

## Tissue Engineering Approaches for Motor Neuron Pathway Regeneration

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### Abstract

During fetal development, a tightly-regulated spatio-temporal pattern of guidance cues directs and maintains motor neuron axonal growth along specific pathways to reach the target cells and tissues. However, an inflammatory environment resulting from an injury (e.g., trauma, stroke) or disease [e.g., amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), and hereditary spastic paraplegia (HSP)] leads to progressive degeneration of motor neurons and destruction of axonal tracts in the adult CNS. Failure to reinstate healthy axonal connections under these conditions can severely compromise locomotor function, resulting in muscle atrophy, paralysis and death. Annually, thousands of people are diagnosed with various motor neuron related injuries and diseases in the United States, and a majority of them succumb to this condition soon after. Efforts to regenerate and accurately re-establish the lost motor neuronal networks using drug delivery, gene therapy or stem cell transplantation yielded valuable information regarding disease progression and functional circuitry formation. Recent investigations using animal models identified the role of various genes and biomolecules involved in motor neuron and related subcerebral projection neurogenesis. These results suggest that developing motor neurons could be guided by a mixture of diffusible growth factors, through varying extracellular matrix environments, to induce robust axonal outgrowth and target identification. This review discusses state-of-the-art tissue engineering approaches and techniques currently employed to promote motor neuron repair and axonal pathway regeneration under injury/ disease conditions.

**Keywords:** Motor neurons; Growth factors; Extracellular matrix; Animal models; Tissue engineering; Regenerative medicine; Drug delivery; Central nervous system

### Introduction

In the mammalian central nervous system (CNS), a complex network of neurons in the pre-motor and motor cortex project their axons to several areas of the CNS, including the striatum and spinal cord. For example, corticospinal motor neurons (CSMN) and other subcerebral projection neurons located in the layer V of mammalian cortex project to targets in the spinal cord and brain stem, and together with spinal motor neurons, they control the most precise aspects of voluntary motor function [1-3]. Motor neurons residing in the ventral hindbrain and spinal cord extend axons into the periphery

to relay commands for effective locomotor movement and function. Individual motor neuron subtypes could be distinguished either by their axon pathway and target choice, or by their soma location within the CNS. These pathways generated during embryogenesis have distinct axonal and soma characteristics, and are critical in the formation of circuits that mediate physical motion [4,5]. Hindbrain motor neurons are clustered together into discrete nuclei that are organized segmentally along the anterior-posterior axis [6]. Developmentally, these neurons occupy the stereotyped positions in the dorsal-ventral plane of the neural tube. Spinal cord subtypes can also be found in continuous columns, spanning a number of segments of the vertebral column [6]. Similar to the hindbrain motor neurons, they also have stereotyped anterior-posterior and dorsal-ventral positions. Additionally, spinal cord motor neurons that share a proximal axonal pathway are co-localized into columns. It is in these columns that one finds the individual subclasses of motor neurons that target specific musculature [6].

Historically motor neurons have been classified based on early studies conducted on the zebrafish. Since zebrafish has a simpler spinal structure than most mammals, it was easy to classify each spinal cord segment into 3 or 4 motor neuron types, labeled after their location (e.g. medial primary, rostral primary, caudal primary, and variable primary) [7]. Recent developmental biology studies using rodent models were more successful in understanding physiological differences based on their tissue type. For example, five LIM homeobox transcription factors were shown to control the fate of motor neurons and potentially regulate the expression of target genes specific to the identity and location in the spinal column [8]. Similarly, Hox genes were shown to play a prominent role in the development and classification of motor neurons, and their suppression in knock-out mice models demonstrated altered motor projection to various limbs [9]. These and other similar studies suggest that motor neuron classification could better be assessed genetically than anatomically.

Genetic diseases and trauma could contribute to changes in normal motor, sensory or autonomic function of these neuronal pathways. Inflammatory environment, particularly resulting from an injury (trauma, stroke) or disease (ALS, PLS, HSP), could lead to progressive degeneration of motor neurons and destruction of neuronal tracts in the adult CNS [10-13]. The epidemiology of these types of ailments is widespread. Annually, thousands of people are diagnosed with various motor neuron related injuries and diseases in the United States and a majority of them succumb to this condition within 3-5 years post-diagnosis [14]. Recent reports suggest that at least a quarter million US citizens have been suffering from spinal cord injuries (SCI) [15], with the cost of managing SCI alone approaching ~\$4 billion per year [16].

ALS is a late-onset progressive neurodegenerative disease affecting motor neurons and nearly 30,000 Americans have currently been diagnosed with this condition [17]. ALS is typically fatal, as those diagnosed have a life expectancy of <5 years [18]. Spinal muscular atrophy is an autosomal recessive neurodegenerative disease primarily in newborns, characterized by the degeneration of spinal cord motor neurons. The incidence ratio is approximately

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one in 10,000 live births, with a carrier frequency of one in 50 [19]. Although not always fatal, there is currently no effective medical treatment for this condition either. Other CNS disorders such as Parkinson's disease and Multiple Sclerosis affects approximately half a million people each, in the U.S. alone [20,21]. Given these incidence rates, it is absolutely vital to restore and regenerate the lost motor neuronal tracts in the CNS on a priority basis, although effective clinical (pharmacological and surgical) treatment options do not exist. Failure to reinstate healthy motor neuronal connections under these conditions can severely compromise locomotor function, resulting in muscle atrophy, paralysis and death.

Current treatment options for neuromuscular diseases can broadly be categorized under symptomatic and supportive treatments, which barely maintain patient's quality of life [22]. Pharmaceutical treatments using FDA approved drugs such as Riluzole® extends life by only a few months, with no major relief in symptoms [23]. Riluzole® supposedly acts by reducing the body's natural production of the neurotransmitter glutamate, which carries signals to the motor neurons. Other drugs which may help with symptoms include combinations of dextromethorphan, quinidine, anticonvulsants and non-steroidal anti-inflammatory drugs, opiates and antidepressants [22]. Physical and occupational therapy, followed by rehabilitation has been shown to improve posture, prevent joint immobility, and slow muscle weakness and atrophy in addition to reducing spasticity by increasing range of motion, and circulation [22]. Additionally, assistive devices such as supports or braces, orthotics, speech synthesizers, and wheelchairs might help retain patient's independence [22].

It is important to note that adult CNS has a limited ability for self-repair, which necessitates among others, cellular reparative strategies focused on ameliorating secondary cellular degeneration, promoting endogenous repair mechanisms, and exogenous cell replacement therapy [24]. In order to overcome the inherent inability of adult CNS for self-repair, tissue engineering approaches have also been explored to treat a wide spectrum of CNS motor neuron anomalies [25]. A critical challenge to regenerating mimics of native motor neuron pathways is the inability to stimulate, sustain and steer axonal outgrowth over a long distance, till they reach their intended targets. Conventional tissue engineering approaches allow us to replace or regenerate motor neuronal cells and their tracts, lost due to injury or disease conditions. Thus, in this article, we will highlight the various cellular, genetic and tissue engineering approaches taken by researchers over the past few years, their successes and limitations, and future directions for research in this area.

