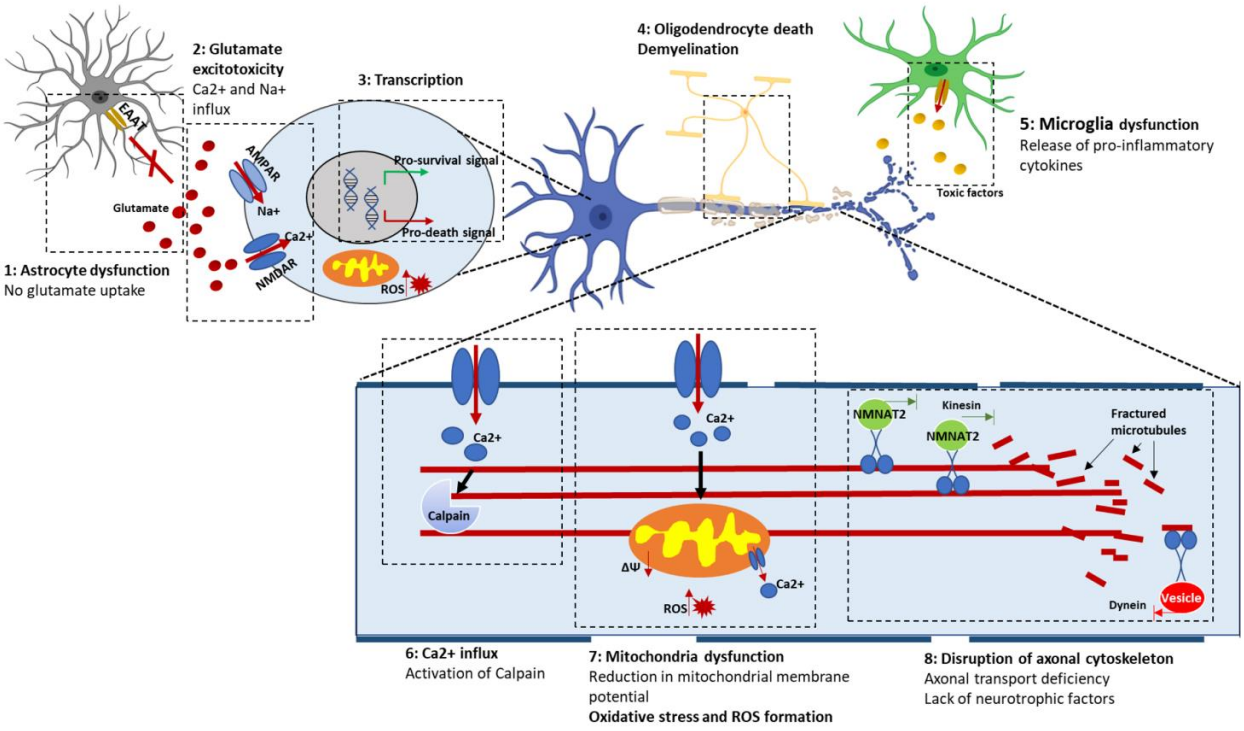

Figure 2: Mechanisms of axon degeneration in WM injuries and diseases. Traumatic injuries or neurodegenerative disorders activate various injury mechanisms in axons. (1) Astrocyte dysfunction and lack of neurotransmitter (glutamate) uptake in synapses overstimulate neurons. (2) Glutamate excitotoxicity results in opening of calcium channels and initiation of calcium influx and ionic imbalance. (3) Under neurotrophic deprivation, induction of pro-death genes (Puma) exceeds expression of pro-survival genes in neurons. (4) Oligodendrocyte dell death and membrane damage disrupts myelin sheath and results in demyelination. Elevation of energy consumption and low ATP production results in Na+/K+ pump dysfunction, excessive Na+ currents and accumulation of calcium ions in the axon. High levels of calcium activate enzymes

including phospholipases and calpains causing more axonal damage by affecting ATP production and impairment of cytoskeleton. (5) Microglia are also activated during injury process through several mechanisms including the release of intracellular components from necrotic or apoptotic cells and presence of heat shock proteins and misfolded proteins (DAMPs). Once activated, microglia release proinflammatory cytokines that further induce activation and proliferation of astrocytes promoting astrogliosis. (6) Axon degeneration initiates with an increase in intra-axonal calcium levels by entry through calcium channels and disrupted membrane spaces. Calcium dependent proteases, such as calpain are activated and cleave microtubules. (7) Calcium overload causes mitochondrial dysfunction that is associated with reduction in ATP generation, membrane potential ($\Delta\Psi$) and generation of reactive oxygen specious (ROS). These features initiate mitophagy and formation of autophagosome to degrade mitochondria. Damage to the mitochondrial membrane results in release of cytochrome C and apoptosis inducing factor AIF), which can induce apoptosis. These changes can negatively affect mitochondria fusion/fission and their transport leading to their accumulation in some spots and reduction of their transport to axonal terminal. (8) Increased calcium influx also induces cytoskeleton breakdown and alteration of axonal transport, which reduces the capacity of delivering soma-derived trophic factors such as NMNAT2. This can lead to activation of SARM-1 pathway and Wallerian degeneration in the distal part of inured axon. Some parts of the figure are depicted by BioRender.com. *Shahsavani N, Kataria H, Karimi-Abdolrezaee S. Mechanisms and repair strategies for white matter degeneration in CNS injury and diseases. Biochim Biophys Acta Mol Basis Dis. 2021;1867(6):166117*

Increase of intracellular levels of Ca^{2+} following axonal injury plays a critical role in induction of degeneration. Ca^{2+} overload can be due to disruption of axonal membrane, voltage gated Ca^{2+} channels or damage to mitochondria or ER. Excessive levels of Ca^{2+} leads to activation of Ca-dependent cysteine proteases, calpains that induce cytoskeleton breakdown. Loss of cytoskeleton integrity leads to disruption of axonal transport which is one of the features of axon degeneration. Disruption of axonal transport contributes to white matter degeneration (WM) degeneration. Energy supply by mitochondria transport and maintaining neuronal activity by transporting neurotransmitters and trophic factors that are essential for neuronal homeostasis (207). Therefore, restoration of axonal transport is critical for axonal integrity and function. Drugs that promote microtubule stability (Taxol) have been widely utilized to prevent axon degeneration. Similar to cell bodies, axons can be negatively affected by glutamate excitotoxicity. Glutamate excitotoxicity results from impaired uptake of excess glutamate from extracellular space following

neurotransmission. Activation of two general classes of receptors, “ionotropic" and G-proteins linked receptors or “metabotropic" are implicated in excitotoxicity. Kainate (KA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and N-methyl-D-aspartate (NMDA) receptors are different groups of ionotropic receptors involved in excitotoxicity (208, 209). Glutamate receptors are widely expressed by soma, axons and dendrites; therefore, neurons are potentially susceptible to glutamate excitotoxicity in their entirety. Accordingly, although glutamate excitotoxicity is considered primarily a somato-dendritic insult, it also occurs in WM myelinated tracts as their axons express AMPA and KA receptors (210) (211). The recent identification of axonal glutamate receptor subunits within axonal internodal nanocomplexes has raised the possibility of direct axonal excitotoxicity (212). Glutamate application to the distal axon of cortical neurons in compartmentalized chambers induces axonal degeneration but in lesser extent compared to application of glutamate to the cell bodies. Thus, current evidence indicates that axons are also a target of glutamate excitotoxicity and modulation of glutamate receptors present and attractive therapeutic targets for WM degeneration. Oxidative stress also leads to axon degeneration. In SCI, antioxidants are significantly reduced concomitantly with an increase in lipid peroxidation (213). Administration of NOX2-specific ROS inhibitor, gp91ds-tat, is shown to significantly reduce oxidative stress and improve functional recovery following SCI (214).

1.2 Significance of neuroprotective strategies

Neurological impairment after SCI reflects extensive cell loss, in particular neurons, and inefficient response of the injured spinal cord in replacing damaged neurons and the poor ability for axon regeneration (215). Unlike, other cell types in the CNS, replacement of neurons is very challenging. In the CNS, neural precursor cells (NPCs) and oligodendrocyte progenitor cells

(OPCs) are two precursor populations with the potential for cell replacement following injury (216). In the brain, there are two regions (subventricular zone of lateral ventricles and sub-granular zone of hippocampus) that have the capacity of neuronal replacement. In contrast, in the spinal cord, NPCs are latent and in healthy condition they only maintain their own population. OPCs are also responsible for oligodendrocyte maintenance during homeostasis and replacement during injury. In injury conditions, NPCs mainly differentiate into astrocytes and in lesser extent to oligodendrocytes. Therefore, without interventions, replacement of lost neurons from endogenous stem cells in the spinal cord is very challenging. Thus, transplantation of NPC to replace neurons has been studied and shows the potential to form new neurons that can anatomically integrate to the host tissue (217-225). However, neuronal replacement through stem cell transplantation has its own challenges including graft survival, functional integrity to the host tissue and maladaptive connectivity and plasticity. Therefore, development of neuroprotective strategies are critical to reduce the initial damage to neurons within the milieu of SCI.

It is well known that following injury, precursor cells have limited capacity to replace neurons and oligodendrocytes which is mainly related to the dysregulated, hostile post-injury microenvironment after SCI (226, 227). Dysregulation of the microenvironment can be partially associated with downregulation of essential trophic factors for cell survival and replacement (226, 227). Neuregulin-1 is one of the neurotrophic factors that is significantly down regulated following SCI (228).

1.3 Neuregulin-1 and ErbB Network in the central nervous system (CNS)

Neuregulins, also known as heregulins, are a family of growth factors encoded by 4 genes (Nrg-1- 4) and contain similar sequences with epidermal growth factor (EGF) (229). Nrg-1 is the

most well-known and well-studied member of this family (229). Nrg-1 is a key mediator in neuronal development and function in the developing nervous system as it is implicated in neuronal differentiation, neuronal guidance, synapse formation, myelination and neuromuscular junction formation (229, 230) (Figure 3). Nrg-1 is encoded by a 2.4mbp gene in mice and 2.6mbp in humans and rats (231). Nrg-1 mRNA undergoes several alternative splicing processes and can generate six different types of proteins (type I-VI) and close to 31 isomers in humans (231, 232). All Nrg-1 isomers share an EGF-like domain that is located in the extracellular domain of the protein (231, 233). This EGF like domain is necessary and sufficient for the activation and signal transduction via ErbB receptor tyrosine kinase (231, 233). EGF-like sequence contains three domains: 1) N-terminal, which is shared among all Nrg-1 isoforms, 2) α or β type exon, and 3) a variable 'stalk' region, which locates in between the EGF-like and transmembrane domains.

The most accepted nomenclature of the identified Nrg-1 isoforms is derived from different exons located downstream of the EGF-like domain. Nrg-1 type I-III are encoded in a wide variety of vertebrates, but type IV is specific to mammals (234, 235) and type V and VI to primates (236). The immunoglobulin (Ig)-like domain, which is usually in between N-terminal and the EGF-like domain contributes to specific interactions with extracellular matrix components (e.g. heparan-sulfate proteoglycans; HSPGs) and define the distance and concentration over which these growth factors act (237). It is hypothesized that HSPGs concentrate Nrg-1 ligand in neighboring ErbB receptors so that they may increase the affinity of ligand- receptor binding (237).

The extracellular N-termini of the type I isoforms (and likely types IV–VI) lack a signal sequence or alternative mechanism to release from the cell membrane. Their expression on the cell surface and proteolytic release of their growth factor domain is determined by their central transmembrane and intracellular domains (238, 239). In contrast, type II isoforms enter the

secretory pathway as they contain the classic signal peptide. Nrg-1 type III isoforms are unique compared to all other types as they include an N-terminal sequence containing a cysteine-rich domain (CRD), an additional transmembrane domain (TM) (238) and their restricted expression to neurons (240, 241). Consequently, Nrg-1 type III isoforms have cytosolic N-termini and an EGF-like domain, which is attached to the cell membrane. This structure limits type III signaling to cell–cell interactions, while it enables bidirectional signaling by acting as a ligand as well as a receptor that modulates the behavior of neurons (242). Nrg-1 isoforms are synthesized as pro-Nrg-1 that is bound to the cell membrane and contains an extracellular EGF domain. Mature Nrg-1 is released upon cleavage of the pro-Nrg-1 at the juxta-membrane region close to the carboxy-terminal of the EGF-like domain. Three types of transmembrane proteases catalyze this cleavage: TACE (TNF-α converting enzyme, aka ADAM17) (243, 244), BACE (β-site of amyloid precursor protein cleaving enzyme, aka memapsin 2) (245) and meltrin beta (aka ADAM19) (246). Following cleavage, Nrg-1 isoforms that lack TM are directly released extracellularly as ligand (247). Recent evidence shows that Nrg-1 type III is the first protein substrate that is not only cleaved by multiple sheddases (BACE1, ADAM10 and ADAM17), but is also processed by three different intramembrane-cleaving proteases (I-CLiPs).

Nrg-1 ligand signals through activation of ErbB receptors. ErbB family of receptors comprises of four transmembrane protein tyrosine kinases, including ErbB1(EGFR, HER1), ErbB2 (Neu or HER2), ErbB3 (HER3) and ErbB4 (HER4) (248). ErbB1 is an EGF receptor and Nrg-1 does not bind directly to this receptor. Among these ErbB receptors, ErbB3 and ErbB4 contain Nrg-1 binding domain. Binding of Nrg-1 EGF-like domain stimulates ErbB3 or ErbB4 receptors, which leads to ErbB4 homodimer or their heterodimers with ErbB2 and then tyrosine phosphorylation (249, 250). Since ErbB4 has both Nrg-1 binding site and intracellular tyrosine

kinase domain, it can both interact with Nrg-1 and become phosphorylated. However, ErbB2 lacks the binding site for the ligand, thus it only acts as a co-receptor for ErbB3 and ErbB4 to form heterodimers (251). On the other hand, ErbB3 possesses an impaired kinase domain and therefore its homodimers are catalytically inactivated (251).

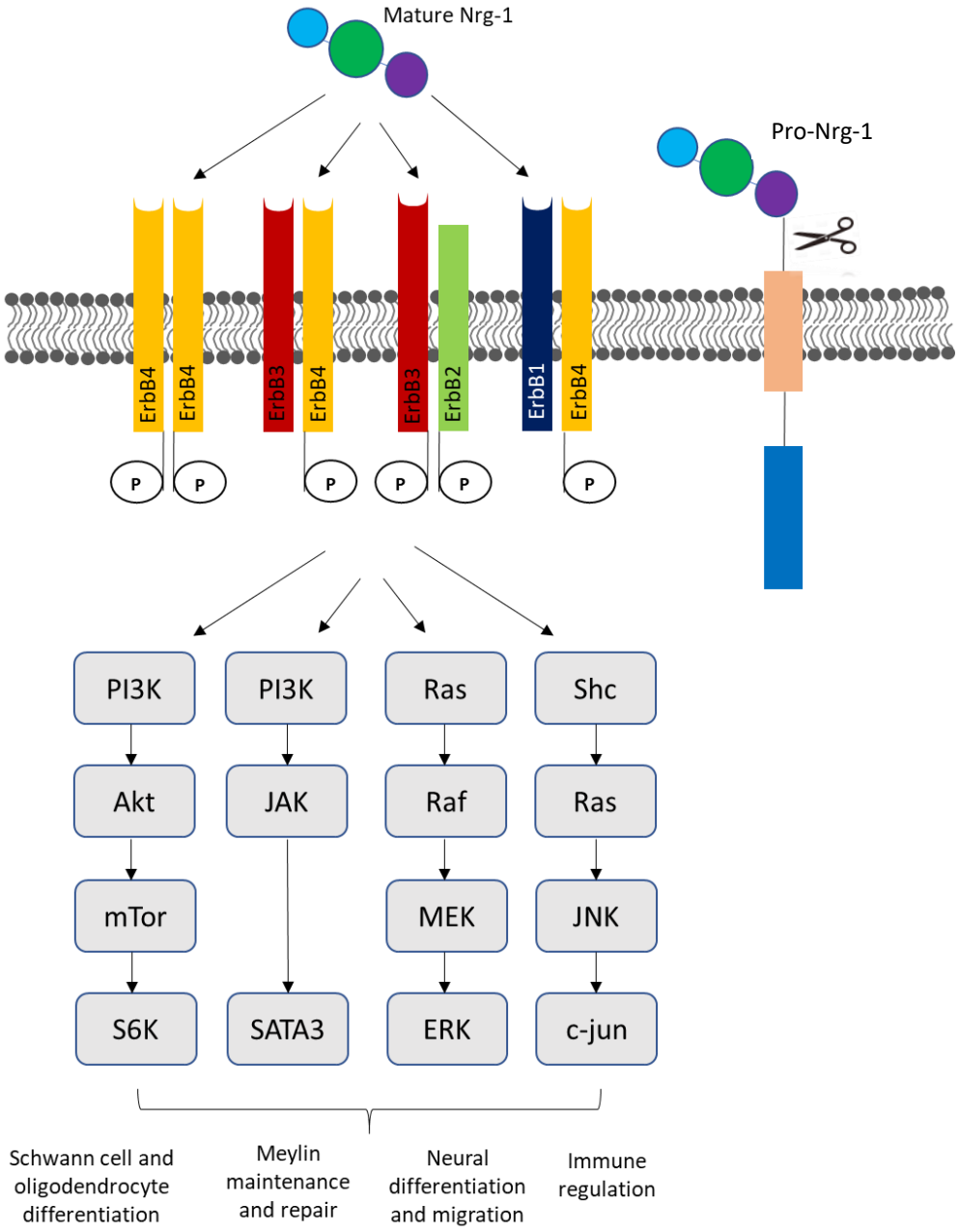

Figure 3: Nrg-1/ErbB signaling and associated downstream pathways. Nrg-1 signals through ErbB protein tyrosine kinase receptors, ErbB-2, ErbB-3 and ErbB-4. Upon binding of Nrg-1, ErbB receptors undergo conformational change and gain higher affinity to other ErbB receptors. They make homo- or heterodimers to activate their kinase domains and transmit the signal to the downstream pathways. ErbB-3 lacks an active kinase domain and ErbB-2 has no ligand binding domain. Therefore, ErbB-2 and ErbB-3 form heterodimers with other ErbB receptors. On other hand, ErbB-4 has both ligand binding and tyrosine kinase that allows functional homo- and heterodimerization. Following activation of intracellular signaling pathways Nrg-1 implicates in Schwann cell and oligodendrocyte differentiation, myelin formation and maintenance, neuronal differentiation and immune response regulation.

1.3.1 Role of Nrg-1 in the normal CNS

Nrg-1 is an important growth factor that plays critical roles during development and adulthood in several organs and systems, including the nervous system (Figure 4). Nrg-1 null mutant mice die embryonically at days E10.5–E13.5 due to cardiac dysfunction indicating it is essential for cardiac development (252). In healthy CNS, Nrg-1 and its receptors are expressed in neurons and glial cells and plays critical roles during development and in adulthood (230, 253).

During development, Nrg-1 is implicated in NPC regulation. NPCs express Nrg-1 type III and their proliferation is regulated by Nrg-1 (254). In addition, Nrg-1 and ErbB receptors are implicated in several functions of NPCs. Of note, ErbB4 is the most studied ErbB receptor and previous research showed that ErbB4 receptors are critical for migration and differentiation of NPCs as its conditional disruption leads to abnormal migration of NPCs to olfactory bulbs (255). Extensive evidence supports the role of Nrg-1/ ErbB signaling in neurogenesis. In zebrafish, Nrg-1 II is important for fate specification of NPCs toward neurogenesis (256). In mice, Nrg-1 also plays an important role in proliferation of NPCs and their capacity for neurogenesis (257). Nrg-1 is also one of the key players of neuronal pathfinding and regulates migration of cerebral cortical neurons, cerebral granule cells (258), glutamatergic neurons and GABAergic interneurons (259, 260). Moreover, Nrg-1 is one of the regulators of neuronal maturity and arborization. Several

studies show the presence of Nrg-1 in cultures of retinal, cerebellar granule and thalamic neurons can promote their neurite outgrowth and complexity (261-263). Role of Nrg-1/ErbB signaling has been extensively studied in the context of synapse formation. ErbB-4 is highly expressed in GABAergic interneurons and interacts with PSD-95 (postsynaptic density protein of 95 kDa) (264). Presence of Nrg-1 in cortical neuron cultures promotes assembly of excitatory synapses specifically in GABAergic interneurons via regulating the stability of PSD-95 (265).

Overexpression of ErbB-4 enhances α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) synaptic currents selectively and increases dendritic spine density, inhibition of Nrg-1/ErbB-4 signaling diminishes synaptic AMPA receptors, synaptic NMDA currents and spines (266). Intriguingly, Nrg-1 signaling directly regulates glutamatergic transmission in dopaminergic neurons by specific modulation of metabotropic glutamate receptor 1 (mGluR1) functioning (267, 268). Nrg-1 also regulates the formation of the neuromuscular junction (NMJs) (244). In heterozygous Nrg-1 mutant mice (deletion in exons encoding the EGF-like domain or the immunoglobulin (Ig)-like domain of neuregulin), the density of postsynaptic AChR is reduced and the neuromuscular transmission is shown to be dysfunctional (229). Using chicken embryo, it is shown that Nrg-1 signaling regulates the density of AChR in innervating motoneurons at early stage of developing NMJs, while its role is not influential at later stages (269). Nrg-1 plays a significant role in the position of "pre- and post-synaptic" components (269). Nrg-1 also plays a critical role in myelination in both the PNS and CNS. In the CNS, Nrg-1 receptors (ErbB 2, 3 and 4) are expressed by OPCs and oligodendrocytes (270). Nrg-1 has been also shown to increase proliferation of O2A progenitor cells, O4+ and O1+ oligodendrocyte precursor cells(271) in addition to survival of oligodendrocytes (271-273). It is further shown that disruption of ErbB signaling suppresses oligodendrocyte differentiation of progenitor cells (274).

