

Stem Cell Biology and Regenerative Medicine

Philip V. Peplow
Bridget Martinez
Thomas A. Gennarelli *Editors*

Regenerative Medicine and Brain Repair

 Springer

Stem Cell Biology and Regenerative Medicine

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Kursad Turksen, Ottawa Hospital Research Institute, Ottawa, ON, Canada

Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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Philip V. Peplow · Bridget Martinez ·
Thomas A. Gennarelli
Editors

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Editors

Philip V. Peplow 
Department of Anatomy
University of Otago
Dunedin, New Zealand

Thomas A. Gennarelli
Professor Emeritus
Department of Neurosurgery
Medical College of Wisconsin
Milwaukee, WI, USA

Bridget Martinez
Department of Pharmacology and Medicine
Reno School of Medicine
University of Nevada Reno
Reno, NV, USA

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Title: "Stroke of Genius" This is an acrylic on canvas piece. Aptly named as most strokes (disease stroke/blood clots in the brain) occur from damage to endothelial tissue in the cardiovascular system (heart). Hence, comparing the equal importance of both side, by side a stroke of genius. Artist, Bridget Martinez, MD PhD

Preface

Neurodegenerative diseases and brain injury are increasing in incidence in adults. Current medications only temporarily reduce some of the symptoms but do not cure or delay progression of the disease or injury. The development of effective treatments would dramatically improve the independent living and quality of life of patients. Cell transplantation strategies offer an approach to facilitating brain repair, but efficacy is often limited by low in vivo survival rates of cells that are injected in suspension. Transplanting cells that are attached to or encapsulated within a biomaterial construct has the advantage of maintaining cell-cell and cell-material interactions and improving cell survival in vivo. A variety of biomaterials have been used in preclinical studies to assist with in vivo cell transfer and incorporation into the host tissue.

It is the goal of this book to provide a forum for both experimental and clinical international experts in the field of stem cell/biomaterials research to present recent data on the latest achievements of new and emerging regenerative technologies for brain repair. Theoretical backgrounds together with tested protocols that reproduce experimental and clinical laboratory methods for educational purposes are presented. It is hoped that the topics covered herein will extend knowledge on the role of neuroregenerative technologies and that this will lead to a more effective approach to clinical management and ultimately benefit patient care.

We wish to express our deep appreciation and gratitude to each of the chapter authors for the time and effort spent on writing informative reviews on their respective areas of clinical and research interest. Also, we wish to thank Dr. Gonzalo Cordova, Senior Editor, Life and Biomedical Sciences Books, Springer Nature, for help and advice in putting together this book.

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Philip V. Peplow
Bridget Martinez
Thomas A. Gennarelli

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Contributors

Christopher Adams Neural Tissue Engineering Group, School of Life Sciences, Keele University, Staffordshire, UK

Nayef A. Amhaz University of Florida, Gainesville, FL, USA

Genevieve Z. Barquet University of Florida, Gainesville, FL, USA

Raja Haseeb Basit Bradford Royal Infirmary, Bradford, West Yorkshire, UK

Julia Bielanin (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Johannes Boltze School of Life Sciences, University of Warwick, Coventry, UK

Divya Maitreyi Chari Neural Tissue Engineering Group, School of Medicine, Keele University, Staffordshire, UK

Parker Cole (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Fatima Dakroub American University of Beirut, Beirut, Lebanon

Luís Pereira de Almeida Institute of Interdisciplinary Research, Center for Neuroscience and Cell Biology, Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

Nadine Didwischus McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

María Fernanda Guerrero-Islas Institute of Translational Neurosciences, Department of Neurosciences, CUCS Universidad de Guadalajara, Guadalajara, Mexico

Zhikun Guo Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang, China;
Henan Key Laboratory of Cardiac Remodeling and Transplantation, Zhengzhou Seventh People's Hospital, Zhengzhou, China

Weiwei He Key Laboratory of Micro-Nano Materials for Energy Storage and Conversion of Henan Province, Institute of Surface Micro and Nano Materials, College of Chemical and Materials Engineering, Xuchang University, Xuchang, Henan, P. R. China

Daniel Henriques Institute of Interdisciplinary Research, Center for Neuroscience and Cell Biology, Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

Bushra Kabiri Neural Tissue Engineering Group, School of Medicine, Keele University, Staffordshire, UK

Olivia C. Kenyon University of Florida, Gainesville, FL, USA

Alena Kisel Department of Radiology, University of Pittsburgh, Pittsburgh, PA, USA;

McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Firas H. Kobeissy Morehouse School of Medicine, Atlanta, GA, USA

Sumasri Kotha Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA;

Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Amanda Li Washington University School of Medicine, Washington University in St. Louis, St. Louis, MO, USA

Zongjin Li Nankai University School of Medicine, Nankai University, Tianjin, China;

Tianjin Key Laboratory of Human Development and Reproductive Regulation, Tianjin Central Hospital of Gynecology Obstetrics, Nankai University Affiliated Hospital of Obstetrics and Gynecology, Tianjin, China

Yong Lin Brain Injury Center, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;

Shanghai Institute of Head Trauma, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Meina Liu Puyang People's Hospital, Puyang, China;

Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang, China;

Henan Key Laboratory of Cardiac Remodeling and Transplantation, Zhengzhou Seventh People's Hospital, Zhengzhou, China

Arun Kumar Mahato Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki, Finland

Xiaobo Mao Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA;

Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA;

Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD, USA;
Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD, USA

Davis M. Maulding (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Liliana Mendonça Institute of Interdisciplinary Research, Center for Neuroscience and Cell Biology, Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

Michel Modo Department of Radiology, University of Pittsburgh, Pittsburgh, PA, USA;

McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA;

Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, USA

Andrew R. Morris University of Florida, Gainesville, FL, USA

Heather L. Morris Target RWE, Durham, NC, USA

Kai Pan Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang, China;

Henan Key Laboratory of Cardiac Remodeling and Transplantation, Zhengzhou Seventh People's Hospital, Zhengzhou, China

Stuti R. Patel University of Florida, Gainesville, FL, USA

Marimelia A. Porcionatto Department of Biochemistry, Neurobiology Laboratory, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

Rodrigo Ramos-Zúñiga Institute of Translational Neurosciences, Department of Neurosciences, CUCS Universidad de Guadalajara, Guadalajara, Mexico

Carlos Isaac Ramírez-Bañales Institute of Translational Neurosciences, Department of Neurosciences, CUCS Universidad de Guadalajara, Guadalajara, Mexico

Mart Saarma Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki, Finland

Mahsa Saeidi (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Zaynab Shakkour American University of Beirut, Beirut, Lebanon

Aruna Sharma (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Hari S. Sharma (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Manjari Sriparna Virginia Commonwealth University School of Medicine, Richmond, VA, USA

Ryan Tian (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Yang Tian (ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas, Fayetteville, AR, USA

Joel Tyson Department of Chemical, Biochemical and Environmental Engineering, University of Maryland Baltimore County, Baltimore, MD, USA

Jessica Wiseman Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield, UK

Laura N. Zamproni Department of Biochemistry, Neurobiology Laboratory, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

Jiepei Zhu Morehouse School of Medicine, Atlanta, GA, USA

Chapter 1

Initiating and Facilitating Brain Repair: Factors, Principles, and Mechanisms



Laura N. Zamproni and Marimelia A. Porcionatto

Abstract *Introduction:* Acute injuries to the brain result in a wide range of sequelae, and several factors contribute to the severity of a brain lesion, including age, overall health, and extension and site of the injury. Complete brain repair after a massive traumatic brain injury or stroke remains challenging. During the acute phase of the injury, the inflammatory microenvironment can induce further neuronal death. The lack of structural support for cellular repopulation, anchoring, and synapse formation reduces the repair chances. After the acute phase, the glial scar formed by reactive astrocytes and oligodendrocytes dramatically decreases the possibility for the surviving neurons to restore damaged synapses. *Methods:* We reviewed studies describing the development of bioengineering techniques, with the production of biocompatible new biomaterials that can become the next step in neurorepair. *Results:* Various natural and synthetic materials have been used to replace damaged tissue, and using induced pluripotent stem cells to originate neurons from the patient dramatically reduces the chances of rejection of biomaterial populated with cells, creating innovative biopatches. Besides using biomaterial constructs to deliver cells, bioscaffolds can deliver genes, nanoparticles, soluble factors, and exosomes to induce neurorepair in a more controllable and permanent manner. *Conclusions:* Understanding the cellular and molecular mechanisms activated in response to brain injury is critical to designing and developing successful brain repair strategies.

Keywords Bioscaffolds · Biomaterials · Brain repair · Tissue engineering · Stem cells

L. N. Zamproni · M. A. Porcionatto (✉)
Department of Biochemistry, Neurobiology Laboratory, Escola Paulista de Medicina,
Universidade Federal de São Paulo, São Paulo, Brazil
e-mail: marimelia.porcionatto@unifesp.br

Abbreviations

3D	Three-dimensional
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMPs	Bone morphogenetic proteins
CCL11	C-C motif ligand 11
CCL2	C-C motif ligand 2
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycan
CXCL1	C-X-C motif ligand 1
CXCL12	C-X-C motif ligand 12
DAMPs	Damaged-associated molecular patterns
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EPO	Erythropoietin
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
FOXA2	Forkhead box A2
GAL1	Galectin 1
GAL3	Galectin 3
GFAP	Glial fibrillary acidic protein
IGFs	Insulin-like growth factors
IL10	Interleukin 10
IL1 β	Interleukin 1 beta
IL6	Interleukin 6
iPSCs	Induced pluripotent stem cells
<i>LMX1B</i>	LIM homeobox transcription factor 1-beta
MAG	Myelin-associated glycoprotein
Micro-TENNs	Microtissue-engineered neural networks
MMPs	Metalloproteinases
MSCs	Mesenchymal stem cells
NGF	Nerve growth factor
NSCs	Neural stem cells
NT3	Neurotrophin 3
NURR1	Nuclear receptor-related 1
O4	Oligodendrocyte progenitor marker
OMGp	Oligodendrocyte myelin glycoprotein
PAX6	Paired box protein 6
PROK2	Prokineticin 2
PRRs	Pattern recognition receptors
RMS	Rostral migratory stream
SGZ	Subgranular zone
SPMs	Specialized pro-resolving mediators

SVZ	Subventricular zone
TBI	Traumatic brain injury
TH	Tyrosine hydroxylase
TNF α	Tumor necrosis factor alpha
TUBBIII	Class III beta-tubulin
VEGF	Vascular endothelial growth factor

Initiation of Neuroinflammation: The Role of Microglia

Neuroinflammation is common among neurological disorders, including brain trauma and stroke [1–3]. It will vary in intensity, range, and duration depending on the context and course of the primary injury. Neuroinflammation can be transient and self-limited, facilitating tissue repair, or persistent and dysregulated, leading to a chronic inflammatory state and tissue degeneration [4].

After a brain injury, the inflammatory process is initiated by debris formed from the cells that died from the acute injury. The molecules released from damaged or dying cells are collectively known as damaged-associated molecular patterns (DAMPs) [5]. DAMPs activate microglia receptors called pattern recognition receptors (PRRs), initiating the inflammatory response. Activated microglia removes cell debris and release inflammatory proteins, such as interleukin 1 beta (IL1 β), interleukin 6 (IL6), tumor necrosis factor alpha (TNF α), and several chemokines, such as C–C motif ligand 2 (CCL2), C–X–C motif ligand 1 (CXCL1) and prokineticin 2 (PROK2) [6–8].

Astrocytes become reactive as part of the inflammatory process and undergo several changes, such as hypertrophy, increased expression of glial fibrillary acidic protein (GFAP), galectin 1 (GAL1), and galectin 3 (GAL3) [9–11]. Also, as part of astrocyte reactivation, metalloproteinases (MMPs) and chemokines such as C–X–C motif ligand 12 (CXCL12) secretion increases [12].

The consequence of increased MMP secretion is the degradation of extracellular matrix (ECM) proteins that will allow for tissue remodeling but also leads to the breakdown of the blood–brain barrier (BBB) due to basal lamina degradation [13]. Circulating immune cells enter the brain parenchyma through the disrupted BBB, thus enhancing the inflammatory process and creating a positive loop [14].

The inflammation resolution, or the end of the inflammatory response, follows the acute inflammation phase and is driven by the release of a class of molecules, the specialized pro-resolving mediators (SPMs), by endothelial cells, macrophages, and neutrophils [15]. Several SPMs have been described, resulting from the metabolism of polyunsaturated fatty acids released by lipoxygenase, cyclooxygenase, or cytochrome P450 monooxygenase. The SPMs resolvins, protectins, maresins, and lipoxins are signaling molecules that play important roles in inflammation resolution [16, 17]. During neuroinflammation resolution, anti-inflammatory cytokines such as interleukin 10 (IL10) and trophic factors are released, favoring

tissue regeneration [18]. However, if the inflammatory process remains unresolved, it can lead to chronic central nervous system (CNS) inflammation and neurodegeneration.

Glial Scar and Neural Tissue Remodeling: The Role of Astrocytes

Along with the resolution of the acute inflammation, molecules derived from reactive astrocytes and oligodendrocytes produce scar tissue known as glial scar [9, 19–21]. The glial scar is composed of neuronal outgrowth inhibitory molecules such as chondroitin sulfate proteoglycan (CSPG), NOGO, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMGp). Figure 1.1 summarizes the phases from acute brain injury to tissue remodeling.

Over the years, there have been many attempts to reduce glial scar as a strategy to reduce injury sequelae. Still, the strategies to inhibit or degrade the scar components resulted in enhanced inflammation and worsening of the pathology, indicating that the glial scar is necessary for the process of inflammation resolution [22–25]. Conditional

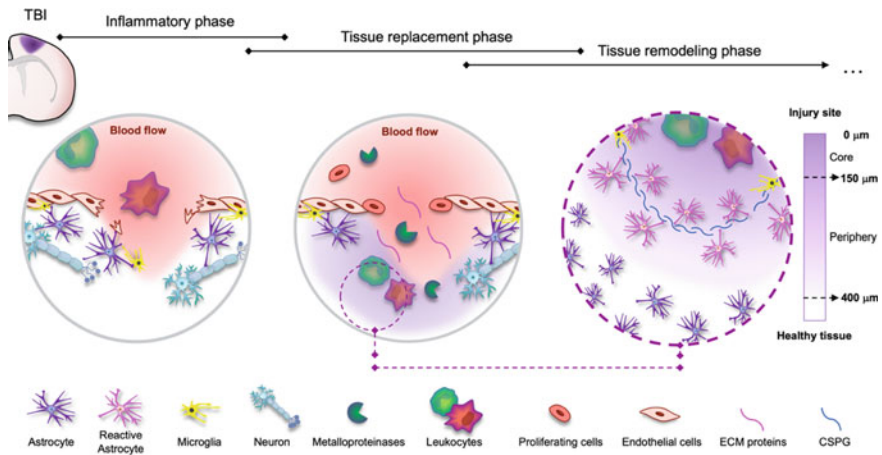


Fig. 1.1 Schematic representation of damaged tissue after traumatic brain injury (TBI) with a timeline of the overlapping phases. The inflammatory phase is characterized by the rupture of the blood–brain barrier (BBB) and infiltration of leukocytes into the brain parenchyma, causing the death of neuronal and non-neuronal cells. The tissue replacement phase begins around 48 h post-injury when proliferating and migrating cells reach the injury site and start restructuring the damaged tissue and blood vessels. The tissue remodeling phase comprises the activation of metalloproteinases (MMPs), incorporation of newly synthesized extracellular matrix (ECM) proteins, myelin-associated proteins, and chondroitin sulfate proteoglycans (CSPG) by reactive astrocytes and oligodendrocytes, culminating with the production of a glial scar (Original illustration by Dr. Julia C. Benincasa)

ablation of reactive astrocytes in mice leads to increased local tissue disruption, severe demyelination, and neuron and oligodendrocyte death [19].

Reactive astrocytes have a dual role in the resolution of brain injuries. On one side, astrocytes produce molecules that surround the primary injury site, avoiding expansion of the injury, and on the other hand, astrocytes secrete molecules that will provide survival signals for the undamaged neurons. The pro-survival molecules include glucose, nutrients, and growth factors such as insulin-like growth factors (IGFs), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3) [25, 26]. Additionally, astrocytes stimulate local angiogenesis by recruiting endothelial cells and fibroblasts into the lesioned area [25].

Post-Injury Neurogenesis and Brain Plasticity

Brain plasticity refers to any process that leads to regaining functions and restoring functional neuronal circuits after an injury. Plasticity involves short-distance axon sprouting, leading to new connections and strengthening existing connections [27]. The plasticity process allows signals to bypass damaged areas by creating new circuits and reassigning new functions to undamaged areas of the CNS. After brain injuries, such as a stroke, neurons in the perilesional area upregulate signaling pathways that promote axonal outgrowth and synapse formation [28]. The turnover of dendritic spines is enhanced, providing a substrate for new connections. Neurons in perilesional tissue can project new axons by several millimeters into nearby cortical areas where new functional synaptic connections are formed [29].

The adult mammalian brain contains two well-defined neurogenic niches: the hippocampus's subgranular zone (SGZ) in the dentate gyrus and the subventricular zone (SVZ) in both lateral ventricles. Extensive work has been done in identifying and characterizing the cellular and molecular components of those niches and searching for new areas in the CNS that can generate new neurons in adults [30–35]. Neural stem cells (NSC) in the SVZ are pluripotent stem cells that can produce astrocytes, oligodendrocytes, and neurons. Newborn neurons, or neuroblasts, produced by NSCs in the SVZ neurogenic niche, migrate through the rostral migratory stream (RMS) to the olfactory bulbs, where they differentiate into mature interneurons [36].

The discovery of adult neurogenesis in mammalian brains shed light on new possibilities for brain repair. In rodent brain trauma and stroke models, there is increased cell proliferation in the SVZ and the recruitment of neuroblasts that migrate along blood vessels towards the injury [37, 38]. Again, the reactive astrocytes are essential players in this process and are critical regulators of adult neurogenesis. Astrocytes are one of the primary sources of molecules such as bone morphogenetic protein (BMP) and WNT, which regulate NSC proliferation and differentiation. NSC-derived neuroblasts are attracted to the injury site by chemoattractive agents like C–C motif ligand 2 (CCL2), C–C motif ligand 11 (CCL11), and CXCL12, mostly produced by astrocytes [6, 12, 39–43].

Although neuroblasts born in the SVZ migrate toward the injury site, most of them cannot penetrate the glial scar due to the presence of chondroitin sulfate proteoglycan (CSPG) that impairs cell protrusions and adhesion dynamics through Rho GTPase inhibition [21]. Neuroblasts that reach the injury site are attracted by CXCL12 secreted by astrocytes and PROK2 secreted by microglia [6] but die by apoptosis induced by partially cleaved CXCL12 [44].

These processes expose the complexity of the cellular and molecular mechanisms involved in attempts to promote brain repair and, so far, indicate that adult endogenous neurogenesis alone is insufficient for complete brain repair [45]. More recently reactive astrocytes have been suggested as a new possibility for endogenous neurogenesis to repair injured tissue due to their capacity to dedifferentiate and acquire NSC phenotype [10, 11, 46].

Strategies to Repair the Injured Brain

The term “repair,” when used to describe the healing of damaged tissue, means to restore tissue architecture and function, and comprises two processes: regeneration and replacement. Regeneration occurs when the damaged tissue grows into new tissue and is restored to its normal state. Replacement occurs when a different tissue, usually connective tissue, is deposited over the damaged tissue, producing a scar [47]. The complexity of CNS anatomy, physiology, and cellular composition makes repair challenging. Rebuilding the brain means rebuilding the complex brain tissue architecture and its intricate and extensive vascular networks [48].

As presented in the previous section, although these processes are limited, the mammalian brain possesses regenerative mechanisms, including endogenous neurogenesis and neuroplasticity [27, 49]. Beyond the discovery and development of new drugs to overcome the obstacles to neuroregeneration, new therapeutic strategies include stem cell transplantation, gene therapy, and tissue engineering. More recently, various combinations of biomaterials, stem cells, and chemical and physical cues have produced bioengineered tissue-like structures to replace *in vivo* tissues and organs [50].

Biomaterials

Biomaterials refer to biocompatible materials that can be from natural sources, such as ECM proteins, for example, collagens and fibronectin, or algae-derived polysaccharides, such as alginate; biomaterials can also derive from non-natural materials engineered to integrate with a biological system and provide beneficial effects by directing or controlling cell interaction [51–55]. In brain injuries, biomaterials are mainly used for two purposes: as scaffolds to provide mechanical support to the injured brain while providing cues for new neural circuits formation or as carriers, to

deliver content such as stem cells, growth factors, exosomes, and gene vectors to the site of injury [53, 56–58]. By replacing the virtual cavity formed after a brain injury, bioscaffolds can provide a tissue-appropriate physical and trophic environment for new neural cells and circuitry to survive and integrate into the host tissue [56].

The biomaterial must be compatible with the tissue in which it will be implanted and interact with the tissue-specific ECM. In the brain, the ECM components regulate several neural processes, including neurite outgrowth, synaptogenesis, synaptic stabilization, and injury-related plasticity, both in development and adulthood [59]. The main components of the brain ECM are glycosaminoglycans (chondroitin sulfate, heparan sulfate, hyaluronic acid), proteoglycans (neurocan, brevican, versican, and aggrecan), glycoproteins (tenascin-R), and low levels of fibrous proteins (collagen, fibronectin, and vitronectin) [59]. Also, brain ECM varies in different compartments of the neural tissue, for example, the vascular basement membrane is composed of collagen, laminin, fibronectin, and proteoglycans; the perineuronal matrix is made primarily of CSPG, and the interstitial matrix contains mainly proteoglycans, hyaluronic acid, and small amounts of collagen, elastin, laminin, and fibronectin [60]. As mentioned, a glial scar is formed after a brain injury, and it contains a higher amount of CSPG than the surrounding healthy tissue. Unlike other tissues in the body, the glial scar is softer than the healthy neural tissue [61], impacting astrocyte response [62].

The brain ECM structure imposes the biomaterial to be used in the brain must possess specific characteristics. Besides being biocompatible, the biomaterial must possess mechanical properties similar to the brain tissue. Stiffer materials lead to increased astrogliosis, and softer materials lead to poor material stability at the implant site. The ideal biomaterial should not induce or worsen neuroinflammation. In this way, long-term implants can cause a chronic inflammatory reaction, degradability is desirable, and degradation products should be non-cytotoxic. Since the brain is confined to the skull, the biomaterial must present minimal swelling to avoid rising intracranial pressure [55, 56]. The rigid skull also imposes in the biomaterial delivery route, making injectable and shape-adaptable materials like hydrogels preferred over solid scaffolds requiring invasive surgical implantation [56].

Hydrogels are formed by physical or chemical cross-linking of hydrophilic polymers or by self-assembly systems. Their mechanical properties are usually similar to the brain tissue, and they can be injected in a liquid form, fill the irregular injury cavity, and then polymerize, forming a gel, a feature extremely relevant for the delivery method of choice [63].

Bioscaffolds for Brain Repair

The ideal bioscaffold must match the brain biochemical environment in terms of water content and pH, the brain biophysical environment such as viscoelastic properties and porosity, the ECM three-dimensional (3D) architecture on a biologically relevant length scale, and also stimulate cells to infiltrate its structure [64, 65]. Bioscaffolds'

mechanical forces can regulate the cellular microenvironment and control cell–cell and cell-ECM interactions [66–68].

Mechanical forces can influence cell functions such as migration, proliferation, differentiation, and apoptosis [69]. For the CNS, bioscaffold electroconductive properties are usually desirable. It is well established that an electroconductive surface can increase neuronal differentiation, stimulate axon growth, and facilitate synapsis formation [70, 71]. Fibrous scaffolds, particularly those with oriented fibers, can regulate and guide axon sprouting and synapse formation [72, 73].

One of the concerns regarding biomaterials implantation in the brain is the possibility of a foreign body response that would lead to an increase in neuroinflammation [74, 75]. Although some materials have been shown to modulate inflammation, for example, high molecular weight hyaluronic acid decreases microglia and glial scarring at the injury site [76], there is a concern of adverse immune reactions resulting in exacerbated inflammation, healing impairment, fibrotic encapsulation, and isolation and rejection of medical devices [74]. One of the strategies to overcome this issue is incorporating bioactive molecules, such as cytokines and growth factors, that could modulate the inflammatory response. Combining bioscaffolds with stem cells, growth factors, and exosomes show great perspectives of positive results for the development of new strategies for brain repair [77] (Table 1.1).

Table 1.1 Examples of studies focusing on bioscaffolds for brain repair

Cell	Scaffold	Outcome	References
Embryonic mouse cortical neural cells	Silk fibers	<ul style="list-style-type: none"> • Increased neurite extension • Guided axonal elongation • Guided cell migration from cellular spheroids along the fibers 	[78]
Rat hippocampal neurons	Graphene	<ul style="list-style-type: none"> • Induced neuronal networks formation • Increased GABAergic activity 	[79]
Mouse NG108-15 cells	Graphene oxide/silk fibers	<ul style="list-style-type: none"> • Increased cell proliferation • Increased neurite extension 	[80]

(continued)

Table 1.1 (continued)

Cell	Scaffold	Outcome	References
Neuroglioma cells	Poly (3,4-ethylene dioxythiophene)/chitosan fibers	<ul style="list-style-type: none"> • Increased cell proliferation • Increased axon density 	[81]
Mouse NG108-15 cells	Poly (3,4-ethylenedioxythiophene)-polystyrene sulfonate (PEDOT: PSS)/silk fibers	<ul style="list-style-type: none"> • Increased cell proliferation • Increased neurite extension 	[82]
Human neuroblastoma cells	Poly- ϵ -caprolactone (PCL) nanofibers	<ul style="list-style-type: none"> • Increased neurite extension 	[83]
Human neuroblastoma cells	Poly(3,4-ethylenedioxythiophene) (PEDOT)/Carbon nanotubes	<ul style="list-style-type: none"> • Increased neuronal markers 	[84]
Rat hippocampal cells	Aragonite skeleton of the coral <i>Trachyphyllia geoffroyi</i>	<ul style="list-style-type: none"> • Promoted elongation of astrocytic processes • Increased GFAP expression in astrocytes 	[85]
Rat adipose tissue-derived neuron-like cells	Corning® PuraMatrix™ hydrogel	<ul style="list-style-type: none"> • Increased cell proliferation • Increased neuronal markers expression 	[86]
Rat pheochromocytoma cells	Porcine brain decellularized ECM	<ul style="list-style-type: none"> • Increased neuronal differentiation 	[87]
Human Glioblastoma cells (U-87MG)	Carbon nanotubes	<ul style="list-style-type: none"> • Reduced cell growth 	[88]
Rat embryonic NSCs	Collagen/heparan sulfate porous scaffolds	<ul style="list-style-type: none"> • Improved regeneration of neurons, nerve fibers, synapses, and myelin sheaths • Reduced brain edema and cell apoptosis • Recovered rat motor and cognitive functions 	[89]

(continued)

Table 1.1 (continued)

Cell	Scaffold	Outcome	References
Human fetal brain- and spinal cord-derived NSCs	Aligned collagen sponge scaffolds	<ul style="list-style-type: none"> • Stem cell long-term cell survival • Stem cell neuronal differentiation • Reduced inflammation • Reduced glial scar formation • Recovered rat locomotor functions 	[90]
Rat adipose tissue MSCs	Self-assembling nano-peptide scaffold RADA4GGSIKVAV (R-GSIK)	<ul style="list-style-type: none"> • Reduced reactive astrocytes • Reduced microglial cells • Reduced TLR4, TNF, and IL6 	[91]
Human umbilical cord MSCs	Collagen hydrogels	<ul style="list-style-type: none"> • No differences in proteomics between treated and control group 	[92]
Human umbilical cord MSCs	Collagen scaffolds	<ul style="list-style-type: none"> • Increased motor scores • Enhanced amplitude, and shortened latency of the motor evoked potential • Reduced injury area in magnetic resonance imaging 	[93]
Adipose-derived MSCs overexpressing BDNF and NT3	Silk fibroin/chitosan scaffold	<ul style="list-style-type: none"> • Reduced scar tissue • Decreased inflammation • Increased nerve fiber formation 	[94]
Rat NSCs	Matrigel	<ul style="list-style-type: none"> • Decreased reactive astrogliosis • Improved functional recovery 	[95]

(continued)

Table 1.1 (continued)

Cell	Scaffold	Outcome	References
Human ESC-derived NSCs	Hyaluronic acid hydrogel	<ul style="list-style-type: none"> • Increased oligodendrocyte differentiation • Improved locomotor function 	[96]
Mouse iPSCs-derived NSCs	FGF and chondroitin sulfate hydrogel	<ul style="list-style-type: none"> • Improved vascular remodeling • Improved cortical blood flow • Improved sensorimotor function 	[97]
Embryonic rat NSCs	3D bioprinted collagen/silk fibroin scaffold	<ul style="list-style-type: none"> • Reduced glial scar • Increased regenerative axons • Improved functional recovery • Improved electrophysiologic tests 	[98]
Mouse CGR8 ESCs	Poly ϵ -caprolactone (PCL)/gelatin scaffolds	<ul style="list-style-type: none"> • Promoted neural differentiation • Promoted efficient secretion of dopamine 	[99]
Human iPSC- and ESC- derived NSCs	Poly(ethylene glycol) diacrylate-crosslinked porous scaffolds	<ul style="list-style-type: none"> • Increased neural cells functional maturity 	[100]
Human iPSCs	Fibrin hydrogel	<ul style="list-style-type: none"> • Increased Olig2, MBP, Sox 10, and PDGFRα expression • Increased oligodendrocyte differentiation 	[101]
Human olfactory MSCs	Chitosan-aniline pentamer/gelatin/agarose scaffolds	<ul style="list-style-type: none"> • Promoted differentiation into motor neuron-like cells 	[102]

(continued)

Table 1.1 (continued)

Cell	Scaffold	Outcome	References
Rat hippocampal NSCs	Poly- ϵ -caprolactone (PCL) fibers	<ul style="list-style-type: none"> • Increased cell proliferation • Increased astrocyte and oligodendrocyte differentiation 	[103]
NSCs	Poly (L-lysine) modified silk fibroin film	<ul style="list-style-type: none"> • Increased cell proliferation • Decreased apoptosis • Increased neuronal differentiation 	[104]
Mouse MSCs	Graphene foam	<ul style="list-style-type: none"> • Promoted dopaminergic neuronal differentiation 	[105]

Bioscaffolds Containing Stem Cells

Stem cell-based therapies are largely explored for CNS repair in neurological pathologies such as TBI, stroke, Alzheimer's and Parkinson's disease, and amyotrophic lateral sclerosis (ALS), among other neurodegenerative disorders [49, 95, 106–112]. Stem cell sources include embryonic stem cells (ESC), mesenchymal stem cells (MSC), induced pluripotent stem cells (iPSC), and NSC [113]. In many stem cell transplant studies published, the expectation was that stem cells would exert therapeutic properties by differentiating into neurons at the injury site, repopulating the injured tissue. Still, the cells often function as bioreactors producing and secreting soluble factors that promote survival, neuroprotection, angiogenesis, and neurogenesis [113].