## Stem Cell Transplantation

### Role of retinoic acid and sonic hedgehog

Before discussing the various types of stem cells investigated for motor neuron repair, it is necessary to highlight two distinct biomolecules shown to promote stem cell differentiation into neural lineage. Retinoic acid (RA) - a metabolite of vitamin A - has been heavily researched by developmental biologists for its role in embryogenesis and in converting stem cells into neural lineages [26]. Studies have shown that RA enhances neurite outgrowth by increasing the number of receptors for neural growth factor (NGF) in both neuroblastoma cells and dorsal root ganglia (DRG) [27]. Additionally, RA concentration can affect how specified the neural tissue will develop with relation to its location in the body

[28]. Given its strong role in the development of the neural crest, it is understood that RA also promotes survival and proliferation of neurogenic precursors [29]. Neurobiology studies have shown that RA, in collaboration with FGFs and Shh, not only controls the onset of neurogenesis, but also significantly contributes to ventral spinal cord patterning and motor neuron specification, by providing transcriptional activation and promoting formation of motor neuron progenitors [30]. Additionally, lentiviral infection of the sensorimotor cortex in adult rats with RA receptor  $\beta 2$ , three weeks prior to spinal cord lesion, induced axonal and functional regeneration of the cortical spinal tracts [31]. Furthermore, in spinal motor neuron development, RA and its receptors help in the recruitment of acetyltransferases crucial for inducing the expression of spinal motor neuron genes [32]. Disruption in RA signaling has been shown to compromise the maintenance of the differentiated state of adult neurons, as well as the degeneration of motor neurons, leading to the onset of motor neuron diseases [33]. Thus, while RA seem to positively modulate several pathological aspects of CNS injury, more studies are needed to elucidate specific targets of RA and its receptors post-injury, and the interaction between RA and other pro-regenerative signals. In conclusion, the studies described here strongly attest to the therapeutic utility of RA in the induction of axon regeneration and the treatment of neurodegeneration.

In mammals, motor neuron differentiation occurs within the ventral neural tube when neural precursor cells come in contact with signaling molecules such as sonic hedgehog (Shh) [34]. Shh is expressed by the notochord underneath the neural plate, and by the floor plate at the ventral midline of the neural tube [35-37]. Shh also appears to regulate the expression of different transcription factors in neural precursors at precise dorsal-ventral positions in the neural tube, which most likely dictates the location and function of the specific types of motor neurons [6]. Shh administration was shown to increase the population of neural precursor cells after spinal cord injury, provide a neuroprotective effect, improve neural function [38], and control cell fate specification and axon guidance [39,40]. When provided together, RA and Shh have been shown to create a favorable environment for stem cell differentiation into motor neurons [28,41-44], which will be discussed further in this section.

### Embryonic stem cells

Stem cells are capable of differentiating into specialized cell types, although not necessarily all cell types. Embryonic stem cells (ESCs), the most pristine form of stem cells, remain totipotent in humans up to the 8-cell stage (3-4 days) after fertilization [45]. Under right conditions, they can replicate indefinitely *in vitro*, offering a novel tool in regenerative medicine based therapies. However, ethical concerns related to sourcing, necessity for immunosuppression, risk of tumor formation and possible chromosomal instability, dampen the enthusiasm generated by ESCs for potential clinical applications [38]. Studies performed *in vitro* have shown that directional differentiation of ESCs, both mouse and human, into motor neurons could be achieved with optimal concentrations of RA and Shh, provided at fixed time points during differentiation and lineage commitment [42,46]. However, the reported efficiency is relatively low under these conditions, with only 20-30% ESCs expressing the motor neuronal marker, HB9. Interestingly, supplementation of Noggin or purmorphamine, in addition to Shh and RA, increased the efficiency of ESC differentiation beyond 50% in both human and monkey ESCs [44,47,48]. Zurn et al. reported on the combined effects of growth

factors (BDNF, GDNF, IGF) on the survival, differentiation, neurite outgrowth and choline acetyltransferase (ChAT) activity of motor neurons resulting from embryonic rat ventral mesencephalon cells *in vitro* [49]. Motor neurons derived from ESCs have also been found to be functionally active *in vitro* as attested by the electrophysiological and synaptic protein analyses [50-52].

Ikeda et al. observed that transfection of mouse ESCs with *MASH1* promoted motor neuron lineage cells, and that implantation of these resulting cells restored neural networks and motor function of a paralyzed limb in hemiplegic mice models [53]. In another study, Peljto et al., have shown that in lieu of exogenous growth factors, mouse ESCs can be directed to differentiate into highly specific motor neuron subtypes using endogenous Wnts, FGFs, and hedgehog (Hh), thus mimicking the native route of motor neuron subtype differentiation [54,55]. In addition, when these ESC-derived motor neurons were grafted into chick spinal cord, they were found to home in appropriate columnar domains and project axonal trajectories, similar to their *in vivo* counterparts. Overall, these studies suggest that ESCs can be differentiated and programmed into specialized motor neurons on demand, both *in vitro* and *in vivo*, using an optimal set of growth factors and culture conditions, which should be explored further for human clinical transplantation purposes.

### Umbilical cord-derived stem cells

Neonatal stem cells, also referred to as umbilical-cord derived stem cells, are isolated from the umbilical cord immediately after birth [38]. These cells can indefinitely be maintained in cultures *in vitro* and also survive long-term cryopreservation. However, they are also immunologically-immature and may still be rejected by the body when transplanted [38], unless the patient's immune system is strongly suppressed, or has been ablated before transplantation. When human umbilical cord stem cells were intravenously injected into presymptomatic mice model of ALS, these cells not only delayed ALS disease progression by 2-3 weeks, but also extended lifespan of diseased mice [56]. The transplanted cells in this study were found to survive for up to 10-12 weeks *in vivo*, with significant migration to regions of motor neuron degeneration in the CNS. Kuh et al., found that direct injection of human umbilical stem cells, along with brain-derived neurotrophic factor (BDNF), resulted in greater axonal regeneration and functional recovery in a rat model of spinal cord injury, compared to treatments which received only umbilical stem cells [57]. Other studies using human umbilical cord-derived stem cells showed similar encouraging results when injected directly into injured or diseased murine models [58-60]. However, the ability of these cells to provide neuroprotective capabilities is dependent on the injected cell density. For example, an optimal density of  $25 \times 10^6$  cells was found to significantly increase lifespan of ALS mice by 20-25% and delay disease progression by 15% [61]. These studies suggest that stem cells derived from umbilical cord may have therapeutic potential in cell-based treatment of ALS by providing cell replacement and protection of motor neurons, probably by the modulation of autoimmune process.

### Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multi-potent cells that can differentiate into various cell types or lineages under tightly-controlled microenvironments, consisting of ECM proteins and growth factors. Human bone marrow-derived MSCs are a desirable cell source for autologous cell replacement therapy to treat CNS injury due to their

plasticity, low immunogenicity, and a lower risk of tumor formation than ESCs. Isolated MSCs can be expanded to a few passages *in vitro*, and over the past decade a few groups have reported on the differentiation ability of mammalian MSCs into motor neurons, although the efficiency of the differentiation process is quite low [38,62]. Despite these limitations, human MSCs have been shown to promote axonal regeneration and in some cases improve functional outcome after SCI and stroke [63,64]. A recent study conducted by Jiang et al., found that differentiating human MSCs extended axonal outgrowth and increased neural lineage commitment, when cultured on PCL nanofibers and in the presence of RA [24]. There is also evidence to suggest that aggregation of human MSCs into 3D spheroids, using a hanging-drop protocol, enhances their anti-inflammatory properties and proliferation when implanted into diseased regions of the body [65]. In an experimental ALS mouse model, direct injection of human bone marrow derived MSCs into lumbar spinal cord resulted in a significant improvement in motor performance and motor neuronal count, compared to controls [66].

In order to boost the efficiency rate of human MSC differentiation into motor neurons, Park et al., genetically modified these cells to express motor neuron-associated transcription factors (Olig2 and HB9) and differentiated them *in vitro*. They not only achieved higher differentiation rates (~30% of total cells) under these conditions, but also found that these cells form functional connections with muscle fibers *in vitro* [67]. When human MSCs were seeded on synthetic poly (lactic-co-glycolic acid) scaffolds in the absence of any growth factors and implanted into transected rat spinal cords, enhanced neuronal differentiation, axonal regeneration and motor-function recovery was evident at 4- and 8-weeks post-implantation [68]. Given the potential that (a) MSCs can be isolated on demand from adult humans without ethical or moral concerns, (b) differentiated *in vitro* into desired motor neuron lineage and (c) clinically-transplanted at the site of injury or disease for enhanced functional recovery, further research in this field could be of great value for tailored patient-specific therapy.

### Neural stem cells

Neural stem cells (NSCs) are self-renewing, multi-potent cells, capable of differentiating primarily into neuronal and glial lineages. Unlike many other cells in the body, neurons do not divide within CNS, which necessitates replacement of lost/ injured neurons with these CNS resident cells. NSCs typically assume a quiescent phenotype till they receive exogenous signals from their microenvironment to proliferate, migrate or differentiate into a specific lineage [69]. Many researchers observed that adult NSCs are not lineage-restricted to their developmental origin, but can generate region-specific neurons *in vivo* when exposed to the appropriate environmental cues [70,71]. Lu et al. observed that when a murine NSC line derived from neonatal mouse cerebellum was grafted into cystic dorsal column lesions within cervical spinal cord of adult rats, the cells supported extensive growth of host axons within two weeks, and also produced higher levels of neurotrophic factors [72]. Murine NSCs have been shown to expand and differentiate into both neurons and glia in the presence of epidermal growth factor (EGF) and bFGF, and via genetic modification, and could be explored as a promising therapeutic treatment for transplantation into those afflicted with spinal cord injuries [73]. When human NSCs were implanted in an ALS SOD1 rat model, it was observed that rats injected with live NSCs lived at least 17 days longer compared to control animals, highlighting the

potential of NSC grafts as future cellular therapy option for motor neuron diseases [74]. However, it is important to note that although NSCs exhibit great potential for the repair and regeneration of motor neurons, current barriers include their limited availability in the CNS, incomplete characterization, and lack of awareness of the signals involved in their proliferation, differentiation and integration within a given neural network [75].

### Diseased or Injured Animal Models

The pathogenesis of neurodegenerative diseases and spinal cord injury typically involves axonal and cellular damage to motor neuron compartment, leading to progressive loss of muscle control and death of motor neurons within the CNS [76,77]. A majority of CNS injuries occur at the cervical (C2-C4) level in close proximity of descending axons to their cell bodies and directly affect locomotion and digit function, thus highlighting the increased clinical relevance for hastened therapeutic intervention [15]. Multi-institutional clinical trials, testing a multitude of pharmacological interventions, demonstrated only limited benefits to the regeneration and functional recovery of motor neurons [78-86]. These human clinical trials also failed due to our limited understanding of the cellular, molecular, and genetic factors that control and direct motor neuron survival, axonal projection and connectivity in healthy and inflammatory conditions.

A variety of animal models have been developed to study the onset and pathological progression of motor neuron diseases (Table 1). The murine corticospinal tract model has been used extensively to investigate regeneration and remodeling of motor axons after injury or disease. In the mouse, CSMN descend within the dorsal funiculus of the spinal cord white matter, to reach the distal cervical spinal cord ~postnatal day 1 (P1), the distal thoracic cord ~P4, and the distal lumbar cord ~P7 [87]. These axons arborize among spinal cord interneurons and spinal motor neurons ~2-3 days after their arrival at their appropriate level [88]. CSMN innervate their final targets via interstitial branching from the axon shaft [89,90]. However, axotomy studies examining CSMN growth and survival requirements in rodents offered conflicting results, due to the difficulty in separating direct versus indirect effects of growth factors on CSMN [91-93].

Murine models are also routinely used to study the cellular and molecular pathways involved, because of the similarity of these pathways in both mice and humans [94]. In select cases, knock-out

zebrafish have proven more useful as they present a more simplistic model of the CNS than murine models [95]. Among murine models, spontaneous mutants, target mutants, and those with an induced spinal cord injury are commonly used [96,97]. Within the spontaneous mutant models wobbler mouse, progressive motor neuropathy (PMN) mouse, and the ENU-induced mutant mouse have been widely adopted [98]. PMN mutant mouse is perhaps the most widely used model for spinal muscular atrophy research. The autosomal recessive mutation causing this pathology has been mapped to chromosome 13, and these mice develop hind-limb paralysis and display progressive degeneration of motor neurons until they die 6-8 weeks later [99]. The wobbler mouse can be used as a more generalized neurodegeneration model as it is characterized by an unsteady gain with degradation in the thalamus, cerebellum and brainstem, until it dies 3 months after birth [100]. The gene mutated in this autosomal recessive disease model has not been identified yet, but has been mapped to chromosome 11 [101]. The ENU-induced mutant was developed by exposure to N-Ethyl-N-Nitrosurea, a chemical mutagen that produces point mutations in the genome, and breeds mice with a later-progressive motor neuron disease characterized by mitochondrial swelling, golgi fragmentation and cytoplasmic inclusions [102-104].