1.3.2 Role of Nrg-1 in pathologic CNS

Since Nrg-1 contributes to several functions in homeostasis of CNS, its role in pathophysiological conditions has also been explored, which will be discussed in following sections.

1.3.2.1 Nrg-1 and endogenous cell response after SCI and demyelinating disease

Our group, for the first time, reported that Nrg-1 level is dysregulated in traumatic SCI and focal demyelinated lesions of the spinal cord (227, 228).. In rat SCI, there was a rapid and persistent downregulation of Nrg-1 with no restoration in its levels in chronic stages (228). In lysophosphatidyl-choline (lysolecithin, LPC) focal demyelinating lesions of spinal cord, Nrg-1 was similarly downregulated (227). In rat SCI, administration of human recombinant Nrg-1 containing EGF-like domain was able to significantly promote oligodendrocyte differentiation and preservation, while reduced astrocyte differentiation (228). Restoration of Nrg-1 levels also promoted tissue preservation after SCI, which was associated with enhanced functional recovery (228). Our group has also demonstrated that Nrg-1 administration promotes oligodendrogenesis, oligodendrocyte maturation and remyelination in LPC induced focal demyelinating lesions (227). These studies revealed a significant role for Nrg-1 in promoting morphological complexity of oligodendrocytes and their maturation into myelinating cells *in vitro* (227). Altogether, these studies have established an important role for Nrg-1 in supporting oligodendrogenesis and remyelination after CNS injury.

1.3.2.2 Nrg-1 and neuroinflammation

Several studies have demonstrated the modulatory role of Nrg-1 in inflammatory responses by decreasing the expression levels of pro-inflammatory cytokines, IL-1β and IL-6 following CNS

injuries (275-277). Our group has demonstrated that Nrg-1 can promote a shift in pro-inflammatory phenotype of immune cells to anti-inflammatory, pro-regenerative phenotype. These studies showed that Nrg-1 can remarkably promote the expression of anti-inflammatory cytokine IL-10 by activated microglia/macrophages and regulatory T cells in SCI and LPC-induced focal demyelination models (227, 278, 279). IL-10 is implicated in enhancing oligodendrogenesis and remyelination (280). It also directs the fate of NPCs towards oligodendrocytes (281). Interestingly, IL-10 levels are significantly reduced in MS patients (282, 283) and administration of IL-10 expressing NPCs to EAE mice mitigates inflammatory responses (284). Our group demonstrated that intrathecal or systemic administration of Nrg-1 in rats with SCI elicits immunomodulatory effects by increasing the population of M2 microglia/macrophages and enhancing the expression level of IL-10 and arginase-1 (279). This was partially due to the inhibition of NF-κB pathway (285). Nrg-1 administration also reduced the expression level of pro-inflammatory cytokines and mediators including NO, IL-1β, TNF-α and MMP-2/ 9 both in primary cultures of microglia and following SCI (279). Studies by our group further revealed that Nrg-1 treatment increases the population of regulatory T regulatory (Tregs) and B regulatory (Bregs) cells in the blood and spinal cord tissue following SCI (279). Our group has also extensively studied the role of Nrg-1 on astrocyte activity in SCI and in cultures of astrocytes. These studies showed that Nrg-1 moderates astrogliosis by reducing the levels of released CSPGs (278) that are shown to inhibit axon regeneration, cell replacement and remyelination (286-288). Immunomodulatory and neuroprotective effects of Nrg-1 has been also reported in ischemic model of CNS injury as well (266, 275, 289).

Recent studies by our group in experimental autoimmune encephalomyelitis (EAE) mouse model of MS have uncovered an important immunomodulatory role for Nrg-1β1 (290). This work

showed Nrg-1β1is significantly depleted in the spinal cord and peripherally in the plasma and spleen during the onset and peak of EAE. Interestingly, Nrg-1β1 was also reduced in the plasma of individuals with early MS symptoms. In both EAE and MS, dysregulation of Nrg-1 was positively associated with disease progression (290). These studies revealed that availability of Nrg-1β1 promotes a more balanced immune response by moderating monocyte infiltration at blood-CNS barrier (290). Altogether, evidence from our group and others suggest a positive role for Nrg-1 in regulating the response of glial cells and infiltrating leukocytes after SCI and demyelinating lesions. The positive immunomodulatory effects of Nrg-1 in SCI and cerebral ischemia suggests its neuroprotective role in CNS pathology.

1.3.2.3 Nrg-1 and neuroprotection

The goal of neuroprotective treatments is to minimize the acute neuronal and oligodendroglial cell death and axon degeneration (291). To meet this expectation, the therapeutic time window is a key factor. Nrg-1 administration prior or up to 1 hour after diisopropylfluorophosphate (DFP) treatment, an organophosphate-like neurotoxin, significantly reduced apoptosis and oxidative damage in neurons, while Nrg-1 did not show any neuroprotective effects at 4hrs post injury (292). In cerebral ischemic and reperfusion models, Nrg-1 treatment has reduced caspase-3 mediated apoptosis in neurons and decreased the expression of pro-inflammatory cytokines such as IL-1β and TNF-α (293, 294). In addition to exogenous Nrg-1 treatment, Noll and colleagues have evaluated the neuroprotective effects of endogenous Nrg-1 in mice that were subjected to middle cerebral artery occlusion (MCAO) (295). Male and female heterozygous Nrg-1 knockout mice (NRG-1+/-) exhibited a significant increase in cortical infarct size compared with wild-type mice (WT) littermates 24 h following MCAO indicating that endogenous Nrg-1 is implicated in protection of neurons (295). In hippocampus of line 41 mouse

model of Alzheimer disease (AD) lentiviral overexpression of Nrg-1 I and III appeared to significantly ameliorate cognitive deficit while increasing the expression level of neuronal marker, MAP2, PSD95 and synaptophysin (296). In an *in vitro* model of AD induced by cytoplasmic terminal 31 (CT31) fragment of amyloid precursor protein (APP) in primary cortical neurons and SH-SY5Y cells, Nrg-1 is shown to reduce the APP-CT31-induced upregulation of p53 and cleaved caspase 3 expression which was diminished by inhibition of ErbB-4 receptor and AKT (297). Activation of CDK5/Akt/PI3 kinase pathway has been identified as one of the mechanisms underlying the neuroprotective effects of Nrg-1 (292, 298). Increase in activation and phosphorylation of Akt has been reported in neurons and glial cells following Nrg-1 administration in cerebral injury animal models (292). Dysregulation of Nrg-1 and ErbB3 /ErbB4 has been observed in motor neurons in Amyotrophic Lateral Sclerosis (ALS) (299). Nrg-1 is normally expressed at C-boutons of cholinergic synapses (300) and in ALS the size and the number of these boutons are dysregulated (301). In superoxide dismutase (SOD)1 model of ALS, Nrg-1 administration enhances the number of synaptic c-boutons and improves motor neuron survival indicating that Nrg-1 is implicated in pathophysiology of ALS disease [382]. Our group has also demonstrated an overall tissue preservation in Nrg-1 treated rats following SCI (228). Altogether, availability of Nrg-1 fosters neuroprotection in a variety of injury conditions.

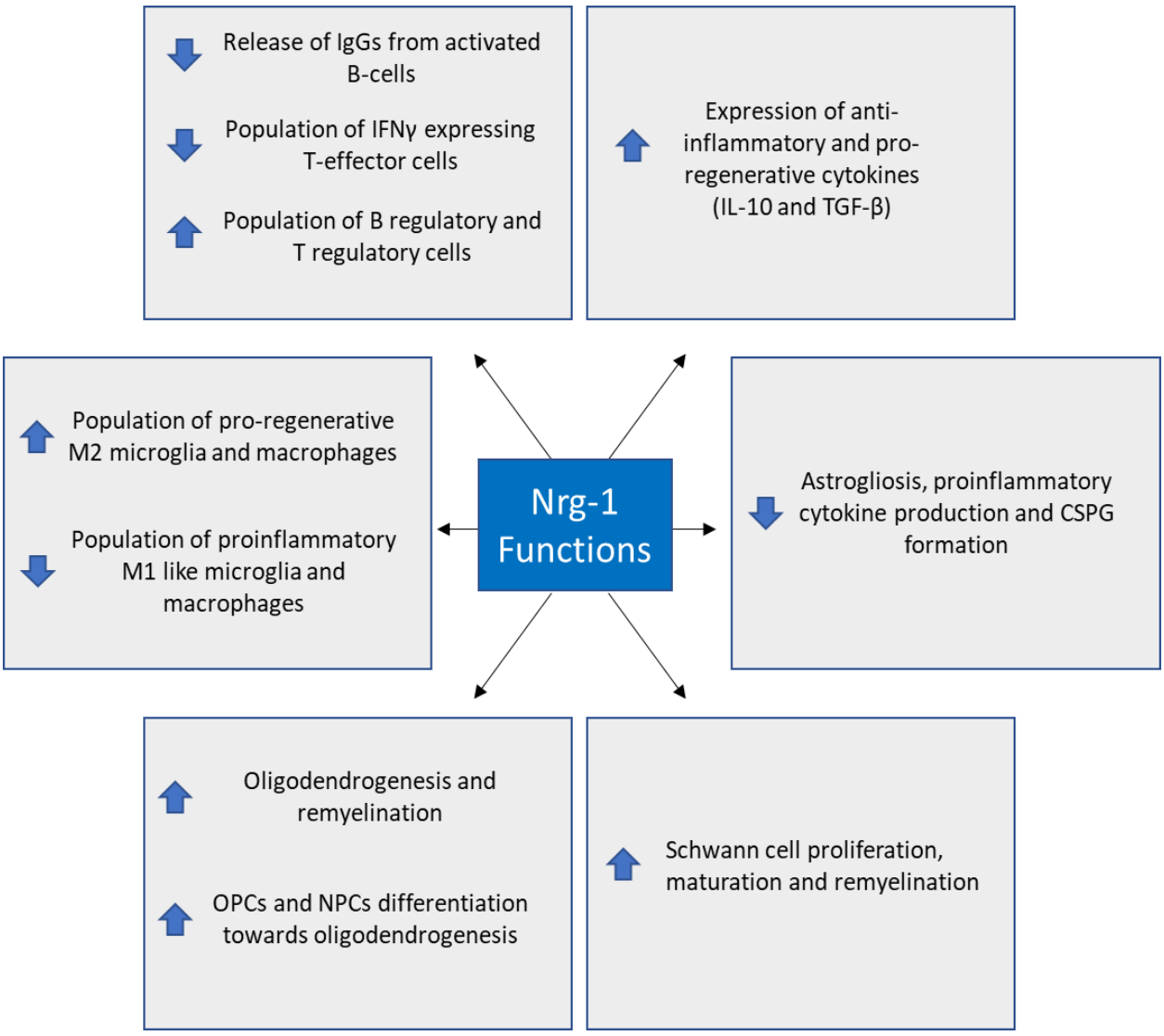

Figure 4. Roles of Nrg-1 in nervous system injury and diseases. Nrg-1 has regulatory role in injury and repair processes of CNS and PNS. Nrg-1 promotes a pro-regenerative milieu in CNS lesions by fostering beneficial innate and adaptive immune responses. Nrg-1 promotes B regulatory (Breg) and T regulatory (Treg) cells population that express anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β). Nrg-1 drives microglia and macrophages towards pro-regenerative M2 phenotype and decreases the population of proinflammatory M1-like microglia and macrophages. Nrg-1 may confer neuroprotection directly or indirectly by increasing IL-10 production by regulatory immune cells. Nrg-1 can also moderate reactive astrocytes and suppress their production of pro-inflammatory cytokine and chondroitin sulfate proteoglycans (CSPGs). Following repair process, Nrg-1 is important for remyelination by promoting differentiation of neural precursor cells (NPCs) and oligodendrocyte precursor cells (OPCs) towards oligodendrocytes.

1.4 Study rational, general hypothesis and research objectives

1.4.1 Study Rationale

SCI results in neurological deficit and motor and sensory dysfunction. SCI leads to extensive cell loss, in particular neurons, and inefficient response of the injured spinal cord in replacing damaged neuron. Therefore, development of neuroprotective and neuroregenerative strategies are critical. Our group has discovered that SCI results in acute downregulation of Nrg-1ß with no restoration in its levels in chronic phases. Nrg-1 is a critical growth factor that is implicated in development and physiology of neurons, axons, and myelin. Our recent therapeutic studies in a clinically relevant model of SCI in rats have shown that bioavailability of Nrg-1 through administration of recombinant human Nrg-1β1 promotes oligodendrogenesis and myelination, attenuates reactive astrogliosis and fosters a regulatory immune response by providing a pro-regenerative anti-inflammatory response in resident glia and leukocytes. Notably, Nrg-1 treatment also remarkably attenuates CSPGs, which are the major barrier to axon regeneration within the injured spinal cord. Altogether, administration of Nrg-1 results in improved motor functions in SCI in the rats which are associated with modulation of SCI microenvironment. Most importantly, the role of Nrg-1 in protection of neurons following SCI has not been explored and my thesis sought to evaluate the effects of Nrg-1 on neuronal protection following SCI.

1.4.2 General Hypothesis

Availability of Nrg-1 protects neurons following spinal cord injury by mitigating cell death and injury.

1.4.3 Research Objectives

1- To evaluate whether Nrg-1 is neuroprotective in SCI.

2- To evaluate the direct effects of Nrg-1 on CNS neurons in response to injury *in vitro*.

3- To identify the underlying intracellular mechanisms by which Nrg-1 regulated neurons in response to injury *in vitro.*

These objectives were addressed in a research manuscript that is reported in Chapter 2 of this thesis.

Chapter 2: Availability of neuregulin-1beta1 protects neurons in spinal cord injury and against glutamate toxicity through caspase dependent and independent mechanisms

This collaborative work is published as an original article in Experimental Neurology (EXNR-21-102R1)

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Authors' contributions: My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. Manuscript preparation was done in collaboration with my supervisor, Dr. Soheila Karimi-Abdolrezaee. Dr. Arsalan Alizadeh contributed by performing spinal cord injury in rats. Dr. Hardeep Kataria contributed by training me for primary rat cortical neuron cultures.

Funding: This work was supported by grants from the Canadian Institute of Health Research (CIHR, Grant #: 133721 and 156218) to SK-A. NS was supported by studentships from Manitoba Graduate Fellowship, Will-to-Win/Manitoba Paraplegic Foundation and the University of Manitoba GETS program.

Conflict of interest: The authors have no conflicts to disclose.

Highlights

- Neuregulin-1β1confers neuroprotection after spinal cord injury by attenuation of oxidative damage and apoptosis.
- Neuregulin-1β1 directly protects neurons from glutamate toxicity by modulating mitochondria associated apoptotic cascades.
- Neuron-specific effects of Neuregulin-1β1is partially mediated by MAP kinase and AKT pathways.

2.1 Abstract

Spinal cord injury (SCI) causes sensorimotor and autonomic impairment that partly reflects extensive, permanent loss of neurons at the epicenter and penumbra of the injury. Strategies aimed at enhancing neuronal protection are critical to attenuate neurodegeneration and improve neurological recovery after SCI. In rat SCI, we previously uncovered that the tissue levels of neuregulin-1beta 1 (Nrg-1β1) are acutely and persistently downregulated in the injured spinal cord. Nrg-1β1 is well-known for its critical roles in the development, maintenance and physiology of neurons and glia in the developing and adult spinal cord. However, despite this pivotal role, Nrg-1β1 specific effects and mechanisms of action on neuronal injury remain largely unknown in SCI. In the present study, using a clinically-relevant model of compressive/contusive SCI in rats and an *in vitro* model of glutamate toxicity in primary neurons, we demonstrate Nrg-1β1 provides early neuroprotection through attenuation of reactive oxygen species, lipid peroxidation, necrosis and apoptosis in acute and subacute stages of SCI. Mechanistically, availability of Nrg-1β1 following glutamate challenge protects neurons from caspase-dependent and independent cell death that is mediated by modulation of mitochondria associated apoptotic cascades and MAP kinase and AKT signaling pathways. Altogether, our work provides novel insights into the role and mechanisms of Nrg-1β1 in neuronal injury after SCI and introduces its potential as a new neuroprotective target for this debilitating neurological condition.

Key words:

Spinal cord injury, Neuregulin-1, cell death, apoptosis, neurons, caspase, mitochondria, rats

2.2 Introduction

Traumatic spinal cord injury (SCI) results in significant tissue degeneration that leads to permanent impairment of motor, sensory and autonomic functions (15). Neurons and glial cells die instantly at the epicenter of the injury as the result of primary mechanical damage. However, cell death continues to spread rostrally and caudally to the penumbra of the injury during the acute and subacute stages of SCI (15, 302, 303). Neuronal death is a convergence of multiple injury mechanisms including vascular injury, ischemia, ionic imbalance, glutamate excitotoxicity, oxidative cell stress, lipid peroxidation and inflammation, among others (15, 304). Therefore, early neuroprotective strategies are of critical importance to reduce the extent of neuronal degeneration after SCI. Given the heterogeneity and complexity of the acute secondary injury cascades, treatment strategies that can target multiple cell injury events would likely be more effective in attenuating neurodegeneration after SCI.

Our group provided the original evidence that SCI induces acute and persistent downregulation of neuregulin-1 beta 1 (Nrg-1β1) in rats (228). Nrg-1β1 is a member of the neuregulin family of proteins that regulate differentiation and function of neurons, axons and glia in the developing and adult nervous system (305). Nrg-1 consists of multiple isoforms through alternative mRNA splicing in which all share an epidermal growth factor (EGF)-like domain that is required to bind and activate ErbB-2, ErbB-3 and ErbB-4 tyrosine kinase receptors in Nrg-1 signaling (305). Developmental studies have identified a well-established role for Nrg-1/ErbB signaling in the formation and maintenance of oligodendrocytes, Schwann cells and myelin, as well as neuronal migration, maturation, axon pathfinding and synaptogenesis (229, 306-309). In the pathologic CNS, emerging evidence from our group and others also highlights a positive role for Nrg-1/ErbB network in modulation of injury microenvironment (266, 285, 295, 310-315). We

recently demonstrated restoring the reduced levels of Nrg-1β1 in the injured spinal cord results in long-term neurological recovery in rats with SCI (227, 228, 278, 279). Our characterization has identified several glia-mediated mechanisms by which Nrg-1β1 treatment promotes beneficial effects in the microenvironment. Nrg-1β1 can enhance oligodendrogenesis and remyelination in SCI and focal demyelinating lesions of the spinal cord (227, 228). Studies from our group and others indicate that Nrg-1 also promotes remyelination after SCI (307) and in animal models of MS; lysolecithin-induced focal demyelination (227) and experimental autoimmune encephalomyelitis (EAE) (316). Interestingly, in mouse SCI, Bartus and colleagues have shown that Nrg-1/ErbB signaling regulates generation of new Schwann cells from resident oligodendrocyte progenitor cells (OPCs) in the injured spinal cord that is associated with enhanced remyelination and functional recovery after SCI (307, 317). Importantly, we found availability of Nrg-1β1 can foster an anti-inflammatory, pro-regenerative response by resident glia and peripherally recruited leukocytes after SCI (278, 279). To date, despite the significance of Nrg-1β1 in neuronal differentiation, maintenance and function in the developing CNS, its role and mechanisms in regulating neuronal injury remain largely unknown in SCI.

In the present study, using parallel *in vivo* SCI and primary *in vitro* glutamate toxicity models, we aimed to determine whether availability of Nrg-1β1 affects neurons during the injury process. We utilized recombinant human Nrg-1β1 peptide, which contains the functional EGF-like domain of Nrg-1. Here, for the first time, we demonstrate that Nrg-1β1 treatment attenuates formation of reactive oxygen species (ROS), lipid peroxidation and necrosis, and protects neurons against caspase mediated-apoptosis in rats with compressive/contusive SCI. Under SCI relevant glutamate toxicity *in vitro*, presence of Nrg-1β1 ameliorates both caspase dependent and independent mediated apoptosis in primary neurons. Mechanistically, we show Nrg-1β1 attenuates

neuronal death by regulating mitochondria associated-apoptotic pathways, and modulation of MAP kinase and AKT signaling. Taken together, we provide new mechanistic insights into the role of Nrg-1β1 in regulating neuronal injury in acute SCI, and show the promise and relevance of Nrg-1β1 as a potential neuroprotective target following SCI.

2.3 Material and methods

2.3.1 Animals

All animal protocols were approved by the University of Manitoba Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines and policies. For *in vivo* SCI studies a total of 69 adult female Sprague-Dawley (SD) rats (8-10 weeks, 250g) were used (Table 1). For *in vitro* experiments, 20 timed pregnant SD rats were obtained. Rats were provided by Central Animal Care at the University of Manitoba

Table 1. Number of animals per experimental group in SCI studies.