As mentioned, the ECM of the injured brain does not offer a proper microenvironment for cell anchoring, proliferating, and differentiating [114, 115], and this poses a challenge for conventional stem cell delivery. Intravenous or intracardiac administration of MSCs after a rat model of TBI showed that <0.0005% of the cells injected were found at the injury site after three days [108]. Even the local administration of MSCs at the injury site does not guarantee that the cells will remain at the administration site as they migrate away, likely attracted by chemokines secreted at further locations [23].

Delivering stem cells in bioscaffolds can surpass this issue once bioscaffolds provide the biomechanical support for cells until they can produce ECM, increasing cell survival [114]. Scaffolds seeded with MSCs improved cell retention compared to MSCs alone in a mouse stroke model [73]. Stem cells in biological scaffolds

can be implanted into damaged sites to secrete neurotrophic factors, enhance axon regeneration, promote myelination, and reduce scar formation [116].

Differentiation of transplanted stem cells into neurons and incorporation into healthy tissue is rarely observed. New bioscaffold compositions have been proposed to direct stem cell fate by providing physical–chemical cues to enhance neuronal differentiation. Scaffolds' physical cues include mechanical properties, pore sizes, porosity, surface stiffness, 3D structures, and mechanical and electrical stimulation [117].

Bioscaffolds Containing Soluble Factors

Biomaterials are promising drug delivery vehicles for their ability to provide local, time-controlled release, particularly important in the brain since the BBB imposes intravenous drug delivery restrictions. Also, a biomaterial platform provides sustained drug release in a single application [118]. Bioscaffolds can be used to deliver growth factors helping to create a pro-regenerative environment. Some of the factors tested include BDNF, erythropoietin (EPO), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) [119].

A combination of collagen/chitosan scaffold with FGF was used in a spinal cord injury model and significantly improved locomotor function and electrophysiological examinations eight weeks after scaffold implantation [120]. Rats receiving collagen/chitosan scaffold containing FGF presented improved regeneration of nerve fibers tract evaluated by magnetic resonance imaging [120].

Another important feature in brain repair is to support the re-establishment of the tissue vasculature lost due to injury. An *in vivo* mouse stroke model was used to assess if the delivery of immobilized VEGF could enhance angiogenesis at an injury site. Histidine-tagged VEGF was immobilized via affinity interaction to a porous laminin-rich sponge and transplanted at the lesion site. In comparison to transplanting the laminin-rich sponge with soluble VEGF, the immobilized factor produced stronger angiogenic activity, indicating this as a viable method to efficiently delivering soluble factors to aid brain repair [121].

Bioscaffolds Containing Exosomes

Exosomes are small membrane vesicles secreted by eukaryotic cells for intercellular communication and signaling [122]. Exosomes cargo includes cytokines, growth factors, signaling lipids, mRNAs, and microRNAs that can influence cell response to injury, infection, and disease [123]. Exosomes have been studied for brain repair and combined with bioscaffolds for therapy. The rationale of incorporating exosomes into a bioscaffold is that the scaffold may sustain exosome cargo delivery at the injury site [124].

Exosomes from MSC cultured in a 3D collagen scaffold enhanced brain trauma recovery more than exosomes secreted by MSC cultured on conventional conditions. The exosomes were delivered intravenously in a mice model of brain trauma, and both exosome types promoted endogenous angiogenesis and neurogenesis, reduced neuroinflammation, and significantly improved rat functional recovery. However, the 3D cultured MSC-derived exosomes provided a better outcome in spatial learning than conventional MSC-derived exosomes [125].

Incorporating bone marrow MSC-derived exosomes into hyaluronan/collagen hydrogel successfully induced angiogenesis and neurogenesis in a mouse TBI model. Upon recruitment of endogenous NSCs, neuronal differentiation, and vascularization was observed and related to increased axonal regeneration, remyelination, synapse formation, and functional recovery [126].

Bioscaffolds for Gene Delivery

Gene therapy has been a candidate therapy for many CNS disorders [127]; however, the use of viral vectors in gene therapy still poses a concern, and the development of new, highly efficient, low cytotoxic gene therapy strategies are required [128]. Biomaterials and bioscaffolds may offer a safe alternative for delivering genetic material to cells and can be, in the future, the key for genetic therapy in humans [57, 129, 130].

MgAl-NO₃ layered double hydroxide was used as a non-viral vector to deliver nucleic acids (pDNA, miRNA, and siRNA) to MSC using a 3D scaffold approach. Nucleic acids were complexed with MgAl-NO₃ layered double hydroxide and incorporated in collagen-nanohydroxyapatite scaffolds. The fabricated platform allowed successful MSC transfection [128].

The bioscaffold-gene therapy combination strategy has been investigated for developing pro-angiogenic platforms [131]. Combining nanoparticles carrying the gene encoding for the chemokine CXCL12 with a collagen-CSPG scaffold enhances MSC angiogenic properties. MSC on the scaffold overexpresses CXCL12 mRNA earlier combined with expressing the angiogenic factor VEGF. In a wound-healing model, the conditioned media from MSC grown on the scaffold increased endothelial cell viability, tubule formation, and cell migration. Pro-angiogenic genes were also upregulated in endothelial cells exposed to conditioned media of MSC grown in the scaffold [132].

Engineered Microtissue

Microtissue-engineered neural networks (micro-TENNs) were developed to support neuronal survival and neurite extension [133]. Micro-TENNs consist of neuronal populations with long axonal tracts entrapped into tubular hydrogels of 180–500 μm diameter and up to 2.0 cm length. Micro-TENNs are fabricated by filling a cylindrical mold with a longitudinally centered needle with liquid hydrogel. Once the gelification (<https://www.google.com/search?sxsrf=ALeKk01ekvuKURAIcty2qmelUVMV80rKJw:1609616609506&q=gelification&spell=1&sa=X&ved=2ahUKewjJoO-Rgf7tAhXtlosKHcTIAGIQBSgAegQIBBAw>) occurs, the needle is removed, creating a hollow micro-column filled with a solution containing ECM components that provides an environment suitable for neuronal adhesion and axonal outgrowth [134]. Micro-TENNs reconstitute the architecture of long-distance axonal tracts. They may be an effective substrate for re-establishing long-distance axonal connections and reconstructing damaged brain pathways. Micro-TENNs have very small diameters and are easily delivered into the brain with minimally invasive procedures.

Micro-TENNs were injected into rats' brains using a stereotaxic device to connect deep thalamic structures with the cerebral cortex. The authors found that micro-TENN neurons survived at least one month and maintained their extended axonal architecture along the cortical-thalamic axis. They also found micro-TENN neurons extend neurites into the host cortex, with successful synapse formation [133]. In another approach, micro-TENNs were used to align an astrocytic network, mimicking the glial tube that forms the rostral migratory stream RMS. Those astrocytic networks successfully improved NSC migration and directly directed the cells from the neurogenic niche to the injured area [135].

Micro-TENNs containing sensory dorsal root ganglion (DRG) neurons were grafted across the region of a spinal cord injury (SCI) in rats, spanning the glial scar. Micro-TENN's capacity to promote axonal regeneration was assessed, and after one-month post-injury, graft-derived axonal outgrowth was observed in the spinal cord. The low astroglial response observed indicates that the engraftment process did not produce large trauma or scar formation, suggesting that micro-TENNs might be used as a therapeutic strategy for repairing SCI [136].

3D Bioprinting

The human brain is the most complex structure in the human body, and in this context, 3D bioprinting may offer a solution for designing specific individualized constructs while controlling tissue architecture. 3D bioprinting allows combining one or more cell types with a supportive bioscaffold, designated bioink, to fabricate structures resembling native tissue topography [137].

Despite the massive advance in the field in the last decade, it is still impossible to print whole organs that can be implanted. Some promising results have been obtained for the spinal cord. A mixture of iPSC-derived spinal NSC and oligodendrocyte progenitor cells was 3D bioprinted in a gelatin and fibrin bioink to fabricate a spinal cord. Bioprinted NSC was able to differentiate and extend axons throughout the scaffold. Physiological spontaneous calcium flux studies confirmed neuronal activity [138]. A 3D silk fibroin scaffold with cavities that simulate the normal spinal cord anatomy was transplanted, combined with NSC, in rats submitted laminectomy. The rats receiving the combination of scaffold and NSCs showed significantly higher neurological scores. They also performed better in electrophysiological studies, and magnetic resonance imaging revealed spinal cord continuity and injury cavity filling. The bioprinted spinal cords also decreased the glial scar, increasing regenerative axons [98].

Although implantable brain tissue is not yet available, producing smaller and less complex brain structures to study physiological cell-to-cell or cell-to-scaffold interactions is feasible. 3D bioprinting can improve in vitro platforms for modeling neurological diseases, neural regeneration, and drug development. For example, a 3D brain-like co-culture construct containing 3D neurospheres was fabricated in an astrocyte-laden resembling the NSC niche environment. Then, the authors used a photo-cross-linkable bioink to bioprint neurosphere layers, in which cells differentiate into neurons [139].

A fibrin-based bioink formulation, drug-releasing microspheres, and human iPSC-derived NSC were used to bioprint neural tissues. Microspheres were loaded with guggulsterone, which promotes NSC differentiation into dopaminergic neurons. Combining these three elements, a highly viable tissue expressing the neural markers GFAP, class III beta-tubulin (TUBBIII), forkhead box A2 (FOXA2), tyrosine hydroxylase (TH), and the oligodendrocyte progenitor marker (O4) was obtained. After 30 days, gene expression analysis showed the expression of LIM homeobox transcription factor 1-beta (*LMX1B*), Paired box protein 6 (*PAX6*), and the nuclear receptor-related 1 (*NURR1*), a gene expressed in midbrain dopaminergic neurons [140].

Conclusion and Future Directions

Although spontaneous tissue regeneration is limited in the CNS, tissue engineering strategies to overcome the biological and physical challenges imposed by brain injury are gradually being developed. Bioengineering already offers a series of commercially available products for tissues like skin, bone, and cartilage, but this is not the case for the CNS, and up to now, there are no suitable bioengineered therapeutic solutions to amend injuries to the CNS.

Since several pathologies can affect the CNS, it is rational to believe that the approaches presented here will complement each other rather than compete. Additionally, the constructs should match the patient's needs. In Parkinson's disease,

for example, a platform that stimulates dopaminergic neuron growth is required, whereas, in amyotrophic lateral sclerosis (ALS), the challenge is to replace long neuronal tracts. The advancement of precision medicine and new scaffold fabrication methods, such as 3D bioprinting, will allow individualized treatment. Bioscaffolds and scaffold-based constructs should evolve with increasing complexity and functionality in the next years, impacting medical research. However, producing fully vascularized tissue units is essential to increase constructs' thickness and complexity while ensuring cell survival, although has not been accomplished yet. Also, biomaterials' long-term effects on the CNS and their interaction with the immune system must be addressed. Finally, it is essential to understand developmental biology to design better "smart bioscaffolds" capable of stimulating neurogenesis and neural network formation.

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Chapter 2

Strategies to Upgrade the Stem Cell Application for Brain Transplantation



Daniel Henriques, Johannes Boltze, Luís Pereira de Almeida,
and Liliana Mendonça

Abstract *Introduction:* Cerebral stem cell transplantation has the potential to replace dead cells and restore lost brain functions in neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's disease. However, success has been highly limited by poor survival, migration, differentiation, and functional integration of the transplanted cells. *Methods:* A thorough literature review was performed on PubMed and Web of Science using the keywords "stem cell brain transplantation", "migration", "survival", and "differentiation". Results were selected based on relevance to the topic of this chapter. *Results:* Several strategies to improve the therapeutic impact of stem cell transplantation in brain diseases have been tested with some success. These include modulation of the extracellular matrix and neuroinflammation, co-transplantation with other cell types such as astrocytes and endothelial cells, cell patterning, reduction of apoptotic cell death, and the use of neurotrophic factors alongside with cell transplantation. These different approaches show promising results in promoting cell survival, migration, differentiation, and functional integration. *Conclusion:* Significant improvements in cell therapy strategies for brain diseases have been reported. However, further research is required to determine whether a single strategy is possible to be implemented or whether these strategies must be customized for each disease to develop safe and effective cell-based treatments for neurodegenerative diseases.

D. Henriques · L. P. de Almeida · L. Mendonça (✉)

Institute of Interdisciplinary Research, Center for Neuroscience and Cell Biology, Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal
e-mail: liliana.mendonca@cnc.uc.pt

D. Henriques

e-mail: dhenriques@cnc.uc.pt

L. P. de Almeida

e-mail: luispa@cnc.uc.pt

J. Boltze

School of Life Sciences, University of Warwick, Coventry, UK

e-mail: Johannes.Boltze@warwick.ac.uk

Keywords Stem cell survival · migration · differentiation · and functional integration · Neurotrophic factors · Apoptotic cell death reduction · Extracellular matrix and neuroinflammation modulation · Cell patterning · Cell co-transplantation

Cell replacement strategies are promising cell therapy approaches that have led to important preclinical progress in different neurodegenerative disorders such as Parkinson's disease (PD) [8, 31, 109], Huntington's disease (HD) [26, 42], and Machado-Joseph disease (MJD) [69] in the last decades. Clinical trials have demonstrated the potential of cell replacement strategies for neurodegenerative diseases treatment [7, 27, 58, 75, 79]. Nevertheless, despite many promising results, there is still much room to improve the therapeutic impact of these therapies since many cell-based strategies show limited survival, migration, and differentiation of the transplanted cells [33].

Several studies have worked on the enhancement of cell engraftment mainly by increasing cell survival and migration upon transplantation, as well as graft differentiation into the correct type of cells [20, 70, 74, 104, 114]. Overall, several strategies have been tested, namely extracellular matrix modulation, co-transplantation with other cell types, neurotrophic factors administration/overexpression, modulation of cell death and inflammation, as well as cell patterning and differentiation (Fig. 2.1).

Extracellular Matrix Modulation

The extracellular matrix (ECM) in the adult brain is a complex network that is critical to different cellular processes including cell survival, proliferation, differentiation, and migration [9]. Thus, the ECM is a promising candidate target when attempting to improve cerebral cell transplantation procedures.

To enhance neural stem cell (NSCs) transplantation, Tate and colleagues [104] transplanted 30,000 primary fetal-derived NSCs within a tissue-engineered scaffold containing extracellular proteins that may provide support to the transplanted cells. The NSCs were transplanted within a **fibronectin** (FN)- or **laminin** (LN)-based scaffold into a mouse model of traumatic brain injury (TBI). Evaluation of cognitive function by Morris water maze testing, 5 weeks post-transplantation, showed a significant improvement in mice treated with the NSCs in the LN scaffolds, whereas the animals only treated with NSCs showed no improvement compared to controls.

This work demonstrated that the use of the scaffolds did not protect against the initial graft decline but resulted in increased long-term cell survival. The number of cells that survived 8 weeks post-transplantation was significantly higher when cells were transplanted within the FN or LN scaffolds although no proliferating cells were detected in any of the tested conditions. Thus, the reported difference in cell numbers was due to increased cell survival rather than enhanced proliferation. Additionally,

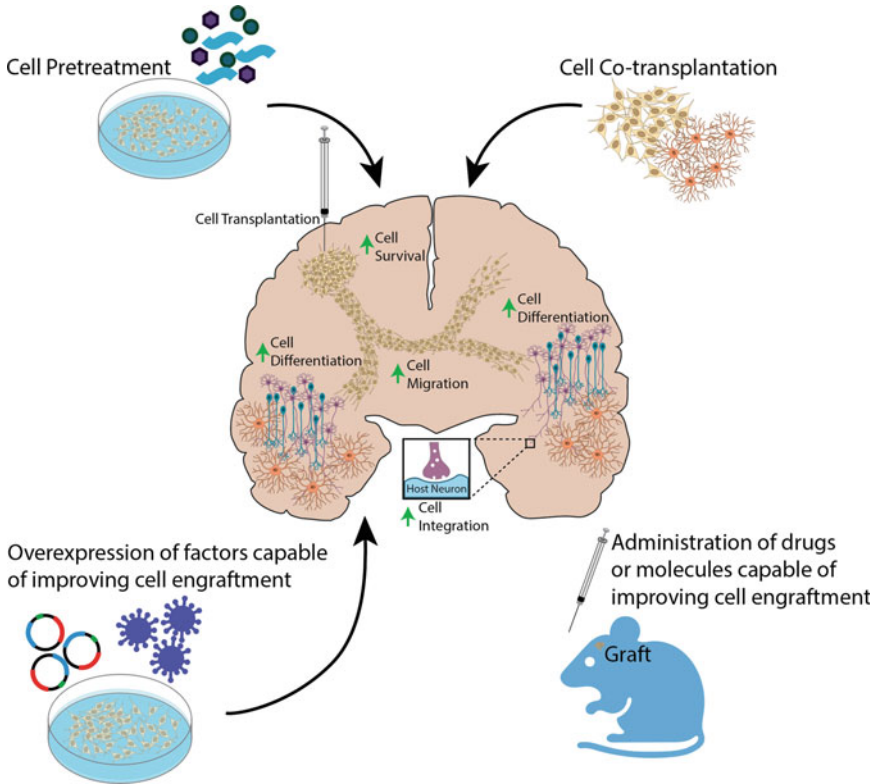


Fig. 2.1 Different therapeutic approaches to improve cell therapy. Strategies to target cell survival, migration, and differentiation, namely, pre-treatment of the cells before transplantation, cell co-transplantation, overexpression of different factors, and administration of drugs/molecules capable of improving cell engraftment are illustrated

cells transplanted within the scaffolds showed enhanced cell spreading in the anterior and posterior direction, though all transplanted cells were present in the hippocampus [104].

Cui and colleagues [19] investigated whether the effect of **neural cell adhesion molecule L1** overexpression, an adhesion molecule shown to promote survival of fetal dopaminergic neurons in vitro [37], could enhance functional regeneration after cell transplantation into the brains of a PD mouse model (Table 2.1). Substrate-adherent embryonic stem cells (SENA) are pre-differentiated embryonic stem (ES) cell-derived neural precursors that differentiate into an almost pure population of neurons in vitro and in vivo [22]. Cui and colleagues transfected murine SENA to overexpress neural cell adhesion molecule L1. These cells were transplanted to the striatum and adjacent to the substantia nigra. One-month post-transplantation, L1-overexpressing SENA resulted in increased graft size and a higher number of

surviving cells when compared to wild-type SENA. Moreover, an increased migration of grafted cells was observed. Interestingly, the better cell survival and migration results were proven to be a consequence of a decrease in apoptosis, whereas no differences were detected regarding cell proliferation when comparing wildtype (WT) grafts to L1-overexpressing grafts 1 month after transplantation. No differences in astroglial activity were detected; nonetheless, a slight decrease in microglial reaction was detected in L1-overexpressing SENA. Transplantation of L1-overexpressing SENA also resulted in a higher percentage of NeuN+ (neuronal nuclear protein) neurons and lower number of GFAP+ (Glial Fibrillary Acidic Protein) astrocytes (1 and 3 months in the striatum and 1 month in the substantia nigra), clearly showing a bias towards neuronal differentiation. Additionally, a higher number of dopaminergic neurons were present in the L1-overexpressing SENA grafts compared to wild-type SENA, while the number of GABAergic neurons was unaltered between groups. To assess motor function improvement after cell transplantation, induced rotation in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned mice was analyzed. L1-overexpressing SENA transplantation resulted in a significant increase in ipsilateral rotation 3–10 weeks post-transplantation, indicating improved motor function. This might be explained by the observed increase in striatal dopamine levels (ipsilateral to the grafted site). Interestingly, the transplantation of L1-overexpressing SENA to the substantia nigra resulted in enhanced number of endogenous TH+ (Tyrosine Hydroxylase) dopaminergic neurons ipsilaterally, reducing the progressive loss of dopaminergic neurons in the substantia nigra. Altogether, these results indicate that the transplantation of L1-overexpressing SENA improves cell survival, migration, and differentiation after intracerebral transplantation.

Nishimura and colleagues [72] identified integrin $\alpha 5$, an adhesion molecule that usually forms heterodimers with integrin $\beta 1$, as being highly expressed and of crucial importance in the innervation of nigral dopaminergic (DA) neurons. It was also demonstrated that administration of **E2B (estradiol-2-benzoate, a E2 derivative)** increased the expression of both integrin $\alpha 5$ and $\beta 1$ and promoted attachment of striatal neurons to fibronectin [72]. Thus, authors hypothesized whether **E2B administration** would increase the functional integration of the grafted neurons into host circuits. Thus, human induced pluripotent stem cell (iPSC)-derived DA progenitors [24] were injected into the striatum of 6-hydroxydopamine (6-OHDA)-lesioned mice and E2B was subcutaneously administered daily. Authors reported enhanced motor phenotype recovery and synapse formation of the grafted DA neurons with host striatal neurons via activation of host neuronal integrin $\alpha 5 \beta 1$. This data indicates that the simultaneous administration of E2B and stem cell-derived DA progenitors can significantly enhance the efficacy of cell therapy in PD.

Galectins are carbohydrate-binding soluble proteins that display many physiological functions such as modulating inflammation, immune response, cell migration, autophagy, and cell signaling [43]. **Galectin-1** (Gal-1) is a soluble lectin expressed in adult NSCs in the subventricular zone and the dentate gyrus [38, 39, 85]. Yamane and colleagues reported that Gal-1 upon intraventricular administration increased neurogenesis in the subventricular zone (SVZ) and improved sensorimotor dysfunction in a mouse model of focal cerebral ischemia [39]. Moreover, they also observed that

Table 2.1 Extracellular matrix modulation strategies

Molecule	Mechanism/ strategy	Condition	Cells	Outcome	References
Fibronectin or Laminin	Tissue-engineered scaffold	TBI	Fetal-derived neural stem cells	↑ Long-term cell survival ↑ Cell migration	[104]
Neural cell adhesion molecule L1	Overexpression	PD	Substrate-adherent embryonic stem cells (SENA)	↑ Graft size, survival, and migration ↑ NeuN+ neurons ↑ Dopaminergic neurons ↑ Survival of endogenous DA neurons	[19]
Integrin $\alpha 5$ and $\beta 1$	Administration of E2B	PD	human iPSC-derived DA progenitors	↑ Motor phenotype recovery ↑ Synapse formation of grafted DA neurons	[72]
Galectin-1	Overexpression	TBI	Human neural stem/progenitor cells	↓ Ischemic region ↑ Motor function recovery	[113]
Nogo-A	Intra-ventricular infusions of neutralizing antibodies	PD	Rat VM tissue	↑ TH+ fibers ↑ Behavior improvement	[94]

TBI Traumatic brain injury, *PD* Parkinson's disease, *DA* Dopaminergic, *E2B* Estradiol-2-benzoate

wild type human Neural Stem/Progenitor cell (hNSPCs) transplantation reduced infarct volume, established synapses between hNSPCs-derived and host neurons, and improved functional outcome in hNSPCs-grafted animals [40]. The effects of hGAL1-overexpressing hNSPCs were also assessed in a non-human primate model of spinal cord injury. hGAL1-overexpressing hNSPCs transplantation resulted in better functional outcome when compared to hNSPCs [113]. hGAL1-overexpressing hNSPCs were also shown to enhance the recovery from TBI in Mongolian gerbils [112]. Accordingly, hNSPCs expressing human Gal1 (hGal1-hNSPCs) were transplanted four days after brain ischemia caused by unilateral carotid artery occlusion. Thirty days after transplantation, lesion volume was significantly reduced in the

hGal1-hNSPCs group when compared to the hNSPCs control group. Three behavioral tests were carried out, elevated body swing test (EBST), bilateral asymmetry test (BAT), and T-maze test. No differences were observed in the T-maze test, with both groups showing a recovery after transplantation. In the BAT, both groups showed recovery after transplantation, with a tendency of larger improvement in the hGal1-hNSPCs ($p = 0.06$). The hGal1-hNSPCs group performed significantly better compared to the hNSPCs group in the EBST test.

Nogo-A has also been targeted in an attempt to improve survival and integration of grafted cells. Myelin associated protein Nogo-A is one of the most potent neurite growth inhibitors in the central nervous system and therefore a prime candidate to be studied. Seiler and colleagues, [94] demonstrated the potential of Nogo-A neutralization in the host brain to improve cell transplantation in PD. Rat ventral mesencephalic (VM) tissue cultures were transplanted into the striatum of 6-OHDA-lesioned mice. Animals were treated for 2 weeks with intra-ventricular infusions of neutralizing Nogo-A antibodies (11C7), while IgG infusions were used as a control. Lesioned animals showed reduced usage of the contralateral paw. The cylinder test revealed that five weeks post-transplantation, the asymmetric forelimb use was significantly mitigated in animals treated with cells and 11C7 antibodies. No differences were detected in graft volume, but 11C7 antibody treatment significantly increased the number of TH+ fibers in the host brain.