There are three types of target mutagen animal models. The SOD1 transgenic model caused by a genetic disruption of the SOD1 (Cu/Zn superoxide dismutase) gene is highly expressed in motor neurons and functions to catalyze the conversion of radicals to hydrogen peroxide [96,105]. These mice develop progressive muscle weakness and atrophy and have a pathology that highly resembles the human disease, including loss of motor neurons and interneurons, reactive astrocytosis, and inclusion bodies [106-108]. Thus, SOD1 mutant mouse is perhaps the most extensively used animal model to study the molecular and cellular processes occurring both before ALS disease onset and during disease progression, and lead to the identification of some therapeutic targets and neurodegenerative mechanisms involved in nearly all forms of neuromuscular disorders [109,110]. Neurofilament and peripherin mouse models work by altering intermediate filament proteins, particularly abundant in large myelinated neurons and responsible for the maintenance of large axonal caliber, similar to those affected in motor neuron diseases [111,112]. Mutant vascular endothelial growth factor (VEGF) mice,

**Table 1:** Animal models widely used to study motor neuron diseases.

Animal	Type	Disease Studied	Ref
Zebrafish	Knockdown of the survival motor neuron	Broad range of motor neuron diseases	[95]
Embryonic Chicken	Transection of the thoracic spinal cord prior to embryonic day (13) of the 21-day developmental period	Spinal cord injury, embryonic development	[118-120]
Mouse	Wobbler Mouse	Broad range of motor neuron diseases	[100,101]
	Progressive Motor Neuropathy (pmn) mouse	Spinal muscular atrophy	[99]
	ENU-induced mutant mouse	Broad range of motor neuron diseases	[102-104]
	SOD1 transgenic mice	Onset of various motor neuron diseases	[105-108]
	Neurofilament and Peripherin Mouse models	Pathogenesis of motor neuron diseases	[111,112]
	Mutant VEGF mice	Human ALS	[113]
	Induced spinal cord injury mouse	Spinal cord injury	[114,115]

on the other hand, carry the deletion of the hypoxia response gene, which mimics the symptoms of human ALS [113].

In order to mimic spinal cord injury, one can cause any type of trauma to a given section of the vertebral column, most commonly performed on mice. Injury models include contusive or hemi-contusive injury, transection or hemi-dissection models, photochemical model, excitotoxic model, clip compression injury, spinal cord displacement, canal stenosis, or spinothalamic tract lesion [114]. Evaluation of injury extent is commonly done by observation of locomotion using the Basso, Beattie and Bresnahn 9 point rating scale, especially in mice. However, limitations include the differences in injury type, specifically between laboratory induced SCI and actual spinal cord trauma [115-117]. Besides, it is difficult to assess the functional outcome in animals, especially as it relates to the pathophysiology of the SCI, and this often leads to misinterpretation of results.

### Role of ECM Components

The pathogenesis of neurodegenerative diseases and spinal cord injury typically involves axonal and cellular damage in motor neuron compartment, leading to progressive loss of muscle control and death of motor neurons within the CNS. Thus, a thorough knowledge of the protein constituents of CNS ECM is required for successful implementation of tissue engineering strategies to regenerate or replace lost motor neuron cells and tracts. Motor neuronal tract regeneration can partially be accomplished by providing a suitable 3D scaffold incorporating micro-architectural facets of CNS ECM, which can deliver the required biomechanical and biochemical cues. It was not until 1971 that the existence of ECM in the CNS was generally acknowledged [121]. Over the past 40 years, developmental biology studies performed both *in vitro* and in animal models have provided abundant evidence that the CNS ECM affects virtually all aspects of nervous system development and function [122,123].

### Allografts

ECM in the CNS is majorly composed of glycoproteins, collagens, GAGs, and other link proteins [122,141,142]. Thus, the ideal scaffolds which can mimic all the native components of CNS ECM are acellular allografts. Guo et al., explored the possibility of removing myelin and cellular components of an intact rat spinal cord, and analyzing its viability as an acellular allograft for implantation purposes. They were successful in removing the cellular components of the spinal cord using hemolysis procedures, resulting in an intact acellular tube consisting of laminin, fibronectin and type IV collagen proteins [142]. During this process, it was observed that the diameter of the spinal cord shrunk by 66-80%, after the removal of the cellular components, leaving behind pores with an average size of 120  $\mu\text{m}$  within the three dimensional scaffold. In another study, Auba et al., studied nerve regeneration obtained using ulnar nerve allografts in nonhuman primates temporarily treated with FK506 (tacrolimus), and compared with that obtained using nerve autografts. Although they found no significant differences between allografts and autografts in terms of neuronal and axonal counts, nerve conduction velocity and maturation of myelinated fibers in allografts was observed to be lower, indicating that allografts were partially rejected even with immunosuppression [143,144]. Similarly, when immunosuppressive therapy was withdrawn from rats implanted with allografts in the sural or saphenous nerve gaps, a majority of axons regenerated in the allograft segments were lost, with minimal survival of axons from smaller-

diameter motor fibers [145]. Although allografts reinstate some of the lost characteristics of ECM tissue, they are limited by short supply of cadaveric tissues, and the insufficiency of current chemical processing methods to completely extract immune-reactive donor-cells and contaminating epitopes/ globular proteins, essential to prevent immune rejection in the host. As highlighted by the aforementioned studies, other disadvantages of such extracted matrices include, adverse impact of extraction process on cell-signaling by the resultant tissue, challenges to infiltration by recipient cells into the often dense matrices, inability to faithfully regenerate lost motor neuron tracts post-implantation, and the critical need to chemically stabilize the matrix, which alters its biochemical and mechanical characteristics.

In conclusion, while the utility of three dimensional ECM-based scaffolds for motor neuron pathway regeneration has been strongly demonstrated by these studies, the practical realization of this concept is crucially contingent on the selection of scaffolds from a sub-set of ECM molecules that facilitate this process *in vivo*. In the following sections, we will focus on the recent developments in employing various types of ECM molecules for enhanced motor neuron survival and axonal regeneration at the site of disease or injury.