Time-point	**Experimental group**	**Assessment**		**Total**
		WB/SB	**IHC**	
3dpi	Uninjured	6-7	-	7
3dpi	SCI/vehicle	8-9	5-9	18
3dpi	SCI/Nrg-1β1	6-7	5-7	14
7dpi	Uninjured	6	-	6
7dpi	SCI/vehicle	6	4-7	13
7dpi	SCI/Nrg-1β1	6	4-7	13

WB/SB, Western or slot blot; IHC, immunohistochemistry.

2.3.2 Compressive model of SCI in rats

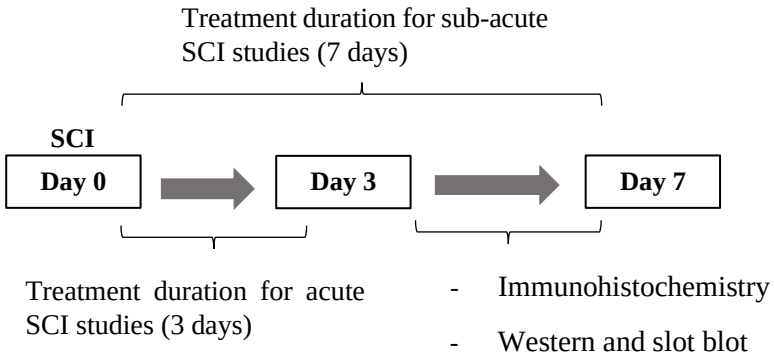
We employed a clip-compression model of SCI in adult female rats, as we used and described extensively (228, 278, 287, 318-321). Briefly, rats underwent laminectomy at thoracic levels T6-T8 under deep isoflurane anesthesia. SCI was induced by compression of the spinal cord at T7 with a 35-g aneurysm clip (University Health Network, Toronto, Ontario) for 1 min. After removal of the clip, the surgical wounds were sutured. For pain management, animals received a pre-surgical injection of meloxicam (Metacam, Boehringer Ingelheim Burlington, Ontario, Canada, 2mg/kg) and a post-surgical injection of buprenorphine (Vetergesic, 0.03 mg/kg Champion Alstoe Animal Health, Whitby, ON, Canada) followed by three additional doses of buprenorphine every 8 h. Rats were housed in a 12:12 h light/dark cycle in standard plastic cages before SCI and afterwards in cages covered with soft paper bedding to prevent skin erosions and urine scalding. Pelleted food and drinking water were available *ad libitum*. To avoid dehydration, animals received subcutaneous injections of 0.9% saline (5ml). To prevent trauma-induced hematuria and bladder infections, Clavamox® (Amoxicillin plus Clavulanic Acid, Pfizer) was added to their drinking water starting 2 days prior to surgery and up to 3 days post-operation. SCI rats received daily inspections for signs of discomfort or illness, and their bladders were expressed three times a day until they regained full voiding function.

2.3.3 Experimental groups and treatments

We used a recombinant human Nrg-1β1 peptide containing the bioactive EGF-like domain of all Nrg-1 isoforms (~8 kDa, amino acids 176–246; Shenandoah Biotechnology, USA). Prior to SCI surgeries, animals were randomly divided into three experimental groups: (1) uninjured control; 2) SCI/vehicle control; (3) SCI/Nrg-1β1 treated, receiving 1μg/day of recombinant human

in vehicle solution containing 0.1% bovine serum albumin (BSA) in 0.9% saline. Nrg-1β1 treatment or vehicle solution was delivered intrathecally by a mini osmotic pump implanted at the time of injury (Alzet® model #1003D for 3 days or model #2001 for 7 days) via an indwelling catheter. The catheter tip was placed inside the subarachnoid space at 2-3mm rostral to the injury epicenter. The catheter was placed parallel to the cord to minimize the compression of the catheter on the spinal cord. Each experimental SCI group included two time points: 3- and 7-days post-SCI. A subset of animals received Dihydroethidium (DHE) (Abcam ab236206) intraperitoneally (10mg/kg) 3 hours before experimental endpoints for assessment of reactive oxygen species (ROS). Information on experimental groups, endpoints and sample size is illustrated in Table 1 and 2.

Table 2. Experimental paradigms for SCI studies



2.3.4 Tissue processing

At each experimental endpoint, SCI rats were deeply anesthetized and transcardially perfused with ice-cold 0.1M phosphate buffered saline (PBS). For molecular biology approaches, five millimeters of the spinal cord tissue centered at the injury epicenter was dissected and homogenized in 1% NP40 buffer (Calbiochem, 492015) with SIGMAFAST^{TM} protease (Sigma,

S8820). For histological analyses, rats were perfused transcardially with 2.5% paraformaldehyde (PFA) in 0.1 M PBS for tissue fixation and submerged in 10% sucrose in 2.5% PFA overnight at 4°C for post-fixation and cryoprotection. Tissues underwent further cryoprotection in 20% sucrose in PBS for two more days at 4°C. A 1.5 cm length of the spinal cord tissue centered at the injury epicenter was excised and embedded in optimal cutting temperature compound OCT (Tissue-Tek®, Electron Microscopy Sciences). Cross sections (35 mm) were cut serially by a cryostat (Leica Biosystems GmbH) and mounted on to Superfrost® Plus Micro Slides (Fisher Scientific) and stored at -80'C until immunostaining procedure.

2.3.5 Immunohistochemistry on tissue sections

Frozen spinal cord cross-sections were processed for immunohistological analysis. We first performed Luxol Fast Blue (LFB) and Hematoxylin and Eosin (H&E) staining to identify the injury epicenter for each rat. The spinal cord cross section with the largest injury area was selected as the SCI epicenter. For immunostaining, air-dried slides were permeabilized with PBS and then incubated with a blocking solution containing 5% non-fat milk, 1% BSA and 0.3% Triton X-100 in 0.1M PBS for 1 h at room temperature. The sections were incubated with primary antibodies overnight at 4°C (listed in Table 3), followed by incubation with appropriate secondary antibodies including goat anti-chicken 488, anti-mouse 568 or anti-rabbit 647 Alexa Fluor (1:400; Invitrogen, Life Technologies Inc, ON, Canada) for 1 h. In a double staining procedure, the tissue sections were sequentially treated with a second primary antibody and then incubated with an appropriate secondary antibody. Following each incubation stage, tissue sections were washed four times with 0.1M PBS for 10min. Finally, all slides were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma D8417) for 30 min at room temperature to label cell nuclei, washed and cover-slipped with

Mowiol mounting media (Sigma, 81381). Specificity of all antibodies was validated through implementation of negative controls (omitting primary antibody from staining protocol) and positive controls (cells or tissues known to express the antigen).

Table 3. List of antibodies used for immunostaining and immunoblotting

Antibody	Targeted Species	Application	Dilution Factor	Source (CAT#)
B tubulin-III	Mouse	ICC	1:300	Sigma (T8660)
NeuN	Mouse	IHC	1:150	Chemicon (MAB377)
MAP2	Mouse	ICC	1:500	Millipore (MAB3418)
NMDAR	Rabbit	ICC	1:500	Abcam (ab17345)
APC	Mouse	IHC	1:50	EMD-Calbiochem (OP80)
Cleaved Caspase-3	Rabbit	WB ICC	1:1000 1:150	Cell Signaling (9664)
GFAP	Rabbit	ICC	1:800	DAKO (Z0334)
Cleaved Caspase-9	Mouse	WB	1:1000	Cell signaling (9508S)
MLKL	Rat	WB	1:1000	Millipore (MABC604)
RIPk1	Rabbit	WB	1:1000	Cell signaling (3493S)
E06	Mouse	SB	1:1000	Avanti (330001S)
4-HNE	Mouse	SB	1:1000	Abcam (Ab48506)
HMGB1	Rabbit	WB	1:1000	Sigma (H9539)
P62	Rabbit	WB	1:1000	Cell signaling (5114S)
LC-3	Rabbit	WB	1:1000	Sigma (L8918)
AIF	Mouse	WB	1:1000	Santa cruz (13116)
Calpain I	Rabbit	WB	1:1000	Cell signaling (2556S)
Bax	Rabbit	WB	1:1000	Cell signaling (5023P)
Bak	Rabbit	WB	1:1000	Cell signaling (12105P)
Phospho-p38	Mouse	WB	1:1000	Santa cruz (sc166182)
p38	Mouse	WB	1:1000	Santa cruz (sc7972)
Phospho-JNK	Mouse	WB	1:1000	Santa cruz (sc6254)
JNK	Mouse	WB	1:1000	Santa cruz (7345)
Phospho-AKT	Rabbit	WB	1:1000	Cell signaling (2965S)
Pan AKT	Rabbit	WB	1:1000	Cell signaling (4691S)

Phospho-ERK1/2	Rabbit	WB	1:1000	Cell signaling (4695S)
Pan ERK1/2	Rabbit	WB	1:1000	Cell signaling (4370S)
GAPDH	Rabbit	WB	1:1000	Santa Cruz (sc-47724)
Actin	Mouse Rabbit	WB	1:400	Sigma (A3853) (A2066)

IHC: Immunohistochemistry, **ICC:** Immunocytochemistry, **WB:** Western blotting, **SB:** Slot blotting

2.3.6 Imaging and tissue analysis

We assessed apoptosis after SCI by co-immunostaining of NeuN (neurons) or APC (oligodendrocytes) with cleaved caspase-3 (ClCasp3). We quantified apoptotic neurons (NeuN+/ClCasp3+/DAPI+) and oligodendrocytes (APC+/ClCasp3+/DAPI+) relative to total neurons or oligodendrocytes (NeuN+/DAPI+ or APC+/DAPI+). Assessments were conducted at 750 μm and 1.5 mm distances rostrally and caudally to the lesion center. In all cell quantifications, DAPI was used to identify cell nuclei. For assessment of ROS using DHE, spinal cord cross sections were stained with DAPI to identify boundary of the spinal cord. Each cross-section was imaged entirely using a 10x objective with Zen tiling software (Zeiss). DHE immunointensity was analyzed based on our previously described protocols (Hart et al., 2020) (278). After background was automatically removed, immunointensity above the threshold was quantified for each tissue cross-section using NIH ImageJ software (imagej.nig.gov), while excluding meninges. Immuno-intensity was normalized to total area of the sections. All samples were processed in parallel under the same condition and imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) under consistent exposure time.

2.3.7 Isolation and culture of primary cortical neurons, glutamate toxicity and Nrg-1β1 treatment

Primary cortical neurons were isolated from cortices of embryonic (E18) SD rat as we described previously (Hart et al., 2020). Cortices were isolated and incubated in papain solution for 15-20 min at 37°C. Once tissues settled at the bottom, the supernatant was discarded and tissues were resuspended in 1ml aCSF and 1x DNase (Sigma, D5025) and mechanically dissociated by fire polished Pasteur pipette. Papain enzymatic activity was neutralized by adding 1ml fetal bovine serum (FBS). Cells were filtered through a 40µm strainer and diluted in 5ml neuronal media consisting of Neurobasal-A (Gibco, 10888-022), 1% B27 supplement (Gibco, 17504-044), Penicillin − Streptomycin − Neomycin (PSN) and L-glutamine, followed by a brief centrifugation. The cells were resuspended in fresh neuronal media and seeded at $1x10^5$ cells per well into Poly-D-lysine (PDL)-coated 8-well glass multi-chambers and 1.2×10^6 cells per well in PDL-coated 6-well plates. Cells were maintained for 10 days in culture to mature, with half the media being refreshed every 2-3 days. Using immunocytochemical analysis, we have confirmed the purity of these neuronal cultures are over 85% (ß Tubulin III positive neurons) with some GFAP expressing astrocytes (Supplementary Figure 1 A-E). Maturity of these neurons was also confirmed by expression of MAP2 and NMDAR (Supplementary Figure 1 F-H).

We induced glutamate toxicity in primary cortical neurons at 10 days post culture. We first performed a concentration study to identify an effective concentration of glutamate (Sigma, G1626), which can significantly induce cell death in neuron cultures. We treated neurons with 5, 10, 25 and 50µM of glutamate transiently for 1h. Then, the media was refreshed with glutamate free media for 8hrs. Glutamate effects on neuronal viability was assessed by LIVE/DEAD assay, lactate dehydrogenase (LDH) assay and Western blotting for cleaved caspase-3 (ClCasp3), as

described in the following sections (Supplementary Fig. 2). Our assessments determined 10μM of glutamate as the most effective concentration for inducing cell death. For Nrg-1 β1 treatment, we tested 10 and 50ng/ml of Nrg-1, representing low and high concentrations. Neuronal cultures were exposed to glutamate (10μM) for 1hr and then the media was refreshed with Nrg-1β1 containing media (no glutamate) for 8hrs. Experimental conditions were 1) Control 2) glutamate (10μM), 3) Nrg-1β1 alone (50ng/ml), 4) glutamate + Nrg-1β1 10ng/ml, 5) glutamate + Nrg-1β1 50ng/ml.

2.3.8 Assessment of overall cell viability and injury in neurons *in vitro*

Cell viability was evaluated either by analysis of LDH in the media of neurons or by LIVE/DEAD assay according to the manufacturer's instructions (LIVE/DEAD® Viability/Cytotoxicity Kit; Thermo Fisher, L3224). Briefly, cells were washed twice with PBS. Then 150 μl of LIVE/DEAD assay reagent including 2mM Ethidium homodimer-1 (EthD-1) and 4mM Calcein AM in PBS was added to each well of 8-well multichamber slides for 15min at 37°C. Then, cells were washed with PBS. Live imaging of 8–10 randomly chosen fields were taken under 20× objective with a Zeiss fluorescent microscope using optical filters optimal for Calcein (485 ± 10 nm, LIVE, green) and EthD-1 (530 ± 12.5 nm, DAED, red). For analysis, the percentage of live cells was determined by quantifying the total number of LIVE cells among the total number of LIVE and DEAD cells, as we described previously (318, 319). For LDH assay, 50μl of reagent (Thermo Fisher, L3224) was added to 50 μl of conditioned media and incubated for 30 min at room temperature. Absorbance at 450 nm with a background correction at 650 nm was measured using Synergy H1 Hybrid Reader (BioTeK Instruments, USA) and the relative cell injury was presented as percentage of control.

2.3.9 Immunostaining procedures *in vitro*

Purity of cortical cultures were assessed by immunostaining against beta tubulin III (ßTIII) for neurons and GFAP for astrocytes. Maturity of neurons was assessed by immunostaining for MAP2 and NMDAR. In all immunostaining procedures, cultured cells were fixed with 3% PFA for 20 min and washed with PBS at room temperature. The cultures were blocked and incubated with antibodies as described in the earlier section for immunohistochemical procedures. To mark nucleus, cells were incubated with 4, 6-diamidino-2-phenylindole (DAPI, Sigma D8417). Fluorescence images were taken at 20X magnification, and 10 random fields were acquired containing an average of 400 cells for each condition. To assess the purity of cultures, percentage of neurons and astrocytes in cultures was determined by counting the number of $BTIII^+$ /$DAPI^+$ neurons and $GFAP^+$/$DAPI^+$ astrocytes normalized to the total number of DAPI+ cells.

2.3.10 Immunoblotting procedures in vitro and in vivo

For all Western and slot blot analyses, protein content was determined in spinal cord tissue, cell lysate and conditioned media by Lowry assay (Sigma, L3540). For HMGB1 assessment, conditioned media was concentrated 30 times using centrifugal column with molecular weight cutoff at 3 kDa (VWR). For Western blotting, a total of 10-40 µg per sample was loaded onto 10-15% Tris-glycine gels and transferred onto nitrocellulose or polyvinyl fluoride (PVDF) membranes using Trans-Blot® Turbo Transfer system (BioRad, 2.5V) for 10 min. Membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tween-20 Tris-buffered saline (0.2% TBST) and incubated for 2 h at room temperature, followed by incubation with primary antibodies diluted in blocking solution overnight at 4°C. A list of the antibodies used in immunoblotting is provided in Table 3. Membranes were washed and incubated with horseradish

peroxidase-conjugated (HRP) goat anti-mouse or -rabbit secondary antibodies (1:4000) for 1 h at room temperature. For slot blot analysis of 4-HNE and E06, 3-10 µg of protein from spinal cord tissue or neuronal conditioned media was loaded onto nitrocellulose membrane using the Bio-Dot® slot blot system and similar blocking and antibody incubation protocols as described above. For Western and slot blot analyses, targeted bands were detected with ClarityTM Western ECL substrate (BioRad, 1705060) using MicroChemi 4.2 (DNR Bio-imaging Systems) following manufacturer's specifications. Densitometry analysis of protein bands was conducted using NIH ImageJ software. To control for variances in protein loading, results were normalized to total protein Ponceau S stain for conditioned media or actin expression for cell lysate.

2.3.11 Mitochondrial morphology

For mitochondrial analysis in neurons, cortical cultures were incubated with 50 nM MitoTracker™ Red CMXRos (Thermo Fisher, M7512) for 15 min at 37 °C, washed once with PBS and fixed with ice-cold 98% methanol for 10 min at − 20 °C. Images were acquired using an LSM710 Zeiss confocal microscope and the brightness and contrast were adjusted using Image J software. Cells containing elongated and highly interconnected mitochondria were categorized as "tubular," while cells that had small and spherical mitochondria were scored as "fragmented." At least 100 cells per condition from three independent studies were scored and the percentage of each category were calculated.

2.3.12 Statistical analysis

Statistical analysis was conducted for all *in vitro* and *in vivo* assessments using GraphPad Prism Software (version 6.07, California, USA). Statistical differences between the treatments were evaluated by One-Way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test. For SCI immunohistological analyses, we used Three-Way ANOVA repeated measures followed by Holm-Sidak post hoc. All data is expressed as mean ± standard error of mean (S.E.M). Significance was noted statistically if $p<0.05$. To ensure integrity of results, proper blinding and randomization was applied for all assessments.

2.4. Results

2.4.1 Nrg-1β1 treatment significantly mitigates SCI-induced cell injury and lipid peroxidation.

We previously demonstrated Nrg-1β1 is acutely and persistently dysregulated after compressive/contusive SCI in rats, and its restoration positively modulates the glial and inflammatory responses and improves neurological recovery (278). Here, we further elucidated the cellular and molecular mechanisms by which Nrg-1β1 treatment promotes the microenvironment of SCI (see Graphical Abstract and Figure 8 for a summary of the studied targets). Here, we further elucidated the cellular and molecular mechanisms by which Nrg-1β1 treatment promotes a neuroprotective microenvironment following SCI. We focused on cell injury mediators at 3- and 7-days post injury (dpi), which represent the peak of cell death in rat SCI during acute and early sub-acute stages of injury, respectively. We delivered Nrg-1β1 intrathecally to the site of injury at the time of SCI at a rate of 1μg per day in a sustainable manner using mini osmotic pumps. Efficacy of this dose was identified in our previous studies (278). We asked

whether Nrg-1β1 treatment can affect SCI-induced necrosis, lipid peroxidation and ROS formation (Fig. 1A-I). We first assessed the tissue level of HMGB1, a general marker of cell injury and necrosis (322). Western blot analysis of the spinal cord lesion showed a significant, progressive increase in HMGB1 levels at both 3 and 7dpi (2.5 and 10-fold, respectively) in SCI vehicle control group compared to uninjured group (Fig. 1A and D). While Nrg-1β1 treatment had no significant effect on HMGB1 levels at 3dpi (Fig. 1A), it significantly reduced HMGB1 by 41% at 7dpi compared to vehicle treated group (Fig. 1D). We also assessed lipid peroxidation using slot blotting for E06 and 4-hydroxy-2-nonenal (4-HNE), two major products of oxidized lipids. At 3dpi, we detected a significant increase in the tissue levels of both E06 and 4-HNE in vehicle treated SCI rats (7.3 and 3.1 folds, respectively) compared to their basal levels in uninjured spinal cord. However, treatment with Nrg-1β1 had no significant effect on lipid peroxidation markers at this time-point (Fig. 1B and C). At 7dpi, Nrg-1β1, however, was able to significantly attenuate the SCI-induced levels of E06 by 60% compared to vehicle treated group, although 4-HNE levels remained unaffected (Fig. 1E-F).

We also assessed ROS production in the injured spinal cord of vehicle and Nrg-1β1 treated rats by intraperitoneal administration of Dihydroxy Ethidium (DHE). Our examination included lesion and perilesional areas (at 750μm and 1.5mm points rostral and caudal to the injury epicenter) as well as distant healthy regions (3mm). Intensity measurement of DHE signal revealed a significant elevation in ROS levels at 3dpi and 7dpi in vehicle SCI rats as compared to its basal expression in healthy regions of the spinal cord (Fig. 1 G-I). Interestingly, Nrg-1β1treatment was able to significantly reduce ROS production at 1.5mm rostral (56%) and 1.5mm caudal (82%) points to the epicenter at 3dpi and 7dpi, respectively (Fig. 1H-I). Taken together, these data suggest availability of Nrg-1β1 can attenuate general cell injury at the sub-acute stage of SCI in rats.