Cell Co-transplantation

Evidence indicates that **endothelial cells** promote NSPCs proliferation [96, 105]. Accordingly, Nakagomi and colleagues [70] transplanted murine cortical stroke-induced NSPCs into severe combined immunodeficiency (SCID) mice. These NSPCs are Nestin+ cells found in the post-stroke cortex but not in the non-ischemic cortex. Seven days after stroke, red fluorescent protein (RFP)-positive NSPCs were transplanted with and without the addition of Green fluorescent protein (GFP)-positive bovine pulmonary microvascular endothelial cells (EC). Five days after transplantation, a significantly higher number of RFP + NSPCs were observed in the NSPCs + EC co-transplantation group as compared to the group receiving NSPCs only. Similar results, as well as higher cell migration, and significantly higher numbers of proliferating graft-derived cells (RFP+/BrdU+) were observed 28 days after EC co-transplantation. EC co-transplantation also increased numbers of RFP+/NeuN+ cells, indicating a higher level of neuronal differentiation. Finally, cortex dysfunction is associated with behavior disinhibition in the presence of light, resulting in increased locomotion in the presence of light. Here, mice that received NSPCs + EC co-transplantation showed improved cortical function during the light phase (suppression of locomotion), whereas no differences were reported during the dark phase.

Astrocytes play a crucial role in maintaining the microenvironment within NSCs niches in the adult brain [93], namely by enhancing NSCs proliferation and differentiation into neurons [41, 97]. **Niche astrocytes (NA)** are astrocytes obtained from P0 (Postnatal day 0) murine brains or from the subgranular zone (SGZ) of adult murine brains. NA can be isolated by sorting GFAP+, epidermal growth factor receptor-negative (EGFR-), and CD24- cells [76]. Luo and colleagues [63] evaluated the effect of NA co-transplantation with NSCs isolated from the neonatal murine SVZ. Cells were transplanted into the striatum of adult male Sprague–Dawley rats following middle cerebral artery occlusion (MCAO). Seven days after transplantation, the number of GFP+ NSCs was significantly higher in the co-transplantation group. The ratio of BrdU+/GFP+ to BrdU-/GFP+ cells was significantly increased after NA co-transplantation, indicating a promotion of NSCs proliferation. Fourteen days after transplantation, grafts containing cells positive for the migrating neuroblasts marker Doublecortin (Dcx), neuronal markers β 3-Tubulin and NeuN, astrocyte marker GFAP, and oligodendrocyte marker oligodendrocyte specific protein (Oli) were increased, demonstrating that NSCs are capable to differentiate into neurons, astrocytes, and oligodendrocytes. Additionally, the NeuN+/GFP+ ratio, *i.e.*, the number of NeuN+ cells in the GFP+ cell population, was significantly higher, indicating increased neuronal differentiation of NSCs co-transplanted with NA.

Glial cells in the olfactory bulb and olfactory nerve are known as **olfactory ensheathing cells (OECs)**. OECs hold properties similar to Schwann cells, such as promoting and assisting axonal growth by releasing cytokines that promote neuronal survival and neurite outgrowth. Consequently, OECs have been considered as excellent cellular co-transplants to increase NSCs survival and engraftment. Liu and colleagues [61], co-transplanted rat NSCs and p75+ OECs (50:50 ratio) following TBI in mice. Motor impairments were rescued 7 and 14 days after cell transplantation in groups with and without co-transplantation but NSCs survival was significantly increased in the NSCs and OECs co-transplantation group. Additionally, co-transplantation significantly increased the number of surviving neurons, and reduced apoptotic cell death. Furthermore, there was a significant downregulation of *Il-6* (interleukin-6) and *Bad* (apoptosis signal molecule) mRNA levels 7 days upon NSCs and OECs co-transplantation.

Neurotrophic Factors

Neurotrophic factors are molecules responsible for supporting and stimulating neuronal survival and growth as well as neurotransmitter production and release. Neurotrophic factors are also associated with neuronal regeneration and differentiation in the developing and adult brain [32]. Several neurotrophic factors have been identified, which can be grouped into three families, neurotrophins (NGF family, BDNF, NGF, Neurotrophin-3/4), glial cell line-derived neurotrophic factor family (GDNF, neurturin, ephrin, etc.), and neuropoietic cytokines (heterogeneous group of molecules from the cytokine family) [45, 81]. Many neurotrophic factors have been

considered as options to aid cell transplantation in cell replacement strategies for brain disorders (Table 2.2).

Dayer and colleagues [20] increased NSCs proliferative and migratory properties by overexpressing **FGF2/bFGF** (Fibroblast growth factor 2/basic fibroblast growth factor) through lentiviral transduction. The FGF2/bFGF-overexpressing NSCs were transplanted into the cortex of Wistar pups at P3. Cell migration velocity was significantly higher for the FGF2/bFGF-overexpressing cells that migrated 60 μm over 12 h, as compared to control NPCs that exhibited a smaller fraction of cells migrating just 20 μm over 12 h. Hence, two weeks after transplantation, FGF2/bFGF-overexpressing NPCs were found at increased distances from the transplantation site as compared to control cells. Interestingly, FGF2/bFGF-overexpressing NPCs (GFP+) and control NPCs (tdTomato-positive) co-transplantation resulted in cell migration enhancement of the FGF2/bFGF-overexpressing cells, while no change in cell migration behavior was observed for the control NPCs. NeuN and doublecortin staining indicated that only a small fraction of cells (<0.5%) was able to differentiate into neurons after transplantation, and no differences were observed between both groups. A gradual decrease in the FGF2/bFGF immunoreactivity was observed up to 60 days after transplantation, demonstrating that FGF2/bFGF expression is transient. Moreover, only a small percentage of cells remained Nestin+ and/or incorporated BrdU. These results indicate that the gradual downregulation of FGF2/bFGF resulted in loss of the migratory and proliferative properties conferred by FGF2/bFGF overexpression in NPCs. On the other hand, long-term expression of FGF2/bFGF, and consequently maintaining cells in their proliferative state, might lead to tumor formation [20, 107]. Hence, the observed gradual decrease in FGF2/bFGF expression might be advantageous. Thus, the initial FGF2/bFGF expression increases the proliferation and migration, allowing for the initial expansion and migration of the transplanted cells, whereas the decrease in FGF2/bFGF expression over time stop cells' proliferation and allows cells to differentiate into glia and neurons.

Takayama and colleagues, being inspired by previous experiments reporting the enhancement of cell survival and neurite outgrowth of dopaminergic neurons promoted by FGF2/bFGF [65, 67, 99], investigated its potential effect to improve dopaminergic neuronal engraftment in a PD rat model [103]. A cell suspension of dopaminergic tissue, extracted from the rat embryonic VM, and FGF2/bFGF-expressing fibroblasts (transduced with FGF2/bFGF-encoding retroviruses) were co-transplanted. Co-administration improved neuronal survival and fiber outgrowth after eight weeks. Moreover, amphetamine-induced rotations were significantly mitigated after co-transplantation, achieving better results than transplantation alone, suggesting a stronger therapeutic effect of co-transplantation.

Brain derived neurotrophic factor (**BDNF**) is one of the most prominent neurotrophic factors triggering neurogenesis and neuroprotective effects in the brain. Ma and colleagues investigated whether the transplantation of mouse NSCs expressing BDNF (BDNF/NSCs) would achieve better results than control NSCs following TBI [64]. NSCs transfected with a plasmid construct to express BDNF and stained with the non-diffusible red fluorescent membrane dye PKH-26 were intracerebrally transplanted after TBI in rats. Two weeks later, PKH-26-labeled cells

Table 2.2 Administration and overexpression of neurotrophic factors strategies

Molecule	Mechanism	Condition	Cells	Outcome	References
FGF2/ bFGF	Overexpression	Wild-type	Rat neural progenitor cells	↑ Cell migration velocity and proliferation	[20]
FGF2/ bFGF	Co-transplantation with FGF2/ bFGF-expressing fibroblasts	PD	Dopaminergic tissue from the VM of embryonic rats	↑ Cell survival ↑ Growth of dopamine neurons ↑ Behavioral improvement	[103]
BDNF	Overexpression	TBI	Rat neural stem cells	↑ Cell survival faster synaptic formation ↑ GAP-43 ↑ Behavioral improvement	[64]
BDNF	Overexpression	Stroke	Human neural stem cells	↑ Cell survival ↑ Behavioral improvement ↓ Proapoptotic proteins and ↑ antiapoptotic proteins	[53]
BDNF	Overexpression	HD	Mouse ESC-derived neural progenitor cells	↑ Cell survival and differentiation ↑ Behavioral improvement	[119]
BDNF	Pretreatment	AD	Mouse neural stem cells	↑ Cholinergic neurons ↑ Synaptophysin and acetylcholinesterase activity ↑ Learning and memory	[57]
GDNF	Overexpression	TBI	Mouse neural progenitor cells	↑ Cell survival and differentiation ↑ migration towards lesion ↑ Learning	[2]

(continued)

Table 2.2 (continued)

Molecule	Mechanism	Condition	Cells	Outcome	References
GDNF	Expression in the brain before or after transplantation	PD	hPSC-derived VM DA cells	Before transplantation: ↑ Survival After transplantation: ↑ TH+ fiber density ↑ Reinnervation of host tissue	[28]
Neurturin	Injection	PD	Rat fetal DA neurons	↑ Survival	[83]

FGF Fibroblast Growth Factor, *PD* Parkinson's Disease, *VM*: Ventral mesencephalic, *BDNF* Brain-Derived Neurotrophic Factor, *DA* Dopaminergic, *ESC*: embryonic stem cells, *GDNF* Glial cell line-derived neurotrophic factor, *hPSC* human pluripotent stem cells

were found in the cortical granular layer, the hippocampus, the pons, and in the cerebellar Purkinje cell layers. A significantly higher number of surviving grafted cells and a higher number of $\beta 3$ -Tubulin+ neurons were observed in the BDNF/NSCs group up to 8 weeks post-transplantation. Nevertheless, a decrease in cell survival over time was seen. At 7 days post-transplantation, the BDNF/NSCs transplanted rats presented significantly reduced latency to move when compared to rats transplanted with control NSCs. Moreover, 1 week post-transplantation, both fore- and hindlimb asymmetry recovery were significantly better in BDNF/NSCs-transplanted rats. However, the performance of all transplanted animals, regardless of the transplanted cell type, returned to pre-surgery levels at 8 weeks post-transplantation. Additionally, Ma and colleagues evaluated the effect of transplanted NSCs on the expression of pre- and post-synaptic proteins (SYP and Shank2). Shank2 mRNA and protein levels were significantly higher at all time points evaluated. However, a significant increase in SYP protein and mRNA levels was only observed at 1 and 2 weeks, indicating that BDNF expression may trigger faster synaptic formation of the grafted cells. Since SYP functions as a marker of brain function, it might be correlated with faster functional recovery in BDNF/NSCs-transplanted rats. Moreover, expression of GAP-43, a protein associated with synaptic regeneration following injury, gradually and continuously increased in BDNF/NSCs-transplanted rats, suggesting that increased GAP-43 levels might mediate enhanced therapeutic effect of BDNF/NSCs.

Lee and colleagues [53] transplanted human NSCs genetically modified to over-express BDNF (BDNF-NSCs) after intracerebral hemorrhage (ICH) in mice. Mice receiving BDNF-NSCs exhibited significantly improved motor performance from 8 days up to 8 weeks post-transplantation, compared to both PBS (Phosphate-buffered saline) and control NSCs administration. Cell survival was 1.6-fold and threefold higher at 2 and 8 weeks post-transplantation, respectively, in animals grafted with BDNF-NSCs when compared to NSCs transplantation. Moreover, proapoptotic p53, p21, and caspase-3 protein levels were reduced in animals transplanted with BDNF-NSCs, while antiapoptotic Bcl-2 and Akt1 proteins were significantly higher

compared to control NSCs and PBS transplantation. These results suggest a possible cerebroprotective role of BDNF mediated by antiapoptotic mechanisms.

A similar approach was tested in a mouse model of HD [119]. Mouse embryonic stem cell (ESC)-derived NPCs overexpressing BDNF and GFP (BDNF-GFP NPCs) were transplanted into the striatum of three different HD mouse models, one chemically generated (quinolinic acid, QA) and two genetically induced (R6/2 and N171-82Q). Locomotor function analysis by apomorphine-induced rotations revealed a significant recovery of QA-lesioned animals at 8 weeks after BDNF-GFP NPCs transplantation, whereas control (GFP NPCs) cells induced no improvement. At 8 weeks post-transplantation, rotarod test data demonstrated that the BDNF-GFP NPCs improved latency to fall, while GFP-NPCs did not ameliorate motor functions. No significant inter-group differences were observed in R6/2 and N171-82Q HD mice. Nevertheless, interesting results were obtained on cell survival. While no difference was observed in the QA and R6/2 models (control NPCs versus BDNF-NPCs), cell survival was significantly increased by the BDNF overexpression in N171-82Q mice. Increased neuronal differentiation of BDNF-NPCs was observed in the QA model, while more S100B+ (S100 calcium binding protein B) astrocytes were detected in control NPCs grafts.

The impact of BDNF on NSCs transplantation was also investigated in models of Alzheimer's disease. Li and colleagues [57] pretreated NSCs with BDNF for 1 h before transplantation into the hippocampus of AD mice displaying cholinergic dysfunction and deposition of plaques containing amyloid β -peptides ($A\beta$) (APP/PS1 transgenic mice injected with the p75 murine immunotoxin). Significant improvement of cognitive function was reported for the group transplanted with BDNF-pretreated NSCs, as evidenced by better Morris Water Maze performance (time spent, swimming distance, and the crossing times of platform quadrant). On the other hand, NSCs transplantation per se resulted in no significant learning and memory amelioration. Further graft analysis revealed a higher number of basal forebrain cholinergic neurons (degenerated in AD) in the BDNF-NSCs group compared to the other groups (non-treated and control-NSCs transplantation). Additionally, SYP expression and acetylcholinesterase activity, reduced in AD patients and in this AD mouse model, were significantly increased in the BDNF-NSCs group when compared to the non-treated and NSCs-treated mice. These results demonstrate the enhanced efficacy of NSCs exposure to BDNF before transplantation into the hippocampus of AD mice.

Glial cell line-derived neurotrophic factor (**GDNF**) is a neurotrophic factor with well-known effects in stem cell-based therapy. Bakshi and colleagues [2] hypothesized that GDNF cerebroprotective effects could enhance NPCs survival after intracerebral transplantation in a TBI modeled by lateral fluid percussion brain injury (LFP). NPCs were transfected with a plasmid to overexpress GDNF, and the expression of GFP was promoted through lentiviral vectors. The obtained GDNF-GFP-NPCs were then transplanted into perilesional areas. Cell survival and migration showed no differences at one week post-transplantation, and cells were observed in all assessed animals. However, 6 weeks post-transplantation, no surviving cells were observed in some animals grafted with control NPCs, whereas all animals transplanted with GDNF-NPCs presented GFP+-surviving cells. Additionally, a large

number of GDNF-NPCs migrated away from the transplantation site and towards the lesion, whereas control-NPCs were detected predominantly at the transplantation site. Phenotypic characterization of the transplanted cells revealed that 6 weeks post-transplantation, control NPC grafts presented no cells positive for neuronal, astrocytes, and oligodendrocytes markers but approximately 5% of GDNF-NPCs were NeuN+ and, to a lesser extent, β 3-Tubulin+. Graft-derived glial cells expressing GFAP and NG2 were also observed. Increased cognitive function was observed 6 weeks post-transplantation in animals receiving GDNF-NPCs.

Gantner and colleagues [28] tested the effect of an early and late exposure to GDNF in the therapeutic outcome of human pluripotent stem cells (PSC)-derived ventral midbrain dopamine progenitor (hPSC-derived VM DA progenitor) grafts in an adult PD athymic mouse model. The GFP+ hPSC-derived VM DA progenitors were transplanted into a GDNF-rich environment (adeno-associated virus encoding for GDNF was injected 3 weeks before the cell transplantation), which resulted in an increased cell survival and a significant enhancement in GFP+ neuronal fibers at the graft-host boundary. However, in the dorsolateral striatum and medial striatum the GFP+ innervation density was significantly diminished in the presence of GDNF. Despite the lower innervation, synapses (human SYP (hSYP) in GFP+ fibers) were detected. Moreover, a reduced number of Ki-67+ grafted cells and a concomitant increase of NeuN+ cells indicated that GDNF enhances neuronal differentiation. Since GDNF expression prior to cell transplantation negatively influenced the ability of grafted DA neurons to innervate the striatum, authors tested the effect of AAV-mediated GDNF expression at 3 weeks post-transplantation, hypothesizing that GDNF expression at this time point would not influence DA neuronal migration while retaining its effect regarding neuronal innervation and differentiation. Data revealed no graft size difference between GDNF-treated and non-treated grafts, not indicating any GDNF effect on cell survival. However, increased TH+ neuronal fiber density and increased graft-derived DA re-innervation of the host tissue were detected. Altogether, these data indicate that early (prior to transplantation) GDNF expression promotes survival of grafted cells, whereas delayed exposure (3 weeks post-transplantation) increases dopaminergic re-innervation.

Neurturin (NTN) is a neurotrophic factor from the GDNF family that supports survival of sympathetic neurons [49]. Rosenblad and colleagues [83] investigated if NTN can also improve cell engraftment after intracerebral transplantation in PD. Rat fetal DA neurons were transplanted into the striatum of 6-OHDA lesioned Sprague-Dawley rats. NTN, or vehicle as control, were intrastrially injected every third day for the first 3 weeks after cell transplantation. Amphetamine-induced rotations were absent in all groups, 2 weeks post-transplantation, while the NTN-treated cell group showed a 1.8-fold increase in surviving DA neurons. However, at 4 weeks post-transplantation, no enhancement of graft volume, TH+ cells, and host neuronal reinnervation was observed compared with control cells. This data indicates that the intrastriatal injection of NTN, over 3 weeks following transplantation of DA neurons, enhances the survival of the transplanted fetal DA neurons but has no effect on neuronal differentiation and reinnervation.

Cell Death Modulation

Cell death following brain transplantation is mostly triggered by apoptosis [115]. Cell survival after transplantation depends on intrinsic and extrinsic signals. Stimulating prosurvival cell programs upon transplantation through the activation of intracellular pathways halting death-signaling molecules present in the injured brain is a promising strategy to enhance cell engraftment (Table 2.3).

Schierle and colleagues [92] hypothesized that apoptosis inhibition could enhance cell survival upon intracerebral transplantation. Authors assessed the effect of **Ac-YVAD-cmk (caspase-1 inhibitor acetyl-tyrosinyl-valyl-alanyl-aspartyl-chloromethylketone)** pretreatment on embryonic rat VM cell engraftment in the striatum of 6-OHDA-lesioned adult Sprague–Dawley rats. A 94% decrease in the number of TUNEL+ (late apoptotic) cells was reported in grafts treated with Ac-YVAD-cmk four days after cell transplantation. Transplantation of Ac-YVAD-cmk-treated VM resulted in enhanced behavioral recovery compared with control treatment (VM only) 2 and 6 weeks after transplantation. Moreover, a 400% increase in surviving dopaminergic neurons, a 400% increase in the volume of innervated host striatum, and a 250% increase in graft volume was observed 6 weeks post-transplantation, with no difference in the number of glial GFAP+ cells. These results indicate that caspase-1 inhibition is a good approach to reduce apoptotic cell death and to improve cell engraftment after intracerebral transplantation.

Yi and colleagues [114] investigated the effect of the pro-neural basic helix-loop-helix (bHLH) transcription factors **Mash1** and **Neurogenin 2** (Ngn2) after cell transplantation. NPCs isolated from the embryonic day 14 (E14) rat cortex were transduced with retroviral vectors inducing the expression of Ngn2, Mash1, or LacZ (Control). Then, 450,000 NPCs were transplanted into the striatum or the hippocampus of adult rats. Regardless of the transplant location, NPCs transduced with Mash1 gave rise to significantly larger grafts when compared to the LacZ-expressing NPCs at 2 and 8 weeks post-transplantation. Additionally, Mash1-expressing grafts showed a significantly higher percentage of NeuN+ neurons compared to control (LacZ-expressing) NPCs. Interestingly, 56% of Ngn2-transduced cells differentiated into NeuN+ neurons, significantly more than compared to control NPCs and Mash1-expressing NPCs. The potential paracrine effect of Ngn2-transduced NPCs and the expression of several neurotrophic factors and receptors were further investigated *in vitro*. A higher expression of NT4/5, nerve growth factor, glial cell-derived neurotrophic factor, and glial cell-derived neurotrophic factor receptor c-ret mRNAs in both Mash1 and Ngn2 NPCs were reported. However, an increase in mRNA and protein levels of NT3 and its receptor (TrkC) was exclusively observed for cultures of Ngn2-transduced cells. Moreover, *in vitro* inhibition of the NT3/TrkC signaling pathway in NPCs abolished improved cell survival, and NPCs treatment with NT3 cytokine resulted in cell survival enhancement. Altogether, these data indicate that the survival-promoting effect of Ngn2 on NPCs is mediated via NT3.

Table 2.3 Cell death modulation strategies

Molecule	Mechanism	Condition	Cells	Outcome	References
caspase-1 inhibitor (Ac-YVAD-cmk)	Cell Pretreatment	PD	Embryonic rat VM cells	↓ Apoptosis ↑ Innervated host striatum ↑ Graft volume ↑ Behavioral improvement	[92]
Mash1 and Neurogenin 2	Overexpression	Wild-type	Rat neural progenitor cells	Mash1: ↑ Graft size ↑ NeuN+ neurons ↑ NT4/5, NGF, GDNF Neurogenin2: ↑ NeuN+ neurons ↑ NT4/5, NGF, GDNF ↑ NT3 and TrkC	[114]
Bcl-2	Overexpression	Stroke	Mouse embryonic stem cells	↓ Cell death ↑ NeuN+ neurons ↑ Behavioral improvement	[108]
Akt1	Overexpression	Stroke	Human neural stem cell	↑ Cell migration and survival ↑ Behavioral improvement	[52]
Prosurvival molecules: Hif1a, Akt1, Bcl2, and Bcl-xl	Overexpression	Wild-type	Human neural stem cells	↑ Cell survival	[48]
Ubc9	Overexpression	Stroke	Mouse neural stem cells	↑ Cell survival ↑ NeuN+ neurons ↑ Dendrite formation ↑ Synapses	[5]
Lazaroids (U-74389G and U-83836E)	Cell Pretreatment	PD	Embryonic rat dopamine neurons	↑ TH+ neurons ↑ Graft volume ↑ Behavioral improvement	[71]
Flunarizine	Cell Pretreatment	PD	Rat VM cells	↑ Cell survival ↑ Graft volume ↑ Behavioral improvement	[44]

(continued)

Table 2.3 (continued)

Molecule	Mechanism	Condition	Cells	Outcome	References
Neurexophilin 3	IV administration	PD	Mouse iPSC-derived neural cells	↑ TH+ neurons	[73]
Minocycline	Cell Pretreatment	Stroke	Fetal rat neural stem cells	↑ Cell survival and differentiation ↑ Nrf2 leading to: ↑ BDNF, GDNF, and VEGF ↑ Behavioral improvement	[87]
Small molecules sodium butyrate and nicorandil (NaB + Nico)	Cell Pretreatment	Stroke	Mouse neural stem cells	↑ BDNF ↑ Cell survival ↑ Neurite outgrowth ↓ Inflammatory response ↓ IL1 β , IL6, IL12, and TNF α ↓ infarct size	[36]

PD: Parkinson's Disease, NGF Nerve growth factor, GDNF Glial cell line-derived neurotrophic factor

Another strategy to decrease apoptotic cell death and to enhance neural differentiation, maturation, and axonal growth is the overexpression of the anti-apoptotic gene *bcl-2* [14, 30, 34, 35, 89, 116]. Wei and colleagues transfected mouse ESCs to overexpress Bcl-2, induced differentiation in neural lineages, and transplanted the cells into the injured cortex after MCAO in mice [108]. Three days after transplantation, the ESC-derived neural cells differentiated into NeuN+ mature neurons, GFAP+ astrocytes, NG2+ oligodendroglial precursors, and APC+ mature oligodendrocytes. Moreover, a significant reduction of cell death in the Bcl-2-overexpressing grafts was observed. Additionally, Bcl-2-overexpressing grafts gave rise to a significantly higher percentage of NeuN+ neurons when compared to control ESCs at 14 and 21 days post-transplantation, indicating that Bcl-2 overexpression not only reduces cell death but also enhances differentiation of the transplanted ESCs into neurons. Mice grafted with both cell types, control and Bcl-2-overexpressing ESC-derived neural cells, performed better than vehicle-treated animals in neurofunctional tests, and functional improvement was better in animals receiving Bcl-2-overexpressing cells at 21 days post-transplantation.

Akt1 mediates cell survival signals in NSCs that are critical for NSCs proliferation, growth, and survival. Overexpression of **Akt1** in granule cells has protective effects against apoptotic cell death during growth factor withdrawal [25] and promotes survival upon free radical exposure or hypoxia in hippocampal neurons [16, 66, 110]. Lee and colleagues [52] used a human neural stem cell line overexpressing Akt1 (F3.Akt1 hNSCs) in an ICH stroke mouse model. Overexpression of Akt1 in hNSCs resulted in an increased cell migration, as well as a 40% and 100% increase in cell survival at 2 and 8 weeks post-transplantation, respectively. Additionally, Akt1-overexpressing hNSCs did not proliferate after transplantation. Motor performance evaluation showed significant functional improvement of mice grafted with Akt1-hNSCs as compared to control-hNSCs, indicating that Akt1 overexpression can increase cell therapy outcomes after ICH.

Korshunova and colleagues [48] selected 4 pro-survival molecules, **Hif1a**, **Akt1**, **Bcl2**, and **Bcl-xl** being previously identified as playing a protective role in developmental cell death. These 4 molecules plus a GFP reporter gene were overexpressed in human H9 and RC17 NSCs through lentiviral transduction. To stimulate Akt1 signaling, myristylated Akt1 (myrAkt1), which is the constitutively active version of Akt1 [1], was used. The genetically modified hNSCs were then transplanted into the right striatum of NOD/SCID- γ (Nonobese diabetic/severe combined immunodeficiency gamma) (NSG) mice, whereas control cells were transplanted to the left striatum. Control cells showed approximately 7% and 5% survival, 1 week and 1 month after transplantation, respectively, while pro-survival genes overexpression resulted in 17-fold increased cell survival at 3 months post-transplantation. In the first week, when most of the cell death occurred, the expression of **myristylated Akt1** was more efficient, but all tested genes caused a significant reduction of cell death. Pro-survival genes also reduced continuous cell death, as they improved the number of surviving cells up to 3 months post-transplantation compared to control hNSCs. Activated caspase-3, a commonly used marker of apoptosis, was barely detected in genetically modified hNSCs, while grafted control NSCs showed significantly increased activity of activated caspase-3. Additionally, Ki-67 staining was performed to detect uncontrolled proliferation. Only a few cells were Ki-67+, with a significantly higher number in myrAkt1-expressing hNSCs at 1 month after transplantation. Therefore, the permanent expression of certain genes is not desired since it can lead to unwanted effects such as continuous proliferation and delayed differentiation. Thus, cells were transduced with a lentiviral vector encoding for inducible (via Dox) BCL2L1 (Bcl-xl protein) expression before transplantation, and the gene overexpression was induced in vivo for 7 days. Interestingly, the upregulation of the pro-survival gene for the first 7 days resulted in a similar pattern of cell survival at 28 days when compared to the permanent expression, suggesting that even shorter expression of BCL2L1 is enough to enhance long-term survival of transplanted hNSCs in the striatum.