### Synthetic and ECM-based scaffolds

To overcome the limitations of allografts and autografts, researchers attempted to implant synthetic or natural scaffolds with robust mechanical properties and biocompatibility. Direct surgical insertion of a three dimensional scaffold, seeded with the cells and necessary growth factors, is perhaps the most common approach employed in the CNS of animal models [146-148]. For example, thermo-sensitive hydrogels such as polyethylene glycol (PEG), have been explored for neuron axonal tract regeneration [149]. Upon injection, these hydrogels supposedly conform to the lesion site and quickly integrate into its surrounding ECM to match the mechanical properties of the host tissue, while promoting axonal in growth [150]. PEG hydrogels are also advantageous because they offer controllable degradation rate, bioinert characteristics, and a 3D microenvironment for neural stem cells to proliferate, differentiate and form neurons that are electro-physiologically responsive to neurotransmitters [151]. However even slight changes in the composition of synthetic materials, such the addition of poly(lactic acid) to a PEG scaffold, can affect the functional use of that material, as the lactic acid released during gel degradation can impact the function of encapsulated neural cells [152]. Additionally, mechanical properties of these gels could affect neurite growth, branching and function. Flanagan et al. fabricated polyacrylamide gels with differing amounts of *bis-acrylamide*, to result in substrates with varying elastic moduli ranging from 500-5500 dyne/cm [153]. When mouse spinal cord neurons were seeded on these gels and allowed to grow over several weeks, it was observed that softer substrates promoted a 3-fold increase in branching of neurites compared to those on stiffer gels.

Numerous studies validate claims to the overall superiority of 3D ECM-based cell scaffolds over 3D synthetic scaffolds or 2D monolayer cell cultures, in efforts to simulate the chemical and physical environment of native tissues [154]. Since cellular gene expression within 3D scaffolds can be modulated by scaffold-derived cues such as cell adhesion molecules, growth factors, and mechanical stimuli, ECM-based scaffolds are more likely to evoke native integrin-ECM interactions and preserve the native cell phenotype. Besides, cells within 3D ECM scaffolds exist in a more natural environment

in which they contact other cells and ECM in three dimensions and are therefore expected to more closely evoke native cell responses than 2D substrates. Natural materials provide a better matrix for cells to migrate and adhere, which is extremely important for a motor neuron network, as re-establishing damaged connections among axons is a top priority. In this section, we will review various ECM based materials (Table 2) used in tissue engineering of motor neuron pathways.

**Laminin:** An important component of the CNS ECM is laminin, a glycoprotein also found throughout the body and identified to play several roles in cellular differentiation, migration and adhesion [122]. The  $\alpha$ ,  $\beta$  - integrins on neuronal surface interact and communicate with laminin, contributing to stabilization of cell-ECM interactions within CNS [124]. Studies have shown that laminin plays a strong role in guiding axons to their target, as well as providing myelination around the cell, which is especially important for the white matter portion of the spinal cord. Transgenic mice deficient in laminin display dramatic abnormalities, supporting the notion that laminins are crucial for neuromuscular development and function [155]. In short-term cultures, rat motor neurons were found to adhere, survive and extend neurites on multiple isoforms of laminin [156]. But in long-term cultures, these rat embryonic motor neurons exhibited preference to the type of laminin; Lmn-1 and Lmn-2/4/8 promoted extensive neurite outgrowth, while Lmn-11 supported only shorter neurite extensions with unusually large growth cones [156]. Wildering et al., investigated surface adhesion and neurite outgrowth of molluscan motor neurons on ECM substrates, and noted that the cells adhered and extended neurites on native laminin and type-IV collagen via RGD peptide-dependent adhesion mechanism, but not on plasma fibronectin [157]. It was later discovered that in an ALS mouse model, there is an initial increase in production of  $\gamma$ 1-laminin in the spinal anterior horn from 15-18 weeks, suggesting that although motor neurons were lost during this period, the capacity for self-regeneration was not curtailed [158,159]. Zhang et al., tested the effect of laminin and fibronectin on sciatic nerve regeneration in rats, and reported on significantly higher motor nerve conduction velocity and muscle action potential amplitude of the anterior tibial muscle in fibronectin- and laminin-treated groups, compared to controls

[160]. In addition, a greater number of motor neurons with larger nerve diameter and higher numbers of myelinated axons were also observed in these two groups, compared to controls. Additionally, the concentration of laminin can also affect neurite outgrowth, as demonstrated by Buettner et al.; neurons showed approximately two-fold increase in neurite initiation and outgrowth and a two-fold decrease in branching, for a corresponding 100-fold increase in adsorbed laminin concentration [161]. These studies suggest that local application of laminin can significantly improve motor neuron survival, axonal regeneration, and maturation, which could be explored for tissue engineering based regeneration of motor neuron axonal tracts.

**Fibronectins and fibrin:** Fibronectins are a broad class of proteins that can exist in multiple forms as they have a number of splice variants, with each splice variation playing a different role within the ECM [162]. Fibronectin is a high-molecular weight glycoprotein that commonly binds to integrins on cellular surfaces, similar to that of laminin. Fibronectin also binds ECM components such as collagen, fibrin and heparan sulfate proteoglycans [163]. Recently, King et al., implanted a fibronectin mat within an injured region of rat spinal cord [126], and found that fibronectin creates a neuroprotective effect on the surrounding CNS, and acts as a bridge for the regenerating axons [126]. When silicone chambers filled with a mixture of laminin/fibronectin were seeded between the severed ends of sciatic nerves in rats, a significant increase in the number of myelinated motor axons in the regenerated region was observed within 4 months, compared to the control group [164]. Fibronectin has also been shown to provide a favorable singular growth surface for motor neurons when combined with antibodies [165]. Kuhn et al., investigated motor neurite growth on fibronectin and poly-D-lysine in relation to the actin cytoskeleton regulator Rac-1, and noted that Rac-1 mutant neurons exhibited surface interaction preferably with the fibronectin surface which attenuated neurite growth, suggesting that the fibronectin coating plays some role in outgrowth of wild type motor neurons [166].

Fibrin scaffolds, on the other hand, are made of a cross-linked fibrinogen and thrombin network, with broad usage in repairing injuries of nerves, heart valves, and bone grafts. Some studies have shown that the combination of growth factors and a fibrin scaffold can

**Table 2:** List of known components of neural motor ECM.

ECM component	Type	Function	Ref
Laminin	Glycoprotein	Migration, adhesion, guidance	[124,125]
Fibronectin	Glycoprotein	Neuron outgrowth	[126,127]
Collagen	Protein	Outgrowth, cellular bridging	[128,129]
Perlecan	Proteoglycan	Regulate growth factors, adhesion	[130,131]
Appican	Proteoglycan	Adhesion, neurite extension	[127,132]
Tenascins	Glycoprotein	Increases neural velocity/ conductance	[122]
Reelin	Glycoprotein	Regulate cell-cell interactions during embryogenesis	[133,134]
Lectican	Proteoglycan	Linking, adhesion, and migration	[135]
Hyaluronan	Glycosaminoglycan	Astrocyte proliferation	[136,137]
Thrombospondin	Protein	Axon outgrowth	[138,139]
Agrin	Proteoglycan	NMJ development	[140]

allow one to create an environment, which selectively releases growth factors to enhance the survival and differentiation of cells seeded within [167-170]. Fibrin is used as a coating for synthetic polymeric scaffolds, to provide a suitable environment for cell adhesion and neurite outgrowth [171]. Liu et al., observed that neural progenitor cells seeded in fibrin gel modified with fibronectin can differentiate into ChAT-positive motor neurons [172]. Lastly, as stated previously, studies have also explored combining fibronectin with laminin as a viable growing surface for motor neurons [160,173].