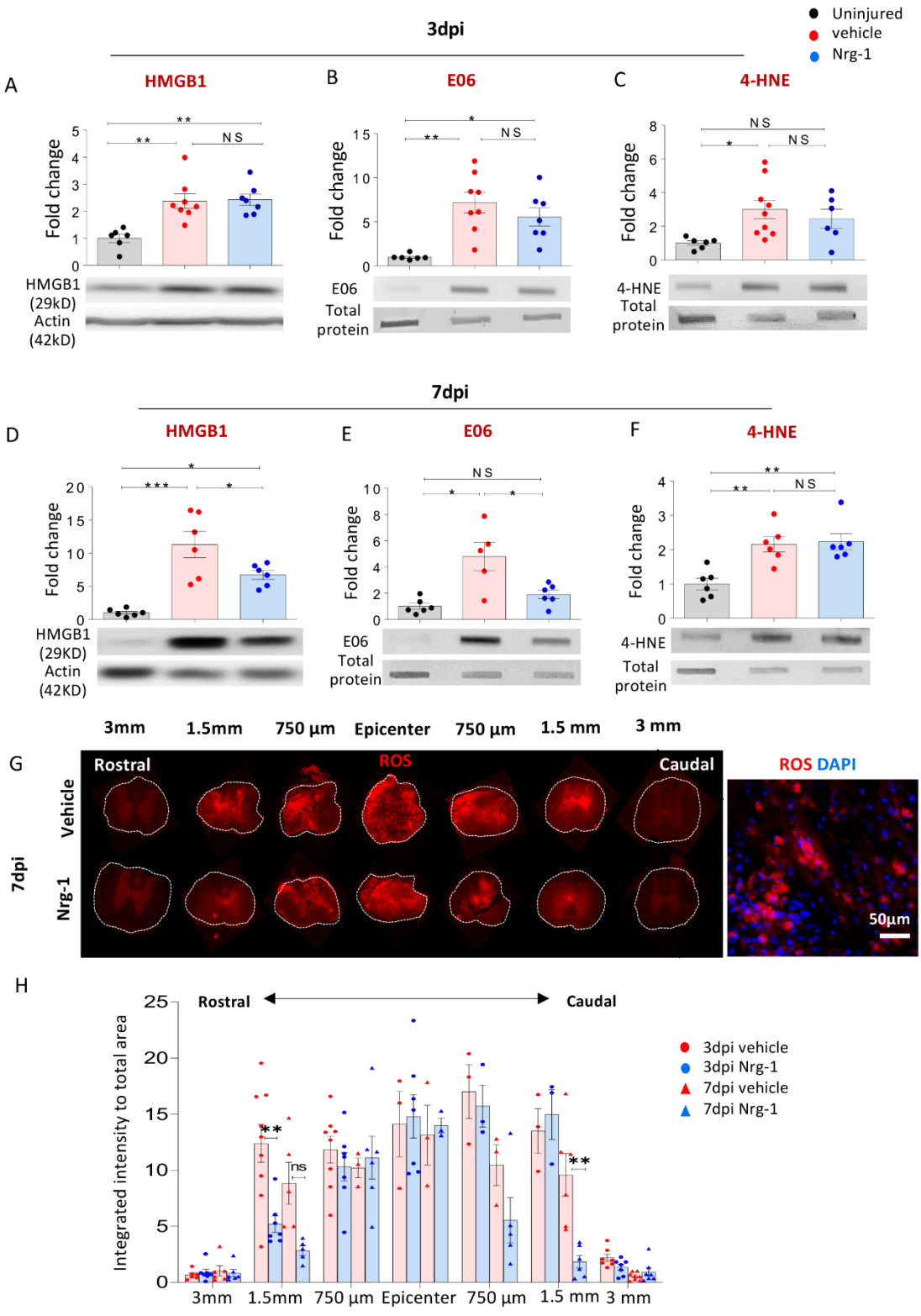

Uninjured
vehicle
Nrg-1
3dpi
A
HMGB1
B
E06
C
4-HNE
Fold change
HMGB1 (29kD)
Actin (42kD)
E06
Total protein
4-HNE
Total protein
NS
7dpi
D
HMGB1
E
E06
F
4-HNE
3mm
1.5mm
750 µm
Epicenter
750 µm
1.5 mm
3 mm
G
Rostral
ROS
Caudal
Vehicle
Nrg-1
7dpi
ROS DAPI
50µm
H
Rostral
Caudal
Integrated intensity to total area
3dpi vehicle
3dpi Nrg-1
7dpi vehicle
7dpi Nrg-1
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Figure 1: Nrg-1ß1 attenuates cell injury and lipid peroxidation after SCI. (A-F) Western and slot blot analyses for general markers of necrosis and lipid peroxidation (HMGB1, E06 and 4-HNE) were performed on tissue homogenate of the injured spinal cord at 3 and 7 day post injury (dpi). (A, D) HMGB1 levels were significantly increased after SCI compared to uninjured baseline. Intrathecal administration of Nrg-1β1(1µg per day) significantly reduced HMGB1 levels at 7dpi, while had no significant effect on its levels at 3dpi. (B-C, E-F) Slot blot analysis also showed a significant increase in both E06 and 4-HNE at 3dpi and 7dpi. Nrg-1β1 treatment significantly reduced the levels of E06 at 7dpi compared to vehicle group but with no effect on 4-HNE levels. At 3dpi, we found no beneficial effects of Nrg-1 treatment on the expression levels of E06 or 4-HNE. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to uninjured group. The data represent mean ±SEM. One-way ANOVA followed by Holm–Sidak post hoc test. *$p < 0.05$ indicates where the data was significant. N=6-9 rats/group. (G) Representative images of reactive oxygen species (ROS) immunolabeling in the cross sections of injured spinal cord are depicted at 750µm, 1.5 and 3mm rostral and caudal to the lesion center at 7dpi time-point. (H) Assessment of ROS immunointensity was performed on the lesion and perilesional areas (epicenter ±3 mm) of both gray and white matter. Nrg-1β1 treatment significantly reduced SCI-induced production of ROS at 1.5mm rostral and 1.5mm caudal points to the epicenter at 3dpi and 7dpi, respectively. The data represent mean ±SEM. Three-way ANOVA followed Holm–Sidak post hoc test where data was significant. *$p < 0.05$ indicates where the data was significant. N=4-9 rats/group.

2.4.2 Nrg-1β1 treatment protects neurons and oligodendrocytes from apoptotic cell death

Apoptosis is a major cell death mechanism after SCI, and neurons and oligodendrocytes are particularly susceptible to caspase-mediated cell death (318, 323). Thus, we examined whether increased availability of Nrg-1β1 can attenuate apoptosis in neurons and oligodendrocytes. We first assessed the overall levels of cleaved caspase-3 (clCasp-3), which is a convergence point of different apoptotic pathways. Western blot analysis at both 3dpi and 7 dpi showed induced expression of clCasp-3 after SCI in vehicle treated rats (4.3- and 16-fold change, respectively) compared to its low basal levels in uninjured tissue (Fig 2A-D). At 7dpi, Nrg-1β1 treatment significantly attenuated apoptosis by reducing the tissue level of clCasp-3 (54%) compared to vehicle SCI group, while it had no significant effect at 3dpi (Fig.2 A, C). We also assessed the expression level of clCasp-9, which is the effector of mitochondrial-dependent apoptosis mechanism. Western blot analysis revealed a significant increase in the levels of clCasp-9 at both acute and sub-acute stages of SCI (4.2 and 3.2 folds, respectively) compared to the basal levels of active caspase 9 detected in uninjured spinal cord tissue (Fig.2 B, D). Similar to clCasp3 expression, while Nrg-1β1 administration had no significant effect on clCasp-9 levels at 3dpi (Fig.2 B), it significantly reduced SCI-induced expression of clCasp-9 by 42% at 7dpi time-point (Fig.2 D). Additionally, we assessed the expression level of clCasp-8, which is the effector caspase within the extrinsic apoptosis pathway. Western blot analysis of the spinal cord tissue at 7dpi showed robust induction in clCasp-8 (24.3 folds) in vehicle treated SCI rats compared to uninjured tissue. However, our analysis showed Nrg-1β1 had no significant effect on the expression level of clCasp-8 compared to vehicle group (Supplementary Fig. 4A).

Since neurons and oligodendrocytes are both affected by apoptosis in SCI, we further determined cell specific effects of Nrg-1β1 treatment on these populations at 3dpi and 7dpi. We

first assessed the effects of Nrg-1β1 treatment on oligodendrocyte apoptosis at 750µm and 1.5mm rostral and caudal points to the injury epicenter in vehicle and Nrg-1β1 treated SCI rats at 3 and 7 dpi. Our cell quantification of apoptotic mature oligodendrocytes marked as APC+/ clCasp3+ cells showed a significant reduction (29.6%) at 1.5mm caudal point under Nrg-1β1 treatment at 3dpi (Fig.2 E). At 7dpi time-point, Nrg-1β1 treatment more prominently protected mature oligodendrocytes as number of apoptotic APC+/clCasp3+ cells were reduced by 41% at 750µm caudal to the epicenter compared to vehicle treated SCI rats (Fig. 2 E). These data indicate availability of Nrg-1β1 protects oligodendrocytes against caspase3-mediated cell death in sub-acute stages of SCI in rats.

Next, we quantified the number of apoptotic neurons (NeuN+/ClCasp3+). Analysis of co-immunolabelled NeuN+/clCasp3+ neurons in dorsal horn (DH) and ventral horn (VH) in the injured spinal cord showed a range of 40-80% apoptotic neurons at 750 to 1.5mm rostral and caudal distances at both 3dpi and 7dpi (Fig. 2L, M). Our cell quantification analysis of apoptotic neurons located at dorsal horn (DH) of the spinal cord revealed no protective effects of Nrg-1β1 on apoptotic neurons at 3dpi (Fig. 2L), while at 7dpi Nrg-1β1 treatment resulted in a significant reduction in the number of apoptotic neurons of DH at 1.5mm caudal to the epicenter by 31.29% compared to vehicle treated group (Fig. 2L). Similarly, assessment of NeuN+/clCasp3+ in ventral horn (VH) neurons showed no effects of Nrg-1β1 at 3dpi while at 7dpi there was a significant reduction (18.55%) in the percentage of apoptotic neurons at 1.5mm caudal to the injury epicenter (Fig 2M). Our analysis of the perilesional areas at 750µm rostral and caudal distances, however, showed no significant effects by Nrg-1β1 on neuronal apoptosis in the areas closer to the injury epicenter (Fig 2M). Additionally, we performed neuronal cell count to quantify the total number of preserved neurons across the rostro-caudal length of the injured spinal cord at the

abovementioned distances including the injury epicenter. At 3dpi, quantification of NeuN+/DAPI+ neurons showed no significant difference in the total number of neurons between Nrg-1β1 and vehicle treated groups (Supplementary Fig. 1A). However, at 7dpi, there was significant neuronal preservation (46.6%) at 1.5mm caudal to the injury in Nrg-1β1 treated animals compared to the vehicle treated rats (Supplementary Fig. 1A). To determine the long-term neuroprotective effects of Nrg-1β1 on neuronal preservation, we further assessed the total number of neurons (NeuN+/ DAPI+) at 6 weeks post injury (6wpi), which represent chronic SCI (Supplementary Fig. 1M). Similar to 7dpi, neuronal quantification revealed significant preservation of neurons at 1.5mm caudal to the epicenter in Nrg-1β1 treated group (30.57%) compared to vehicle treated animals with no significant effect at other distances (Supplementary Fig. 1M). These data suggest that Nrg-1β1 treatment can protect neurons at the sub-acute and chronic stages of SCI in caudal regions to the injury center.

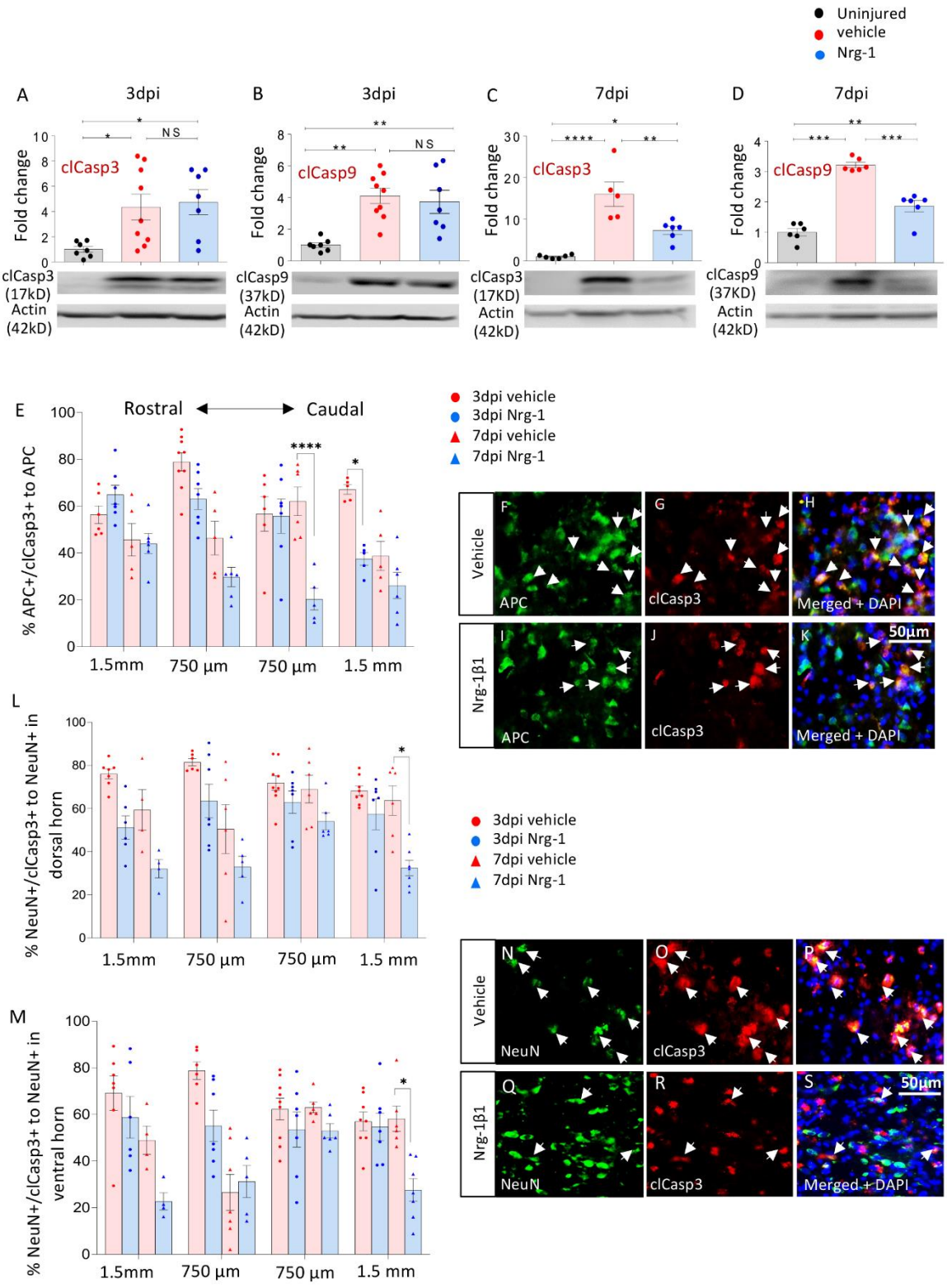

Uninjured
vehicle
Nrg-1
A
3dpi
B
3dpi
C
7dpi
D
7dpi
Fold change
clCasp3
clCasp9
NS
clCasp3 (17kD)
clCasp9 (37kD)
Actin (42kD)
E
Rostral
Caudal
% APC+/clCasp3+ to APC
1.5mm
750 µm
750 µm
1.5 mm
3dpi vehicle
3dpi Nrg-1
7dpi vehicle
7dpi Nrg-1
Vehicle
Nrg-1β1
F
G
H
I
J
K
APC
clCasp3
Merged + DAPI
50µm
L
% NeuN+/clCasp3+ to NeuN+ in dorsal horn
M
% NeuN+/clCasp3+ to NeuN+ in ventral horn
N
O
P
Q
R
S
NeuN

Figure 2: **Nrg-1ß1 significantly attenuates apoptosis following SCI.** (A-D) Western blot analyses of cleaved-caspase-3 (cl-Casp3) and cleaved-caspase-9 (cl-Casp9) were performed on the injured spinal cord at both 3dpi and 7dpi. Nrg-1β1 treatment significantly reduced the SCI-induced levels of clCasp-3 and clCasp-9 at 7dpi, while had no significant effects at 3dpi. The data represent mean ±SEM, One-way ANOVA followed by Holm–Sidak post hoc test, *$p < 0.05$ indicates where the data was significant. N=6-9 rats/group. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to uninjured group. (E) Apoptosis of oligodendrocytes was performed in the injured white matter of spinal cord. Nrg-1β1 treatment significantly protected oligodendrocytes, as number of clCasp-3+/APC+ cells were significantly reduced at 750µm and 1.5mm caudal points to injury epicenter at both 3dpi and 7dpi. (F-K) Representative images depict localization of clCasp-3 in oligodendrocytes in white matter region of the injured spinal cord (APC+/clCasp-3+, white arrow) in vehicle versus Nrg-1β1 treated groups at 7dpi time-point and 1.5mm caudal to the epicenter. (L, M) Quantitative immunohistological analysis of neuronal apoptosis was conducted in dorsal horn (DH) and ventral horn (VH) at 750µm and 1.5mm distances rostral and caudal to injury epicenter in both vehicle and Nrg-1β1 treated SCI rats at 3dpi and 7dpi. Assessment of NeuN+/clCasp3+ neurons at DH and VH indicated that Nrg-1β1 treatment significantly attenuated the percentage of apoptotic neurons at 1.5mm caudal points to the epicenter relative to vehicle group. There was no significant change in the number of apoptotic neurons at 3dpi. Nrg-1β1 treatment also had no significant effect on apoptosis in distances closer to the epicenter (i.e., 750µm rostral and caudal). (N-S) Representative images depict localization of clCasp-3 in neurons (NeuN+/clcasp-3+, white arrow) in vehicle and Nrg-1ß1 treated groups at VH and 1.5mm caudal to the epicenter and at 7dpi. Green color represents NeuN/ APC signal and red color represents clcasp3 signal. The data represent mean ±SEM, Three-

way ANOVA followed by Holm–Sidak post hoc test where the data was significant. *$p < 0.05$ indicates where the data was significant. N= 4-9 rat/ group.

2.4.3 SCI- induced necroptosis and autophagy was unaffected by Nrg-1β1 treatment in acute and early subacute stages

We further examined whether Nrg-1β1 treatment influences necroptosis and autophagy, two other cell death mechanisms in the injured spinal cord. For necroptosis, we assessed the expression levels of two major necroptosis markers, receptor-interacting serine/threonine-protein1 (RIP1) and mixed lineage kinase domain-like (MLKL). At 3dpi and 7dpi, Western blot analysis of spinal cord lesion showed a significant increase in the expression levels of RIP1 (3.44 and 3.64 folds, respectively) in vehicle treated rats compared to its basal levels in uninjured rats (Fig. 3A, C). Likewise, MLKL expression was also induced after SCI (Fig. 3 B, D). However, Nrg-1β1 treatment had no significant effect on the tissue levels of these necroptosis markers after SCI (Fig. 3 A-D).

Autophagy is an essential function of the cell in maintaining the balance of cellular components and damaged organelles (324). Studies in mice SCI suggest autophagic flux is disrupted in the first week post-SCI as indicated by accumulated LC3-II/I (microtubule-associated protein 1A/1B light chain 3-II/I (LC3-II/I) in autophagosomes) and p62/ sequestosome1 (SQSTM1) (325, 326). Our Western blot analysis of spinal cord tissue also confirmed the blockage of autophagy in rats with SCI. At 3dpi, while there was no significant increase in the expression of p62 after SCI, we found a significant rise (2.6 folds) in the protein expression of LC3-II/I in vehicle SCI rats compared to their uninjured counterparts (Fig. 3E-F). However, Nrg-1β1 treatment had no significant effect on SCI-baseline autophagy flux recovery at 3dpi time-point. At

7dpi, we also found a significant increase in both p62 (3.23 folds) and LC3 -II/I (9.30 folds) in SCI rats comparing to uninjured tissue; however, similar to 3dpi, these effects remained unchanged under Nrg-1β1 treatment (Fig. 3 G-H). Altogether, our results suggest Nrg-1β1 does not play a significant role in modulating necroptosis and autophagy flux in rat SCI, while its presence can attenuate necrosis and apoptosis.