A cytoprotective process involved in ischemic stress resistance is **SUMOylation**, a posttranslational modification of proteins by Small Ubiquitin-like Modifier (SUMO) [54, 55]. Bernstock and colleagues investigated whether mouse NSCs engineered to overexpress **Ubc9** (SUMO E2 conjugase) results in increased graft survival after MCAO [5]. Survival of NSCs transplanted into the ischemic lesion was assessed

5 days post-transplantation. A 1.9-fold increase in grafted Ubc9-NSCs was observed, likely due to the decreased cell death (reduction of TUNEL+ cells) and increase in cell proliferation detected. Additionally, a larger number of grafted Ubc9-NSCs differentiated into NeuN+ neurons while an increase in dendrite formation (Microtubule-associated protein 2, MAP2+ cells) and integrated synapses (PSD95+ cells) were reported. These data indicate that SUMOylation in NSCs increases cell survival after transplantation, and fosters neuronal differentiation, suggesting that SUMOylation is a valuable strategy for improving NSCs-based cell therapy in stroke and, potentially, other neurodegenerative diseases.

Lazaroids are a class of lipophilic steroids (21-aminosteroids) that inhibit lipid membrane peroxidation, serving as free radical scavengers and reducing cell death. Their therapeutic potential has been studied in several central nervous system diseases [17, 29]. Nakao and colleagues investigated the impact of two lazarooids, U-74389G and U-83836E, on the engraftment success of embryonic rat dopaminergic neurons in the striatum of 6-OHDA-lesioned Sprague–Dawley rats [71]. U-74389G and U-83836E (0.3 μM) were added to the cells' isolation and culture medium. Six weeks after transplantation, the number of TH+ neurons was increased by 265% and 263% in animals receiving U-74389G- and U-83836E-treated cells, respectively. Moreover, the graft volume was significantly increased in both animals receiving U-74389G- and U-83836E-treated cells. Additionally, the rats transplanted with lazarooids-treated cells present a faster functional recovery. At 2 weeks post-transplantation, these rats showed a significantly reduced motor asymmetry, whereas at 6 weeks post-transplantation all animals (including controls) showed complete reversal of rotational asymmetry. The absence of differences at 6 weeks post-transplantation can be explained by the overall improved graft survival. Although a lower percentage of surviving TH+ dopaminergic neurons (14–18%) was observed in control grafts, these grafts still contained approximately 1100 TH+ neurons, enough cells to promote functional recovery. Additionally, lazarooids serve as free radical scavengers. Thus, they are prime candidates for improving cell therapies for neurodegenerative disorders characterized by excess oxidative stress.

Flunarizine is a selective calcium entry blocker with calmodulin-binding and histamine H1-blocking properties. Flunarizine has been shown to protect neurons against neuronal death from oxidative stress, serum deprivation, and ischemia [91]. Flunarizine is European Medicines Agency (EMA)-approved and has a particular therapeutic potential since the overactivity of calcium channels is associated with excitotoxicity, a known contributor to neurodegeneration during trauma and disease [90, 91]. Schierle and colleagues [44] investigated if flunarizine can reduce cell death in grafted DA neurons. 6-OHDA-lesioned Sprague Dawley rats transplanted with 1 μM flunarizine pretreated syngeneic E14 VM cells showed better functional recovery compared to animals transplanted with untreated cells. Graft evaluation revealed a 160% increase in the number of surviving dopaminergic neurons in the flunarizine-pretreated grafts, as well as 100% increase in graft volume and host striatum innervation. Altogether, these results indicate flunarizine can enhance cell therapy efficacy.

Nishimura and collaborators [73] evaluated potential gene candidates to support grafted neurons by studying the differences in brain environments and evaluating potential patterns in gene expression as well as functional outcomes after transplantation of iPSC-derived DA neurons. **Neurexophilin 3 (NXPH3)** was chosen for supporting cell survival. NXPH3 is an endogenous peptide binding to a neuronal cell surface receptor (NRXN) and has been associated with increased synaptic transmission, plasticity, as well as survival and maturation of newborn neurons [3, 10, 18, 111]. Mouse iPSC-derived neural cells were transplanted into the striatum of C57BL6 mice in the presence or absence of NXPH3 (1 $\mu\text{g}/1 \mu\text{l}$ injected adjacent to the graft). Eight weeks after transplantation, the ratio of TH+ neurons *per* graft volume was increased in the presence of NXPH3, suggesting promotion of survival of grafted iPSC-derived DA neurons, and demonstrating a positive effect of NXPH3 on cell engraftment.

Minocycline has been used as an antibiotic and anti-inflammatory drug in clinical practice. In vitro experiments demonstrated a cytoprotective effect on rat fetal NSCs through upregulation of antioxidant genes (Nrf2, NQO1, and HO-1) and increased expression of BDNF, GDNF, NGF, and VEGF [87]. Sakata and colleagues [87] investigated whether the preconditioning of NSCs with minocycline can improve cell engraftment upon brain transplantation. NSCs harvested from the SVZ of fetal rats were treated with minocycline (0, 1, 3 or 10 μM for 24 h) before transplantation into the cortex of MCAO mice. Minocycline pretreated NSCs exhibited increased survival and differentiation into neurons (β 3-Tubulin+ and MAP2+), astrocytes (GFAP+), and oligodendrocytes (NG2+) on day 28. BDNF, GDNF, and VEGF levels were increased in the brain of mice transplanted with minocycline pretreated NSCs. A significantly improved functional recovery was also reported. Importantly, authors demonstrated that the beneficial minocycline effect on NSCs is mediated by upregulation of erythroid 2-related factor 2 (Nrf2), a regulator of cell resistance to oxidants [59].

NSCs treatment with **sodium butyrate** (NaB), a fatty acid that has been linked to neural differentiation of stem cells [118], and with **nicorandil** (Nico), a stimulator of the APE1 pathway presenting antiapoptotic activity [102], enhance cell survival and provide protection against oxidative stress [102]. Hosseini and colleagues evaluated the effect of NaB and Nico pretreatment on NSCs survival in C57BL/6 mice after MCAO [36]. Mouse NSCs were pretreated with NaB and Nico (for 1 week at 1 mM and 12 μM , respectively), and transplanted into the right lateral ventricle of MCAO mice. A significantly higher BDNF expression in the group treated with a combination of NaB and Nico was observed as compared to untreated mice and mice treated with NaB or Nico alone. Moreover, increased cell survival and neurite length outgrowth were observed after NSCs combination exposure to NaB and Nico. Additionally, NaB plus Nico pretreated NSCs grafts attenuated the inflammatory response following stroke, in particular decreasing microglial and astrocyte activation, and reducing the expression of the proinflammatory interleukin (IL)-1 β , -6, -12, and TNF α (Tumor necrosis factor). Moreover, infarct size was significantly reduced in mice receiving pretreated NSCs, being accompanied by a preservation of neurological function.

Modulation of Inflammation

A neuroinflammatory response within the brain or spinal cord is a common hallmark of acute and chronic neurodegenerative disorders. It is mediated by cytokines, chemokines, reactive oxygen species, and other secondary messengers produced by CNS cell populations including microglia, astrocytes, and endothelial cells. Neuroinflammation has a detrimental impact on disease progress and potential recovery [23, 60, 100]. On the other hand, many neuroinflammation mediators also play a role in plasticity, tissue repair, as well as neuroprotection and neurogenesis (Table 2.4).

Stromal cell-derived factor 1 alpha (SDF-1 α) upregulation in the ischemic brain stimulates regeneration [82]. Chau and colleagues promoted SDF-1 α (and GFP) overexpression in mouse iPSC-derived neural progenitors [13] by using lentiviruses. Seven days after MCAO, 100,000 or 300,000 cells were transplanted into the peri-infarct region of mice that were injected daily with BrdU. Fourteen days after cell transplantation, the number of NeuN+/BrdU+ cells (new neurons) and Glut-1+/BrdU+ (new endothelial cells) were quantified, and the SDF-1 α -iPS-NPCs group presented a higher amount of both cell types. However, most GFP+ cells did not co-label with BrdU (both control and SDF-1 α -iPS-NPCs) suggesting that the observed increase was due to endogenous cell recruitment. The transplantation effect of two cell doses (100,000 and 300,000 cells) and two cell types (control and SDF-1 α -iPS-NPCs) on functional outcome were also tested 7 and 14 days after cell transplantation. The lower dose of control cells did not improve locomotor function at any of the time points tested but the low dose of SDF-1 α -iPS-NPCs induced significant functional improvements at both time points. The higher dose of control cells also showed significant functional improvements compared to the untreated group, but benefits were larger after transplantation of SDF-1 α -iPS-NPCs.

In diseases such as PD, a major factor interfering with cell therapy is the inflammatory environment limiting transplanted cell survival. **Nuclear receptor-related factor 1 (Nurr1)** overexpression can inhibit the pro-inflammatory iNOS, TNF α , and IL-1 β expression in microglia and astrocytes, which protect neurons from inflammation-mediated neurotoxicity [84]. Qian and colleagues tested VM neural stem cells (mNSCs) and microglia (MG) overexpressing Nurr1. Cells were transduced with a lentivirus encoding for Nurr1 and for copGFP in mNSCs (NNSCs) or copRFP in microglia (NMG) [80] and transplanted into the striatum of 6-OHDA-lesioned mice. Amphetamine-induced rotations were measured at 3, 6, 9, and 12 weeks. Five experimental groups were evaluated: sham, NSCs, NNSCs, NNSCs plus MG, and NNSCs plus NMG. All groups except the sham group exhibited functional recovery at 3 weeks post-transplantation. Importantly, the NNSCs plus NMG presented significantly better motor performance compared to all the other groups at 12 weeks post-transplantation. Brain histological analysis revealed a significantly increased number of TH+ neurons in the striatum of mice transplanted with the NNSCs plus NMG group compared to all the other groups, as well as a significant reduction in Iba1+ (ionized calcium binding adaptor molecule 1) cells (microglia); except when compared to NNSCs group. These data indicate that the transplantation

Table 2.4 Strategies to modulate neuroinflammation

Molecule	Mechanism	Condition	Cells	Outcome	References
Stromal cell-derived factor 1 alpha (SDF-1 α)	Overexpression	Stroke	Mouse iPS-derived neural progenitor cells	↑ New neurons ↑ New endothelial cells ↑ Behavioral improvement	[13]
Nuclear receptor-related factor 1 (Nurr1)	Co-transplantation with Microglia overexpressing Nurr1	PD	Rat VM neural stem cells + Rat microglia	↑ Behavioral improvement ↑ TH+ neurons	[80]
Tumor necrosis factor-alpha (TNF- α)	Cell Pretreatment	TBI	Human neural progenitor cells	↑ Survival ↓ Infarct volume ↑ Behavioral improvement	[46]
Interleukin 6	Cell Pretreatment	Stroke	Mouse neural stem cells	↑ Survival ↓ Infarct size ↓ Inflammatory response ↑ Angiogenesis ↑ Behavioral improvement	[86]
Adjudin	Cell Pretreatment	Stroke	Mouse neural stem cells	↓ Infarct size ↑ Resistance to oxidative stress Rescue BBB destruction ↑ BDNF ↑ Angiogenesis ↑ Behavioral improvement	[117]
CTLA4-ig + ANTI-cd154 antibodies	Administration	-	Mouse glial-restricted progenitors	↓ Allograft rejection	[56]

PD Parkinson's Disease, TBI Traumatic brain injury

of Nurr-1-overexpressing NSCs and microglia can improve the therapeutic impact of cell therapies in PD.

Tumor necrosis factor-alpha (TNF- α) is a pro-inflammatory cytokine expressed in response to pathological processes such as brain trauma and neurodegenerative diseases. TNF- α also has important protective effects against apoptosis and aids

differentiation, proliferation, and survival of NSCs in the brain [4, 51, 77]. Kim and colleagues hypothesized that pretreatment of hNPCs with **TNF- α** increases cell survival after intracerebral transplantation [46]. TNF- α -preconditioned (20 ng/mL for 24 h) hNPCs were transplanted into mice with hypoxic-ischemic (HI) brain injury (condition arising from reduced blood flow and oxygen deprivation), showing higher viability than untreated cells. Moreover, the expression of cIAP2 (cellular inhibitor of apoptosis 2) was found enhanced in TNF- α -pretreated hNPCs both in vitro and in vivo. Additionally, functional outcome was better and infarct volumes were lower in animals receiving TNF- α -pretreated hNPCs. Finally, a significant reduction of apoptotic cells was seen 7 days (cleaved caspase-3+) and at 4 weeks (TUNEL+) after TNF- α -hNPCs transplantation in vivo.

Interleukin 6, an important pro- and neuroinflammatory cytokine is enhanced in several neurodegenerative diseases [98, 101], while also increasing endogenous progenitor cell proliferation and survival in the brain [11]. Sakata and colleagues pretreated NSCs with IL-6 to assess whether this improves the therapeutic impact of NSCs transplantation after MCAO [86]. NSCs harvested from the fetal SVZ were exposed to IL-6 (20 ng/ml for 24 h) before transplantation. Two days after transplantation, the number of late-apoptotic, grafted cell death (TUNEL+) was reduced in the IL-6 pretreated NSCs group. Transplanted NSCs were capable of differentiating into neurons (β 3-Tubulin+) and astrocytes (GFAP+). Twenty-eight days after transplantation, no differences regarding the percentage of differentiated cells were reported between groups. Nonetheless, a higher number of surviving cells and reduction of infarct size were detected in animals receiving IL-6 pretreated NSCs. Transplantation of IL-6 pretreated NSCs also mitigated proinflammatory microglial responses. Additionally, IL-6 pretreated NSCs promoted angiogenesis, increasing blood vessel density compared to all other groups. Finally, animals receiving IL-6 pretreated NSCs showed superior functional outcomes. In vivo and in vitro data indicate that IL-6 protective effects are triggered through the activation of STAT3- (signal transducer and activator of transcription 3), which is mediated by SOD2 (superoxide dismutase 2) expression.

Adjudin is under development as a potential non-hormonal male contraceptive. It is an indazole derivative and, as many derivatives of indazole, has anti-inflammatory properties [6, 12, 21]. Adjudin was also reported to possess neuroprotective properties [62, 95]. Preconditioning NSCs with Adjudin was investigated to assess whether it can improve therapeutic NSCs' effect in stroke [117]. Adjudin-pretreated mouse NSCs were intrastrially transplanted 24 h after MCAO. Adjudin preconditioned NSCs significantly reduced infarct volume and ameliorated neurological deficits 3 days after transplantation. NSCs transplantation also reduced levels of IL-6, IL-1 β , and TNF- α and reduced microglial activation (CD11b) after MCAO. Adjudin-pretreated NSCs even further reduced neuroinflammation cytokine expression. There was also an increased resistance to oxidative stress mediated by enhanced levels of catalase and SOD2 as well as reduced blood-brain barrier (BBB) damage, indicated by decreased leakage of Evans Blue (EB) and IgG, increase in BDNF levels, and

increased angiogenesis (indicated by numbers of CD31+/BrdU+ cells). This translated into improved functional outcome. These data confirm the ability of Adjudin cell preconditioning as a useful tool for cell-based therapies in stroke.

The use of immunosuppression protocols to prevent cell rejection can also lead to adverse effects. Li and colleagues [56] explored an alternative to conventional immunosuppression protocols by applying a co-stimulation blockade (CoB) strategy using anti-CTLA4-antibodies, thus preventing full T-cell activation, as well as anti-CD154 antibodies (MR-1) that prevent allograft rejection. Mouse glial-restricted progenitors (mGRP) expressing β -actin-luciferase for bioluminescent imaging (BLI) were transplanted into the forceps minor of the corpus callosum, a location known for strong mGRP allograft rejection. Three groups were tested: control (immunocompetent mice+PBS), CoB group (immunocompetent mice + CoB), and immunodeficient animals (SCID mice). CoB resulted in a significant reduction of phagocyte reactivity and T-cell infiltration around mGRP grafts compared to control mice. All groups showed a decline of BLI signal within 3 weeks post-transplantation. There was a complete signal loss in the control group, while both the Cob and SCID groups exhibited a residual BLI signal over a 203-day follow-up period with no differences between groups. Importantly, similar results were observed when human GRPs were transplanted (up to 19 days), proving the strategy to be effective in protecting GRP xenografts. Further tests indicated that the co-stimulation blockade strategy improved the survival of GRP and preserved their remyelination capacity.

Cell Modulation: Brain Tissue-Specific Patterning and Differentiation

Many neurodegenerative diseases are characterized by the loss of a specific brain cell types or cell degeneration in a specific brain region. For instance, a hallmark of PD is the loss of dopaminergic neurons. For successful cell transplantation strategies, the cells need to survive, integrate, and differentiate into the correct type of cells at the right place after transplantation. Choosing the appropriate cells for transplantation is a critical step in determining the success of cell replacement strategies.

Noggin, a bone morphogenetic protein (BMP) antagonist involved in neural differentiation during brain development, promotes neural differentiation from ectodermal cells and serves as a rostral induction factor during brain development [68, 88]. Noggin has also been studied in cell therapies for PD. Chiba and colleagues [15] transplanted naive or Noggin-exposed (500 ng/ml over 4 days) hESC-derived DA neurons into 6-OHDA-lesioned rats. By 4 weeks after transplantation, animals receiving hESC-derived DA neurons exposed to Noggin showed better functional outcome than animals receiving naive hESC-derived DA neurons or sham treatment. Histological analysis revealed a fivefold increase in TH+ neurons, with the number of surviving TH+ cells correlating with the reduction of functional deficits. These results indicate that Noggin preconditioning can improve the efficacy of cell therapy.

Transplantation of human PSC-derived DA neurons showed limited *in vivo* benefits in several studies. These results might be due to incomplete cell specification of the transplanted cells. Thus, Kriks and colleagues developed a new strategy for the derivation of human DA neurons, which also allows cell engraftment enhancement upon intracerebral transplantation [50]. hESCs and hiPSCs were subjected to a modified dual SMAD-inhibition protocol (with timed exposure to LDN193189, SB431542, SHH C25II, purmorphamine, FGF8, and CHIR99021) to generate floor-plate (FP) precursors that were later differentiated into DA neurons (FP-derived DA neurons). Rosette-derived DA neurons were also tested. Those were obtained by the most widely used strategy for deriving DA neurons, i.e., a stem cell co-culture with MS5 cells plus timed exposure to SB431542, Noggin, SHH, and FGF8 to generate neural rosettes, followed by manual isolation and culture with timed exposure to SHH, FGF8, BDNF, and ascorbic acid [78]. Rosette-derived DA and FP-derived DA neurons were transplanted into the striatum of 6-OHDA-lesioned NOD-SCID IL2Rgc null mice. Four and a half months after transplantation, graft evaluation revealed a well-defined graft containing TH+ cells co-expressing FOXA2 and human-specific NCAM, whereas rosette-derived DA grafts gave rise to fewer TH+ neurons. Additionally, FP-derived DA neuronal grafts promoted a complete rescue of amphetamine-induced rotations, whereas rosette-derived DA grafts produced no significant reduction of these rotations. Rosette-derived DA grafts also showed massive neural overgrowth, proliferating cells were almost absent in FP-DA-derived grafts. FP-derived DA neurons were also transplanted into 6-OHDA-lesioned rats subjected to immunosuppression by cyclosporine A. Five months after transplantation a robust graft survival of more than 15,000 TH+ cells was observed and functional outcome was also improved. These data suggest that previously reported poor performance transplanted DA neurons in PD may be due to incomplete/inadequate specification of cells prior to transplantation and highlights the importance of generating the correct type of cells to optimize cell replacement strategies.

The discovery of the FP origin of mesencephalic dopaminergic (mesDA) neurons instead of the neuroepithelial progenitors [74] allowed establishing new protocols to pattern hPSCs towards VM progenitors [47]. Nolbrant and colleagues [74] published an optimized protocol to generate caudalized VM progenitors that give rise to mature and functional mesDA neurons upon transplantation into the adult rat brain. The caudal patterning of FOXA2+/LMX1+-expressing VM progenitors was optimized by the timed addition of FGF8b, and authors provide critical information for monitoring and adjusting the patterning process for the successful protocol using different cell lines. This protocol efficiently resulted in the production of billions of DA progenitors, with high batch-to-batch reproducibility. Additionally, cell product created by this newly developed protocol (STEM-PD) is in the pipeline for a PD clinical trial by the Lund and Cambridge Universities (EudraCT number 2021-001366-38) [106].

Conclusion

In this chapter, we highlight several strategies that have been employed to increase safety and efficacy of cell transplantation strategies for brain repair and regeneration. While significant improvements have been reported using these strategies, there are still several limitations to be overcome. Further research is required to optimize cell survival, migration, differentiation, and integration after intracerebral transplantation. Nevertheless, several promising avenues for improving the success of cell-based therapy have already been described and tested in translationally meaningful setups.

Disclosure of Interests

All authors declare they have no conflict of interest.

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List of Abbreviations

6-OHDA	6-Hydroxydopamine
A β	Amyloid β -peptides
BAT	Bilateral asymmetry test
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
BLI	Bioluminescent imaging
BMP	Bone morphogenetic protein
CoB	Co-stimulation blockade
DA	Dopaminergic
Dcx	Doublecortin
E14	Embryonic day 14
E2B	Estradiol-2-benzoate
EB	Evans Blue
EBST	Elevated body swing test
EC	Endothelial cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
ERDF	European Regional Development Fund

ES	Embryonic stem
ESC	Embryonic stem cell
FGF2/bFGF	Fibroblast growth factor 2/basic fibroblast growth factor
FN	Fibronectin
FP	Floor-plate
Gal-1	Galectin-1
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green fluorescent protein
HD	Huntington's disease
HI	Hypoxic-ischemic
hNSPCs	Human Neural Stem/Progenitor cell
ICH	Intracerebral hemorrhage
IL-6	Interleukin-6
iPSC	Induced pluripotent stem cell
LFP	Lateral fluid percussion
LN	Laminin
MAP2	Microtubule-associated protein 2
MCAO	Middle cerebral artery occlusion
mesDA	Mesencephalic dopaminergic
MG	Microglia
mGRP	Mouse glial-restricted progenitors
MJD	Machado-Joseph disease
mNSCs	VM neural stem cells
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
myrAkt1	Myristylated Akt1
NA	Niche astrocytes
NaB	Sodium butyrate
NeuN	Neuronal nuclear protein
Ngn2	Neurogenin 2
Nico	Nicorandil
NOD/SCID- γ	Nonobese diabetic/severe combined immunodeficiency gamma
NSCs	Neural stem cells
NTN	Neurturin
NXPH3	Neurexophilin 3
OECs	Olfactory ensheathing cells
Oli	Oligodendrocyte specific protein
P0	Postnatal day 0
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PSC	Pluripotent stem cells
QA	Quinolinic acid
RFP	Red fluorescent protein
S100B	S100 calcium binding protein B
SCID	Severe combined immunodeficiency

SDF-1 α	Stromal cell-derived factor 1 alpha
SENA	Substrate-adherent embryonic stem cells
SGZ	Subgranular zone
SOD2	Superoxide dismutase 2
STAT3	Signal transducer and activator of transcription 3
SUMO	Small Ubiquitin-like Modifier
SVZ	Subventricular zone
TBI	Traumatic brain injury
TH	Tyrosine Hydroxylase
TNF α	Tumor necrosis factor
VM	Ventral mesencephalic

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Chapter 3

Neural Stem Cell Intervention in Traumatic Brain Injury



Andrew R. Morris, Heather L. Morris, Genevieve Z. Barquet, Stuti R. Patel, Nayef A. Amhaz, Olivia C. Kenyon, Zaynab Shakkour, Jiepei Zhu, Fatima Dakroub, and Firas H. Kobeissy

Abstract *Introduction:* Traumatic brain injury (TBI) is a major cause of death and disability worldwide. The secondary brain damage post-TBI encompasses oxidative stress, metabolic changes, necrosis, excitotoxicity, and neuroinflammation. These processes further intensify the neural injury induced by TBI. Several treatment strategies that are aimed at different TBI features have been proposed and tested in clinical trials. Yet no approved effective drug has been identified for TBI treatment. Failure of conventional drug interventions suggests a need for research on novel stem cell-based therapies to treat TBI. *Methods:* Citing the most up-to-date literature, we used the MEDLINE/PubMed database for review articles, research papers, books, systematic reviews, and case studies and the ClinicalTrials.gov database for human clinical studies; both provided by the U.S. National Library of Medicine. *Results:* Neural stem cell (NSC)-based neurotherapy is used in numerous diseases to promote functional recovery and tissue reconstruction. This chapter will discuss treatment options by both endogenous NSCs and exogenous stem cells. Preclinical TBI therapeutic strategies have demonstrated limited translational success so far. Moreover, they have led to the combination of multiple therapeutic approaches among researchers. Additionally, we will discuss treatment strategies with the use of drugs showing beneficial effects, and those that may have synergistic effects when used in combination with other complementary strategies. *Conclusions:* The success of this approach

Andrew R. Morris and Heather L. Morris: These two authors are equally contributed.

A. R. Morris (✉) · G. Z. Barquet · S. R. Patel · N. A. Amhaz · O. C. Kenyon
University of Florida, Gainesville, FL 32610, USA
e-mail: morrisar@ufl.edu

H. L. Morris
Target RWE, Durham, NC 27703, USA

Z. Shakkour · F. Dakroub
American University of Beirut, Beirut, Lebanon

J. Zhu · F. H. Kobeissy (✉)
Morehouse School of Medicine, Atlanta, GA 30310, USA
e-mail: frasko@gmail.com

for other medical conditions presents another driving force for exploring combination TBI therapies. It is expected that many cell-based innovations and translational applications will continue to manifest in the future.

Keywords Brain trauma · Neural stem cell · Pathophysiology · Regenerative Medicine · Neurotherapy

Introduction

Traumatic brain injury (TBI) is a major cause of death and disability worldwide with an estimated 27–69 million new cases per year. TBI is extremely heterogeneous due to differences in the severity of the primary injury and the secondary neurochemical events it triggers, such as mitochondrial and metabolic dysfunction, cerebral edema, oxidative stress, and disruption of the blood–brain-barrier (BBB). The type of primary injury and the secondary responses to the injury have a great influence on recovery patterns and overall outcomes. Several animal models have been developed and used to explore potential neurotherapies for TBI. Although animal models are limited in their resemblance to human anatomy/physiology, they can still provide important information regarding the effects of TBI and the effectiveness of different treatment methods.

TBI Impact and the Need for Neurotherapy

The secondary brain damage that occurs (oxidative stress, metabolic changes, necrosis, excitotoxicity, and neuroinflammation) forms the basis of TBI and intensifies the neural injury process. Several treatment strategies, each aimed at different features of TBI, have been proposed and applied to clinical trials, yet no approved effective drug has been identified for TBI treatment. Progesterone has been shown to have neuroprotective effects against several parts of the secondary injury cascade and to strengthen motor and cognitive outcomes, but these effects did not translate well during phase III clinical trials. The failure of conventional drug interventions suggests a need for research on novel stem cell-based therapies to treat TBI.

The Role of Oxidative Stress

Considering that activation of the oxidative stress response is a major part of the secondary injury cascade, multiple antioxidative stress drugs have been used for early-stage intervention. *Nimodipine*, a calcium channel blocker, and *Tirilazad*, a

lipid peroxidation inhibitor both made it to the clinical trial phase but failed to show a significant increase in positive therapeutic outcomes.

Two double-blind trials were conducted to test the effectiveness of *Nimodipine* as a TBI treatment. In the first study, this drug was given within 24 h following TBI for seven days [14] and, in the second, it was administered for two days [228]. Both studies failed to show an increase in positive outcomes following treatment when compared to the control group.

Although results from preclinical studies with *Tirilazad* were promising, the clinical trials did not show a significant increase in favorable outcomes. In a mouse weight drop model of TBI, *Tirilazad* was given five minutes and 90 min after injury. The survival rate in the treatment group was 78.6% compared to 27.3% in the placebo group [87]. Another study reported reduced mortality and cerebral edema in groups that received the medication, 15 min and three hours post-TBI [164]. Phase III clinical trials involving both moderate and severe TBI patients failed to demonstrate the effectiveness of *Tirilazad* in TBI treatment [112, 132].