**Collagens:** Within the context of neural tissue engineering, collagen-1 based scaffolds have been extensively used to investigate cellular growth, differentiation, proliferation, migration, axonal extensions, and tissue targeting. Collagen scaffolds can be easily manipulated to control the concentration (biomechanics), density (porosity, pore size) and pH, and motor neurons and biomolecules could be mixed into these scaffolds to assess changes in neuron survival and neurite outgrowth in response to the microenvironment [174]. However, the precise role of collagens in motor neuron biology is still not entirely known. Collagen matrices have been shown to provide suitable properties to repair and guide an injured nerve *in vivo*. Collagen's function in promoting axonal outgrowth of motor neurons has become a bit of an oddity, as some studies show that bridges containing collagen create a meshwork that promotes growth [128,129], while paradoxically acting as a barrier for axonal growth after trauma or injury to CNS [175]. Allodi et al. observed extensive neurite outgrowth from motor neurons, when cultured within collagen matrix *in vitro*, in the presence of different growth factors (BDNF, NT-3) [176]. When collagen scaffold-filled collagen tubes were implanted in a spinal cord injury rat model, either standalone or loaded with cells and growth factors, favorable alignment of the reparative tissue with the long axis of the spinal cord was observed, with significant reduction in the formation of fluid-filled cyst [177]. Motor neuron guidance, attraction, and repulsion studies have been performed in collagen gels demonstrating the ability of these cells to grow in 3D through the matrix [178,179]. When collagen-associated amino acids were measured in patients afflicted by ALS disease, reduced collagen levels in the spinal cord were found to be related to the degeneration of upper and lower motor neurons, thus proving the crucial role collagen plays in maintaining motor neuron function [180]. Gingras et al., developed a 3D model of motor neuron regeneration, by culturing mouse spinal motor neurons in 3D collagen scaffolds in the presence of Schwann cells, fibroblasts and a cocktail of growth factors, and observed formation of dense myelin sheaths surrounding motor fibers after 28 days culture *in vitro* [181]. Furthermore, collagen scaffold also facilitated significant motor neuron survival and robust neurite outgrowth (>850  $\mu\text{m}$ ) in 3D over 14 days, compared to control cultures in this study. Taken together, these observations suggest that 3D biodegradable collagen matrices with tunable properties could potentially be used in clinical applications, not only for delivering cells and/or drugs to the desired regions in CNS, but also to physically support growing motor neurites.

**Other components:** In addition to the materials discussed above, a few other ECM proteins have proven viable for the survival and outgrowth of CNS neurons. When polysaccharides (e.g. dextran, chitosan, methylcellulose, agarose) have been used either standalone or in combination, to form scaffolds with tunable mechanical and surface properties, they facilitated greater neuronal attachment and neurite extension, with potential applications in regenerating motor

neuronal tracts [182-185]. Although glycosaminoglycans (GAGs) are a major constituent of the embryonic CNS ECM, some types of GAGs such as chondroitin sulfate (CSPG) and heparin sulfate (HSPG) have been shown to be excessively secreted and deposited at the site of injury or disease, and contribute significantly to axonal outgrowth inhibition both *in vitro* and *in vivo* [186,187]. In an ALS disease model of transgenic rats, Mizuno et al., found a significant upregulation of CSPGs such as neurocan, versican, and phosphacan in the ventral spinal cord, creating a non-permissive microenvironment for motor neuron regeneration [188]. HSPGs such as syndecan-1 and glypican-1 have been shown to play a crucial role in the survival of injured motor neurons and in nerve regeneration after injury, by significantly upregulating their mRNA expressions [187,189]. On the other hand, recent studies suggest that the expression of CD44, a receptor for another GAG molecule hyaluronic acid, increased at the onset of ALS disease in SOD1 mouse model, suggesting that the inflammatory responses involving CD44 may play a role in this disease [190]. These studies suggest that matrices or scaffolds made of GAG molecules such as CSPG and HSPG should sparingly be used for tissue engineering efforts towards motor neuron survival and pathway regeneration. Numerous reports suggest that GAGs can not only promote neurogenesis *in vitro*, but also promote axonal regeneration following peripheral nerve axotomy, possibly by regulating the interaction between IGF-I and the IGF binding proteins on neuronal surface [191,192].

It is clear from above studies that from a regeneration standpoint, there are multiple scaffolding materials that a bioengineer can choose from, to support motor neuron survival, neurite growth and guidance under disease/ injury conditions. These scaffolds can be fine-tuned for desired attributes such as biomechanical, biochemical, architectural and biocompatible needs. Thus, a thorough understanding of the microenvironment (cellular and ECM components, biomolecular cues and their gradients, mechanotransduction) within that particular CNS region is crucial before deciding the therapeutic approach to be employed for ultimate repair of the injury site. In conclusion, multiple components of the ECM surrounding motor neurons in the CNS play an important role in disease or injury pathogenesis. In order to properly engineer a solution to repair these pathways, it is crucial to understand and carefully consider what combinations and amounts of these molecules will help in successful regeneration and restoration of normal function under these conditions.

## Role of Growth Factors and Biomolecules

During fetal development, a tightly-regulated spatio-temporal pattern of guidance cues (e.g. neurotrophic factors) direct and maintain motor neuron axonal growth along specific pathways to reach the target cells and tissue [193,194]. A neurotrophic factor is typically defined as the biomolecule involved in the regulation of neuron survival, migration, neurite outgrowth and guidance during development [195]. Specifically in the context of motor neurons, various growth factors in the CNS have shown both positive and negative effects, depending on the spatio-temporal location, developmental stage and function of the neuron studied. Numerous neurotrophic factors and signaling molecules have been shown to promote and sustain developing motor neurons and encourage survival of postnatal motor neurons after damage [106,196-199]. Here, we will review (Table 3) the roles of prominent growth factors proven to affect the longevity and quality of motor neuron.