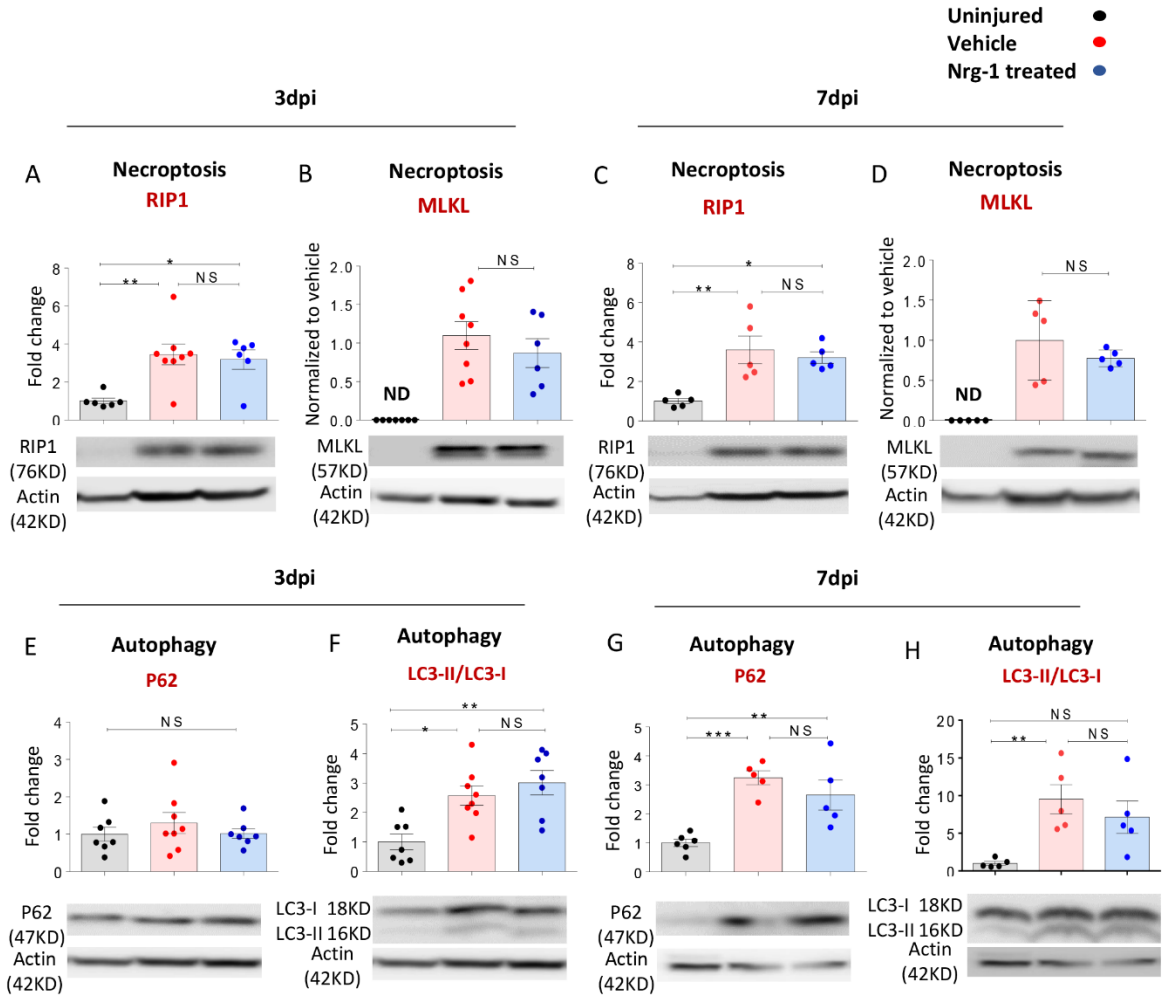


Figure 3: Nrg-1ß1 availability does not influence autophagy and necroptosis at acute and early subacute SCI. (A-H) Western blot analysis show the tissue levels of necroptosis markers, receptor-interacting serine/threonine-protein 1 (RIP 1), mixed lineage kinase domain-like (MLKL) and autophagy, p62 and Microtubule-associated protein 1A/1B-light chain 3 (LC3), in normal versus vehicle and Nrg-1β1 treated SCI rats at 3dpi and 7dpi. (A-D) SCI resulted in a significant induction in necroptosis at 3dpi, which remained upregulated at 7dpi. However, Nrg-1β1 treatment did not change the expression level of necroptosis markers at 3dpi and 7dpi. (E-H) Similarly, Western blot analysis of autophagy machinery also revealed its upregulation at both time-points; however, Nrg-1β1 treatment had no significant effect on expression level of these markers. The data represent mean ±SEM. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to uninjured or vehicle group. *$p < 0.05$, One-way ANOVA followed by Holm–Sidak post hoc test). N=4-6 rats/group.

2.4.4 Nrg-1β1 abrogates glutamate induced cell death in primary cortical neurons

Since we identified Nrg-1β1 attenuates ROS production, necrosis and apoptosis after SCI, we next asked whether it can directly and specifically regulate neuronal injury. We addressed this question in an *in vitro* model of glutamate toxicity in primary neurons. Glutamate excitotoxicity represents a major acute injury mechanism that causes cell death after SCI (327). We employed a primary cortical neuron culture with over 85% purity (Supplementary Fig. 2A-E). Neurons were prepared from E18 cortices and maintained in cultures for 10 days to mature. We first performed a concentration–response study to determine an effective concentration of glutamate that would induce statistically significant cell death in primary neurons. At 10 days post culture, neurons were treated with different concentrations of glutamate (5, 10, 25 and 50µM) for 1h. We confirmed

cortical neurons were mature and responsive to glutamate, as they expressed mature neuronal marker, microtubule associated protein-2 (MAP-2) and glutamate receptor, N-Methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA) (Supplementary Fig. 2F-H). In our glutamate concentration study, we used several outcome measures including LIVE/DEAD assay, lactate dehydrogenase (LDH) measurement, and Western blotting for clCasp3 to determine the extent of neuronal injury following glutamate toxicity (Supplementary Fig. 3A-H). Our assessments collectively identified that 10 μM of glutamate was effective to significantly decrease overall cell viability (30%, LIVE/DEAD assay), increase cell injury (6.5 folds, LDH), and induces clCasp3 expression (2.5 folds) in neurons compared to control condition. Using this system, we next evaluated whether availability of Nrg-1β1 can affect glutamate-induced neuronal injury. We treated neurons with two concentrations of Nrg-1β1, 10ng/ml (low) and 50ng/ml (high), following glutamate challenge (10μM) and maintained cultures for 8 h.

Quantitative analysis of LIVE/DEAD assay showed glutamate exposure resulted in a significant (45.87%) reduction in viability of cortical neurons compared to control condition (Fig. 4A-F). Cortical neurons that received 10 and 50 ng/ml of Nrg-1β1 for 8h after glutamate exposure had a significantly higher survival rate (10.4% and 12.1%, respectively) compared to non-treated glutamate condition. Notably, Nrg-1β1 treatment had no apparent effects on neuronal viability under non-glutamate control condition (Fig. 4A). We additionally examined cell injury by assessing the release of LDH into the conditioned media of neurons after glutamate exposure with or without Nrg-1β1. Glutamate treatment resulted in significant release of LDH by cortical neurons (2.76 folds) compared to control condition, and treatment with Nrg-1β1 (10 and 50 ng/ml) ameliorated cell injury significantly by 37.68% and 36.59%, respectively, compared to non-treated glutamate condition (Fig. 4 G). We also assessed HMGB1 release from neurons under glutamate

toxicity. While HMGB1 was undetectable in the conditioned media of neurons in control condition, its level was highly induced after glutamate challenge. Treatment with Nrg-1 resulted in a significant reduction in glutamate-induced release of HMGB1 in neurons by 60% and 53% in 10 and 50ng/ml of Nrg-1β1, respectively (Fig. 4 H). These *in vitro* studies indicate Nrg-1β1 can directly influence neurons under injury conditions and highlight its beneficial effects in attenuating glutamate-mediated injury in neurons.

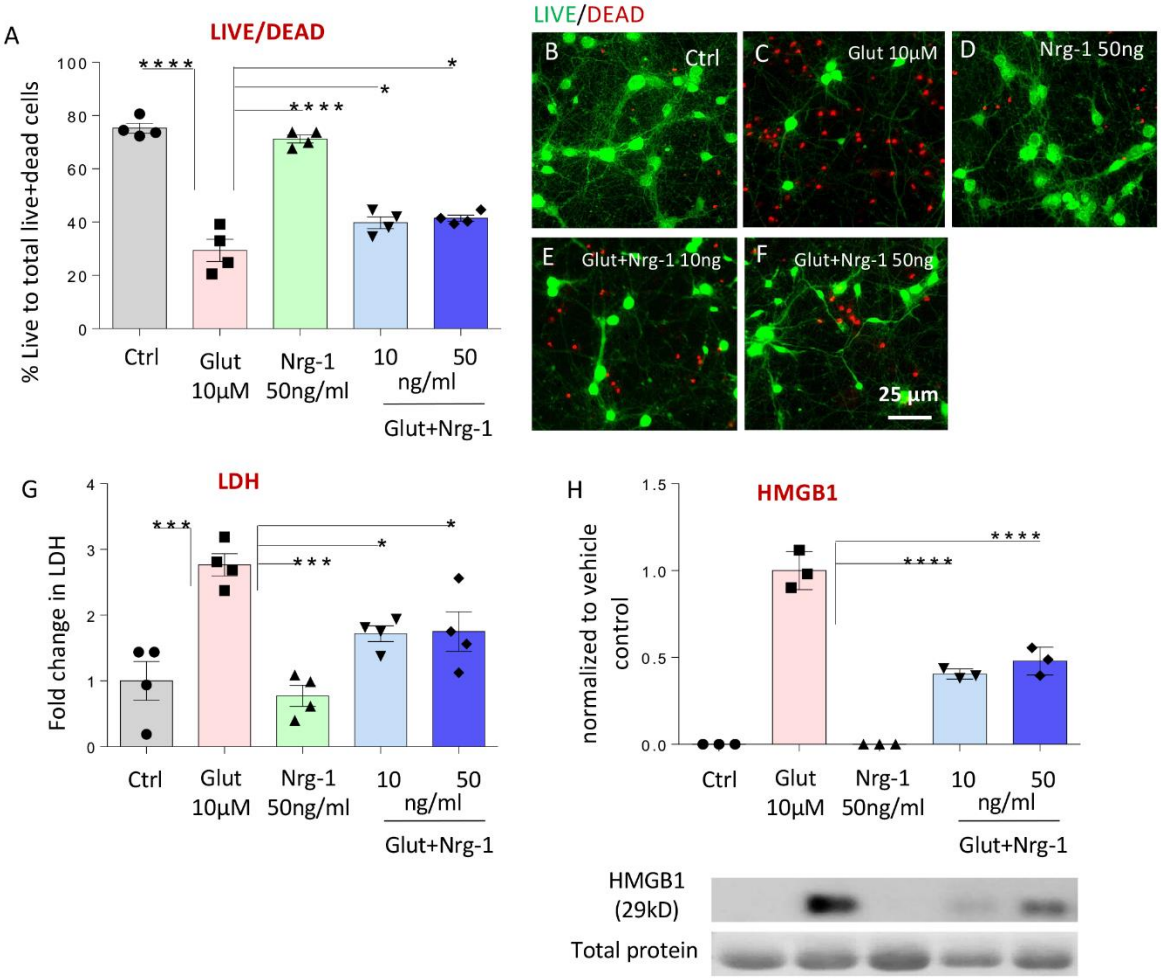


Figure 4: Nrg-1ß1 protects cortical neurons against glutamate mediated cell injury *in vitro*. (A-H) Nrg-1β1 treatment for 8h reduced cell death and injury in primary rat mature cortical neurons after exposure to glutamate. (A) LIVE/DEAD assay showed that Nrg-1ß1 treatment at 10 and 50 ng/ml was able to significantly enhance survival of neurons under glutamate (10μM) challenge for 1h. (B-F) Representative images of LIVE/DEAD assay under different conditions. (G) LDH assay was performed on conditioned media of neurons under various treatments. Treating neurons with Nrg-1ß1 for 8h following glutamate exposure resulted in a significant decrease in LDH release by neurons. (H) Western blot analysis showed a significant reduction in glutamate-induced release of HMGB1 in neurons that were treated with Nrg-1ß1 compared to non-treated glutamate condition. In all experiments, Nrg-1β1 treatment did not affect neurons under normal condition. The data represent mean ±SEM, *$p < 0.05$, One-way ANOVA, followed by Holm–Sidak post hoc test. Ponceau S staining of total protein in conditioned media was used as a loading control for Western blot analysis of HMGB1, and results are shown as fold change relative to control or glutamate conditions for LDH and HMGB1, respectively. N= 3-4 independent culture experiment.

2.4.5 Nrg-1β1 confers protection against glutamate toxicity through caspase-dependent and independent mechanisms.

Excess glutamate induces caspase-3 mediated apoptosis and DNA fragmentation by up regulation of Bcl-2-associated X protein (Bax) and mitochondrial signaling in primary cortical cells *in vitro* (328, 329). Our studies in SCI showed administration of Nrg-1β1 reduces the overall tissue expression of clCasp3 in the injured spinal cord and specifically in neurons. Thus, we sought to identify intracellular mechanisms by which Nrg-1β1 regulates glutamate-mediated cell death in

neurons. First, we performed Western blotting for clCasp3 in cortical neuron cultures following glutamate challenge. Our results confirmed glutamate treatment significantly promotes caspase 3 activation (4.1 folds) compared to control neurons. Nrg-1β1 treatment at 10 and 50ng/ml markedly reduced clCasp-3 expression by 55.4% and 55.7% in glutamate treated neurons, respectively (Fig. 5A). Since activation of caspase 3 is a convergence point of intrinsic and extrinsic apoptosis pathways, we next assessed the expression of clCasp-9 and clCasp-8 that are involved in extrinsic and intrinsic pathways, respectively. Assessment of clCasp-9 in glutamate treated neurons showed a 4.4-fold increase at 8h post glutamate challenge, which was attenuated with 10 and 50ng/ml of Nrg-1β1 by 60.5% and 49.4%, respectively (Fig. 5B). Interestingly, we found no significant change in the expression levels of clCasp-8 either in glutamate or Nrg-1β1 treated neurons compared to control condition (Supplementary Fig. 4B). This suggests glutamate challenge in our studies did not induce extrinsic apoptosis pathway in primary cortical neuron cultures, while it significantly promoted activation of intrinsic apoptosis pathways through caspase 9 and caspase 3 mediated mechanisms.

Apoptosis can also occur through caspase independent and mitochondrial mechanisms. It is known that reduction in mitochondrial membrane potential and mitochondrial dysfunction can result in the release of several pro-apoptotic factors such as apoptosis inducing factor (AIF) to cytoplasm that trigger caspase independent apoptosis (330, 331) (332, 333) (334). Therefore, we next asked whether Nrg-1β1 can regulate apoptosis by modulating the releasee of AIF from mitochondria to cytoplasm. Our Western blot assessments on cytoplasm fraction of neurons confirmed glutamate toxicity induces a significant increase in cytoplasmic AIF (3.5 folds) compared to control condition. Interestingly, treating neurons with 10 and 50ng/ml of Nrg-1β1 remarkably reversed the glutamate-induced elevation of cytoplasmic AIF to the levels observed in

non-treated glutamate condition (Fig. 5C). It is well-established that excess glutamate leads to Ca^{2+} overload in neurons (335, 336) and stimulates the expression of calcium-dependent proteases such as calpains that can result in mitochondrial dysfunction and AIF release (335, 337). Interestingly, our assessment of revealed Nrg-1β1 treatment also reversed glutamate-induced expression of calpain I in neurons to its baseline levels in control condition (Fig. 5D). Thus, our data suggest Nrg-1β1 can attenuate glutamate-induced apoptosis in neurons by regulating mitochondria. Based on these results, we further studied the effect of Nrg-1β1 on mitochondrial morphology and membrane permeabilization.

Permeabilization of the mitochondrial outer membrane is regulated by multidomain proapoptotic Bax and Bak (338). Our Western blot analysis on total cell lysate showed glutamate toxicity resulted in significant upregulation of Bax (1.8 folds) and Bak (1.74 folds) in neurons compared to control condition (Fig. 5 E-F). Treatment with Nrg-1β1 was able to reverse glutamate-induced upregulation of Bax and Bak to their baseline levels in neurons. We next investigated the involvement of mitochondrial voltage-dependent anion channel 1 (VDAC1) in glutamate mediate cell death and Nrg-1β1 effects. VDAC1 is located in the outer mitochondrial membrane and plays a key role in mitochondrial mediated apoptosis and intrinsic pathway through its interactions with Bax and Bak (339, 340). Our assessment showed glutamate toxicity significantly promoted the expression level of VDAC1 (2.5 folds) in neurons compared to control condition, which was reduced to its baseline levels under 10 and 50ng/ml of Nrg-1β1 (Fig. 5G). Notably, in all the above assessments, addition of Nrg-1β1 peptide *per se* had no effect on neurons compared to control baseline condition.

Mitochondrial fragmentation is a hallmark of apoptotic cell death in primary neurons (341). Therefore, we further evaluated the effects of Nrg-1β1 on mitochondrial morphology by using

MitoTracker, which its uptake depends on mitochondrial membrane potential. Under control condition, 67%% of neurons showed tubular mitochondria, while 13% displayed fragmented structure (Fig.6 A, B). Glutamate toxicity significantly reduced tubular mitochondria by 42%, while increased fragmented mitochondria by 44.3% (Fig.6 A, C). The highest concentration of Nrg-1 (50ng/ml) was able to significantly preserve the tubular structure of mitochondria (45.63%) comparing to glutamate condition (24.51%) (Fig.6 A, D). Number of neurons which exhibited fragmented mitochondria structure was also significantly reduced by 26.2% and 23.5% with 25ng/ml and 50ng/ml of Nrg-1 conditions, respectively (Fig. 6A).

In conclusion, we demonstrate availability of Nrg-1β1 can attenuate glutamate mediated cell death in neurons through modulation of both caspase-dependent and -independent pathways, which can result from reduction in expression levels of mitochondrial membrane permeabilizers.

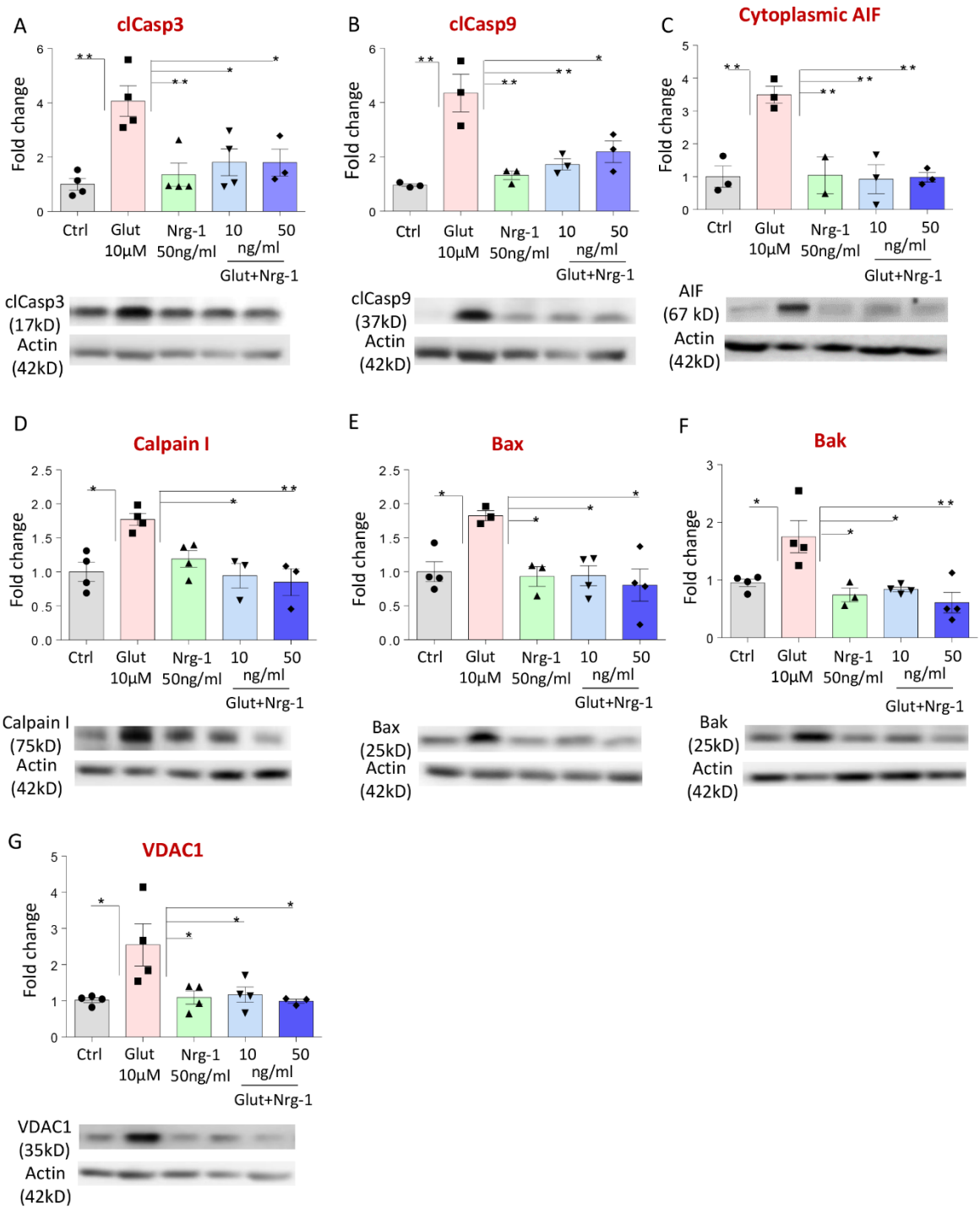


Figure 5: Nrg-1 ß1 attenuates glutamate-induced apoptosis in neurons through caspase and mitochondrial mediated mechanisms. Western blot analysis of apoptosis was performed on cultured primary rat mature cortical neurons treated with Nrg-1ß1 (10 and 50ng/ml) following 1h

glutamate challenge (10µM). (A) Glutamate treatment resulted in significant increase in cleaved caspase 3 (clCasp3) expression in neurons, and both low and high concentrations of Nrg-1ß1 significantly reduced caspase 3 activation. (B) Nrg-1ß1 also significantly reduced the expression of clCasp9 in glutamate treated neurons suggesting that Nrg-1ß1 mediates its effects through intrinsic apoptosis pathway. (C) Western blot was also performed to assess caspase-independent pathway of apoptosis under glutamate and Nrg-1ß1 treatment. Analysis of cytoplasm fraction in cortical neurons demonstrated a significant increase in the expression of apoptosis inducing factor (AIF) in glutamate condition, which was significantly decreased after Nrg-1ß1 treatment. (D) Glutamate challenge also significantly enhanced the expression level of calpain I, which was reduced to the baseline level in Nrg-1ß1 treated neurons. (E-F) Western blot analysis of Bax and Bak, factors involved in mitochondrial membrane permeabilization showed a significant increase in their levels after exposure to glutamate. Both concentrations of Nrg-1ß1 significantly reversed the glutamate-induced level of these factors to the normal levels. (G) Expression of voltage dependent anion channel 1 (VDAC1) protein was also induced by glutamate challenge and Nrg-1ß1 treatment returned its levels to the normal baseline levels. The data represent mean ±SEM. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to control condition. *$p < 0.05$, One-way ANOVA, followed by Holm–Sidak post hoc test. N=3-4 independent experiments).