Halting the Inflammatory Response

Three drugs, Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), *Selfotel*, and *Cyclosporine*, were assessed in clinical trials for their ability to halt the TBI-induced inflammatory response. However, these drugs failed to show any significant improvement in comparison to the control groups.

A study involving the treatment of cats with PEG-SOD following fluid percussion injury showed that the treatment significantly reduced the occurrence of microvascular dysfunction. Microvascular dysfunction is associated with superoxide anion, whose production is increased directly after TBI [125]. A phase III clinical study was later conducted that explored the effect of treating moderate to severe TBI patients with PEG-SOD within eight hours of injury. However, this treatment had no significant clinical impact with the survival rate and neurological recovery remaining roughly the same between the treatment group and the placebo group [172].

The treatment of TBI rats with N-methyl-D-aspartate (NMDA) antagonists halted secondary inflammatory mechanisms and the activity of excitatory amino acids at the injury site [66]. This prompted the clinical investigation of the NMDA antagonist, *Selfotel*. Phase III clinical trials were initiated with severe TBI patients but were later discontinued due to safety concerns. During these clinical trials, *Selfotel*-treated patients experienced increased mortality and psychoactive behavioral side effects [170]. The blockade of the synaptic transmission mediated by NMDA receptors such as *Selfotel* hinders neuronal survival pathways, thus explaining the failure of *Selfotel* as a TBI treatment.

Cyclosporine was tested clinically for its immunosuppressive properties. It was administered within eight hours of injury in a randomized double-blind controlled study involving patients experiencing a diffused axonal injury. The study showed that the *Cyclosporine* treatment did not significantly improve cognitive function [7].

Hormones and Neuroprotection

Progesterone and erythropoietin (EPO) are important hormones that possess neuroprotective properties. Progesterone is a hormone synthesized by the brain, ovary, and placenta. EPO is a hormone essential for the differentiation and proliferation of red blood cells [16]. Several preclinical studies have demonstrated positive effects associated with Progesterone utilization in TBI. Following these, two phase III clinical trials proved to be successful. However, two other phase III clinical trials were halted and deemed futile [218]. The failed results could be due to inadequate drug dose when converting between rodent drug dosage and human doses [218].

EPO has exhibited antioxidative, angiogenic, neurotrophic, anti-inflammatory, and anti-apoptotic properties in several stroke and seizure models induced by kainite [26, 79]. A double-blind randomized clinical trial investigating the effects of EPO on moderate to severe TBI patients did not yield significant positive outcomes [178].

Overall, the aforementioned drugs have proven to be clinically ineffective, regardless of the promising preclinical studies. Additionally, most available TBI drugs only target a specific part of the TBI cascade [56, 121]. Although 411 clinical trials have been conducted so far, there are currently no FDA-approved drugs available for TBI treatment [175].

Stem Cell-Based Therapies

Neural stem cell (NSC)-based neurotherapy is used in numerous disease models due to the neuroprotective abilities of stem cells, which promote functional recovery and tissue reconstruction [217, 247]. In rodent TBI models, NSC-based therapy by cell engraftment has achieved functional recovery of neurological deficits and has demonstrated clinical translational potential for humans [18, 34, 108, 250]. Optimal conditions for NSCs require efficient and controlled cell proliferation, migration, and differentiation. Moreover, the treatment should be associated with a reduced risk of eliciting a host immune response, a high capacity for integration of the NSCs into the host neuronal network, and an enhanced recovery of neurologic deficits after transplantation. This section discusses TBI treatment options by both endogenous NSCs and exogenous SCs.

Endogenous Stem Cells

Adult NSCs (ANSCs) are a type of endogenous stem cell that is multipotent and can generate new neurons and glia [199]. ANSCs are found along the walls of the lateral and third ventricles of the brain [4]. They are localized in the subependymal zone (SEZ) of the lateral ventricles' lateral walls and in the subgranular zone (SGZ)

of the hippocampal dentate gyrus [4, 23]. During ANSC differentiation and migration, the neuroblasts generated in the SEZ—also known as the adult subventricular zone (SVZ)—travel along the rostral migratory stream (RMS) to the olfactory bulb, where they fully differentiate into inhibitory neurons [75, 114, 150, 248]. The capability for neurogenesis highlights the potential of ANSCs in endogenous neural cell replacement therapy after TBI-induced neuronal loss.

Neurogenesis occurs through the stages of cell proliferation, differentiation, and migration. These stages are regulated by biochemical factors such as steroids, neurotransmitters, and growth factors, as well as environmental factors like stress and physical exercise. Accordingly, pathophysiological conditions may serve as a regulatory factor. For instance, the disturbance of normal brain function due to disease or TBI reduces the proliferative capacity of SEZ, thereby hindering neurogenesis [10, 201].

Clinical trials have not yet demonstrated the successful use of endogenous NSCs in humans. NSCs combined with pharmacological therapy may prove useful [49, 186]. Experiments with rodent models have demonstrated success. A 2022 study found that a combination of NSCs with other treatments leads to improved axonal regeneration, synaptogenesis, and synaptic plasticity in the cerebral cortex of treated TBI-mice [143]. Furthermore, a 2021 study explored the Sonic hedgehog (SHH) pathway—an NSC pathway—in TBI. It reported that increased SHH signaling resulted in increased endogenous cell proliferation and significantly improved motor function recovery in TBI-treated rodents. However, the insufficient data about the SHH pathway in humans with TBI limit the prospects of conducting clinical trials [194].

Regulation of Neurogenesis

In adults, neurogenesis is regulated by intrinsic, extrinsic, and environmental factors. Collectively, these influence the niche, proliferation, differentiation, migration, survival, and maturation of SVZ stem and progenitor cells [67]. Intrinsic factors are expressed by precursor cells and include morphogens such as the Notch pathway and the Sonic hedgehog pathway (SHH). The Notch pathway maintains stem cells by promoting their self-renewal. The SHH pathway maintains and influences the proliferation of adult progenitor cells [96, 106, 144, 152].

Transcription factors expressed in the SVZ and migrating neuroblasts are other intrinsic factors that regulate neurogenesis [83, 144]. *Pax6* is one such transcription factor—it is essential for the generation of periglomerular cells and granule cells, which are subpopulations of olfactory bulb (OB) neurons [124]. *Pax6* may also play a role in tissue adhesion and the development of radial glial cells in the developing forebrain [82, 219]. Vascularization regulates neurogenesis through the diffusion of circulating factors such as cytokines and growth factors [53]. Endothelial cells produce factors that promote self-renewal of NSCs [209], including the Pigment Epithelium-Derived Factor (PEDF), which activates the Notch pathway [9].

Extrinsic factors are expressed by surrounding tissues and regulate neurogenesis. Serotonin is an extrinsic factor that enhances proliferation of SVZ stem cells [25]. Growth factors are also extrinsic factors that can increase cell proliferation in the SVZ and promote neuroblast migration. Examples include the Brain-Derived Neurotrophic Factor (BDNF), the Vascular Endothelial Growth factor (VEGF), the Fibroblast Growth Factor (FGF) and the Epidermal Growth Factor (EGF) [144, 171, 212]. BDNF enhances survival and differentiation in SVZ-derived cells in damaged rat brains [94, 115]. Moreover, in a 2020 study, mesenchymal stem cells (MSCs) with or without BDNF-medication were administered to TBI-treated rodents. The BDNF-medicated MSCs exercised better promotion of neurogenesis than regular MSCs, demonstrating BDNF's ability to promote neurogenesis after TBI [252].

Lastly, environmental factors like drug and alcohol use regulate neurogenesis. Antidepressants and antipsychotics impact serotonergic and dopaminergic transmissions. Thus, they have the capacity to modify SVZ neurogenesis [53]. Alcohol dependence can lead to a lasting suppression of cell proliferation in the SGZ, due to its impact on the GABAergic system [88]. In a 2019 experiment, rodents that consumed binge alcohol prior to TBI treatment exhibited decreased cell proliferation and neuroblasts in the SVZ and RMS. This demonstrated an overall negative impact of alcohol on neurogenesis in TBI [230].

Response of Endogenous NSCs to Brain Damage and Their Potential in Brain Repair

Cell proliferation in the subependymal layer adjacent to the caudate nucleus is elevated in patients with Huntington's disease (HD) [50]. However, this alteration was not demonstrated in mouse models of HD [122]. Patients with Parkinson's disease (PD) demonstrated a decrease in the brain neural precursors, caused by dopaminergic denervation [98]. Likewise, human and transgenic mouse models of Alzheimer's disease (AD) showed impairment in the proliferation of precursor cells [198]. In contrast, progenitor proliferation in SVZ and SGZ regions significantly increased post-TBI [199]. This is of particular importance because of the regenerative capacity of SCs within these regions. These cells provide new neurons that can be administered to an injured brain and integrated into the host circuitry. Some advantages of endogenous stem cells are being less invasive, thus preventing host rejection. Moreover, they facilitate averting ethical issues [201]. Robust research efforts should focus on mobilizing SCs in the adult brain to attain an endogenous repair mechanism.

Neurogenesis was shown to be induced in eleven adult human brain specimens with TBI. The samples displayed an elevated number of cells expressing endogenous markers of neural stem/progenitor cells (NS/NPCs) [270]. In an adult rat model of ischemic injury, immature SVZ neuroblasts were shown to be recruited at the site of the injured striatum, to which they differentiated into mature neurons. Despite

this, 80% of newly formed neurons died after two to six weeks. Ultimately 0.2% of injured striatal neurons were replaced [11].

Interestingly, another study determined that Calretinin-expressing cells survived, while DARP-32-expressing spiky neurons died in rat-damaged striatum [142]. SVZ neuroblasts have also been recruited and differentiated into mature neurons in the cortex of the same lesion model, surviving for 35 days [130]. In an experimental TBI model, SVZ cells migrated to the lesion site and expressed nestin and glial fibrillary acidic protein (GFAP) proteins. However, they did not express markers of mature neurons such as NeuN [202]. In a bilateral common carotid artery occlusion model, cell proliferation in the dorsolateral SVZ doubled in ischemic immature mice after 48 h, without significant regeneration [145].

Additional research revealed that the development and integration of neurons into an existing circuit takes roughly 10–14 days [199]. For example, proliferating cells within the SVZ and SGZ of the lesion group in a rat fluid percussion injury (FPI) model increased immediately. However, these multiplying cells were not differentiating into a lineage of neurons [43]. Adult mice's functional neuroblast recruitment following injury demonstrated that the administration of stromal cell-derived factor 1 beta (SDF1 β) and angiopoietin, which enhance neurogenesis, help to prevent the loss of sensorimotor functions following cortical ischemic injury [181]. These findings indicate that SVZ neuroblasts can engage in a potential therapeutic target for post-lesional functional recovery.

Furthermore, several factors that regulate neurogenesis in normal physiological conditions play a role in the brain's reaction to TBI-associated conditions. Growth factors and signaling molecules such as VEGF and SHH have been shown to encourage SVZ cell proliferation and mobilization to the injured adult cortex of mice [6, 239]. Other factors that influence neurogenesis and neuroblast migration have been explored. TBI lesions induce an inflammatory response that promotes the secretion of cytokines, which modulate neuroblast recruitment. Some drugs may influence endogenous neurogenesis. Metformin was recently shown to increase endogenous neurogenesis in a hypoxia–ischemia injury model. Metformin treatment increased the size of the endogenous NPC pool and promoted migration and differentiation in injured brains of newborn mice [51]. It additionally promoted NPC migration into the surrounding parenchyma and enhanced sensory-motor functions [51]. As a result, stimulating factors may offer a promising therapeutic approach for successfully repairing the brain after cortical injuries.

The above studies suggest that endogenous stem cell mobilization could be used for cellular therapy. Still, several findings are controversial because the results from various studies are not always consistent. This is likely due to differences in the cellular and molecular mechanisms involved in the various injury models utilized, and the complex environment surrounding the cells [80]. Furthermore, the SVZ cellular niche is spatially heterogeneous, giving rise to distinct neuronal subtypes in the OB [69], a process that may be affected differently after various types of injuries [6, 51, 239].

In summary, numerous preclinical studies have highlighted the potential of endogenous stem-cell therapy in successfully repairing brain damage [199]. Producing enough functional and mature neurons capable of surviving and integrating into the damaged brain neural network remains a significant challenge. After trauma, primary brain injury combined with secondary tissue loss creates an unfavorable environment that inhibits the survival and/or integration of recruited cells. Consequently, alternative sources capable of providing larger numbers of self-proliferating precursors with similar neurogenic potential, such as exogenous stem cells, should be considered [199].

Exogenous Stem Cells

Stem cell engraftment has demonstrated therapeutic potential for TBI. Embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), bone marrow stromal cells (BMSCs), adult neural stem cells (ANSCs), and induced pluripotent stem cells (iPSCs) have been successfully transplanted.

Embryonic Stem Cells (ESCs)

Found in the blastocyst stage of early embryonic development, ESCs can be extracted and cultured in an undifferentiated state. The vast utility of ESCs has led to their use in neural grafting experiments. ESCs can migrate and form proper innervations following transplantation into normal or damaged central nervous systems (CNS) [95]. Moreover, they can promote the formation of neurons and glial cells [102]. Over the past 20 years, ESCs originating from fetal brains have been considered a pinnacle in TBI animal models featuring cell transplantation. The use of ESCs in animal models has been correlated with the survival of transplanted cells, [92] and motor function recovery [105].

Furthermore, human fetal brain ESCs were able to survive and differentiated into neurons and astrocytes following the migration to the contralateral cortex of an injured rat brain [245]. Animal studies have indicated that transplanted ESCs ameliorated the injury, while expressing glial or neuronal markers [97, 105]. NSCs isolated from a mouse fetal brain and grafted into damaged adult mouse brains stimulated a significant recovery of both motor functions and spatial learning by differentiating into both neurons and glial cells [213]. Extrinsic factors impact the incorporation and synaptic connectivity of transplanted cells following TBI [84]. Human ESCs that were transplanted into mice and successfully induced into NSCS, resulted in major improvements in cognitive and motor functions in animals that were treated [140]. However, when differentiated human ESCs were transplanted into a controlled cortical injury (CCI) rat model of TBI, a transient increase in angiogenesis and neuronal survival was detected. This was accompanied by a reduction in astrogliosis

and lesion volume within the injured area [213]. The benefits of establishing an enriched environment (EE) with cortical ESCs transplantation following TBI were investigated in a rodent model. The treated animals in the EE group performed better than animals with lesions exposed to either EE or ESCs treatment alone [189]. Human embryonic stem cell-derived cerebral organoids (hCOs) have also revealed promising benefits in TBI mouse models. These comprised a significant reduction in neuronal cell death and the promotion of post-TBI neurogenesis. The transplantation of hCOs cultured at 8 weeks led to the reconstruction of the damaged cortex, as well as neurogenesis in the hippocampus and improved motor functional recovery [118, 241].

Stem cells have emerged as one potential for cell replacement therapy in neurodegenerative diseases and spinal cord injury (SCI). This is due to their ability to differentiate and integrate into the host's neural parenchyma. Animal models of PD showed that fetal tissues that had been grafted ectopically into the striatum can survive and re-innervate the striatum, reversing multiple behavioral deficits [70]. ESCs were also shown to survive and differentiate into astrocytes, neurons, and oligodendrocytes in SCI [142]. An *in vitro* protocol was developed using mouse ESCs to generate cortical neurons that imitated/mimicked the main stages of corticogenesis and cortical neuronal diversity and were then transplanted into the cerebral cortex of newborn mice. These mice subsequently developed axonal projections like cortical neurons, especially targeting the visual cortex [74]. Exposure of SCs to the Wnt and bone morphogenic protein (BMP) signals resulted in the production of cortical neuronal cells [242], while exposures to morphogens (i.e., FGF-8 or FGF-8 antagonist) modulated the rostrocaudal identity of cortical neuronal cells that were generated *in vitro* [63].

Cortical neuronal precursors derived from mouse ESCs and transplanted into other cortical regions, namely motor and visual cortices, formed projections with specific targets [104]. Cortical neurons derived from human ESCs transplanted into the brains of newborn mice led to the integration and development of axonal projection within the motor and visual cortices [64]. Similarly, cortical neurons from mouse ESCs aided in the repair of distinct cortical injuries in adult mice. This was mediated by generating occipital progenitors to establish projections over long distances corresponding to the circuits of the visual cortex [167]. No significant integration was observed after the transplantation of the same type of cells in the damaged motor cortex, nor after grafting motor cortical neurons in the damaged visual cortex. However, the transplanted neurons received afferents from the visual thalamus and responded to the visual stimuli of the retina *in vivo* [167]. This indicates their capacity for functional restoration of the damaged visual cortex circuits. These results suggest the presence of regional specificity associated with distinct cortical areas for the repair of cortical neurons [167]. In 2018, engrafted human ESCs differentiated into visual cortical neurons and integrated into lesioned mouse visual cortices. Moreover, they expressed molecular markers of the cortical layers and developed projects toward visual cortex-specific targets [65].

In an hESC study using cells from long-term cryostorage, transplantation following a moderate TBI led to improved spatial learning and recall in the Morris

Water Maze. Moreover, it resulted in a reduction in risk taking in an elevated plus maze. These findings further support the potential use of hNSCs post-TBI [13]. In a severe TBI model, the use of transplanted hESC in brain lesions of CCI mice improved spatial learning and memory following organoid grafting [15].

The results summarized herein provide an updated perspective for cell therapy in the reconstruction of the injured brain from neuroanatomical and functional points of view. Despite the high plasticity and enhanced survival capacity displayed by ESCs within neural transplantation experiments, the use of ESCs still faces challenges that limit its generalizability. This also limits their clinical application in the treatment of various brain injuries. ESC research is reliant upon the successful integration of transplanted cells as well as the tight regulation of their rate of proliferation and differentiation to prevent the risk of teratomas' growth. Additionally, the derivation of human ESCs is restricted by ethical and logistic issues inherent in obtaining human blastocysts.

Mesenchymal Stem Cells (MSCs)

MSCs are adult multipotent stromal cells that can be extracted from bone marrow and other adult tissues [33]. MSCs can differentiate into osteogenic, adipogenic, chondrogenic, and neurogenic lineages and express various surface markers (i.e., CD73, CD105, and CD90) [86, 235]. MSCs are capable of proliferation, unlimited self-renewal, migrating to the injury site, and differentiating into neural cell lineages [32, 204]. The characteristics that make MSCs ideal for TBI treatment include their ability to cross the blood–brain-barrier (BBB) through paracellular pathways, [161, Schmidt 2006]. Moreover, they can restore BBB integrity by upregulating the expression of tissue inhibitor of matrix metalloproteinase-3 [33, 166] (Beyer 2006).

MSCs have been shown to migrate selectively to injury sites of TBI and improve motor function following transplantation [240]. This process is mediated by signaling pathways associated with the release of chemokines and growth factors [193]. MSCs additionally promote neuronal development and enhance motor and sensory functional recovery by differentiating into neurons and astrocytes in a rat TBI model [8]. Factors that have been secreted by MSCs can enhance self-repair of existing tissue cells [153], stimulate NSC proliferation in vitro, and increase the expression of GFAP [36].

Transplanted MSCs were found to alter the concentrations of inflammatory cytokines including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), and (IL-1 β) [71]. Following TBI, IL-1 β , IL-17, and IL-6 were reduced in the injured cortex of rats after transplantation, while IL-10 and transforming growth factor beta 1 (TGF- β 1) expressions were elevated [267]. Frontal bone MSCs (Fb-MSCs) used in a mouse model reduced behavioral and cognitive deficits following TBI and promoted neural regeneration [196].

The use of MSCs shows promise in TBI repair, but there is still limited knowledge of how these cells target specific tissues. More research is needed to further understand the beneficial role of targeting the treatment ability of MSCs shown by paracrine factors [231]. Additionally, researchers need to better understand the impact of time on the efficacy of using MSC therapy is vital, since improvements were obtained when performed shortly after injury [229].

Reprogramming Exogenous Cells

MSCs can be reprogrammed by adding media to the culture. The Dibutyryl cAMP-medium has shown the highest potential for neural differentiation [22]. The human amnion-derived neural stem-like cells (AM-NSC) showed cognitive improvement and recovery in brain tissues and elevated expression levels of neurotrophic factors including BDNF, NGF, neurotrophin-3 and glial cell line-derived factor (GDNF) shortly after transplantation [255]. However, AM-NSCs did not survive one month following transplantation in the brain injury site. There exists limited evidence regarding the long-term integration of these cells into host tissues [126, 187, 251, 268]. Although MSCs-based therapies appear to be feasible, more research is needed to better elucidate their underlying regenerative properties, particularly their capacity for long-term survival.

Bone Marrow Stromal Cells (BMSCs)

The bone marrow contains non-hematopoietic stem cells that have multipotent attributes [37]. BMSCs are appealing, having the ability to differentiate into MSCs. When exposed to various conditions, they can differentiate into other cell types such as neurons and glial cells [85, 204]. Functional recovery was demonstrated following the use of human and rat BMSCs engraftments in focal brain ischemia models [37, 269]. In a TBI rat model, BMSCs that were grafted into an injured area survived and migrated to the site of the injury, where an increase in the expression of GDNF was detected [37]. In a rat population subjected to cortical TBI, transplantation of Notch-induced BM-derived MSCs located at the site of the injury led to the formation of a cellular bridge at the nearby neurogenic SVZ. This transplantation facilitated the remodeling of tissue and the induction of host cells, even in the event of a lack of long-term graft persistence [223].

A distinct advantage of BMSCs is that they can be administered to damaged brain tissue non-invasively and with fewer immunological problems. A 2017 clinical trial (NCT 0157540) was conducted as an extension of a similar clinical trial in pediatric patients with severe TBI [47]. It reported the safety of BMSCs treatment and its efficacy in protecting critical regions of the brain and improving functional outcomes [48].

Adult Neural Stem Cells (ANSCs)

ANSCs present an opportunity in TBI treatment as they have an unlimited self-renewal capacity. Moreover, they differentiate into mature neural cell types like embryonic or fetal stem cells without the technical and ethical problems coupled with the risk of tumor formation and immunological rejection [236]. ANSCs are commonly harvested from the SVZ/SEZ in the adult brain in the surrounding walls of the lateral ventricles, the SGZ of the hippocampal dentate gyrus (DG); and the non-canonical sites of adult neurogenesis (i.e., hypothalamus, amygdala, substantia nigra, and cerebral cortex) [68, 151].

During the process of adult neurogenesis, various cell cycle regulators exhibit distinctive functional properties in the control of stem cells, particularly inside the SEZ [184]. The use of inducible deletions targeted to ANSCs, the Retinoblastoma (Rb) tumor suppressor has shown evidence of specifically controlling progenitor proliferation in the DG and the SVZ. The loss of Rb was associated with enhanced neurogenesis in both the hippocampus and OB in the short term [174, 234]. Unlike in development, the deletion of Rb did not cause major defects in differentiation or neuronal migration [76, 107]. Thus, the Rb pathway could potentially be targeted by genetic manipulation to amplify the pool of neurogenic population without altering the outcomes of ANSCs. Transplanted ANSCs in a rat TBI model migrated to the injured site while also expressing markers of both mature oligodendrocytes and astrocytes. This resulted in improved survival capacity for an extended period [221]. ANSCs that had been transplanted into an injured rat cortex were associated with improved motor function [90]. The transplanted cells migrated to damaged boundary zones and reduced the cerebral lesion size, while promoting functional recovery [213]. These positive effects could be due in part to the enhanced angiogenesis found near the injury site [213]. However, these adult cells showed less plasticity compared to ESCs. Moreover, only a small portion of human ANSCs survived up to four months after engraftment in a rat ischemic injury model [182]. In summary, the clinical application of ANSCs is limited for TBI treatment and still faces important challenges.

Positive and negative outcomes have been linked to induced adult neurogenesis in injured brains [177, 208, 220, 261]. Additionally, the perilesional cortex becomes an anti-neurogenic environment following TBI [27]. This favors the proliferation and replacement of oligodendrocytes after CCI in a mouse model [55]. Isolation of SVZ-derived neuroblasts (neuroblast markers like Tuj1, Dcx, and Gad65/67) and their transplantation into the ectopic brain regions led to cells converting into a glial phenotype (astrocytic or oligodendrocytic) [207]. A recent study has contradicted these findings through the transcription regulatory network analysis of the perilesional cortex using an FPI model. Results showed a 6.7-fold increase in the expression of the neurogenic marker Pax6 and 30% of the target genes. Moreover, it reported the upregulation of Tp73, Cebp, and Spi1 expressions [141]. The latter genes have been shown to regulate apoptosis, microglia, and inflammation, which are known to contribute to secondary damage following TBI [141].

Another complication post-TBI is the loss of hippocampal neurons, mainly DG newborn adult neurons [72]. This results from unresolved or intense stress on the endoplasmic reticulum through CCAAT-enhancer-binding protein (C/EBP) homologous protein activation [99]. One study assessing endogenous neurogenic mechanisms following FPI found that the number of immature neurons increases on day seven post-FPI along with ectopic migration of doublecortin (DCX+) immature neurons into the dentate gyrus region [208]. A post-rat FPI model revealed an increased level of neurogenesis. However, this was accompanied by a persistent decrease in neurogenesis compared to the control group. The immediate increase in elevated neurogenesis led to a long-term decline in neurogenic capacity [177]. These findings challenge the concept that post-injury neurogenesis is beneficial. It might cause long-term adverse outcomes by exhausting neurogenic capabilities that deplete the neural precursor population and enhance epileptogenesis incidents [177].

The intravenous delivery of recombinant TIMP3 was found to ameliorate ANSC loss in the DG post-TBI by activating the Akt-mTORC1 pathway [77]. This pathway also mediates the increase in neural stem cell proliferation and neurogenesis in the DGI post-TBI [237], while also mediating the synaptic reorganization and mossy fiber sprouting post-CCI injury [30]. Although these findings are promising, the activation of the Akt-mTORC1 pathway may increase seizure frequency following TBI [117]. The inhibition of the mTOR pathway by rapamycin is neuroprotective against post-traumatic epileptogenesis [117]. Alternate neurorestorative pathways should be considered to salvage ANSC-derived neuronal loss post-TBI.

Future research efforts should focus on uncovering the molecular mechanisms responsible for establishing a balance between enhanced stem cell/progenitor proliferation and the plasticity of newborn neurons. This will aid in treating the TBI sequela without exposing the brain to adverse effects such as post-traumatic epilepsy.

Induced Pluripotent Stem Cells (iPSCs)

The use of specific cell types from a patient's stem cells provides an immunological advantage. Non-autologous cell transplantation can be rejected by the host immune system. Consequently, somatic cell reprogramming was investigated as an alternate source of pluripotent stem cells [224]. Adult somatic cells such as fibroblasts can generate iPSCs through the forced induction of the expression of stem cell-specific transcription factors. These comprise Oct4, Sox2, c-Myc, and Klf4 [224, 259]. iPSCs potentially represent an infinite cellular source with abilities like ESCs without the need to sacrifice embryo(s). This finding has unraveled new opportunities within the field of regenerative medicine, which include generating patient-specific iPSCs (i), differentiating iPSCs into specific cell types (ii), and engrafting the iPSC progeny (iii).

Multiple investigations of these possibilities have shown that iPSCs generate motor neurons [59, 62], cochlear neurons [179], peripheral neurons [134], dopaminergic neurons [89, 246], and retinal cells [185]. Neuroepithelial cells can also be

produced from iPSCs and induced to differentiate into glutamatergic cortical neurons [210, 264].