**Table 3:** Selected growth factors and their effects on motor neurons.

Growth factor	Observed effects	Cell/ Tissue type	Ref
Nerve growth factor	Promote survival and outgrowth of neurons	Primarily sensory neurons	[200-202]
Neurotrophin-3	Motor neuron survival and outgrowth as well as sensory axon growth	Motor and sensory neurons in the dorsal root ganglia	[203,204]
Ciliary neurotrophic factor	Motor neuron survival, outgrowth and sprouting, also involved in inflammatory responses	Spinal cord motor neurons and nerves of the PNS	[205,206]
Bone morphogenetic protein	Architecture, organization	Subventricular neurons, spinal cord motor neurons, as well as neural tissue in the hindbrain	[207-209]
Basic fibroblast growth factor	Maintain and increase the number of neurons	Wide variety of tissue types though brain and spinal cord, also plays a strong role in the wound healing and the maintenance of multiple tissue types	[210-212]
Epidermal growth factor	Differentiation of stem cells, maintenance of generation of astrocytes, involved in cellular migration		[210,213]
Retinoic acid	Differentiation of stem cells and extension and organization of axons	Dorsal root crest, additionally in the dorsal root ganglia	[26,28,29]
Sonic hedgehog	Motor neuron guidance and protection	During development, and midline structure of brain and spinal tissue	[38,40]

### Nerve growth factor

Nerve growth factor (NGF) is perhaps one of the most extensively studied neural growth factors. Following spinal cord transection, invading immune cells deposit NGF at both the distal and proximal stumps at the injury site, suggesting the critical role of NGF in neuron recovery and axonal regeneration [200]. Such information could be particularly applicable to neurodegenerative diseases such as ALS, where beneficial axon-target interactions are disrupted early in the disease pathogenesis [214]. NGF has been shown to guide differentiation and survival of neuronal populations during development, modulate neural plasticity in the mature CNS, and eliminate damaged neurons and glial cells under pathological conditions [215]. When neural stem cells were implanted adjacent to the spinal cord sections, they not only secreted NGF which promoted motor neuron survival, axonal outgrowth and targeting toward them, but also protected motor neurons from experimentally induced excitotoxic damage both *in vitro* and *in vivo* [216]. These studies strongly suggest a therapeutic role of NGF, based on their differentiability into motor neurons as well as their capacity to provide trophic support to endangered host motor neurons, under diseased (e.g., ALS) or spinal cord dysfunction conditions.

However, elevated NGF levels have been implicated in the progressive death of motor neurons in ALS, as the oxidation or nitration of secreted NGF might also enhance its apoptotic activity toward motor neurons [215,217]. An undesired effect observed from NGF delivery *in vivo* is that neural tissue begins to over-extend its axons beyond the specified target, resulting in chronic pain and unnatural neural reflexes [218].

### Brain-derived neurotrophic factor

Studies have shown that brain-derived neurotrophic factor (BDNF) promotes neuron survival while also making them vulnerable to toxicity [219-221]. In ALS patients, BDNF mRNA expression and production increased early in the onset of disease, in response to partial denervation and to promote neuromuscular junction stability [222,223]. Jeong et al., have shown that motor neuron survival was promoted by BDNF stimulation of either the cell body or axons/

dendrites, via activation of tyrosine kinase receptors *TrkB* on axons/dendrites, endocytosis of the BDNF-*TrkB* complex and *de novo* protein synthesis crucial for mutant SOD toxicity of motor neurons [224,225]. These findings were supported by other observations that subcutaneously injected BDNF is transported to motor neurons to activate ERK signaling pathway in the spinal cord and exhibit neurotrophic effects in animal models [226]. BDNF has also been shown to rescue motor neurons from naturally-occurring and axotomy-induced cell death, besides supporting their survival *in vitro* [227,228]. In an animal model of neonatal axotomy, locally delivered BDNF saved 40–70% of motor neurons which would have potentially died after axotomy in lumbar and cranial motor pools, although BDNF did not prevent the decrease in choline acetyltransferase (ChAT) activity in L<sub>4</sub> and L<sub>5</sub> ventral roots resulting from sciatic nerve transection [229].

### Neurotrophins

While neurotrophins (NTs), members of the NGF protein family, have been known to prevent neuronal degeneration and regulate neuronal phenotype during development or adulthood, they have differential effects on motor neurons. NTs were initially considered to have no direct effects on motor neurons *in vitro* [230,231]; subsequent studies, however, have shown that they can promote motor neuron survival *in vitro* [232]. NT-3 has been shown to be a potent trophic factor for motor neurons by promoting their survival and axonal outgrowth in cultures as well as in animal models [204,232]. It should be of interest to note that NT-3 and BDNF share more than 50% nucleic acid sequence homology with NGF, and all the three biomolecules could readily be retrogradely transported by motor neurons from their targets in a specific, receptor-mediated manner [229,233]. NT-4/5, on the other hand, prevented axotomy-induced death of facial motor neurons in neonatal rats, by using functional receptors such as *Trk-B* to retrogradely transport the drug [234]. It was also observed that these tyrosine kinase cellular receptors are also expressed in facial motor neurons of adult animals as well, suggesting that motor neurons can respond to NT-4/5 throughout their lifetime [234]. Interestingly however, mRNA expression of NT-4/5 was evident in facial muscles of both embryonic and neonatal rats,

but not in adult rats in these studies. Taken together, the effects of NTs along with those of BDNF discussed above, suggest a prominent physiological role in motor neuron biology, and raise the possibility of their potential use as a therapeutic agent for human motor neuron diseases.

### Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) is known as a potent survival factor for neurons and oligodendrocytes, and to reduce tissue destruction during inflammatory attacks. Preliminary studies suggested that local application of CNTF prevented the degeneration of cell bodies of neonatal rat motor neurons, whose axons have been transected [235]. This study also suggested that CNTF might be exerting higher influence as a lesion factor than NGF, based on the developmental time course of CNTF expression, regional tissue distribution and its cytosolic localization. Nevertheless, CNTF has been shown to promote spinal motor neuron survival, phenotype maintenance, and axonal sprouting and outgrowth following injury [206], via effective utilization of CNTF receptor- $\alpha$  [236,237]. CNTF also plays an important role in the *in vitro* survival of spinal motor neurons, and reduces their *in vivo* death resulting from apoptosis, genetic mutations, or nerve lesions [238]. CNTFR $\alpha$ -mediated retrograde transport of CNTF by sciatic motor neurons was observed to increase following adult sciatic nerve lesion, which demonstrates the effectiveness of this growth factor in spinal motor neuron regeneration and recovery [239]. Studies have shown that mRNA levels of CNTFR $\alpha$  in spinal motor neurons increased after 1-7 days of nerve lesion, suggesting their importance in CNTF protein synthesis as well as their survival and functional recovery [240]. In another study, CNTF has been shown to dramatically reduce the functional and morphological changes in mouse model of mutant progressive motor neuronopathy, prolong survival and improve motor function of these mice, and greatly reduce the loss of motor axons in the phrenic nerve and degeneration of facial motor neurons [241]. Finally, Schorr et al., performed systematic immunoreactivity studies to show that CNTF is expressed in a majority of upper and lower motor neurons in the human CNS, and its expression is maintained even in ALS patients over a wide age range [242]. In conclusion, the protective and restorative effects from these studies raises hope for a new paradigm in the treatment of human degenerative motor neuron diseases with CNTF.