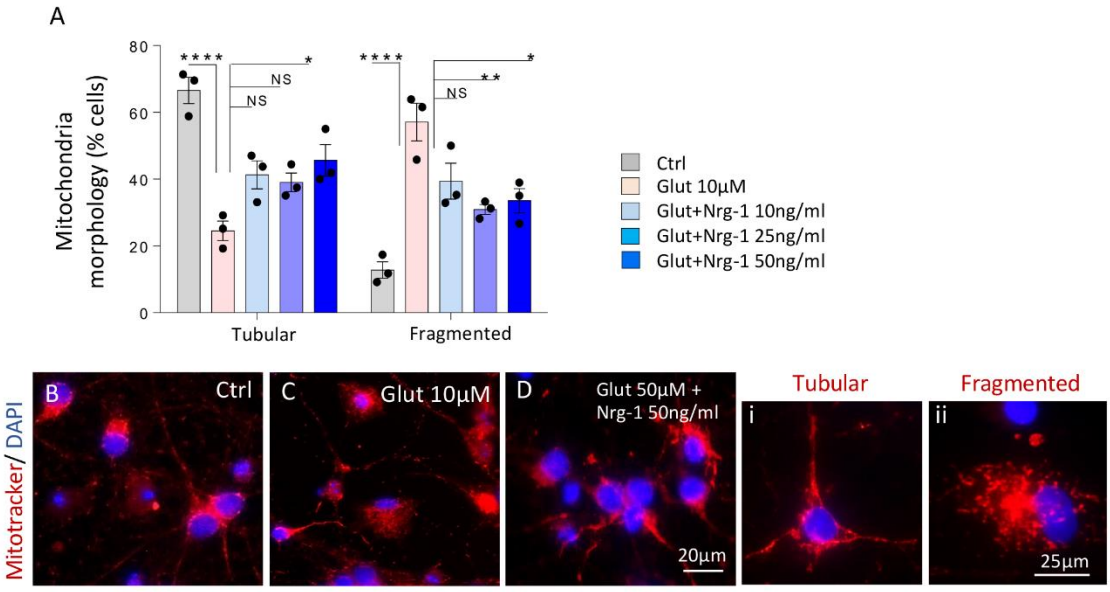


Figure 6: Nrg-1ß1 attenuates glutamate-induced mitochondrial fragmentation. (A) Morphometric assessments of mitochondria showed a significant increase in mitochondria fragmentation following glutamate toxicity. Nrg-1ß1 treatment at 25 and 50ng/ml significantly preserved mitochondrial tubular structure compared to glutamate-treated condition. (B- D) Representative images of mitochondrial morphology under different conditions are depicted. The magnified views of tubular (i) and fragmented (ii) morphology are shown. Mitotracker and DAPI signals are shown in red and blue, respectively. The data represent mean ±SEM. Two-way ANOVA, followed by Holm–Sidak post hoc test. *p < 0.05 indicates where the data was significant. N=3

2.4.6 Neuroprotective effects of Nrg-1β1 against glutamate toxicity is mediated by MAP kinase and AKT pathways.

We sought to identify the intracellular pathways involved in neuroprotective effects of Nrg-1β1 on neurons. We first focused on the mitogen-activated protein kinase (MAPK) pathway

including its major signaling cascades c-Jun N-terminal kinase (JNK) and P38 that are known to mediate the activation of downstream pro-apoptotic Bax and Bak (342). Using Western blotting, we assessed phosphorylation (activation) status of JNK and P38 in neurons after glutamate exposure with and without Nrg-1β1 treatment. Analysis of the ratio of phosphorylated to total P38 and JNK showed glutamate significantly induced phosphorylation of both P38 and JNK (2 folds and 3.5 folds, respectively), while the total amount of these proteins remained unchanged. Treatment with 50ng/ml of Nrg-1β1 significantly attenuated glutamate-induced phosphorylation of P38 by 51.7% (Fig. 7 A). Likewise, increase in JNK phosphorylation after glutamate exposure was significantly attenuated under 10 and 50ng/ml of Nrg-1β1 treatment by 60% and 43.7%, respectively (Fig. 7 B).

We further evaluated involvement of the extracellular-signal-regulated kinase (ERK) pathway, which is another major cascade in MAPK signaling. ERK1/2 pathway is also a known downstream effector in Nrg-1 signaling cascade (343, 344). Western blot analysis demonstrated a significant decrease in phosphorylated form of ERK1/2 following glutamate treatment (37%). Treatment of cortical neurons with 10ng/ml of Nrg-1β1 significantly recovered the reduced phosphorylated levels of ERK1/2 after glutamate exposure by 43.5% (Fig. 7 C). Furthermore, we assessed the effect of Nrg-1β1 on the AKT (serine/threonine protein kinase B), which is also a major downstream pathway in Nrg-1 signaling (278, 305, 345). Glutamate exposure resulted in a significant reduction in AKT phosphorylation (37%) in neurons compared to the baseline control condition, and Nrg-1β1 treatment at 50ng/ml of Nrg-1β1 was able to significantly restore the phosphorylation level of AKT to the normal level (Fig. 7 D). These results indicate that Nrg-1β1 mediates its neuroprotective effects by regulating several intracellular pathways affected by glutamate-mediated injury in neurons.

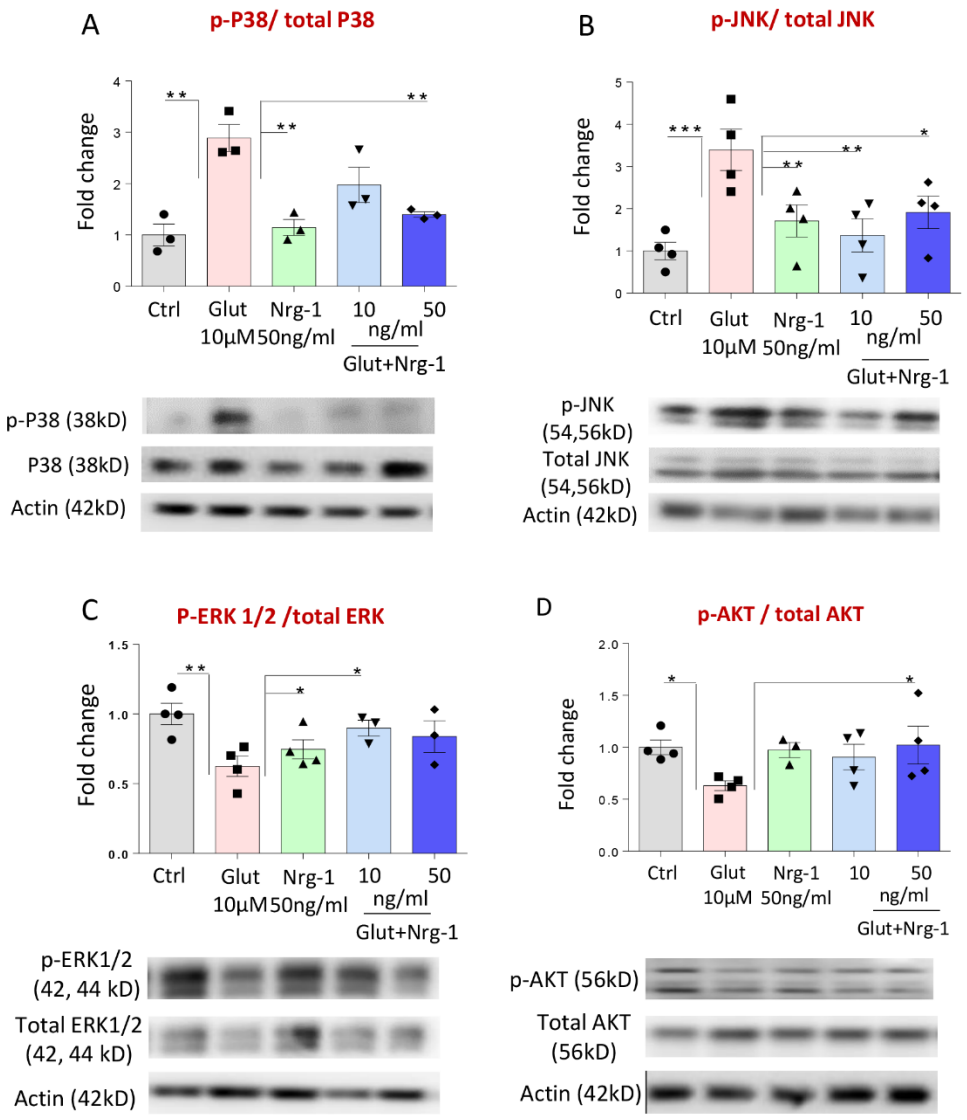


Figure 7: Nrg-1ß1 protection of neurons against glutamate toxicity is mediated by MAPK and AKT pathways. (A-D) Immunoblotting was performed to assess phosphorylation levels of P38, JNK, ERK and AKT in cortical neurons under various conditions. (A-B) Ratio of phosphorylated P38 and JNK to total P38 and JNK was significantly increased following glutamate challenge, and treatment of neurons after glutamate exposure with Nrg-1ß1 significantly reduced their phosphorylated levels compared to the non-treated glutamate condition. (C, D) Conversely,

ratio of phosphorylated ERK1/2 and AKT to total ERK1/2 and AKT was reduced following glutamate challenge, and 10ng/ml and 50ng/ml of Nrg-1ß1 treatment significantly increased phosphorylation of AKT and ERK1/2, respectively. The data represent mean ±SEM. For each condition, the ratio of phosphorylated levels of the protein to total protein is depicted. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to control condition. * $p < 0.05$, N=3-4, One-way ANOVA, followed by Holm–Sidak post hoc test.

2.5 Discussion

Traumatic SCI represents a complex dysregulated microenvironment, which induces significant neurodegeneration. Neurons in particular are susceptible to their environmental changes after injury due to their high metabolic demands and dependency on a large cellular and molecular network that regulates their homeostasis and function (346). Unraveling molecular mechanisms that directly impact neurons in the hostile milieu of the injured spinal cord would allow development of targeted neuroprotective strategies for SCI.

In the present study, we report for the first time that injury-induced dysregulation of endogenous Nrg-1β1 contributes to neuronal injury, and its therapeutic restoration can protect neurons from oxidative damage and cell death in rats with compressive/contusive SCI. Using parallel primary *in vitro* studies, we provide direct evidence that Nrg-1β1 can support neurons against glutamate induced toxicity by modulation of various mitochondrial and intracellular pathways in neurons. Mechanistically, Nrg-1β1 attenuate both caspase-dependent and AIF mediated apoptosis that is partially mediated through modulation of MAPK and AKT pathways.

Collectively, our work has uncovered a protective role for Nrg-1β1 in SCI that can directly affect several injury mechanisms in neurons.

Nrg-1β1 is well-known for its critical roles in the developing central and peripheral nervous system (305). In recent years, growing evidence from our group and others has uncovered important roles for Nrg-1β1 in the pathologic CNS. Our original studies in SCI discovered a pronounced decline in the transcript and protein levels of Nrg-1β1 in the spinal cord lesion within one day after SCI that persisted into chronic stage (228). Nrg-1β1 is highly expressed by neurons and oligodendrocytes in the spinal cord and is mainly localized in axons (228); therefore, its reduced levels within the SCI lesion likely reflect the loss of Nrg-1β1 expressing cells. We showed downregulation of Nrg-1β1 has functional impact on the repair process, as its partial restoration through intrathecal or systemic delivery was sufficient to promote oligodendrogenesis and remyelination (227, 228), and positively modulated astrocyte response and glial scar formation after SCI (278). Most notably, Nrg-1 has emerged as a novel immunomodulatory mediator in CNS injury and diseases. Our previous studies in rat SCI showed Nrg-1β1 treatment can convert the pro-inflammatory response of microglia and infiltrating leukocytes to a pro-regenerative, anti-inflammatory phenotype (279). Intriguingly, Nrg-1β1 treatment remarkably induced the release of interleukin-10 in SCI that is a key pro-regenerative cytokine (278). Importance of Nrg-1 in immune regulation has been also reported in other neurological conditions such as MS and Schizophrenia (310, 316, 347). In MS models, our recent findings identified an association between downregulation of Nrg-1β1 and immune dysregulation and disease progression (310). Nrg-1β1 is also shown to promote neuronal survival in models of cerebral ischemia (293) as well as motoneuron degeneration *in vitro* (348) and *in vivo* (349, 350). Taken together, current evidence suggests a beneficial immunomodulatory role for Nrg-1β1 that seems to indirectly protect neurons

from inflammatory-induced injury. In the present study, we have provided new evidence that Nrg-1β1 can also directly regulate neuronal response to injury.

Our current study unraveled that Nrg-1β1 attenuates the SCI-induced levels of HMGB1 in the spinal cord. HMGB1 is a non-histone nuclear protein released by glia and neurons upon injury or inflammation, and activates receptors for advanced glycation end products (RAGE) and toll-like receptor (TLR) 4 on the target cells (351). HMGB1/TLR4 axis is a key initiator of neuroinflammation in many CNS injuries including SCI and traumatic brain injury (351, 352). Increased expression of HMGB1 in the injured spinal cord of mouse triggers microglial activation and neuronal death in the ventral horn (353). HMGB1 inhibition has also reduced acute edema and glial activation in a rat model of SCI (354). Moreover, early administration of anti-HMGB1 antibodies within six hours after SCI in mice can promote functional recovery through protection of blood-spinal-barrier and suppression of inflammation (355). Importantly, HMGB1 is elevated systemically in patients with acute and chronic SCI (356). Thus, the efficacy of Nrg-1β1 in attenuating HMGB1 in our current work provides a putative mechanism for its immunomodulatory effects in our previous studies (279), and suggests its promise as a therapeutic target for minimizing neurodegeneration after SCI.

ROS production and oxidative stress have a significant role in the pathophysiology of SCI (213, 357, 358). Excessive generation of free radicals under pathophysiological conditions such as SCI can enhance ROS production that results in alteration of proteins, DNA and poly unsaturated fatty acids in membrane lipids (213, 357). Lipid peroxidation is a common and deleterious type of ROS-induced cellular oxidation (359). The efficacy of Nrg-1β1 in reducing ROS in the injured spinal cord is intriguing, as targeting oxidative stress has been considered a viable neuroprotective strategy for SCI (360). While the underlying mechanisms by which Nrg-1β1 regulates ROS

production in SCI needs further elucidations, recent studies in thyroid cancer suggest Nrg-1 modulates ROS levels by regulating ERK pathway and nuclear factor E2-related factor 2 (NRF2) (361). Interestingly, our present data identified ERK as a downstream mediator of Nrg-1β1 effects in neurons. Thus, it is plausible that NRF2 could be a putative downstream mediator for Nrg-1β1/ERK to maintain neuronal redux status in injury, which requires further investigations. Increased oxidative stress after SCI also leads to upregulation of matrix metalloprotease-9 (MMP-9) and disruption of blood-spinal-barrier that trigger apoptosis (362). Interestingly, our previous studies showed Nrg-1β1 administration can remarkably attenuate MMP-9 activity in SCI (278), which can provide a mechanism by which Nrg-1β1 reduced ROS formation and lipid peroxidation after SCI in the present study.

Another major finding of this work was identifying the role and mechanisms of Nrg-1β1 in regulating apoptosis in the context of SCI and under glutamate toxicity. Apoptosis is a key cell death pathway in rodent and human SCI that impacts both neurons and glia (363, 364). Inhibition of apoptosis has been an attractive protective strategy for a variety of neurodegenerative diseases and traumatic injuries including SCI (365-367). For example, peptide-based caspase inhibitors prevent neuronal loss in animal models of head injury and stroke (366-369). Protective role of Nrg-1 against neuronal injury and apoptosis has been also shown in motoneuron degeneration (348) and focal cerebral ischemic models (293, 295, 298), suggesting a broader role for Nrg-1β1 in CNS injuries. In our studies, Nrg-1β1 treatment ameliorated caspase-3 mediated apoptosis in neurons after SCI and *in vitro* under glutamate toxicity. Interestingly, these *in vivo* and *in vitro* data collectively determined that Nrg-1β1 seems to inhibit apoptosis primarily through modulation of intrinsic apoptotic pathway as it reduced caspase 9 activation with no significant effects on the activity of caspase 8. It is well-established that activation of Fas receptors and caspase-8 drives

extrinsic apoptosis pathway following SCI that contributes to cell death (370) (371-375). Our study also showed a dramatic increase in the expression levels of cleaved caspase 8 after SCI; however, Nrg-1β1 had no significant effects on caspase 8 activity. Interestingly, in our direct *in vitro* studies, glutamate injury did not induce caspase 8 activation in neurons, while it increased cleaved caspase 9 and caspase 3, which were attenuated by Nrg-1β1. The unchanged level of active caspase 8 was, however, expected as inflammation is considered the main trigger of extrinsic apoptosis pathway in SCI. Therefore, our *in vitro* injury model did not allow to directly assess the effects of Nrg-1β1 on extrinsic apoptosis pathway in neurons. Nonetheless, our SCI studies indicate that Nrg-1 does not affect the overall activation of caspase 8 in the injured spinal cord tissue.

Our findings showed neuroprotective effects of Nrg-1β1 were more pronounced at 7-day and 6 weeks post-SCI with no significant effects on cell death at the 3-day time-point during the acute stage of SCI. This can be due to the robust pro-inflammatory responses in acute SCI that may override the protective effect of Nrg-1β1. This observation is in line with another study from our group in which we demonstrated that Nrg-1β1 treatment can significantly subsidize the pro-inflammatory response at 7 days and 42 days after SCI but had no significant effects on the levels of pro-inflammatory mediators at 3 days after injury (Alizadeh 2018). Therefore, neuroprotective effects of Nrg-1β1 in subacute and chronic SCI could be, at least in part, due to the immunomodulatory effects of Nrg-1 at these time-points. Our findings also revealed that Nrg-1β1 confers neuroprotection mainly at distances further from the epicenter. While further elucidation is needed, this may reflect the severity of our injury model and the force of primary mechanical injury that results in extensive neuronal damage at the injury epicenter due to the trauma-induced mechanical injury. However, cell death spreads rapidly in the gray matter rostrally and caudally in acute SCI and continues during the subacute stage. Our data suggest that Nrg-1β1 treatment

appears to be most effective in preventing the extent of neuronal and oligodendroglia cell death in the adjacent areas that are not impacted mechanically by the primary trauma. Additionally, axon degeneration in descending and ascending tracts and loss of connectivity with their target neurons within the spinal cord can potentially contribute to neuronal cell death in distant areas to the epicenter. Our previous studies showed that Nrg-1β1 treatment preserves axons and white matter after SCI (228, 278). Thus, it is plausible that the positive effects of Nrg-1β1 in protecting spinal cord neurons may be partially due to the preservation of innervating axons in the white matter distant to the injury epicenter, which needs further investigations. Interestingly, our findings showed significant neuronal preservation at 1.5 mm below the lesion under Nrg-1β1 treatment. It is well known that integrity of neurons caudal to the spinal cord lesion is critical for re-establishment of spinal cord circuitry (376, 377) and successful regain of spinal cord reflexes and locomotion circuitry below the level of the injury (378, 379). Importantly, preservation of neurons caudal to the SCI lesion also provides the essential neuronal targets for re-innervation by regenerating and/or sprouting axons of descending supraspinal and ascending sensory pathways as well as segmental spinal interneurons (376, 380).

Mechanistically, we provide direct evidence that Nrg-1β1 protects neurons by regulating mitochondria associated apoptotic events. Apoptosis can be elicited by various stimuli. Pathologic accumulation of ROS is a key trigger of apoptotic cell death by targeting mitochondria (381). As discussed earlier, our SCI studies revealed a positive correlation between reduced levels of ROS and a decrease in neuronal apoptosis in Nrg-1β1 treated rats. Upon oxidative injury, ROS is generated excessively in mitochondria, which induces lipid peroxidation and increases mitochondrial membrane permeability. Translocation of the pro-apoptotic factors Bax and Bak to mitochondria outer membrane facilitates membrane permeability and promotes the release of

cytochrome C and AIF to the cytoplasm that consequently trigger caspase-dependent and independent apoptosis, respectively (382). Accumulation of ROS also promotes oxidation of mitochondrial permeability transition pore (MPTP) components such as VDAC (383). VDAC1 is a major channel that facilitates calcium transport, and its dysregulation has been associated with apoptosis in many neuropathological processes (384). Blockade of VDAC1 oligomerization decreases the number of apoptotic cells after SCI (385). For the first time, we demonstrate Nrg-1β1 ameliorates mitochondrial outer membrane permeabilization in neurons under glutamate induced injury by reducing the elevated expression of Bax, Bak and VDAC1. This is further supported by a reduction in the cytoplasmic levels of AIF and calpain I in neurons under Nrg-1β1 treatment. AIF is known to induce apoptosis independent of caspase 3 activation, suggesting Nrg-1β1 can also modulate caspase independent cell death in neurons. Our findings also revealed that Nrg-1β1 attenuates cell death by modulating several pro-survival and pro-death intracellular pathways in neurons. We found Nrg-1β1 inhibits pro-death JNK and p38 pathways, while promoting pro-survival ERK and AKT signaling in neurons. JNK activation can inhibit anti-apoptotic factors such as Bcl-2 and Bcl-X_L, and instead enhance phosphorylation and activation of pro-apoptotic members of this family (381). Taken together, our findings suggest a comprehensive role for Nrg-1β1 in regulating multiple intracellular apoptosis mediators and pathways in injured neurons.