Human iPSCs can also differentiate and generate cortical neurons by recapitulating the main stages of corticogenesis without the addition of morphogens [64]. Integrative retroviral vectors [224] and lentiviral vectors [259] were used to introduce reprogramming factors into adult differentiated cells. Subsequent studies have attempted to create “safer” iPSC lines using polycistronic lentiviral vectors [216]. Moreover, iPSCs were created by overexpressing specific types of microRNAs [110] and using recombinant proteins [271]. Transplanted human iPSC have been shown to survive and subsequently differentiate into all three neuronal lineages without evidence of tumorigenesis in an SCI model using adult marmosets. This resulted in the enhancement of axonal regeneration and prevented demyelination after SCI through the exertion of immunomodulatory and neuroprotective effects [120]. The best cognitive recovery and motor performance were surprisingly found in a rat TBI model using a combination of iPSC transplantation and an enriched environment in contrast to groups transplanted with either iPSCs only or a standard environment [61].

Endogenous Cells Reprogramming

Despite the common belief that the brain cannot repair itself post-injury, some researchers were encouraged by the fact that dormant stem cells in conjunction with growth factor stimulation can be induced into maturity [146]. Attempts to activate ANSCs to fully repair injured brain tissue face numerous challenges, including the limits of endogenous neurogenesis’ potential itself. Neuronal replacement was of little value and 80% of incipient neurons were short-lived (≤ 2 weeks) and made no contact with targets, despite massive CNS injury [11, 169, 215]. Nonetheless, some researchers attempted to reprogram endogenous glial cells into neuroblasts directly or via iPSCs [2, 21, 28, 44, 73, 93]. Despite its promise, iPSC-based therapy still faces significant challenges. These include the cells’ proclivity to form tumors at an early stage of differentiation. Before considering any clinical approach, the functionality and safety of neurons derived from iPSCs must be assessed [95]. A thorough understanding of properly controlling the fate of iPSCs is required to profit from their potential use in cell-based therapies. Various preclinical studies using stem cell-based therapies are discussed below. The reported anatomical, behavioral, and sensorimotor outcomes and the sites of stem cell engraftment are also summarized.

Clinical Applications of Stem Cells in CNS Injury

Recently, clinical research on stem cell treatment of CNS injuries has received extensive attention. Different stem cells have been assessed in clinical trials such as mesenchymal stem cells and neural stem cells. The two key obstacles affecting the progression of stem cell therapy are the optimal transplantation time and the transplantation route [3]. Various time intervals, such as 1-, 2-, 7, and 14-days post-injury were investigated. Moreover, routes of administration such as intracranial, intrathecal, intravenous, and intra-arterial were evaluated. Still, the ideal time and route are yet to be determined [3]. One of the current concerns in the field of neuroscience is the injury repair and neuroregeneration processes [54]. Since this is a form of neuroplasticity, one of the goals is to promote or reinstall functionality in the injured area [54]. Particularly, SCI and TBI have been the focus of extensive neuroscience research [78]. This encouraged the establishment of various hypotheses and clinical trials that are attempting to ameliorate the lives of patients with CNS injuries.

TBI Clinical Trials

While the pace of TBI research has accelerated, the treatment options remain limited. Clinical trials are yet to come up with new innovative strategies to help patients overcome the consequences of TBI [3]. Ma et al. [151] revealed the potential therapeutic effects of rat embryonic NSCs in TBI and their possible underlying mechanism [3]. They extracted NSCs from the forebrain of a 14-day-old rat embryo and injected them directly into the injured brain of a CCI rat model 72 h post-trauma [3]. Eight weeks after transplantation, neuronal function was improved, NSCs survived, and their differentiation into neurons was observed. NSCs not only maintained the neural tissue but also provided expanded plasticity to the key brain structures that are vital for learning and memory [3]. Although transplanted embryonic or adult NSCs can survive, migrate, differentiate, and improve neurologic and cognitive functions in preclinical TBI models, the realistic and ethical sources of NSCs for autologous clinical translation have not yet been achieved.

Mesenchymal stem cells (MSC) are multipotent, exist in adults, and can self-replicate and differentiate into various cell types [3]. MSCs can differentiate into neural cells including neurons. However, their differentiation without any stimulation is not strongly effective. To improve the survival and differentiation of MSCs into nerve cells, they are usually reprogrammed before transplantation [222]. To investigate the efficacy and safety of bone marrow MSCs in the treatment of subacute TBI, Tian et al. [229] conducted a prospective, non-randomized, and open-label clinical trial [3]. The study included 97 patients aged between 18 and 50 years with a persistent vegetative state or motor disturbance post-injury [3]. The patients were injected intrathecally with 4×10^6 autologous BM-MSC within two months after trauma and followed up for 14 days [3]. Improvement in motor function was reported

in 27 out of 73 patients, and 11 out of 24 patients experienced an improvement in the consciousness level. There were no serious transplantation-related complications or adverse effects [3]. Younger and earlier-treated patients benefited more from the treatment. The field of stem cells is constantly growing and leading to new findings. The growing number of clinical trials has furthered the advancement of research in the treatment of patients with TBI utilizing stem cells.

Combination Therapy

Preclinical TBI therapeutic strategies have had limited translational success, prompting researchers to explore the combination of multiple therapeutic approaches. Several studies have previously shown the importance of combining multiple strategies in other CNS-related conditions and non-CNS conditions such as Crohn's disease and AIDS [12, 46, 162, 203]. These findings are particularly relevant for TBI studies because of the heterogeneity and complexity associated with brain insult and secondary injury sequelae [159]. Treatment strategies geared with the aim of targeting post-TBI symptoms rely upon the use of drugs showing beneficial effects, and those that may have synergistic effects when used in combination.

One example of potential synergistic effects is the utilization of *Lithium* and *valproate* in a CCI mouse model. Lithium monotherapy shows evidence of rescuing motor deficits and alleviating anxious behaviors following injury by reducing microglial activation and cyclooxygenase-2 induction [257]. Furthermore, cerebral edema was reduced by the downregulation of interleukin-1 β . This finding is translated behaviorally by enhanced learning in a Water Maze test [273]. The administration of *valproate* to rats with TBI enhanced both motor and cognitive function by improving BBB integrity [52].

When used in combination, sub-therapeutic doses of valproate and Lithium in a CCI mouse TBI model reduced the provoked lesion space and enhanced BBB integrity while attenuating hippocampus neuronal degeneration. Monotherapy did not replicate these protective effects [258]. The natural differentiation of transplanted human fetal NSCs, or human iPSC-derived NSCs among injured CNS may take upwards of a year [147–149, 200, 217]. Due to this lengthy time until effect, novel compounds [i.e., P7C3 class of aminopropyl carbazole and human multi-neurotrophins (MNTS1) show potential benefits to stimulate neurogenesis and aid both endogenous and exogenous NSCs into a neuronal replacement in both an efficient and timely manner [19, 20]. Valproic acid (VPA) treatment in combination with cell grafting stimulated axonal regeneration and synaptogenesis following TBI by increasing neurons induced by VPA treatment [143]. Furthermore, MSCs combined with low-intensity transcranial ultrasound (LITUS) have shown that it is more effective in treating TBI compared to MSCs alone. The combination therapy promoted neuronal proliferation and differentiation while reducing the inflammatory response and edema [256]. Combination therapy of both regulatory T cells and MSCs diminished microgliosis 14 days post-TBI in vivo in rats [31]. The following sections

present the findings associated with combining TBI treatment strategies with stem cell therapies.

Progesterone and Stem Cells

The use of progesterone in combination with other treatments, particularly stem cells, is promising in animal models. Progesterone induced significant neurobehavioral improvements in rats, assessed in water mazes, open field, and roto-rod tests [180]. Transplanted NSCs in combination with progesterone and an enriched environment led to enhanced neuronal migration, survival, and differentiation [180]. When progesterone is administered in combination with Endothelial Progenitor Cells (EPC) after TBI, it resulted in increased oxygenation, decreased edema, and the elimination of toxic molecules [160]. The use of progesterone has improved the restoration of BBB integrity and brain water content, and both the vessel density and expression of occludin resulted in enhanced neurological function following TBI. However, this was alleviated by progesterone thus emphasizing the importance of using progesterone with EPCs [260].

Statins and Stem Cells

Fenofibrate, a peroxisome proliferator-activated receptor α (PPAR- α) agonist, was found to reduce edema following TBI and stimulate neuroprotection and behavioral improvements [40]. Statins were first used in combination with fenofibrate resulting in increased neurological rescue and beam-walk performance and a parallel decrease in lesion volume and edema [41]. The promising findings of fenofibrate led to the use of atorvastatin in combination with BM stromal cells in a rat CCI model of TBI. This combination therapy significantly ameliorated the functional cognitive outcome and neurological severity score. Moreover, it increased the MSCs number in the brains of mice, subsequently leading to increased cellular proliferation, differentiation and vascular density in the hippocampus and the site of the injury [154].

Combining *Simvastatin* and MSCs significantly rescued behavior in all tested groups. The group with a low dose of *Simvastatin* and a high dose of MSCs had the best outcome and showed synergistic effects [155]. The same study reported significant proliferation of endogenous cells in all groups. Donor MSCs were detected in the lesion boundary zone and contralateral cortex. The use of Statins in combination with MSCs showed significantly more neuroprotection than either monotherapy used alone, particularly at the functional level [155].

Stimulating Factors and Stem Cells

Erythropoietin (EPO) induces the proliferation of red blood cells [16] in the treatment of anemia and is often studied as a stimulating factor in TBI. EPO shows antioxidative, neurotrophic, angiogenic, anti-inflammatory and anti-apoptotic properties in models involving strokes and seizures induced by kainite and TBI preclinical studies [26, 79]. Despite a lack of evidence in terms of mortality and neurological function rescue, [178], EPO was considered potentially useful as a combination therapy. When EPO was combined with *Simvastatin*, an additive effect was observed and reached the levels of sham controls in cognition as measured by the water maze test. Additionally, there was a significant and pronounced additive outcome in cellular proliferation and axonal integrity strengthening the validity of EPO as a combination therapy [35].

The combination treatment using EPO with CD34 MSCs showed a significant decrease in ischemic damage following TBI compared to monotherapy [232]. This effect was mediated by EPO activity and an increase in neurologic rescue measured by bidirectional inclined plane test in rats that underwent TBI following combination therapy [232].

Granulocyte-colony stimulating factor (G-CSF) when used with human umbilical cord blood cells (hUCBCs) has exhibited anti-inflammatory properties, increased neurogenesis, reduced the expression of amyloid proteins, and improved cognition and synaptogenesis following the occurrence of stroke or the onset of Alzheimer's Disease [205]. hUCBCs injected with G-CSF one-week post-TBI was the most potent at rescuing behavioral outcomes compared to monotherapy treatments [1]. This study showed that G-CSF combined with stem cell therapy may assist in the recovery of motor function and alleviating inflammation [1].

Growth Factors and Stem Cells

Growth factors (GFs) regulate key functions such as growth and differentiation. Thus, they are likely to be beneficial in the treatment of TBI. Several studies have demonstrated the beneficial effects of GFs, including the halting of oligodendroglia death and enhancing the differentiation of progenitor cells into oligodendrocytes [206]. VEGF treatment promotes angiogenesis and enhances neurological recovery, while inhibiting apoptosis in the cortex and basal ganglia glia [29, 265]. Insulin-like growth factor 1 (IGF-1) induced at the sites of acute TBI injury markedly decreased the expression of cell injury markers. Moreover, it improved the recovery of motor activity and can significantly improve nutritional and metabolic endpoints [91, 113, 138]. IGF-1 additionally promotes MSCs development, supporting the proliferation and differentiation into neuronal lineages [101] Transplantation associated with IGF-1 transduction improved spatial learning deficits. Moreover, it reduced astroglia activation and microglial/macrophage accumulation within a hippocampal mechanical injury in a mouse model [129].

Other studies have shown that nerve growth factors (NGFs) activate NGF-transduced NSCs and lead to improved cognitive and motor functions [190]. Modified NSCs that express BDNF demonstrated increased neuroprotective effectiveness and significant improvement in motor function through the enhancement of survival and proliferative properties [39]. When administered intravenously, prostaglandin F2 (PGF-2) and moderate hypothermia significantly improved motor function measured by the beam-walk test. This was not consistent with spatial learning tasks [254]. The combinatory treatment was found to be more neutral than additive, [254] but the absence of negative effects is promising for the combinatory treatment with GFs.

ROCK Inhibitors and Stem Cells

The Rho-Rock pathway blockade is a potential neuroprotective strategy in TBI, since its activation inhibits axonal growth in vivo [24, 131]. *Fasudil* (HA-1077) and *Y-27632* of the 4-aminopyridine, both ROCK inhibitors, have anti-inflammatory, anti-oxidative, anti-apoptic, and neuro-generative properties that exert anti-dementia effects [57, 100, 103, 128, 137, 233, 249]. *Fasudil* was found to stimulate neurite growth in C12.2 cells, [38] and could mobilize NSCs and migration to the site of the injury after a hypoxic injury [38, 58]. When used in combination with the priming of Wharton's jelly-derived MSCs, it resulted in the expression of neuronal markers in an in vitro stroke model and led to enhanced functional performance [135]. The use of *Y-27632* promoted cellular differentiation of stem cells into cortical and basal telencephalic progenitors. Moreover, it inhibited the anti-neurogenic and anti-neuro-differentiation of chondroitin sulfate proteoglycan found in glial scar tissue. This supported the notion that ROCK inhibitors may be promising as a combination therapy for post-TBI treatment [139, 243, 253].

Scaffolds, Transplantation Enhancers, and Stem Cell Therapy

Scaffolds and mildly invasive films in combination with stem cells have been used to ensure proper proliferation, differentiation, and migration of transplanted/ endogenous stem cells to aid in TBI recovery [214]. Fibronectin added to chitosan served as a link to FGF-2 and led to radial glial cell (RGC) expressing nestin three days after transplantation [214]. Fibroblast Growth Factor (bFGF) used with sodium hyaluronate collagen scaffold significantly induced differentiation of NSCs into functional neurons after two weeks [60]. When used in a rat model following TBI, the transplantation increased synaptic formation and function of the transplanted NSCs and showed significant rescue of learning and memory tested by the Morris Water Maze [60].

Hydrogels are polymeric networks containing a large amount of water that can recapitulate the native extracellular matrix. Synthetic polymers, much like their natural counterparts, can be used to create hydrogel scaffolds but with improvements in immune response [42]. In combination with drug delivery or stem cells, hydrogels may synergistically improve therapeutic treatment.

Nimodipine, [133] was used alone and in combination with umbilical cord-derived MSCs. In both situations the treatment rescued the decrease in body weight and both the locomotor and cognitive behavioral deficits [237]. The combination treatment led to a significant amelioration of the rescued phenotype. Moreover, mice that received the combination treatment had higher levels of Bcl-2 and lower p53 and Bax [237]. Another study showed that a cluster of microRNAs (miR-17-92) combined with NSC led to an improvement of motor coordination in a mouse model through the promotion of cell differentiation, increased regeneration and reduced astrogliosis [157, 158]. Preconditioning mouse neural progenitor cells (NPCs) to activate survival pathways attenuated neuropsychiatric defects and suppressed the inflammatory cascades following transplantation in both transplanted cells and the host tissue [244].

Preconditioning with nanoparticles delivering short hairpin RNAs that silenced proinflammatory chemokine CCL20 and chemokine receptor CCR6 prior to transplantation was shown to increase hMSC efficacy [163]. Other studies have investigated the means of enhancing the migration and homing of stem cells following TBI treatment. The CXCL12/CXCR4 axis led to the enhanced migration of neuroblasts after the administration of exogenous CXCL12 by increasing the expression of matrix metalloproteinases MMP-2 [157, 158]. Alternatively, treatment with SDF-1-loaded nanoparticles led to increased NSCs at the lesion site post-TBI [263].

Human neural stem/progenitor cells (hNS/PCs) were assessed in combination with curcumin-loaded noisome nanoparticles (CM-NPs). The treatment led to significant improvement in locomotor activity and a reduction in edema following a TBI in rats [173]. This indicates the potential benefit of CM-NPs when used with stem cells post-TBI.

Combinatory Treatments with Stem Cells and the Need for Optimization

A strong rationale for the use of combination therapies is brought upon by the complexity of the TBI insult. Moreover, the success of this approach for other medical conditions presents another driving force for exploring combination therapies in TBI.

MSC therapy as a stand-alone or combination treatment creates a nearly limitless set of research opportunities. As such, MSC therapy represents a fertile area for future research. It is likely that many cell-based innovations and translational applications will continue to manifest in the coming years.

Routes of Administration

The route of administration of stem cell therapy is heavily dependent on two factors: effectiveness and minimal invasiveness. The most relevant ones are the intravenous, intra-arterial, and direct routes. The intravenous route is a minimally invasive procedure. However, it has been shown that a very modest percentage (1.48–3.69%) of infused MSCs reach arterial circulation. A lower amount of the infused cells (0.0005%) makes its way to and remains at the site of injury in the brain.

Intra-arterial injection is also a minimally invasive approach that is performed by injecting the cells directly into the internal carotid artery ipsilateral to the side of the ischemic lesion. Although previous data have linked this route to an increased risk of damage caused by decreased cerebral blood flow (CBF) and inflammation, new data have shown that intra-arterial injection of NSCs using a micro-needle technique does not cause micro embolic strokes.

Finally, direct stem cell implantation, though invasive, is promising and maximizes the number of stem cells at the site of injury. Studies have shown that MSCs topically applied to the brain surface can migrate to a TBI site in a rat model with a higher proliferation rate as compared to intravenous administration. Moreover, transplanting NSCs into a nearby uninjured site rather than directly at the site of injury seems to provide a much better opportunity for the transplanted stem cells to proliferate away from the inflammation at the TBI site. In summary, each of the presented routes has its advantages and disadvantages, and the best route of administration is not yet evident. Future comparative trials and studies regarding administration would provide more information on the route of administration with the fewest side effects and highest efficiency.

Conclusion

TBI is a major health concern with heterogeneous underlying pathologic events and secondary sequelae. Currently, FDA-approved drugs for TBI treatment are still lacking due to limited efficacy or the presence of adverse events in clinical assessments. In several neurological and neurodegenerative disorders, stem cells are considered as a treatment option. This prompted their investigation in several pre-clinical studies as a therapeutic option for TBI. In this chapter, we discussed the results of these studies and the advancements achieved by transplanting stem cells in different TBI in-vivo models. This approach resulted in various neuroprotective effects that encompassed the amelioration of inflammatory markers, as well as motor and cognitive functions. However, translating pre-clinical results into clinical assessments is not always successful. Accurate dosage determination and the choice of a proper stem cell type are key determinants of outcomes post-TBI. Moreover, two key obstacles still affect the progression of stem cell therapy, namely the determination of

the optimal transplantation time and the transplantation route. In all cases, clinical studies utilizing stem cells as a treatment option for TBI are still scarce and highly needed. Future trials should have a robust design and should consider the findings from previous in-vivo and clinical studies. This is not an easy task considering the heterogeneity of animal models utilized so far. Moreover, different studies utilize different types and severities of injury, and the treatment options are highly variable. One approach that is highly promising is the assessment of combination therapy. Stem cell transplantation can be co-administered with other interventions such as neuroprotective drugs and transplantation enhancers. Finally, research is also needed to achieve a better understanding of the underlying regenerative properties of stem cells, particularly their capacity for long-term survival. This may allow future studies to manipulate the factors that improve the proliferation and maintenance of transplanted stem cells in the TBI brain (Table 3.1).

Table 3.1 Bioactive biomaterials in the Central Nervous System

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Scaffold and cellular based hydrogels	Growth factors BDNF, bFGF, VEGF	In vitro studies	Neurodegenerative diseases	Signal cellular pathways	Not available yet. Clinical long-term results	[45]
BioScaffold and stem cells in trauma	Mesenchymal stem cells	Animal models	Spinal cord and brain trauma	Pathophysiology of injury repair	Viability to clinical strategies. Risk of tumourigenesis, ethical issues, immunogenicity	[123]
Bioactive scaffold membranes	Regenerative activity vs tissue repair	Mouse model	Vascular brain ictus. Angiogenic factors	Advances in neuroregeneration in stroke	Degradability of the scaffold	[168]
Bioscaffold and neural stem cells	Neurogenesis process through growth factors in stem cells niches	Mouse and in vitro models	Growth factors in vivo. Brain injury	Active repair and neurogenesis and differentiation promotion	Long term effect. Not yet available in humans	(Zhen et al. 2022)
Scaffolds evolution	Stem Cells, gene therapy and growth factors	In vitro and mouse models	Oriented to neurorepair and neurogenesis. Brain injury	Diversity of choices in neurorepair	The level of functional response	[262]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Mesenchymal stem cell-derived exosomes	Neurogenesis and cognitive function recovery	Mouse model	Alzheimer's disease	Production of neurotrophins Neuroregeneration and functional recovery	No clinical studies yet to quantify the efficacy of this approach	[197]
IKVAV-PA, VKIVA-PA self-assembled nanofibres	Neural transdifferentiation of human BMSCs	Human BMSCs culture on IKVAV-PA and VKIVA-PA gels and laminin PDL-coated surfaces	Traumatic injuries in the central nervous system	<ul style="list-style-type: none"> Induction of neuroectodermal lineage commitment Self-assemble into supramolecular nanofibres 	Significant decrease in viability without spreading on the VKIVA-PA gel No clinical studies yet to quantify the efficacy of this approach	Ji et al. (2018)
Fibrous scaffolds and hydrogel matrices	Promote cell survive	In vivo 3D microenvironments	Ischemic stroke	-Emulation of ECMs	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[225]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
NT-3/fibroin complex	Decreased IBA-1 positive cells	Canine spinal cord	SCI	<ul style="list-style-type: none"> Inhibits inflammation Enhances nerve fibre regeneration Improves motor function 	<ul style="list-style-type: none"> No clinical studies yet to determine the optimal concentration of NT-3 used for humans 	[136]
Injectable hydrogels in combination with stem cells	Stem cell adhesion, proliferation	In situ	Ischemic stroke	Site-specific stem cell biomolecule delivery	Lack of control, nonuniform cell distribution and low cell penetration capacity	[81]
Chitlac-THICK	Neural network reconstruction	Primary cultures of hippocampal neurons from rat pups	CNS injured systems	<ul style="list-style-type: none"> Promote neuronal growth, differentiation, maturation and formation of synapses 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[165]
PCL/iPSC-NSCs	Differentiation into neural stem cells	Rat spinal cords	SCI regeneration Cell transplantation	<ul style="list-style-type: none"> Could promote motor function 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[272]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Fungal metabolites	-Scaffolds for the design and synthesis of new multi-target ligands	-In vitro	Alzheimer's Disease	-Antioxidant and anti-amyloidogenic activity	<ul style="list-style-type: none"> No clinical or animal models studies yet to quantify the efficacy of this approach 	[191]
P(TMC-CL)/Ibuprofen	<ul style="list-style-type: none"> Decrease on RhoA activation Favors CD163 + cell recruitment Increases βIII-tubulin and GFAP positive cells 	Dorsal hemi section rat SCI sub-cultured on ND7/23 cells	SCI	<ul style="list-style-type: none"> Contributes to resolution of the inflammatory process Supports axonal growth (βIII-tubulin cells) in the lesion periphery and cellular infiltration 	<ul style="list-style-type: none"> The folding of an electrospun fibrous fabric impaired nerve regeneration 	[195]
Cell interaction with extracellular matrix IKVAV peptide	Neuronal differentiation	In vivo	Traumatic brain injury	Promote axonal regeneration	Not effect on astrocyte proliferation	[176]
Tenascin-C mimetic peptide amphiphile nanofibre gel	Neurite outgrowth	P19 embryonal carcinoma cells	-Spinal cord injuries -Repopulation of dysfunctional areas of the brain	-Self assembled nanofibre -Transition from a solution to gel upon exposure to physiological divalent cations	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[17]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Injectable non-toxic silanized-hydroxypropyl methylcellulose hydrogel	Neuronal differentiation of grafted cells	3D environment	Neuronal damage	Secretion of tissue repair factors of stem cells	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[111]
(RADA)4-KVAV-CPI-MVG	Neurite outgrowth	In vitro PC-12 cells	Neuroinflammatory and neurodegenerative conditions	<ul style="list-style-type: none"> Neuroprotection Neurogenesis 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[127]
3D multichannel/LN silk fibroin	Stimulates growth, development, and the extension of primary hippocampal neurons	Culture of primary hippocampal neurons in vitro, to then implant them in a SCI of rats	SCI regeneration	<ul style="list-style-type: none"> Promotes axonal extension Mediates cell migration Neuronal adhesion promotion Stimulates blood capillary formation 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[266]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
(RADA)4-GG-BMHP1	<ul style="list-style-type: none"> • Axon regeneration and lamination • Less reactive astrocytes (GFAP expression) and gliosis • Motor neuron recovery 	hEnSCs implanted into rats	Chronic SCI	<ul style="list-style-type: none"> • Improvement in motor function • Neural differentiation • Higher myelination 	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	[226]
poly(ethylene glycol) hydrogel with Platelet-derived growth factor-AA	Differentiation of neural stem/progenitor cells (NSPCs)	Neural stem/progenitor cells culture in foetal bovine serum	Spinal cord injury	NSPC oligodendrocyte differentiation	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	Elliot et al. (2015)
RADA 16-FGL	Promoted SC-NSC proliferation and migration into the 3-D scaffold	SC-NSCs from neonatal rats	SCI regeneration Tissue engineering	<ul style="list-style-type: none"> • Self-assemble into nanofibrous structure and support SC-NSC adhesion and survival 	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	[238]
Radially aligned electrospun PLA70/30 fibres release L-lactate	3D organization and supportive function of radial glia embryonic neural stem cells	Radial scaffolds implanted into cavities made in the postnatal mouse brain	Traumatic injuries in the central nervous system	<ul style="list-style-type: none"> • Reactivation of embryonic neurogenic and angiogenic programs • Maintenance of pools of neuronal and glial progenitor cells 	<ul style="list-style-type: none"> • Specific scaffolding topology • Risk of induction of neuroglial tumours 	[5]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Human mesenchymal stem cells	Neuronal differentiation	In vitro	Brain damage	Produce neuronal progenitor and mature neural cell markers	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[183]
Hydrogel	Neuronal differentiations	In vitro	Injury environment and scarring	Greater axonal infiltration, astrocyte migration and myelinated axon growth	Determine the most relevant biological features, and size scale and determine how to create similar architectures within biomaterials	[116]
Transplantation of stem cell in the urinary bladder matrix	Cell differentiation	In rats	Traumatic brain injury	Reduce neuron tissue loss Repair damaged neural network	Previous studies have shown a significant neuronal loss	[240]
NT-3/Chitosan	<ul style="list-style-type: none"> Axon regeneration 	Spinal cord of rats	SCI regeneration	<ul style="list-style-type: none"> Restoration of locomotor function 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[240]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
Electrospun PCL-Spirulina nanofibre mat	Improves the growth and metabolic activity of primary astrocytes	Rat primary astrocytes exposed to Spirulina extract	CNS injured systems	<ul style="list-style-type: none"> Alleviated astrocyte metabolism without cytotoxicity Could reduce inflammation induced by gliosis 	<ul style="list-style-type: none"> Spirulina extract is rarely released from the PCL-Spirulina mat to the media within 24 h 	[119]
Extracellular matrix	Neuronal differentiation as well as neuronal outgrowth	PC-12 cells	Injury of the CNS	Rich in axonal growth inducer proteins	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[156]
Nerve Growth Factor Coated -poly(2-hydroxyethyl methacrylate) Hydrogel-	Enhancing of neuron survival and sprouting	Dorsal root ganglion cultures	Interfacing SNC electronic devices	-Controlled local neurotrophin delivery	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[109]
3D aragonite coralline matrices	Neuronal tissue restoration	Hippocampal (dentate gyrus) neurons and astrocytes of rat pups	Neuronal tissue development in 3D	<ul style="list-style-type: none"> Proliferation of neuronal, astrocytes and other glial cells Support mesenchymal stem cells differentiation into osteoblasts 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[188]

(continued)

Table 3.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Authors
NT-3 from fibrin-HBDS scaffolds	<ul style="list-style-type: none"> Enhances the sprouting of CGRP, ChAT, 5-HT of the raphe spinal tract 	Spinal cord of adult rats	SCI regeneration	<ul style="list-style-type: none"> Neuronal fibre sprouting Reduction of glial scar formation 	<ul style="list-style-type: none"> Motor function does not improve 	[227]
IKVAV-PA	Promote neurite sprouting and to direct neurite growth	Murine neural progenitor cells	Neuroinflammatory and neurodegenerative conditions	-Rapid differentiation into neurons	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	[211]
Hydrogels Containing Peptide or Amino sugar Sequences	Promotion of cellular adhesion and proliferation	Rat brain and spinal cord	Injury of the CNS	-Promotion of cellular infiltration and angiogenesis	-The astrocytes could express factors that inhibits the axonal growth	[192]

Compliance with Ethical Standards

Disclosure of Interests All authors declare they have no conflict of interest.