### Insulin-like growth factor

Insulin-like growth factor (IGF-1) has been shown to actively regulate spinal motor neuron development, maturation, survival and axonal outgrowth [243-245], as well as maintain muscle integrity and regeneration after injury and denervation [246]. The serum concentrations of IGF-1 are slightly decreased in ALS patients, although no significant change in IGF-1 expression was observed in these patient's spinal cords [247,248]. IGF-1 over-expression *in vivo* was able to mitigate ALS pathological phenotype and minimize phenotypic manifestation in a mouse model of spinal muscular atrophy (SMA) [249]. When recombinant human IGF-1 complexed with recombinant human IGF-1 binding protein-3 was administered to SMA mice, it reduced motor neuron degeneration, increased muscle fiber size, and improved motor functions [250]. Shababi et al., have shown that delivery of IGF-1 cDNA, through intracerebroventricular injection, minimized SMA phenotype in a more severe SMA mouse model [251]. In similar studies, transgenic expression of mouse IGF-1

in skeletal muscle of a SMA mouse resulted in an increase in myofiber size and a modest improvement in median survival [252]. Dodge et al., have recently shown that stereotaxic injection of an adeno-associated virus vector encoding IGF-1 to the DCN led to reduced neuropathology and improved muscle strength in ALS mice [253]. Subcutaneous administration of 1 mg/kg IGF-I reduced forelimb muscle atrophy and loss of strength in wobbler mouse motor neuron disease [254].

However, for reasons not completely understood, clinical trials in Europe and North America did not demonstrate any advantage of systemically-administered recombinant IGF-1 in ALS patients, although no adverse side-effects were observed either [255,256]. Nevertheless, a thorough understanding of the underlying pathobiology of IGF-1 delivery [257,258] suggests a possible therapeutic strategy for ALS treatment, and should be explored as a priority.

### Fibroblast growth factor

Fibroblast growth factor (FGF) family, consisting of 22 members, plays an important role in CNS development, neuronal survival and repair, axonal growth and synapse formation [259,260]. The critical role of FGF (specifically, bFGF or FGF2) in stem cell differentiation into motor neurons has been reviewed in the above sections. Moyer et al., have shown that FGF2 treatment significantly rescues neurons and improves functional recovery after experimental head trauma or ischemia in animal models, although the mechanism by which these benefits are exerted is not clear [98]. Teng et al., microinjected FGF1 or FGF2 into the injury site (at T8) of a rat spinal cord contusion injury model at 5 min post-injury, and observed that both the molecules not only promoted survival of autonomic preganglionic neurons in the intermediolateral column and somatic motor neurons in the ventral horn, but also enhanced their functionality as evident from cholinergic phenotype [261]. FGF2 treatment also prevented the respiratory abnormalities produced by thoracic spinal cord injury, and reduced the loss of preganglionic sympathetic motor neurons after injury [262]. This result is especially relevant because FGF2 mRNA levels and protein expression adjacent to the injury epicenter was found to increase over the first 24 h post-injury to spinal cord [263,264]. In ALS animal models, FGF-1 has been shown to modulate a complex interaction of motor neurons with astrocytes, to influence neuronal survival. Specifically, motor neurons release FGF-1 in response to the surrounding inflammatory environment, to signal astrocytes to proliferate and become reactive [265]. FGF-1 was also shown to stimulate transcription factor Nrf2 activation, which promotes expression of antioxidants and cytoprotective enzymes crucial for preventing motor neuron degeneration [265]. Taken together, these results suggest that FGFs demonstrate long-term effects in improving respiratory function, protecting motor neurons, and preserving their function after an injury to the spinal cord. Lastly, two other growth factors - Netrins and Slits - are gaining recognition for their role in axonal outgrowth and guidance as well as for other functions within the CNS [266,267].

### Other biomolecules

Due to an injury or disease in the CNS, cells experience endoplasmic reticulum stress, and thereby activate a signaling pathway termed "unfolded protein response (UPR)" to alleviate this stress and restore homeostasis by decreasing the extent of protein misfolding [268,269]. Under these conditions, neurons tend to exhibit rapid activation of

UPR markers compared to glial cells. Recent studies have shown that small molecule modulators or gene therapy strategies to deliver active forms of XBP1 and ATF4 genes effectively attenuated this stress and tissue damage due to a spinal cord injury or CNS disease, and enhanced locomotor recovery [270,271]. The outcomes from such *in vivo* studies suggest the potential utility of these novel therapeutic strategies to promote myelin sheath regeneration, motor neuronal survival, and axonal outgrowth under an injury or disease in the CNS.

In summary, we discussed a few prominent neurotrophic factors proven to influence motor neuron differentiation, survival, axonal extension and guidance, under both healthy and inflammatory (disease, injury) phenotypes. Results from the studies outlined here suggest that post-injury, developing motor neurons could be guided by a mixture of diffusible biomolecular gradients through varying ECM environments, either at a close range or over a distance, to induce robust axonal outgrowth and target identification. Since considerable functional integration *in vivo* is evident, these studies strongly attest to the role of growth factors, either secreted by cells or supplied exogenously, to influence pathogenesis of the inflammatory CNS and promote growth of injured axons. Nevertheless, these attempts amplify the prevailing notion that rejuvenation of corticospinal tracts and motor neuron axonal outgrowth are fundamentally vital for awakening lost motor functionality, albeit difficult to achieve.

## Concluding Remarks

Tissue engineering technologies offer the promise of nearly limitless organs and tissues to treat a wide variety of injuries and diseases, although their benefits are yet to be fully realized in the area of CNS motor neuron regeneration. The field of tissue engineering is rapidly expanding, and numerous tissue-engineered products and platforms have already been successfully implemented to repair and regenerate lost or damaged tissues in the human body. The field relies heavily on progress made in developmental biology, engineering, molecular biology, and physiology. In this review, we highlighted the utility of various growth factors, biomolecules, synthetic and biological scaffolds and stem cells, either stand-alone or in combinations, to promote differentiation, survival, axonal outgrowth and targeting of motor neurons. We also highlighted a few examples representing the important trend towards an integrated approach that builds on the exciting recent advances in gene therapy and localized drug delivery. The outcomes from further research in this area will permit development of clinically-relevant therapies, including regeneration of lost axonal tracts in CNS injury, cell replacement, clinical transplantation in patients suffering with ALS, PLS or muscular dystrophy, and in fundamental developmental biology. Such a tissue/ cellular engineering approach can be extended to selectively regenerate or repair other rare and delicate neuronal types in mammalian CNS.

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