In conclusions, our parallel *in vivo* and *in vitro* studies have identified an important role for Nrg-1β1 in modulating oxidative damage and neuronal injury in early stages of SCI. We propose that Nrg-1β1 is a pro-survival factor for neurons and its acute dysregulation contributes to neurodegeneration after SCI. Increasing the tissue levels of Nrg-1β1 in the injured spinal cord can protect neurons against oxidative stress and apoptosis directly, and potentially by indirect

mechanisms through its known immunomodulatory effects. Of note, Nrg-1β1 peptide holds promising characteristics as a treatment strategy for SCI due to its desirable property that allow its entry to the spinal cord through the blood-spinal barrier (386) after intrathecal or systemic administration (228, 278, 279). Hence, our findings suggest the relevance and efficacy of Nrg-1β1 treatment as a potential neuroprotective strategy for SCI.

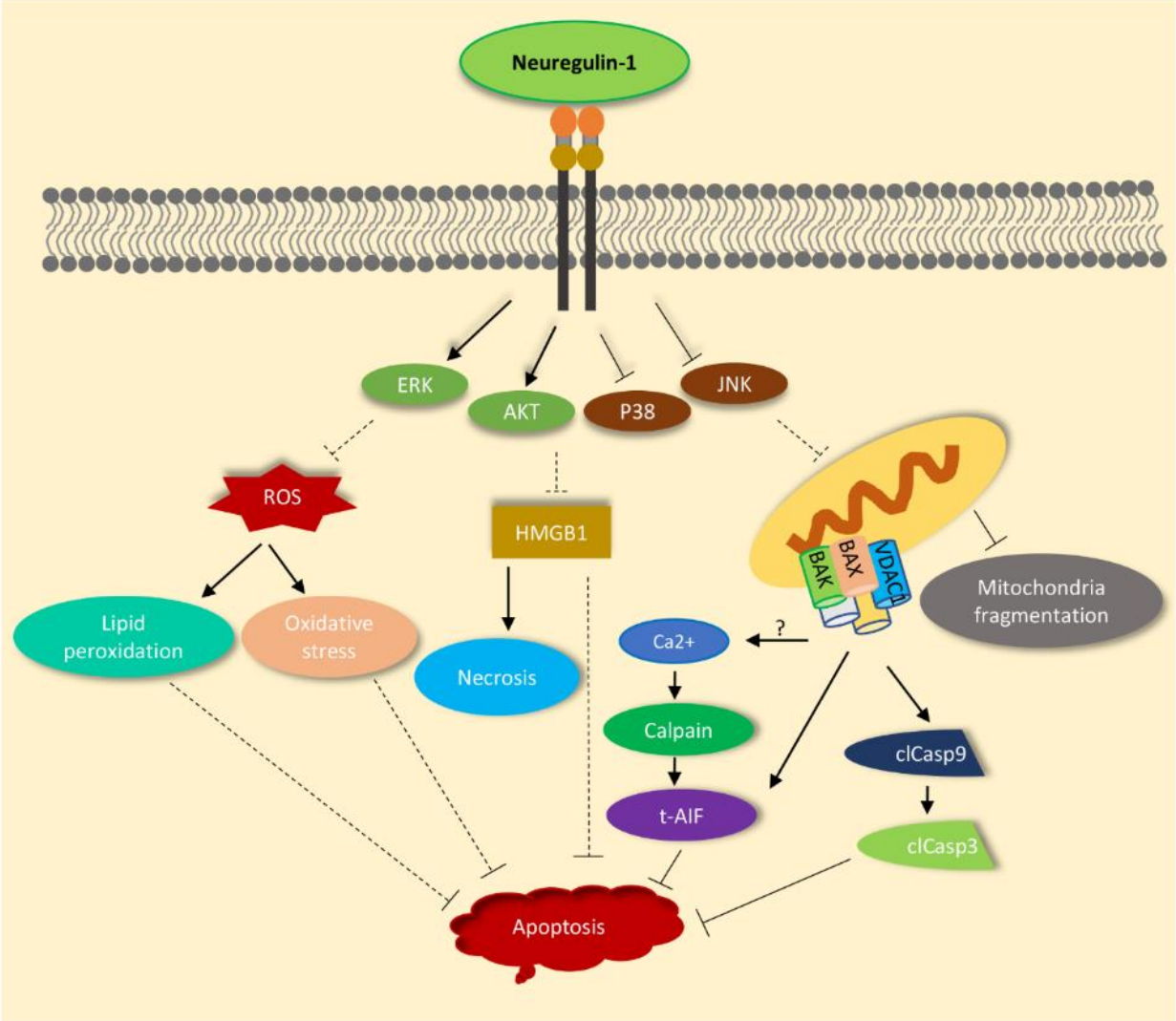
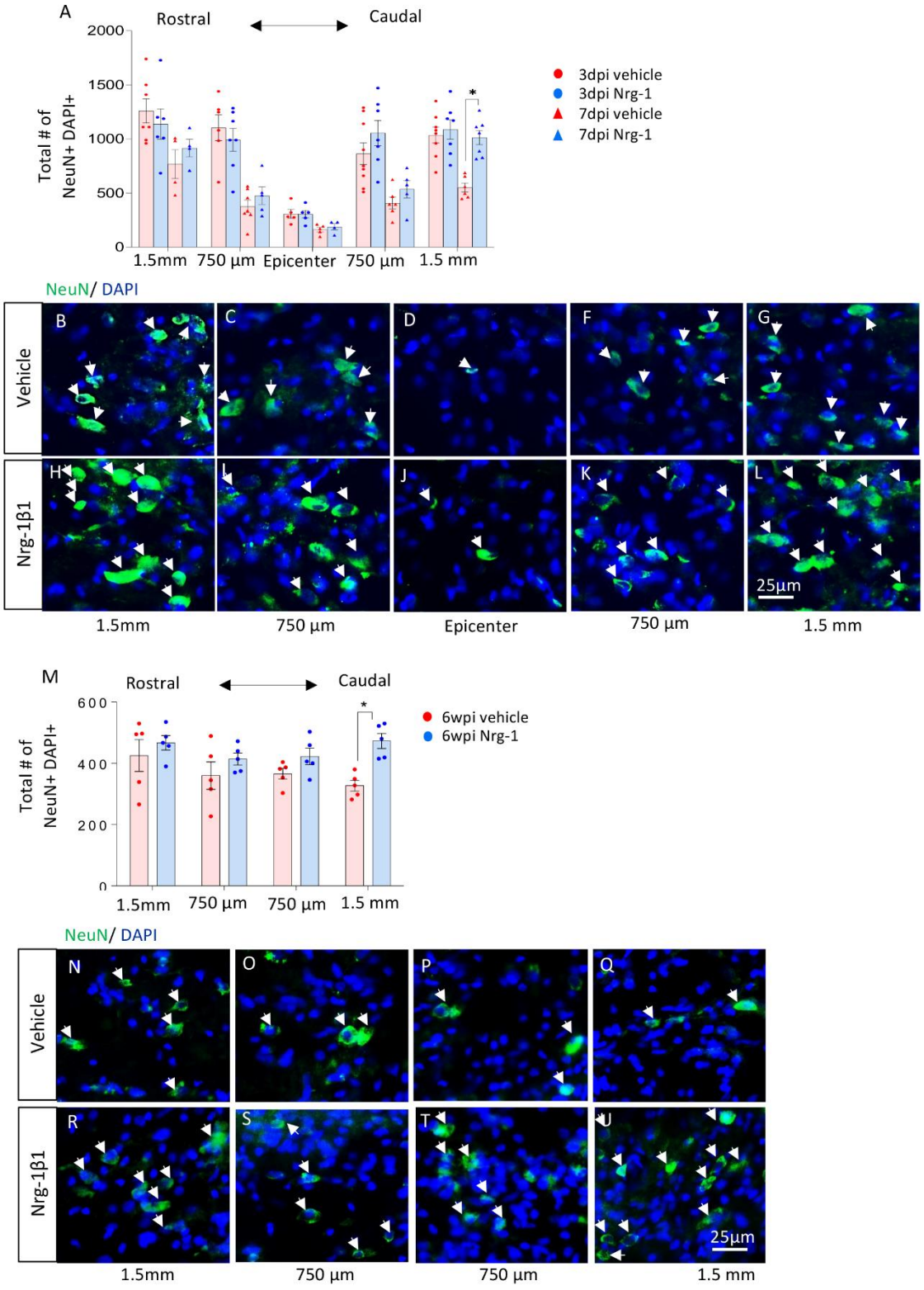


Figure 8: Summary of identified neuroprotective mechanisms of Nrg-1ß1 in this study.

Our *in vivo* and *in vitro* studies collectively show that bioavailability of Nrg-1ß1 attenuates formation of reactive oxygen species (ROS), which reduces lipid peroxidation and oxidate damage. This can also indirectly result in reduced apoptotic cell death. Presence of Nrg-1ß1 also decreases the injury-induced levels of high mobility group box1 (HMGB1). Nrg-1ß1 protects neurons through caspase-dependent and caspase-independent apoptosis following glutamate toxicity. This can be partially due to preserving mitochondrial morphology and reducing the expression levels of mitochondrial outer membrane permeabilizers (MOMPs), Bax, Bak and voltage dependent anion channel1 (VDAC1). Reduction in mitochondria permeabilization prevents cytosolic translocation of proapoptotic factors resulting in lower levels of apoptosis inducing factor (AIF) and cleaved caspase-3 (clCasp-3). We have demonstrated that Nrg-1ß1 provides neuroprotective effects through MAPK signaling pathway by inhibiting JNK and p38 pathways and promoting AKT and ERK pathways.


A
Rostral
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Total # of NeuN+ DAPI+
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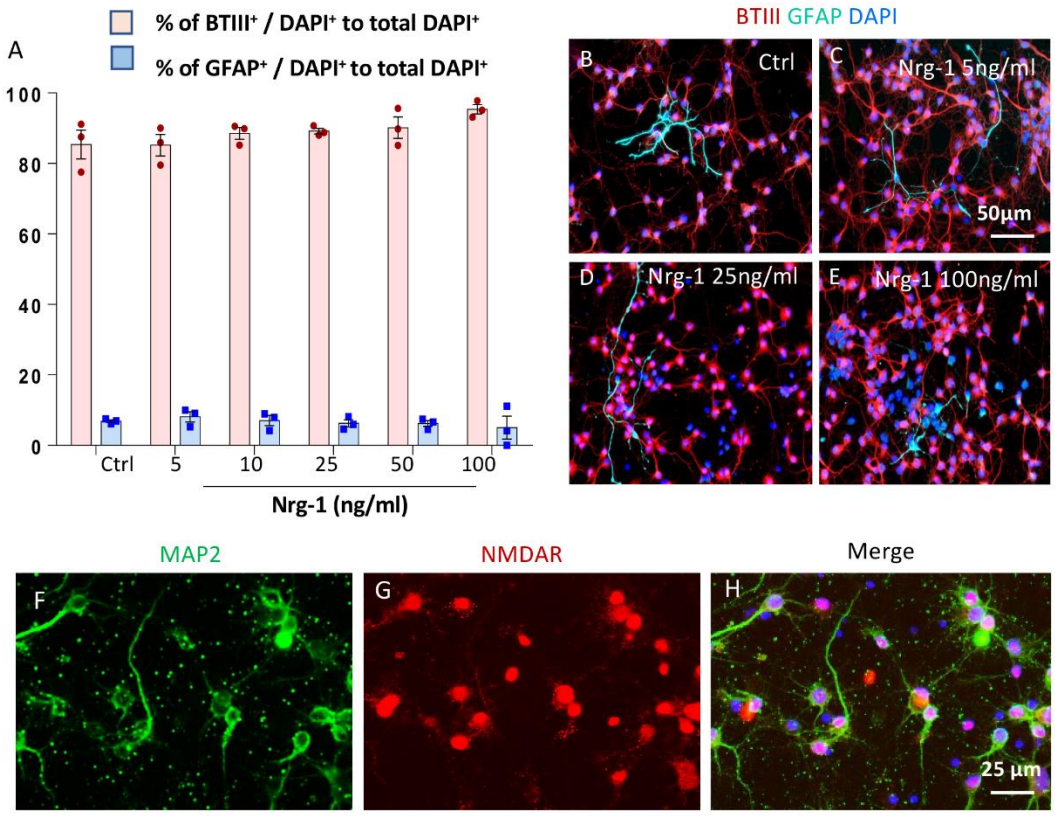
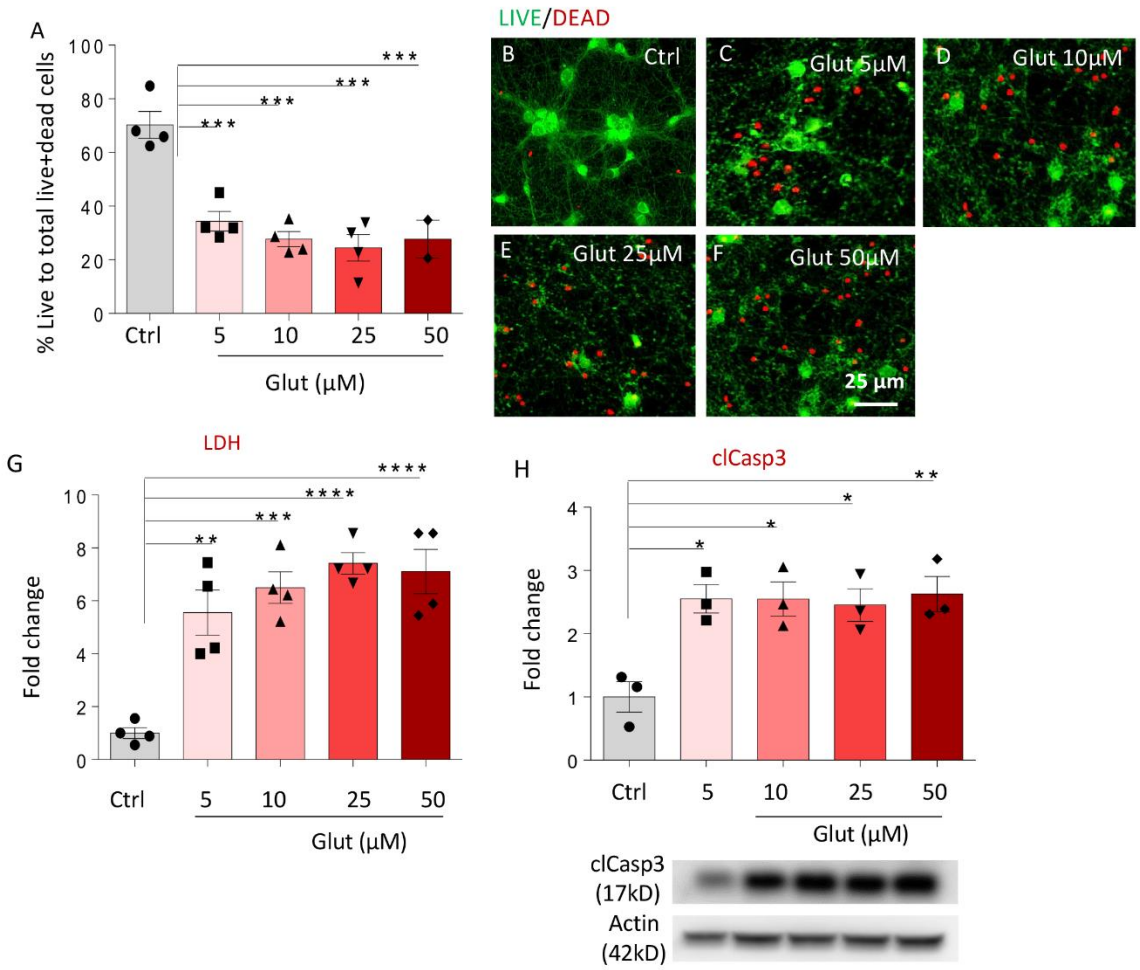
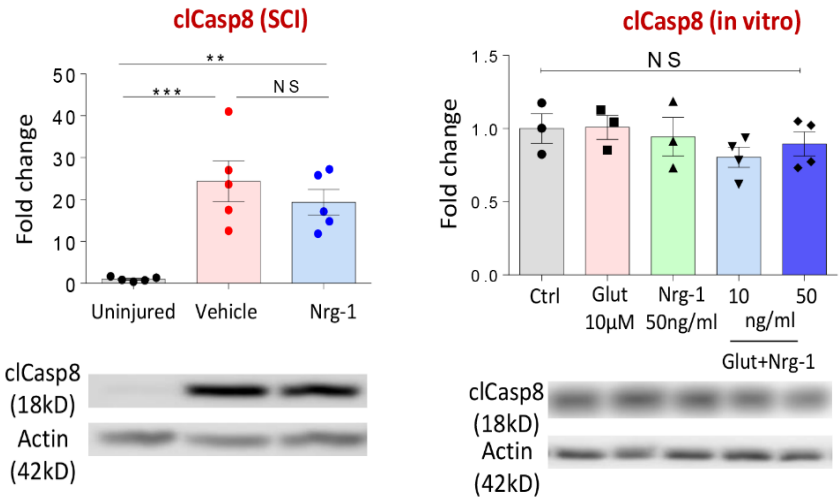
Supplementary Figure 1: Nrg-1β1 treatment promotes neuronal preservation below the level of injury at subacute and chronic stages of SCI: (A-U) Immunostaining of spinal cord cross sections at 3day, 7day and 6week post injury at epicenter, 750µm and 1.5mm rostral and caudal to the injury center in vehicle and Nrg-1β1 treated animals for the neuronal marker NeuN (neuronal nuclei). (A) Neuronal cell count ($NeuN^+/DAPI^+$) revealed Nrg-1β1 treatment significantly protects neurons at 1.5mm caudal to the injury epicenter at 7dpi with no significant changes at 3dpi. (B-L) Representative immunostaining images of NeuN and DAPI are depicted for vehicle and Nrg-1β1 treated animals at 7dpi. Green color represents NeuN signal. The data represent mean ±SEM. Three-way ANOVA followed by Holm–Sidak post hoc test and $*p < 0.05$ indicates where the data was significant. N= 4-8 rat/ group. (M) Neuron count was performed in spinal cord cross sections at 750µm and 1.5mm rostral and caudal to the epicenter in vehicle and Nrg-1β1 treated SCI rats at 6wpi. Nrg-1β1 treatment significantly promoted neuronal preservation at 1.5mm caudal to the injury epicenter at this time-point. (N-U) Representative immunostaining images of NeuN and DAPI are depicted. The data represent mean ±SEM. Two-way ANOVA followed by Holm–Sidak post hoc test. $*p < 0.05$ indicates where the data was significant. N= 5 rat/ group.



Supplementary Figure 2: Characterization of primary cortical neuron cultures. (A-E) Primary cultures of cortical neuron were immunostained for the general neuronal marker beta tubulin III (ßTIII) and the astrocytic marker glial fibrillary acidic protein (GFAP). (A) Cell quantification confirmed over 85% $BTIII^{+}/DAPI^{+}$ neurons under control and Nrg-1ß1 treated conditions (5-100ng/ml). (B-E) Representative immunostaining images of ßTIII and GFAP are depicted. (F-H) Maturity and responsiveness of cortical neurons to glutamate was also confirmed by immunostaining for mature neuronal marker, microtubule associated protein-2 (MAP-2) and N-Methyl-D-aspartic acid or N-Methyl-D-aspartate receptor (NMDAR). The data represent mean ±SEM, * $p < 0.05$, N=3, One-way ANOVA, followed by Holm–Sidak post hoc test).



Supplementary Figure 3: Proof-of-concept data confirm the efficacy of glutamate treatment in inducing cell death and injury in cortical neurons. We performed a pilot study to determine an effective concentration of glutamate that can significantly induce neuronal death and injury *in vitro*. (A-H) Neurons were exposed to various concentration of glutamate (5, 10, 25 and 50 µM**)** for 1h. After 8h, LIVE/DEAD and LDH assays as well as Western blotting for clCasp3 were performed. Our quantitative analyses showed that 10µM of glutamate is sufficient to significantly induce cell injury and apoptosis in neurons. The data represent mean ±SEM. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to control condition. *p < 0.05, One-way ANOVA, followed by Holm–Sidak post hoc test. N=3-4 independent experiments).