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Chapter 4

Neurotrophic Factors in Parkinson's Disease: Clinical Trials



Arun Kumar Mahato and Mart Saarma

Abstract *Introduction:* Parkinson's disease (PD) is characterized by the progressive loss of midbrain dopamine (DA) neurons that regulate voluntary movement and cognitive functions. Despite notable progress in the development of symptomatic therapy, the development of disease-modifying therapy for PD still holds big challenges. Neurotrophic factors (NTFs) are considered as potential regenerative therapy for PD because they can protect DA neurons and promote neuron survival both in vitro and in vivo. Although preclinical studies with the neurotrophic factors have proved to be efficient in animal PD models with mild lesions, the effect of NTFs on PD patients in clinical trials has been modest. In this review, we discuss the current state of NTFs therapies on PD treatment from preclinical and clinical studies, challenges with growth factors (GFs) therapy, and alternative approaches for further development of disease-modifying therapies. *Methods:* We formulated the questions and based on that, literature search was carried out using the PUBMED database. Further, we extracted the data, analyzed and summarized the findings. *Results:* GDNF family ligands GDNF and neurturin and CDNF/MANF neurotrophic factors are extensively studied in preclinical and clinical studies. In preclinical studies, these NTFs have demonstrated neurotrophic activities on DA neurons and have potential disease modifying properties in animal models of PD. However, the effect of NTFs in PD patients has been modest. Importantly, the results from clinical studies show beneficial effects of NTFs including improvements in motor symptoms and quality of life. *Conclusion:* NTFs still remains a potential candidate in the treatment of PD. However, development of GDNF based treatment has many difficulties related to effective delivery, poor diffusion and optimum dose. Regardless of the challenges, efforts to develop neurotrophic factors-based therapies should be continued towards clinical application in PD.

Keywords Parkinson's disease · Growth factors · Neurotrophic factors · Dopamine neurons · Glial cell line-derived neurotrophic factor family ligands · Cerebral dopamine neurotrophic factor

A. K. Mahato · M. Saarma (✉)
Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki, Finland
e-mail: mart.saarma@helsinki.fi

Introduction

PD is globally the second most common neurodegenerative disease, and its prevalence has been projected to double over the next 30 years. The global prevalence of PD is estimated to be 0.3% overall, more frequent in elderly people [118]. It is a progressive neurodegenerative disease which is characterized by the loss of DA neurons in the substantia nigra pars compacta (SNpc) [29, 48]. The pathological changes due to the degeneration and loss of neurons lead to clinical motor symptoms such as bradykinesia combined with resting tremor, rigidity, or both. PD is not limited to the brain's DA system. In PD, other systems affected are the olfactory system, enteric system, cholinergic system, serotonergic system, and noradrenergic system, which might lead to the development of non-motor symptoms [115, 121]. The clinical presentation of PD encompasses a wide range of non-motor symptoms such as constipation, sleep disorder, depression, pain and cognitive decline [15]. Although most non-motor symptoms are thought to develop in the prodromal stage of the disease, their impact is considerably more significant in the later years [130].

PD is a multifaceted disease which is caused by a combination of different factors such as complex genetics, environment, and aging. The histopathological hallmark of PD is the presence of intraneuronal inclusions called Lewy bodies (LB) in DA neurons. LB consists of cellular proteins such as α -synuclein, damage products of various organelles and vesicles [132, 133]. However, the contribution of LB in the initiation and progression of neurodegeneration in PD remains unclear. PD is diagnosed based on clinical motor symptoms, it is the time when a significant number of DA neurons are already degenerated, and the lack of biomarkers hampers the detection of the early stages of the disease. Recent findings show that α -synuclein seed amplification assays (SAAs) could be the potential tools for identifying high-risk individuals at the earliest phases of the disease [134]. This will help in the development of new disease-modifying therapies for PD.

Treatment of PD has been carried out using combinational approaches such as medications, supportive treatment (e.g., diet, exercise, physical therapy, occupational therapy, and speech therapy), and surgical interventions. Current conventional medications are levodopa, catechol-O-methyltransferase (COMT) inhibitors, monoamine oxidase-B (MAO-B) inhibitors, and DA agonists. Levodopa is the first-line drug for managing PD motor symptoms, often combined with MAO-B and COMT inhibitors. It is effective in treating bradykinesia and rigidity. Deep brain stimulation (DBS), a surgical procedure, is often considered in the late-stage patient who are resistant to medical therapies and have significant medication side effects [32]. The current treatment only relieves the symptoms but does not change the course of disease progression. More importantly, the available drugs cannot slow down or stop neurodegeneration and control non-motor symptoms such as constipation, sleep disturbance, anxiety, depression, sexual dysfunction, cognitive dysfunction, and problems in olfaction. Therefore, new disease-modifying approaches are being tested to stop neurodegeneration and disease progression and cure non-motor symptoms. In this

regard, regenerative proteins and gene therapies (described later in the text) or cell-based approaches may be valuable tools to restore or repair the affected DA neurons. Cell-based therapies aim to replace the degenerated DA neurons and restore the DA tone to treat PD. Clinical trials with DA neurons (mesDA) obtained from human fetuses have been conducted in PD patients. The cells were directly transplanted into the striatum of patients. The grafted cells survived and improved the symptoms in a subset of PD patients. However, fetal cells for replacement therapy faced ethical issues, cell availability, and significant side effects such as graft-induced dyskinesia [91]. Alternatively, human embryonic stem (ES) and induced pluripotent stem (iPS) cells are being explored as the source of DA neurons for treating PD. The cells derived from ES and iPS have been tested in animal models of PD and have survived in transplanted grafts. Further, the results obtained from the animal models showed improved motor asymmetry and transplanted DA neurons innervation to the host striatum [53, 54, 56, 68]. The promising results from preclinical studies led to the initiation of several clinical studies with cell transplantation with the hope of developing safe and restorative cell therapy for PD [140].

α -synuclein, one of the critical components of Lewy bodies, is considered a prion-like protein and is thought to be involved in the pathogenesis of PD. Mutations (A30P, E46K and A53T) in α -synuclein gene can cause a familial form of PD accompanied by neuronal cell death due to mitochondrial impairment, lysosomal dysfunction, and alteration of calcium homeostasis. However, the role of α -synuclein in triggering the pathology remains to be explored. To limit its prion-like activities, α -synuclein is considered to be one of the therapeutic targets for treating PD. In this regard, therapeutic interventions should target the point of post-translational modifications, misfolding and aggregation of α -synuclein. Several treatment strategies such as to sequester, degrade or lower α -synuclein expression in specific brain region(s) are being investigated at pre-clinical and early clinical stages to target α -synuclein and mitigate its neurotoxic effects [101].

Another approach for developing disease-modifying therapy is using GFs because they can promote the regeneration and survival of degenerating DA neurons and protect them from injury and toxins. In the rest of this book chapter, we will discuss both pre-clinical and clinical aspects of different GFs, their limitations and their alternative approach in the context of PD treatment.

Neurotrophic Factors

GFs have been used in animal models of PD and tested in clinical trials [98, 135]. Why have GFs been tested in clinical trials? There are several reasons, and among them, perhaps the first, most important is the unique property of certain GFs to keep DA neurons alive and protect them from injury and lesions in vitro and in animal models of PD. It should be noted in this context that many proteins and GFs affect DA neurons, but only a few GFs support the survival of DA neurons. Glial cell line-derived neurotrophic factor (GDNF) family ligands GDNF and neurturin (NRTN) [98,

135], and cerebral dopamine neurotrophic factor (CDNF)-mesencephalic astrocyte-derived neurotrophic factor (MANF) family ligands CDNF and MANF [65, 88] have neurorestorative potency in PD models where neuroprotective effects were obtained after the lesion, thus recapitulating the situation in PD patients. Also, platelet-derived growth factor B (PDGF-B) has shown potency to protect DA neurons both in vitro and in vivo [105, 161], but clinical trials on this factor have been terminated due to lack of efficacy in PD patients [113], therefore, we do not discuss these data. The second reason is that there is also strong evidence that GDNF and NRTN, but also CDNF and MANF, can induce neurite outgrowth and branching of DA neurons, indicating that these GFs have neuroregenerative activity. Thirdly, GDNF and NRTN can increase the functional activity of DA neurons inducing tyrosine hydroxylase (TH) levels, DA transporter (DAT) activity and regulating DA uptake and release. CDNF and MANF also have effects on DA neuron functional activity. Differently from GDNF family ligands, they can reduce neuroinflammation by decreasing the synthesis and release of pro-inflammatory cytokines. Finally, genetic studies have shown GDNF and NRTN, and CDNF and MANF knockout mice show neurodegeneration-related phenotypes. GDNF conditional knockout mice have a dramatic loss of nigral DA neurons in one genetic background [112] but severe functional defects in another [73]. GDNF family ligand receptor RET conditional knockout mice show an age-dependent loss of nigral DA neurons [78]. MANF knockout mice exhibit strong endoplasmic reticulum (ER) stress in DA neurons and increased vulnerability to toxins [106, 107]. CDNF knockout mice show age-dependent deficits in the function of the midbrain DA system. Furthermore, an age-dependent loss of enteric neurons occurring selectively in the submucosal plexus results in a defect in the function of the gastrointestinal tract. The deficiencies of *Cdnf*^{-/-} mice recapitulate some symptoms seen in the initial stages of PD patients [85].

When listing the beneficial properties of the GFs, a question arises: what are the limitations of using GFs as drugs to treat PD? One of the main limitations is that they do not penetrate through the blood–brain barrier (BBB), so their delivery requires expensive and risky brain surgery. Because of that, only later-stage patients with small amounts of DA neurons left can be treated. GFs also have limited diffusion in the brain tissue and generally poor pharmacokinetic properties. When injected into the brain GFs cannot treat most of the non-motor symptoms of PD, and finally, producing, storing and transporting GFs is expensive. One can overcome all these limitations using new technologies.

GDNF Family Ligands (GFLs), Their Receptor Systems, and Effects on Cells

GDNF is a protein discovered in the early 1990s which is essential for the growth, survival and maintenance of both central and peripheral nervous systems [12, 84]. Since the discovery of GDNF, several other members have been discovered, such

as NRTN (Fig. 4.1a), artemin (ARTN), and persephin (PSPN). GFLs are the distant members of the transforming growth factor β (TGF β) family, which signals to the receptor tyrosine kinase RET receptors [79]. However, GFLs cannot directly bind to the RET receptor but requires GDNF family-like receptor α (GFR α) to function. GFLs have their respective co-receptors. GDNF prefers GFR α 1 but can also function via GFR α 2. NRTN, ARTN and PSPN can bind and function via GFR α 2, GFR α 3, and GFR α 4, respectively [1]. NRTN can signal to RET also via GFR α 1. GFRAL is a distant member of GFR α s and can exclusively bind to GDF15, a protein which is a distant member of GFL and also functions via RET regulating appetite [61, 83].

GFR α s attach to the cell surface by a glycosylphosphatidylinositol (GPI) anchor and are located in the lipid rafts, whereas RET that is outside the rafts signals differently. GFLs, together with GFR α s, bring RET to the lipid rafts and form a complex with RET, which then stimulates RET dimerization and further activates its intracellular tyrosine (Tyr) kinase domain. The phosphorylation of Tyr residues serves as the docking sites for several adapter or effector proteins necessary for the activation of downstream pathways such as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), Src kinase and phospholipase C- γ (PLC γ) pathway. The phosphorylation of RET at Tyr 981 serves as the docking site for Src, activating the Src pathway necessary for cell survival and neurite outgrowth. The phosphorylation of Tyr 1062 and Tyr 1096, mainly present in the long isoform of RET, is critical for most of the RET functions. The phosphorylation of Tyr 1062 can activate both the Ras/MAP kinase and PI3K/AKT pathways (Fig. 4.1c).

Similarly, the phosphorylation of Tyr 1096 can also activate PI3K/AKT pathway [1, 96, 97, 135]. Tyr 1015 seems to activate PLC γ pathway mainly in the oncogenic forms of RET [17]. However, data demonstrating the activation of the PLC γ pathway by GDNF is not available. Furthermore, RET can also be activated both in *trans* and *cis* by the complex of soluble GFR α 1 and GDNF. It is believed that the complex of soluble GFR α 1-GDNF interacts with RET, subsequently activating RET outside the lipid rafts, followed by the translocation of the complex to the rafts. The translocation of the activated complex both inside and outside the rafts allows the activation of different signaling pathways [109, 142]. Nigral infusion of soluble GFR α 1 potentiates DAergic neuron function in aged rats indicating that GFR α 1-GDNF trans signaling may also have therapeutic effects [120]. GFLs also function through other receptors, such as neuronal cell adhesion molecule (NCAM) and syndecan-3 [13, 110].

The cell biology of GDNF and its receptors in the context of DA neurons is modestly studied. Understanding the cell biology of GDNF is crucial for developing potential therapeutic interventions for neurodegenerative disorders. GFLs are synthesized as long precursor proteins, which are further processed and secreted as mature proteins. Human GDNF consists of six exons, and alternative splicing of the third exon results in the deletion of 78 bp, producing two different splice forms: full-length pre- α -pro-GDNF (α -GDNF) and the shorter pre- β -pro-GDNF (β -GDNF). The processing of these isoforms to mature GDNF is unaffected by the pro-region difference. However, it is thought that pro-region might play a role in the

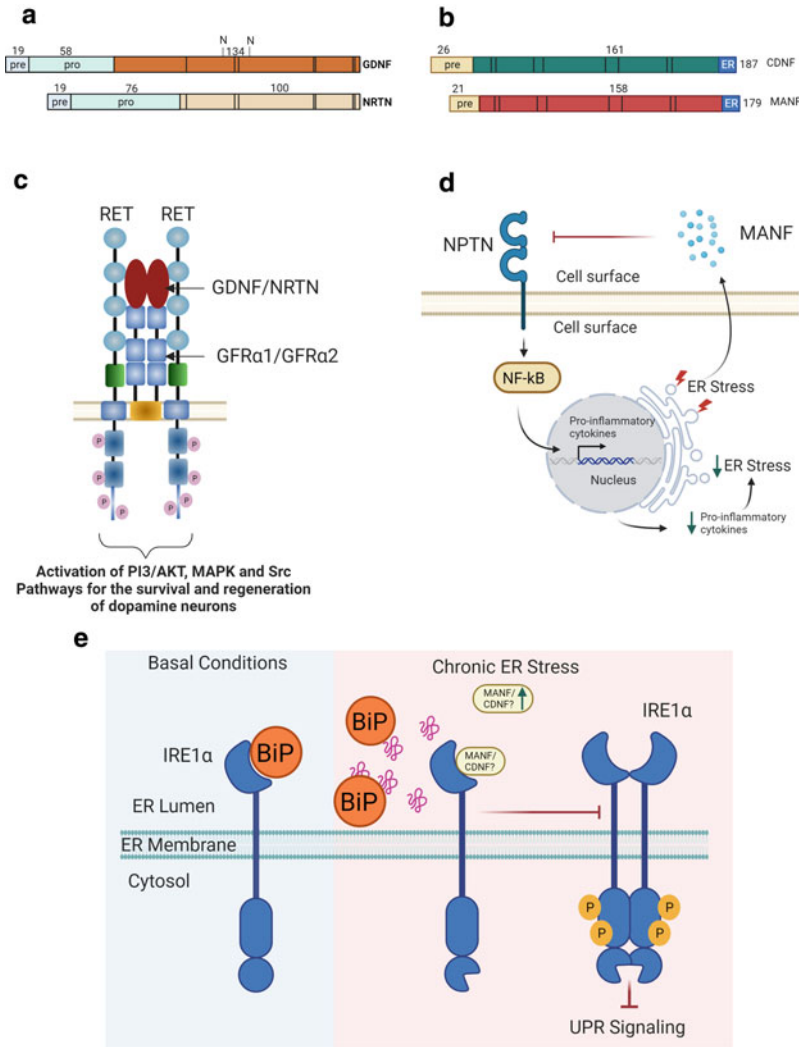


Fig. 4.1 Structure of GFs and their signalling receptors. **a** primary structure of GDNF and NRTN encodes signal peptides (light blue), followed by a pro-region (light green) cleaved to form a highly conserved mature domain containing seven conserved cysteines. **b** primary structure of CDNF and MANF containing signal sequence (yellow) targeting endoplasmic reticulum (ER). MANF/CDNF contains eight conserved cysteine residues followed by ER retention signal (blue) in the C-terminus. **c** GDNF Family ligands (GFLs) signal via receptor tyrosine kinase RET. Homodimeric GDNF or NRTN binds to GPI-anchored coreceptors GFRα1/2 and activates RET receptors and various downstream signalling pathways essential for neuronal survival, regeneration, and neuronal differentiation. **d** neuroplastin (NPTN) is a cell surface receptor for MANF. The binding of MANF that is secreted in ER stress to NPTN suppresses NF-κB signalling, reducing the production of pro-inflammatory cytokines and protecting cells against ER-stress-induced inflammation and cell death. **e** IRE1α ER receptor for MANF. At the basal conditions, BiP is bound to IRE1α and prevents MANF from interaction with IRE1α. However, upon chronic ER stress, BiP dissociates, and MANF binds to IRE1α and decreases ER stress-induced oligomerization and phosphorylation of IRE1α, leading to attenuation of UPR signalling

secretion of GDNF. In the PC-6.3 cell line, it has been shown that different mechanisms regulate the secretion of these isoforms. α -GDNF is secreted constitutively, while β -GDNF is secreted activity-dependently [94]. GDNF is expressed in the brain mainly by neurons and is secreted in a regulated manner. This means that GDNF secretion is under strict neuronal activity control. We will reach an unphysiologically high concentration when overexpressing GDNF by gene therapy. Gene therapy using β -GDNF can achieve physiological pulsatile concentration of GDNF due to activity-dependent secretion.

GDNF is a glycosylated protein, and the glycan moiety is essential for its processing and stability [116]. GDNF, upon binding to its receptor complex GFR α 1 and RET, is internalized [51, 149] and retrogradely transported from the axons to the cell bodies in DA neurons and motor neurons [162]. The internalization of the complex is thought to be mediated by the cell surface sorting receptor called SorLA, leading to the degradation of GDNF in the lysosome or further recycling of the receptors to the cell surface [27, 51, 64, 147]. Interestingly, in the absence of GDNF, SorLA/GFR α 1 complex also targets RET for endocytosis. However, after the endocytosis, RET is not targeted to the lysosome for degradation, affecting the subcellular distribution of RET receptors and GDNF-induced neurotrophic activities [51]. Besides, the binding of GDNF to heparan sulfate proteoglycans limits its biodistribution and availability [13], highlighting that the bioavailability of secreted GDNF is tightly regulated to ensure its proper delivery to target tissues and neurons.

GDNF and NRTN are expressed in neurons and non-neuronal tissues, suggesting that they have a role in the development and maintenance of various organs. In the central nervous system, both proteins are detected in the striatum, substantia nigra, hippocampus and cerebellum [79]. During embryonic development, very high levels of GDNF can be detected in the kidney and gastrointestinal tract. However, this developmental expression of GDNF is transient. The serum level of GDNF is around 400–500 pg/ml in healthy humans [145].

The global deletion of GDNF in mice caused substantial loss of motor neurons, dorsal root ganglion sensory neurons, and sympathetic and parasympathetic neurons. GDNF-deficient mice also lack kidneys, ureters, and enteric nervous system. However, no changes were observed in hindbrain noradrenergic or midbrain DA neurons, indicating no critical role of GDNF in the early development of these neuronal populations [102, 117]. The importance of GDNF in adult DA neurons was studied by the generation of conditional knockout mice by deleting the GDNF gene in the DA system. The outcomes from these studies were controversial, GDNF is either required for the survival of catecholaminergic neurons or not [73, 112]. Recently, new GDNF conditional knockout mice were created where GDNF deletion was obtained by crossing *Gdnf* floxed mice with UBC-Cre-ERT2 mice. The GDNF deletion resulted in an 80% reduction of both GDNF encoding mRNA and protein in the striatum and septum with significant loss of catecholamine neurons in the SN and locus coeruleus, suggesting that GDNF is essential for the maintenance of adult catecholamine neurons [36]. Despite controversies, GDNF clearly is required for the proper function of adult DA neurons and for maintaining DA homeostasis [73, 74]. Similarly to GDNF full KO mice, no apparent changes were observed in

the CNS of GFR α 1 [35, 144] and RET full knockout mice [131]. NRTN-deficient mice also show no defects in the DA system, suggesting its role in maintaining the adult neuronal population. However, NRTN- and its co-receptor GFR α 2 deleted mice have defects in the enteric nervous system and sensory and parasympathetic neurons [59, 124].

GDNF and NRTN support the survival and protect the cultured DA neurons from neurotoxin-induced cell death [79, 125, 126], presumably reducing the apoptosis of these cells [23, 24]. GDNF can also influence DA synthesis, metabolism and DA uptake in cultured DA neurons [84]. The exposure of GDNF increased DA release and promoted the establishment of new functional synaptic terminals in cultured DA neurons [18, 119]. Recently, GDNF was found to prevent α -synuclein aggregate formation in the DA neurons [21]. Interestingly, GDNF can also protect stem cell-derived human DA neurons from 6-OHDA-induced cell death and elicit their neurite outgrowth [160].

CDNF-MANF, Their Receptors and Effects on Cells

MANF protein was purified and characterized by the group of Dr. Commissiong. They demonstrated that MANF is a new neurotrophic factor that explicitly promotes the survival of DA neurons [114]. CDFN was biochemically characterized a few years later and exhibited 59% amino acid sequence homology with the MANF protein [90]. Since MANF and CDFN carry the N-terminal hydrophobic signal sequence, they were initially considered typical secretory proteins. Mature MANF is 158 and CDFN 161 amino acids long, respectively (Fig. 4.1b). Both proteins have the N-terminal signal sequence that takes them to the ER and the secretory route. However, since CDFN and MANF have C-terminal four amino acids long KDEL-like ER retention signal, they are retrieved back from the Golgi to the ER. Although most MANF and CDFN proteins are located inside the cells in the ER lumen, current data clearly show that in the case of ER stress, both proteins are also secreted into the extracellular space. Vertebrates have CDFN and MANF, whereas invertebrates such as fly *Drosophila melanogaster* and nematode *Caenorhabditis elegans* have only one member of the family with the slightly more homologous amino acid sequence to mammalian MANF [108].

The three-dimensional structure of MANF and CDFN was first analyzed by X-ray crystallography, revealing that these proteins consist of two domains [111]. In the first crystallographic analysis, the C-terminal domain structure of MANF was not deciphered, but it was later solved by solution NMR analysis [58]. Initially, only the crystal structure of the N-terminal domain of CDFN was reported [111], and then the whole structure was determined by NMR [82]. Based on the nucleotide and amino acids sequence, no genes and proteins were found to be homologous to CDFN and MANF. However, the three-dimensional structures of mature CDFN and MANF proteins revealed structural homology with several interesting proteins. CDFN and

MANF amino-terminal (N-terminal) domain is a saposin-like domain, and a carboxy-terminal (C-terminal) domain is like SAF-A/B, Acinus and PIAS (SAP) proteins. In line with the property of saposin-like proteins that bind to lipids or membranes, it was postulated that the N-terminal domain mediates the CDNF/MANF interaction with lipids [89, 111]. MANF, but not CDNF, was recently shown to bind sulfoglycolipid 3-O-sulfogalactosylceramide (sulfatide [74] directly. GDNF family members have seven conserved cysteines, and biologically active GDNF family members form homodimers. In contrast, CDNF and MANF have eight conserved cysteine residues with identical spacing, and both proteins are monomers. In addition, GDNF is a glycoprotein, whereas CDNF and MANF are not glycosylated. Interestingly, CDNF and MANF have a conserved CXXC motif in the C-terminal SAP-domain, and a disulphide bridge can be formed in this sequence. The saposin-fold of the N-terminal domain harbors three intramolecular disulphide bonds that stabilizes the structure of CDNF and MANF [58, 82].

MANF expression is ubiquitous, and its tissue levels are, on average, 100 times higher than that of CDNF. Exceptionally high MANF levels can be detected in the secretory organs, reaching 0.87 $\mu\text{g}/\text{mg}$ of total protein in the pancreas and 1.17 $\mu\text{g}/\text{mg}$ of total protein in the testis. The highest levels of CDNF can be detected in the muscle (19.2 ng/mg protein) and in the testis (10.8 ng/mg of total protein. In the brain, MANF protein concentration was around 100 ng/mg total protein and CDNF concentration around 1 ng/mg total protein [28]. The mean serum MANF concentration in healthy humans was 7.0 ng/ml [40], whereas CDNF concentration was 33 pg/ml [41].

The primary location in the ER and simultaneous secretion of CDNF-MANF proteins predict that they have a dual function—one inside the cells and the other extracellular. Consequently, it is logical to assume that both proteins have two receptors. Indeed, this has been demonstrated for MANF. Transmembrane protein neuropilin (NPTN) was shown to act as the cell surface receptor for MANF (Fig. 4.1d). Biochemical analysis demonstrated that MANF directly binds to the extracellular domain of NPTN, inhibiting NF- κ B signaling and alleviating inflammatory response and apoptosis. Interestingly, NPTN does not serve as the receptor for CDNF [158].

Since both MANF and CDNF regulate ER stress and unfolded protein response (UPR) [88], it was logical to assume that one of their receptors is in the ER membrane. UPR is activated when ER is overloaded with unfolded or misfolded proteins. UPR in mammalian cells occurs via three ER transmembrane receptors: IRE1 α (inositol-requiring enzyme 1 α), PERK (PKR-like endoplasmic reticulum kinase), and ATF6 (activating transcription factor 6). According to the current view, a major ER chaperone-binding immunoglobulin protein (BiP alias GRP78) is binding to these receptors preventing their activation and signaling in homeostatic conditions [153]. During its initial stages, UPR restores cellular homeostasis by triggering pro-survival signaling pathways, but its chronic activation leads to apoptotic cell death. Recent data show that MANF directly interacts with the luminal domain of ER-transmembrane UPR receptor IRE1 α (IRE1 α LD) and with lower affinity with UPR sensors PERK and ATF6. By binding to its primary ER receptor, IRE1 α MANF alleviates UPR signaling by decreasing IRE1 α oligomerization, phosphorylation, and

splicing of Xbp1, Atf6 and Txnip levels (Fig. 4.1e). Wild-type MANF, but not MANF mutant deficient in IRE1 α binding, protects neurons from ER stress-induced death [77]. CDNF can also protect neurons from ER stress-induced cell death. Inhibiting UPR signaling in the neurons by adding either PERK inhibitor GSK2606414 [6] or IRE1 α RNase inhibitor 4 μ 8C [26] or IRE1 α kinase inhibitor KIRA6 [47] to the culture medium completely blocks CDNF anti-apoptotic actions in neurons. These data suggest that the survival-promoting effect of CDNF requires the activity of both IRE1 α and PERK pathways [30, 33].

Using different methods, it was shown that MANF interacts with the major ER chaperone BiP [50] and other chaperones, including PDIA6 and GRP170 [34]. MANF binding to BiP prolongs the interaction of BiP with its client proteins, thereby regulating protein-folding homeostasis [159]. CDNF also interacts with BiP and GRP170 [33]. Interestingly, MANF and CDNF interaction with ER chaperone BiP was not required for their survival-promoting activity [33, 34, 77]. In addition to ER chaperones, CDNF directly interacts with α -synuclein, a major protein of Lewy bodies characteristic for DA neurons neuropathology of PD. This interaction inhibits α -synuclein cell entry and, in a rodent α -synuclein preformed fibril (PFF) model of PD, reduces α -synuclein phosphorylation and improves locomotor activity [3].