Supplementary Figure 4: Assessment of clCasp-8 expression *in vitro* and *in vivo*: (A) We performed Western blot analysis to assess the expression level of clCasp-8 at 7dpi. Our results revealed a significant increase in the levels of clCasp-8 (24.35 folds) at 7dpi in vehicle treated rats after SCI compared to uninjured spinal cord tissue. Nrg-1β1 treatment had no significant effects on the expression level of clCasp-8. (B) We further assessed clCasp-8 expression in Nrg-1β1 (10-50ng/ml) treated cortical neuron cultures following glutamate exposure (10μM). Our findings showed no significant changes in the expression levels of clCasp-8 in glutamate and Nrg-1β1 treated conditions comparing to non-treated control condition. Actin was used as a loading control for Western blot analysis and results are shown as fold change relative to control condition. * $p < 0.05$, N=3-4, One-way ANOVA, followed by Holm–Sidak post hoc test.

Chapter III: Discussion

3.1 General overview of findings and discussion

The purpose of this study was to determine the neuroprotective effects of Nrg-1β1 in SCI and more specifically, role and mechanisms of Nrg-1β1 in regulating neuronal response to injury. We have employed a pre-clinical compressive/contusive model of SCI in rats in parallel with an SCI relevant *in vitro* model of glutamate toxicity in primary cortical neurons. In SCI, we have demonstrated that availability of Nrg-1β1 ameliorates oxidative stress by attenuating of ROS formation and lipid peroxidation. Moreover, increased bioavailability of Nrg-1β1 significantly reduces the tissue level of HMGB1, a marker of cell damage and necrosis. Our SCI and *in vitro* glutamate injury studies also identified that Nrg-1β1 can remarkably attenuate apoptosis by reducing clCasp3 expression. Interestingly, the anti-apoptotic effects of Nrg-1β1 were primarily mediated by the inhibition of mitochondria mediated intrinsic apoptosis pathway because it decreased clCasp9 expression but not clCasp8 (the caspase activated in the extrinsic pathway) . In our studies, we found no beneficial effect of Nrg-1β1 on autophagy or necroptosis suggesting that modulating apoptosis is one of the specific mechanisms of Nrg-1β1 in its neuroprotective effects in SCI and specifically in neurons. Interestingly, our findings in SCI indicated that Nrg-1β1 effects are more pronounced in the subacute stage of injury, where apoptosis is underway in the injured spinal cord. Importantly, the neuroprotective effects of Nrg-1β1 are long-lasting and results in significant preservation of neurons in chronic SCI.

Given the significant anti-apoptotic effects of Nrg-1β1, we further dissected the underlying mechanisms by which it regulates apoptosis using our *in vitro* glutamate induced injury model in primary cortical neurons. Our study revealed that Nrg-1β1 modulates both caspase-dependent and caspase-independent mitochondrial apoptosis pathways. We found that Nrg-1β1 preserves the

structure of mitochondria and attenuates the mitochondrial outer membrane permeabilization that collectively leads to reduced apoptosis in neurons. Mechanistically, protective effects of Nrg-1β1 is partially mediated through modulation of MAPK and AKT pathways. Collectively, our work has uncovered a protective role for Nrg-1β1 in SCI that can directly affect several injury mechanisms in neurons.

3.1.1 Nrg-1 and neuronal injury

In the past few years, Nrg-1β1 has emerged as an important modulator of the nervous system injury and repair. To date, Nrg-1β1 has shown neuroprotective effects in several types of CNS injuries such as SCI (228, 278), cerebral ischemia (345, 387), ALS (348), AD (311) and PD (388, 389), and MS (290). Most of these studies, including previous reports from our own lab in SCI and MS, described an immunomodulatory, anti-inflammatory role for Nrg-1 that resulted in tissue preservation following injury. One study before this thesis also demonstrated that Nrg-1 attenuates apoptosis by reducing the levels of caspase-3 in neurons following ischemic injury in the brain (293). In ALS disease, administration of Nrg-1 type III improves motoneuron survival and protects them from degeneration (350). Administration of Nrg-1 also increases preservation of dopaminergic neurons in PD (388, 389). *In vitro* studies suggest that Nrg-1 mediate neuroprotective effects through activation of PI3 kinase/Akt activation by enhancement of autophagy (348, 390). Phosphorylation of Akt has been also reported in neurons and glial cells following cerebral ischemia and in response to Nrg-1 treatment (292). Altogether, protective roles of Nrg-1 have been observed in several injury models *in vitro* and *in vivo*. However, prior to this thesis, the potential role and mechanisms of Nrg-1 in regulating oxidative stress and cells death in

SCI was largely unknown. In following section, I will specifically discuss the role of Nrg-1 on apoptotic cell death.

3.1.2 Role of Nrg-1 in regulation of apoptosis

One major finding of this work was identifying the role and mechanisms of Nrg-1β1 in regulating apoptosis in the context of SCI and under glutamate toxicity. Apoptosis is a key cell death pathway in rodent and human SCI that impacts both neurons and glia (363, 364). As described earlier, the ratio of Bax/Bcl2 determines inhibition or progression of apoptosis (391). Within 24hrs after SCI, the expression level of Bax is significantly increased while Bcl2 is drastically reduced (392). Reduced expression of Bcl2 can lead to less physical binding to pro-apoptotic protein and more Bax/Bak oligomerization resulting in permeabilization of mitochondrial membrane and progression of apoptosis. Therefore, it is plausible that Nrg-1β1 may be involved in regulation of gene expression of pro/ anti- apoptotic proteins. Nrg-1 can also indirectly regulate apoptosis via its immunomodulatory functions. Within minutes following SCI, the expression level of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 is dramatically increasing in the injured spinal cord rostro-caudally, which together triggers the inflammatory responses (393). The expression level of both TNFR1 and TNFR2 are also increased as early as 15min and peak at 8hrs post SCI (394). The presence of TNF-α further exacerbates the glutamate mediated neuronal death via inhibition of glutamate transporters (EAATs) and by enhancing the expression of NMDA and AMPA receptors and $Ca2^{+}$ influx (395).

Regardless of the triggers, inhibition of apoptosis has been an attractive protective strategy for a variety of neurodegenerative diseases and traumatic injuries including SCI (365-367). For example, peptide-based caspase inhibitors prevent neuronal loss in animal models of brain injury

and stroke (366-369), in motoneuron degeneration (348) and focal cerebral ischemic models (293, 295, 298), suggesting a broader role for Nrg-1β1 in CNS injuries. In our studies, Nrg-1β1 treatment ameliorated caspase-3 mediated apoptosis in neurons after SCI and *in vitro* under glutamate toxicity. Interestingly, these data collectively determined that Nrg-1β1 seems to inhibit apoptosis primarily through modulation of intrinsic apoptotic pathway as it reduced caspase 9 activation with no significant effects on the activity of caspase 8. It is well-established that activation of Fas receptors and caspase-8 drives extrinsic apoptosis pathway following SCI that contributes to cell death (370) (371-375). We also showed a dramatic increase in the expression levels of cleaved caspase 8 after SCI; however, Nrg-1β1 had no significant effects on caspase 8 activity. Interestingly, in our direct *in vitro* studies, glutamate injury did not induce caspase 8 activation in neurons, while it increased cleaved caspase 9, which was attenuated by Nrg-1β1. The unchanged level of active caspase 8 in our *in vitro* model was, however, expected as inflammation is considered the main trigger of extrinsic apoptosis pathway in SCI. Although our non-inflammatory *in vitro* injury model did not allow to directly assess the effects of Nrg-1β1 on extrinsic apoptosis pathway in neurons, our SCI *in vivo* model confirmed that Nrg-1β1 does not affect the activation of caspase 8 mediated apoptosis in the injured spinal cord tissue.

3.1.3 Nrg-1 indirect effects on apoptosis through modulation of HMGB1 release and oxidative injury

In addition to apoptosis, our study unraveled that Nrg-1β1 attenuates the SCI-induced levels of HMGB1 in the spinal cord. HMGB1 is a non-histone nuclear protein released by glia and neurons upon injury or inflammation, and activates receptors for advanced glycation end products (RAGE) and toll-like receptor (TLR) 4 on the target cells (351). HMGB1/TLR4 axis is a key

initiator of neuroinflammation in many CNS injuries including SCI and traumatic brain injury (351, 352). Increased expression of HMGB1 in the injured spinal cord of mouse triggers microglial activation and neuronal death in the ventral horn (353). HMGB1 inhibition has also reduced acute edema and glial activation in a rat model of SCI (354). Moreover, early administration of anti-HMGB1 antibodies within six hours after SCI in mice can promote functional recovery through protection of blood-spinal-barrier and suppression of inflammation (355). Importantly, HMGB1 is elevated systemically in patients with acute and chronic SCI (356). Thus, the efficacy of Nrg-1β1 in attenuating HMGB1 in our current work provides a putative mechanism for its immunomodulatory effects in our previous studies (279), and suggests its promise as a therapeutic target for minimizing neurodegeneration after SCI. Intracellularly, HMGB1 controls autophagic/ apoptotic cell death following inflammation (396). *In vitro* studies suggest that HMGB1 preserves pro-apoptotic protein beclin-1 and autophagic protein ATG5 from calpain mediated cleavage that leads to attenuation of apoptosis and autophagy in inflammatory conditions (396). Studies in myocytes reveals that HMGB1 and TNF-α leads to myocardial apoptosis through JNK activation under ischemic condition (397). Therefore, a decrease in the HMGB1 levels in response to Nrg-1 treatment can indirectly result in attenuation of apoptosis.

ROS production and oxidative stress have a significant role in the pathophysiology of SCI (213, 357, 358). Excessive generation of free radicals under pathophysiological conditions such as SCI can enhance ROS production that results in alteration of proteins, DNA and poly unsaturated fatty acids in membrane lipids (213, 357). Lipid peroxidation is a common and deleterious type of ROS-induced cellular oxidation (359). The efficacy of Nrg-1β1 in reducing ROS in the injured spinal cord is intriguing, as targeting oxidative stress has been considered a viable neuroprotective strategy for SCI (360). It is widely shown that ROS formation can indirectly induce apoptotic cell

death in different injury conditions (398-400). While the underlying mechanisms by which Nrg-1β1 regulates ROS production in SCI needs further elucidations, recent studies in thyroid cancer suggest Nrg-1 modulates ROS levels by regulating ERK pathway and nuclear factor E2-related factor 2 (NRF2) (361). Interestingly, our present data identified ERK as a downstream mediator of Nrg-1β1 effects in neurons. Thus, it is plausible that NRF2 could be a putative downstream mediator for Nrg-1β1/ERK to maintain neuronal redox status in injury, which requires further investigations. Increased oxidative stress after SCI also leads to upregulation of MMP-9 and disruption of blood-spinal-barrier that trigger apoptosis (362). Interestingly, our previous studies in SCI and MS showed that Nrg-1β1 administration can remarkably attenuate MMP-9 activity (278), which can be partially due to less ROS formation and lipid peroxidation that we demonstrated in the present study. Mechanistically, we have provided direct evidence that Nrg-1β1 protects neurons by regulating mitochondria associated apoptotic events. Pathologic accumulation of ROS is a key trigger of apoptotic cell death by targeting mitochondria (381). As discussed earlier, our SCI studies revealed a positive correlation between reduced levels of ROS and a decrease in neuronal apoptosis in Nrg-1β1 treated rats. Upon oxidative injury, ROS is generated excessively in mitochondria, which induces lipid peroxidation and increases mitochondrial membrane permeability. Translocation of the pro-apoptotic factors Bax and Bak to mitochondria outer membrane facilitates membrane permeability and promotes the release of cytochrome C and AIF to the cytoplasm that consequently trigger caspase-dependent and independent apoptosis, respectively (382). Accumulation of ROS also promotes oxidation of mitochondrial permeability transition pore (mPTP) components such as VDAC (383). VDAC1 is a major channel that facilitates calcium transport, and its dysregulation has been associated with apoptosis in other neuropathological processes (384). Blockade of VDAC1 oligomerization

decreases the number of apoptotic cells after SCI (385). For the first time, we demonstrate Nrg-1β1 ameliorates mitochondrial outer membrane permeabilization in neurons under glutamate induced injury by reducing the elevated expression of Bax, Bak and VDAC1. This is further supported by a reduction in the cytoplasmic levels of AIF and calpain1 in neurons under Nrg-1β1 treatment. AIF is known to induce apoptosis independent of caspase 3 activation, suggesting Nrg-1β1 can also modulate caspase independent cell death in neurons. Altogether, mitigation of ROS formation and lipid peroxidation could be possible indirect mechanisms underlying the anti-apoptotic effects of Nrg-1β1 in our *in vitro* and *in vivo* studies.

3.1.3 Intracellular mechanisms of Nrg-1

Our *in vitro* findings revealed that Nrg-1β1 attenuates cell death by modulating several pro-survival and pro-death intracellular pathways in neurons that are known to be activated following SCI. We found Nrg-1β1 inhibits pro-death JNK and p38 pathways, while promoting pro-survival ERK and AKT signaling in neurons. JNK has different isomers that are phosphorylated and activated following SCI peaking at 3 days post-injury (401, 402). JNK activation induces cell death by inhibiting anti-apoptotic factors such as Bcl-2 and Bcl-X_L, while enhancing phosphorylation and activation of pro-apoptotic members of this family (381).Activated JNKs also phosphorylate Bcl-2 and prevent their survival effects. Moreover, JNK triggers activation of c-Jun that can further activate Fas mediated cell death (403). The other function of JNK is phosphorylation of the pro-apoptotic protein Bad that can start apoptosis (404).

Other major pathways that regulate cell death in SCI are PI3K/ Akt and ERK/ MAPK (405). Akt is shown to regulate the expression of Bcl-2, cytochrome C and a number of pro-apoptotic proteins (406). It is demonstrated that expression of PI3K and Akt significantly increases

following SCI, which peaks at 1dpi and slowly deceases over the course of the injury but remains upregulated comparing to the normal condition (407). Phosphorylation of glycogen synthase kinase 3β (GSK-3β) that is the downstream effector of PI3K/ Akt pathway prevents the opening of mPTPs and release of cytochrome C to the cytoplasm (408). Following SCI, ERK/ MAPK is also upregulated which in turn results in upregulation of the survival related genes such as Bcl-2 family members (409). Upregulation of ERK/MAPK genes has been associated with reduction in mitochondrial permeability and cytochrome C release (409). Although growing body of evidence indicates the neuroprotective role for ERK following SCI (410, 411), there are other studies that suggest the involvement of this pathway in promoting injury mechanisms. For instance, blockage of ERK1/2 using RNA interference approach attenuates the expression of downstream calpain 1, which a reduction in astrogliosis following SCI (412). Nevertheless, ERK pathway seems to play differential roles after SCI in a context dependent manner. Interestingly, our previous studies in SCI showed that Nrg-1β1 treatment promotes ERK1/2 phosphorylation in the acutely injured spinal cord tissue (278). In the current study, we have confirmed the neuron specific effects of Nrg-1β1 in restoring the injury-induced inhibition of ERK1/2 *in vitro*. Taken together, our findings suggest that Nrg-1β1 regulates multiple intracellular apoptosis mediators and pathways in injured neurons.

In conclusion, our parallel *in vivo* and *in vitro* studies have identified an important role for Nrg-1β1 in modulating oxidative damage and neuronal injury in subacute and chronic stages of SCI. We propose that Nrg-1β1 is a pro-survival factor for neurons and its acute dysregulation contributes to neurodegeneration after SCI. Restoring the declined tissue levels of Nrg-1β1 in the injured spinal cord can protect neurons against oxidative stress and apoptosis directly, and potentially by indirect mechanisms through its immunomodulatory effects identified in previous

studies in our laboratory. Of note, Nrg-1β1 peptide holds promising characteristics as a treatment strategy for SCI due to its desirable property that allow its entry to the spinal cord through the blood-spinal barrier (386) after intrathecal or systemic administration (228, 278, 279). Hence, our findings suggest the relevance and efficacy of Nrg-1β1 treatment as a potential neuroprotective strategy for SCI.

3.2. Study limitations

Similar to all other research studies, our work also had some limitations that could be discussed to properly interpret the data. Addressing these limitations also provides directions for future studies to uncover new aspects of Nrg-1β1 protective mechanisms.

Limitations of this study are mainly related to our *in vitro* settings. We used glutamate excitotoxicity as a neuronal injury model in our *in vitro* system because of its relevance to the pathophysiology of SCI. However, it does not mimic the whole injury condition *in vivo*. Inflammation, ischemia and hypoxia also contribute to neurodegeneration after SCI. Therefore, other *in vitro* injury conditions such as oxygen-glucose deprivation and TNFα-induced cell death could also be tested individually or in combination to confirm neuroprotective effects of Nrg-1 treatment.

Another limitation of this study is related to the use of cortical neurons for *in vitro* studies. Although upper motoneurons in the cortex project to the spinal cord as the descending corticospinal tract and are subject to cell death due to damage to their axons, they are not directly affected by SCI. Therefore, use of spinal cord specific neurons would be more logical as a complementary *in vitro* model to specifically assess the impact of Nrg-1β1 on neurons in the injured spinal cord. We attempted this approach at the beginning; however, culture of postnatal

spinal cord neurons was not feasible due to the high degrees of contaminating glia and minimal number of neurons. To develop spinal cord neuron cultures, our laboratory has recently prepared spinal neurons (motoneurons and interneurons) from human directly reprogrammed neural precursor cells (NPCs). These NPC derived spinal cord neurons will be studied in future projects.

Another limitation was the use of primary embryonic cortical neurons since culturing postnatal cortical neurons is also challenging due to high levels of contaminating cells including astrocytes. However, these neurons allowed to mature in culture, and we confirmed their maturity.

It would have been informative to also specify the ErbB receptors that mediate the effects of Nrg-1β1 on neuronal cell death. However, previous studies in our laboratory showed targeting ErbB receptors individually is challenged by the lack of specificity of existing inhibitors for ErbB 2, 3 and 4 receptors. Most ErbB inhibitors show affinity for multiple ErbB receptors. Importantly, all these inhibitors also target ErbB1 or EGF receptor that disrupts EGF signaling resulting in non-specific results. Therefore, studying ErbB receptors would require genetic knockdown of each receptor or their combination.

3.3. Future directions:

In this study, we have characterized the role and functional impact of Nrg-1β1 in SCI and *in vitro* using human recombinant peptide that contains the functional EGF-like domain of Nrg-1 ligand. This pharmacological approach allowed us to partially restore the declined levels of Nrg-1 in SCI and also activate Nrg-1 signaling *in vitro*. Recently, we have generated a tamoxifen -inducible Cre^{ER} Nrg-1 knockout (KO) mouse model to study the importance of endogenous Nrg-1 in pathophysiology of SCI including its neuroprotective effects. $Nrg1^{fl/fl}$ mice have been bred with Cre mice to generate Nrg1fl/fl: CAG-Cre-^{ERTM} mice (Nrg-1 cKO). Importantly, the loxP sites flank

exons 6–8 encoding EGF-like domain, the functional domain of all Nrg-1 isoforms. With TAM, recombination results in ablation of EGF-like domain. Moreover, exogenous Nrg-1β1 treatment in Nrg-1 cKO animals can confirm the effect of exogenous Nrg-1 treatment without the presence of endogenous Nrg-1. In addition to future *in vivo* studies in Nrg-1 cKO, *in vitro* studies will be performed to evaluate the response of Nrg-1 KO neurons to injury condition. These loss of function studies will be complementary to our current studies and will help unravel the upstream and downstream factors that are functionally associated with Nrg-1.

To date, we have delivered Nrg-1 peptide at the time of SCI for various duration. However, delayed treatments, within hours, days or weeks, are more feasible for clinical management of SCI. Thus, future studies are needed to evaluate the efficacy of delayed Nrg-1β1 treatment after SCI. These studies can identify the window of opportunity for Nrg-1β1 treatment during the course of SCI. Since Nrg-1 exerts both neuroprotective and neuroregenerative effects, it is anticipated that its delayed administration would be still effective but with various outcomes. For example, neuroprotective treatments are mostly effective if given within hours after injury, while neuroregenerative treatments can be delayed further. It would be also interesting to assess the potential of Nrg-1 as an adjunct treatment with other cellular and pharmacological approaches.

3.4 Clinical implications of this study

Currently, there is no therapeutic strategy for SCI. The most effective approach is surgical decompression and administration of methylprednisolone sodium succinate (MPSS) as the only clinically- approved treatment for SCI patients (413-415). However, the long-term efficacy of this treatment has been questioned in the last two decades (416, 417). Given the complexity of the SCI

microenvironment, a multi-faceted approach is required to target several barriers to repair and protection (100, 418).

Our *in vivo* and *in vitro* studies have determined an important neuroprotective role for Nrg-1β1 in modulating oxidative damage and neuronal injury in subacute and chronic stages of SCI. Administration of Nrg-1β1 to the injured spinal cord can protect neurons against oxidative stress and apoptosis directly, and potentially by indirect mechanisms through its immunomodulatory effects. Therefore, our findings suggest the relevance and efficacy of Nrg-1β1 treatment as a potential neuroprotective strategy for SCI. Previous studies in our laboratory also identified beneficial effects of Nrg-1β1 in neuroregeneration as it can promote oligodendrogenesis and remyelination after SCI. Collectively, these findings suggest the therapeutic potential of Nrg-1β1 as a solitary or adjunct treatment strategy for SCI. Of note, a clinical grade of the recombinant human Nrg-1 peptide that is used in our studies has already received safety approval from the US Food and Drug Agency (FDA) for its evaluation in human trials for cardiac dysfunction. Nrg-1β1 can also readily enter to the CNS tissue through the blood-spinal barrier (386) after intrathecal or systemic administration (228, 278, 279). Therefore, Nrg-1β1 treatment has suitable characteristics that increases its translational feasibility for CNS therapeutics.

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