MANF and CDNF primarily operate inside the cells, and their antiapoptotic and cell proliferative effects are mainly regulated via their intracellular receptors [58]. In the case of MANF, this regulation occurs via UPR sensors IRE1 α , PERK and ATF6. It is now quite well established that CDNF and MANF have almost no effects on naïve cells in vitro [33, 34, 90] and in vivo [2, 151]. Our recent data on MANF can give insights into this puzzle. Interestingly, MANF and BiP binding sites in IRE1 α LD are overlapping. Therefore, MANF can bind to IRE1 α LD only if BiP is dissociated from IRE1 α LD, which occurs only in stressed, and not in naive cells [77]. Extracellular MANF can act on cells via NPTN receptor, or in cells lacking it, MANF can be endocytosed by binding to sulfolipids and then move to the locus operandi, the ER. Intracranially injected CDNF and MANF can be detected in brain neurons, supporting the view that both GFs are internalized by the cells. In some cells, such as pancreatic beta cells with intrinsically elevated ER stress due to insulin synthesis, MANF can act readily, promoting their proliferation and protecting them from toxins [86]. Also, CDNF and MANF act directly on stressed or injured neurons, promoting their survival and inducing their neurite outgrowth. As mentioned, these effects are blocked by IRE1 α and PERK inhibitors [30, 33, 34], indicating that their signaling via UPR pathways is essential. These in vitro results are supported by analysis of MANF-deficient mice that have activated of UPR pathways in many cells and tissue, including pancreatic beta cells [28, 86] and in neurons [106]. CDNF knockout mice have ER stress in tissues where MANF is expressed at a lower level [85, 107]. There is also evidence that MANF and CDNF can activate pErk [141] and Akt [55, 163] and these pathways are known to contribute to cell proliferation and neurite outgrowth. Differently from other neurotrophic factors, CDNF and MANF can regulate ER stress and, through that, protein homeostasis. Importantly, CDNF and MANF can also regulate the synthesis and release of pro-inflammatory cytokines and, via that, reduce inflammation [146]. Counteracting apoptosis, regulating protein

homeostasis, reducing inflammation, and mainly acting on stressed or injured cells make CDNF and MANF therapeutically desirable proteins.

Preclinical Studies of Neurotrophic Factors

The potential therapeutic values of GFLs and CDNF/MANF neurotrophic factors were tested by direct infusion of the protein to the brain as they do not pass the BBB. GDNF and NRTN showed remarkable neuroprotective and neurorestorative effects in neurotoxin-induced rodent and primate models of PD [19, 45, 60, 125]. In the neurorestorative model of PD, GDNF has been shown to restore the function of remaining DA neurons by increasing their sprouting and enhancing the formation of new synapses [44, 46, 69, 138]. Interestingly, GDNF administration to the striatum promotes the functional recovery and regeneration of DA striatal fibers and protects cell bodies, underlying the importance of the delivery site for optimum effects of GDNF [70, 71]. Further, GDNF is retrogradely transported to the cell bodies when given to the striatum [143]. These findings also suggest that early-stage PD patients with sufficient number of fibres in the putamen can receive maximum benefits from trophic factors therapy (Fig. 4.2a, b).

The therapeutic effect of GDNF was also tested using gene therapy in animal models of PD. The continuous expression of GDNF was influential in the regeneration of neurons and functional recovery in neurotoxin-based PD models [11, 14, 87]. Lentiviral delivery of GDNF into the striatum and SN of non-lesioned rhesus monkeys which were subsequently treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was shown to prevent degeneration and induce regeneration of DA neurons in the primate model of PD [75]. Further, the adeno-associated virus (AAV) serotype 2 vector expressing GDNF (AAV2-GDNF) was also associated with functional recovery after 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal system. The study showed that fine DAergic fibers were observed in the AAV2-GDNF-administered striatum, suggesting neuroprotection against 6-OHDA neurotoxin lesion in monkey [37]. AAV2-GDNF was also tested in MPTP-treated macaques using convection-enhanced delivery of AAV2-GDNF into the putamen. The study demonstrated that the expression of GDNF promotes the restoration of the DA system. Further, the expression of GDNF in the putamen increased the biodistribution of GDNF and anterograde transportation of GDNF to the substantia nigra [67]. Overall, this study indicates both neuroprotection and neurorestoration can also be achieved by gene therapy, therefore, GDNF gene therapy is under clinical investigation in PD patients (clinicaltrials.gov identifier NCT04167540).

The importance of trophic factors has also been extensively studied in the naive brain. The administration of GDNF increases DA synthesis by increasing the phosphorylation of TH [72, 129]. Nigral injection of GDNF in rats and monkeys increased the spontaneous locomotor activity, which was associated with increased DA turnover [10, 44, 62]. Further, nigral administration of GDNF increased amphetamine-induced locomotor activity and DA release. Grondin et al. showed that GDNF increased

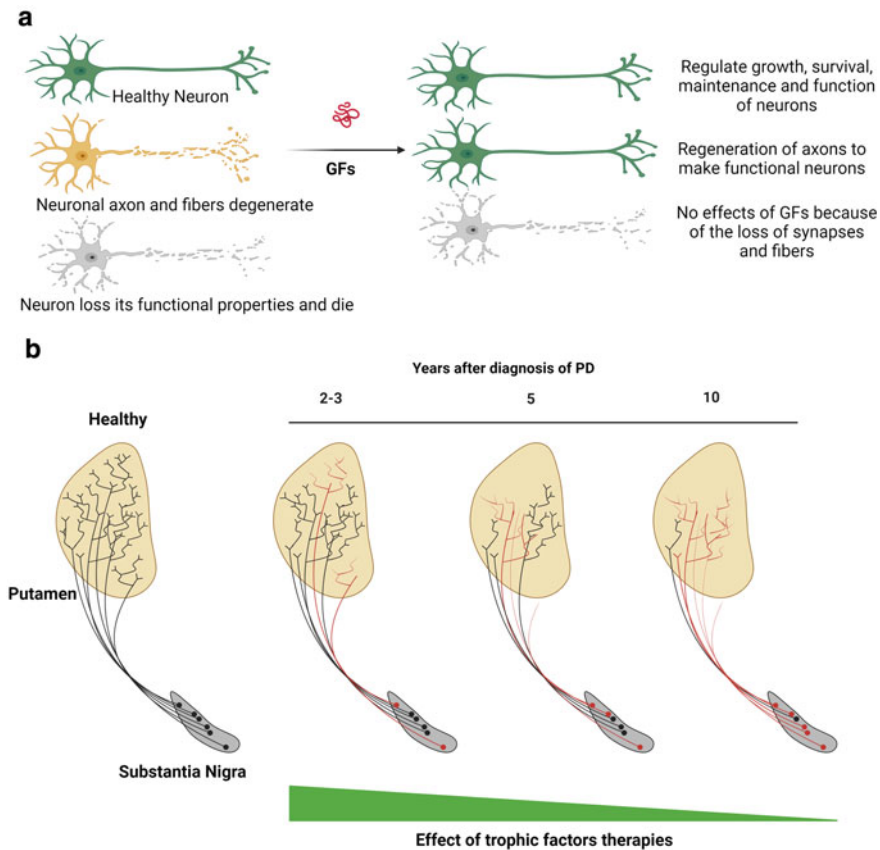


Fig. 4.2 GFs are essential for the survival, maintenance and regeneration of axons of DA neurons, cell bodies and fibers. **a** GFs promote the survival and maintenance of naive DA neurons (green), help to regenerate the axons and increase their functional activity (yellow), and no effect of GFs is observed once DA neurons lose their functional properties (grey). **b** nigrostriatal DA system in healthy and PD's brain. DA neuron's cell bodies are in the substantia nigra, which projects their fibers to the putamen. Gradual loss of DA fibers and cell bodies occurs in PD patients with increased disease progression. GFs therapies are effective early in the disease when fibers remain in the putamen (green arrow)

amphetamine stimulated DA release in the striatum and improved motor functions when delivered to the aged monkey [52]. Recently, it has been shown that twofold overexpression of GDNF from its native locus increased the DA level and enhances the DA release in the striatum after amphetamine administration [80, 148]. Similar to GDNF, direct administration of NRTN to naive rats also increased DA turnover and amphetamine-induced locomotor activity [60]. A single injection of NRTN also increased DA release in male rats, comparable to GDNF, supporting similar trophic activities like GDNF [20].

CDNF and MANF have been tested in three different animal models of PD in rodents and non-human primates. In one of the models, degeneration of DA neurons was induced using neurotoxin 6-OHDA, in a second with MPTP and the third with α -synuclein PFFs. The first in vivo study was conducted with CDFN where a single intrastriatal injection protected and regenerated nigral DA neurons either when injected together with the 6-OHDA neurotoxin, but notably also in a “clinical setting” when CDFN was injected 4 weeks after the neurotoxin [90]. In the first MANF in vivo study, the protein restored the nigrostriatal DA neurons' function when injected 6 h before or 4 weeks after 6-OHDA administration in the striatum. MANF diffused significantly better than GDNF in the striatum [151]. In the comparative study, CDFN, MANF and GDNF were infused for two weeks into the striatum in a rat 6-OHDA model. GFs were infused 2 weeks after 6-OHDA, and CDFN protected lesioned nigral DA neurons and their fibers in the striatum. Interestingly, MANF and GDNF had no statistically significant neuroprotective effect in this relatively severe 6-OHDA lesion model [150]. These studies also showed that MANF diffused in the brain better than CDFN and both GFs significantly better than GDNF. Several gene therapy studies demonstrated that CDFN and MANF can effectively protect and repair DA neurons in the rodent 6-OHDA model. In the two first studies, the effect of AAV2-CDFN was investigated. In one of the studies, CDFN only partially protected DA neurons [7], whereas in the other study, AAV2-CDFN showed eminent neuroprotection [122]. Better expression level and diffusion of the CDFN can explain this difference in the latter study. Striatal delivery of AAV9-MANF produced prominent protection for nigral DA neurons and their fibers and increased striatal and nigral levels of DA [55]. Similar results were obtained when CDFN was overexpressed by the AAV8 vector, but a neuroprotective effect was obtained only in the case of mild or moderate striatal 6-OHDA lesions [154]. Different results were obtained when CDFN and MANF were expressed in the nigra using lentiviral vectors. CDFN overexpression reduced amphetamine-induced rotational behavior and increased striatal TH-positive innervation. MANF overexpression in a similar setting only protected nigral DA neurons. Simultaneous overexpression of CDFN and MANF in the SN significantly decreased amphetamine-induced rotations and protected DA cells and their fibers [25]. Nigral overexpression versus striatal overexpression in other studies and lack of information about CDFN-MANF levels in the case of lentiviral expression may describe the differences between these studies. In the following rodent studies, an additive neurorestorative effect of CDFN and GDNF was observed in the 6-OHDA model of PD in rats [152], further supporting the notion that these GFs have different modes of action. This study also provided the first in vivo evidence that CDFN can regulate ER stress and UPR. In another study, CDFN significantly reduced neuroinflammation in the 6-OHDA model of PD [103]. In cell therapy experiments, [66] investigated the therapeutic effects of CDFN-expressing bone marrow-derived mesenchymal stem cells and reported neuroprotective effects of CDFN-MSC grafts in a rat 6-OHDA model of PD [66].

Until now, only one study has reported the effects of CDFN in a non-human primate 6-OHDA model of PD. In the unilateral 6-OHDA lesion model of PD in marmoset monkeys, the neuroprotective effects of CDFN were compared with the

effects of GDNF [42]. Using *in vivo* imaging with ^{123}I -FP-CIT (a DAT ligand), the authors reported a prominent increase in DAT binding activity in CDNF-treated monkeys. Still, no significant difference was observed in the GDNF-treated group.

The number of studies where CDNF and MANF effects in MPTP models of PD were investigated is somewhat limited. In the first study, intrastriatal delivery of CDNF had both neuroprotective and neurorestorative effects on the DA neurons in a mouse MPTP model of PD [2]. Surprisingly, CDNF had no effect on nigral DA neurons of naïve, healthy animals, which is very different from the effect of GDNF inducing axonal sprouting and other effects in naïve animals. The lack of effect of CDNF was recently explained based on *in vitro* experiments suggesting that in naïve animals, MANF (and possibly also CDNF) receptors could be occupied by another ligand [34, 77]. In the following study, the MANF effect was first investigated in the mouse neurorestorative MPTP model of PD. MANF delivery two weeks after the lesion significantly reduced the loss of DA neurons and increased the striatal DA level in PD mice. Interestingly, MANF treatment also increased the superoxide dismutase activity and glutathione production, indicating that MANF may regulate oxidative stress [92].

In a recent study, CDNF neurorestorative effects were compared with GDNF in a unilateral rhesus monkey MPTP model of PD. CDNF efficiently restored monkey global and fine motor behavior more efficiently than GDNF. Moreover, CDNF, but not GDNF, significantly alleviated non-motor symptoms increasing motivation [139].

Until now, neuroprotective effects of CDNF were investigated in the α -synuclein model of PD only in one study [3]. Importantly, several earlier studies have demonstrated that gene therapy of GDNF is not protecting DA neurons in a rodent model where α -synuclein is overexpressed by viral vectors [31, 93]. *In vitro* studies somewhat unexpectedly demonstrated that CDNF can directly interact with α -synuclein, confirmed by NMR spectroscopy and cellular studies. Interestingly, CDNF reduced the neuronal entry of α -synuclein fibrils and the formation of insoluble inclusions of phosphorylated α -synuclein. *In vivo*, experiments showed that striatal CDNF administration reduced motor deficits in the mouse α -synuclein PFF model of PD [3]. These encouraging results support the notion that CDNF-administration may represent a therapeutic strategy for treatment of PD and perhaps in other synucleinopathies. Taken together, animal experiments demonstrate that CDNF and MANF protect and restore the function of DA neurons by novel mechanisms regulating ER stress, neuroinflammation, and inhibiting cell death.

Clinical Trials with Neurotrophic Factors

Over many decades, there has been considerable interest in trophic factor-based therapies for treating PD. The neurotrophic factors are impermeable to the brain; therefore, they must be delivered intracranially. The promising outcomes from different preclinical studies led to several clinical trials with different trophic factors. GDNF was the first neurotrophic factor to be tested in PD patients. First, a randomized,

double-blind trial with GDNF delivered by intracerebroventricular (i.c.v.) catheter did not improve the motor performance of the PD patients but resulted in adverse effects [104]. It was speculated that the protein might not have reached the target tissues sufficiently, possibly due to GDNF's high affinity binding to the extracellular matrix or proteolysis [76]. Due to the failure to reach the relevant target tissues, two phase I studies were carried out by directly administering GDNF to the putamen. The study conducted by [49] reported significant improvement in the off-medication motor sub-score of the Unified PD Rating Scale (UPDRS) as well as activities of daily sub-score. The clinical improvements in these PD patients were also associated with the significant increase in fluorine-18-dihydroxyphenylalanine (^{18}F -DOPA) uptake on positron emission tomography (PET) scans indicating the neuroprotective and neurorestorative effect of GDNF [49]. In another study by Slevin et al. [137], unilateral infusion of GDNF demonstrated significant improvement in both the on and off states at 24 weeks compared with the baseline scores [137]. Interestingly, the clinical effect of GDNF still persisted until 9 months after the washout period, and unilateral infusion of GDNF had sustained bilateral effects [136]. Both studies reported no significant adverse side effects demonstrating the safety of protein infusion into the human brain [49, 137]. The positive outcomes from these open-label trials were followed by a phase II randomized placebo-controlled clinical trial in PD patients with the bilateral intraputamenal infusion of GDNF (liatermin). However, the study reported no clinical benefit of GDNF in PD patients compared to placebo groups despite increased ^{18}F -dopa uptake. The treatments were well tolerated. However, some adverse events, such as paresthesia, headache, and upper respiratory tract infections, were also reported. In addition, three patients from this study developed neutralizing antibodies GDNF indicating peripheral leakage of the GDNF during administration [81]. Unlike the previous open-label trial, the phase II study used a different drug delivery system and GDNF infusion protocol. This study used the continuous infusion of GDNF to both hemispheres with a single dose. Later, the same delivery system and infusion protocol were tested in monkeys to ensure GDNF bioavailability could mask its potential therapeutic effect. The outcomes from this study showed that GDNF was concentrated near the tips of the catheter, which indicates that drug bioavailability may have been limited to 2–9% of the putamen in human subjects [128]. Recently, a new randomized, placebo-controlled, double-blind phase II clinical study was conducted using intermittent convection-enhanced intraputamenal delivery of GDNF protein to avoid limitations of the previous drug delivery system and infusion protocol. Phase II clinical trial results demonstrate that intermittent intraputamenal administration of GDNF was well tolerated without any drug-related serious adverse events. Although the study did not reach the primary endpoint, there was a significant increase in ^{18}F -dopa uptake throughout the putamen only in the GDNF group, indicating the wide distribution of the protein-producing tissue engagement effect. Interestingly, a post hoc analysis showed clinically significant motor improvement (≥ 10 points) in the off-state in nine (43%) patients in the GDNF-treated group [156, 157].

Gene therapy approach is also being tested in the clinics as an alternative delivery strategy that will potentially help produce correctly folded, posttranslational modified and biologically active GFLs following a one-time dosing procedure. First, GFL gene therapy was initiated with AAV2-NRTN (CERE-120) by direct injection into the putamen. The open-label phase I clinical trial demonstrated that the administration of AAV2-NRTN was safe and well-tolerated, with no adverse events observed in PD patients [100]. The positive outcome from this trial led to the initiation of a multicentre, double-blind, sham-surgery controlled phase II trial in patients with advanced PD where AAV2-NRTN was administered bilaterally to the putamen. However, the study demonstrated that AAV2-NRTN was not superior to sham surgery when assessed using the UPDRS motor score at 12 months. The study was followed up to 18 months and showed small benefits of AAV2-NRTN treatment [99]. Post-mortem analysis of the two PD patients' brains treated with AAV2-NRTN showed that expression of NRTN covered at least 15% of the targeted putamen by volume. Still, minimal NRTN deposits were found in the substantia nigra. This indicates that the impaired axonal transport to the cell bodies of DA neurons in the substantia nigra might have masked the beneficial effect of the NRTN [9]. Later, a multicenter, double-blind trial in patients with advanced PD was carried out with bilateral AAV2-NRTN injection into the substantia nigra and putamen. However, the groups had no significant difference [155]. Post-mortem studies on two patients having advanced PD with AAV2-NRTN gene therapy reported persistent expression of NRTN in both putamen and substantia nigra. Importantly, this study demonstrated that NRTN can activate MAPK and AKT pathways in human brain DA neurons, however, due to the expression of NRTN covering a limited area, its antiparkinsonian benefits remained masked [22].

Following the NRTN clinical trial, the phase I AAV2-GDNF study was initiated with advanced PD patients. Using magnetic resonance imaging (MRI)-guided, CED intraputamenal infusions AAV2-GDNF together with gadolinium were administered to the putamen. The methods allowed to deliver enormous volume (450 μ l/hemisphere) of AAV2-GDNF to cover a large putamen area. The outcome of the trial demonstrated that the treatment was safe and well tolerated, without any adverse events assigned from the delivery system or the test substance. Increased [18 F]DOPA uptake was observed after 6- and 18-month time points after the treatment. The volume distribution of the test substance covered was 26% of the putamen which was much less than anticipated. The limited coverage was due to the cannula's positioning perpendicular to the putamen's long axis [57]. Therefore, to increase the coverage area, a follow-up study is planned with even larger infusion and posterior surgical approach of positioning the cannula along the axis of the putamen [8].

GDNF and NRTN have been tested on PD patients in phase I and phase II clinical trials [98, 135]. Why is there a need to test another neurotrophic factor, such as CDNF? Firstly, CDNF was neuroprotective and neuroregenerative for DA neurons in rodents and in non-human primate models of PD. Secondly, the mode of action of CDNF is entirely different from that of GDNF and NRTN. Most importantly, CDNF has a dual mode of action operating in the ER, regulating ER stress and UPR, and acting extracellularly [88]. CDNF could reduce protein aggregation possibly by

regulating UPR and most prominently reduces α -synuclein aggregation [33, 77] and improves locomotor activity in the rodent α -synuclein model of PD [3]. One of the notable properties of CDNF is its ability to reduce inflammation by decreasing the levels of expression and secretion of pro-inflammatory cytokines [146]. Moreover, CDNF does not affect naïve and healthy cells and therefore has practically no effects when injected into the brain of healthy rodents. This means that in clinical settings, CDNF should have minimal side effects. Finally, one explicit limitation of using GDNF and NRTN in treating PD has been their poor diffusion in the brain tissue caused by their binding to heparan sulphate proteoglycans. CDNF does not bind to heparin and spreads significantly better than GDNF and NRTN [150, 151].

The main goal of the phase I trial was to assess the safety and tolerability of monthly intraputamenal CDNF infusions in patients with advanced PD. The study used an investigational drug delivery system (DDS) with four catheters from Renishaw company. This clinical trial was conducted in three Scandinavian medical centers and was divided into a placebo-controlled, double-blind 6-month main study. This was followed by active treatment for another 6 months. Of 17 patients, 6 were on placebo, 6 received 0.4 mg CDNF/month, and 5 patients received 1.2 mg CDNF/month. The safety and tolerability of CDNF and DDS were the study's primary endpoints. This phase I study met the primary endpoint because drug-related adverse events were mild to moderate, with no difference between placebo and treatment groups [63]. There were no significant changes in efficacy endpoints between the placebo and the two CDNF treatment groups. Still, a minimal clinically significant difference in UPDRS II (off) was found in the CDNF dose groups indicating the possibility that CDNF may affect disease progression. In PET studies, increased DAT availability in the putamen was observed in some patients that received CDNF, which indicates either an increase in DAergic function or neuroprotection. Considering that patients in this study had rather extensive neurodegeneration in the DAergic system because, on average, their disease was diagnosed 10.5 years before the CDNF treatment, and taking into account that CDNF works poorly in animals with severe lesions of the DAergic system [150], the functional improvements induced by CDNF in some patients are very encouraging.

Although the CDNF treatment with intracranial delivery was safe and showed drug effects in some patients, the main limitation (such as intracranial delivery) of this type of treatment remains. To avoid intracranial delivery, several strategies have been used. Recently a BBB penetrating CDNF fragment was discovered, and authors have demonstrated that this fragment recapitulates the main functional properties of CDNF—prevents cell death, regulates ER stress and reduces inflammation [127]. Their recent results demonstrate that peripheral delivery of the CDNF fragment can protect and repair DA neurons in animal models of PD. This opens an entirely new avenue for the disease-modifying treatment of PD. The University of Helsinki has licensed the technology to Herantis Pharma Plc., who is now developing a BBB-penetrating CDNF-fragment-based drug for PD treatment.

Clinical Limitation of Neurotrophic Factors and Alternative Approaches

Despite being very potent in various preclinical studies for PD, neurotrophic factors offer various challenges that make their clinical translation difficult. Neurotrophic factors do not cross BBB; therefore, they must be administered through invasive procedures. Due to ethical concerns, only moderate to advanced-stage PD patients are selected for the clinical trials with GFs. Preclinical studies demonstrate that neurotrophic factors are promising in various PD models when there is still a significant population of surviving DA neurons (Fig. 4.2a). However, in the advanced stages of the disease, there are fewer surviving DA neurons to be rescued, which limits the effectiveness of neurotrophic factor therapy (Fig. 4.2b). In addition, in the advanced disease stages, due to impaired axonal transport, neurotrophic factors might not reach the substantia nigra when given to the putamen making the treatment less effective.

The optimum dose of neurotrophic factors is affected by several factors. Neurotrophic factors, especially GFLs, are sticky proteins as they bind to heparan sulfate proteoglycans, affecting their biodistribution and bioavailability. The doses used in the clinical trials are calculated based on data obtained from preclinical PD models. Notably, the GF shows a biphasic response curve, which means that too little might not have any therapeutic benefits. In contrast, a higher dose might show inhibitory effects or tend to aggregate. So far, in most clinical trials, GDNF has been used in excess amounts, which might also explain the insignificant effect of GDNF in clinics.

Another explanation for failing to translate preclinical findings to clinics is the lack of adequate knowledge of human disease progression. PD is heterogeneous, and individual patients' responses to neurotrophic factor treatment might differ. Moreover, in the preclinical settings, the neurotrophic factors are tested in chemically induced animal models of PD. Here, the treatments are tested soon after the motor symptoms appear. However, in clinical trials, patients selected are at the advanced stage of the disease. Due to the slow progression of the disease, not only DA neurons but the other population of neurons are also affected which might not be reversed by neurotrophic factor treatment [4]. We also observed that in the neurotoxin model PD, where there is more than 80% loss of DA neuron, none of the neurotrophic factors had any therapeutic effects [150].

The consideration of neurotrophic factors for the therapeutic intervention for PD is still debated. However, changing the treatment strategy (described below) might help to achieve a positive outcome after treating with trophic factors. The patients who can only benefit from being treated with trophic factors are early-stage PD patients, with most DA fibers still intact and responsive to L-Dopa treatment. However, due to ethical constraints, early-stage patients are not being selected for clinical trials. To overcome this, either a gene therapy approach or changing the GFs delivery route should be used. Different preclinical and clinical studies show that NTF genes delivered using viral vectors show anti-parkinsonian effects [5]. However, more research should be focused on developing non-invasive ways to deliver genes

to the target. The nanoparticles approach to deliver genes should be considered for treating PD. Nanoparticles are designed to encapsulate the neurotrophic factors gene and can target DA neurons. Several studies have shown that gene transfer by neurotensin (NTS)-polyplex nanoparticles [38] or cationic microbubbles [39] could restore motor function and protect DA neurons from 6-OHDA-induced cell death. Moreover, [38] have shown that expression of GDNF in DA neurons can be controlled by supplying tetracycline as the nanoparticle contains a bifunctional plasmid that codes for the reverse tetracycline-controlled transactivator advanced and tetracycline-response element [38]. The use of nanoparticles to deliver neurotrophic factors has been reviewed extensively elsewhere, suggesting that their use can help penetrate through the BBB and increase stability and bioavailability [16]. The study conducted by [43] showed that GDNF encapsulated in a chitosan-coated nanostructured lipid carrier, when administered via an intranasal route, has an anti-parkinsonian effect [43]. Thus, using nanoparticles to deliver either NTF genes or proteins might help solve delivery issues related to brain surgery.

GDNF and NRTN being sticky proteins, their bioavailability and distribution should be improved. Preclinical studies with GDNF and NRTN mutants have been shown to increase their biodistribution and their stability. NRTN mutants are biologically active and regenerate DA fibers and alleviate the motor symptoms of rats in a 6-OHDA model [125] and MPTP primate model of PD (Mahato and Saarna, Manuscript in preparation).

Another optimal approach is to develop small molecules mimicking the action of the neurotrophic factors for clinical use. The small molecule can penetrate BBB, has better spreading ability and has the potential to cure motor and non-motor symptoms of PD. We have developed a small molecule for GFLs receptor (GFL receptor agonist) and showed that the compound can bind and activate RET receptors, promote cultured DA neurons' survival, and protect the neurons from neurotoxin toxicity. We also showed that GFL receptor agonists can pass BBB, stimulate DA release and alleviate motor symptoms in the 6-OHDA model of PD [95, 123].

Conclusions and Future Directions

Although the clinical application of neurotrophic factors remains challenging, the preliminary evidence from the clinical studies encouraged the further development of trophic factors-based therapy for treating PD. The brain is protected by a highly selective semipermeable border of endothelial cells. This brings significant challenges for trophic factors-based therapy, such as drug delivery to the brain, selecting doses, and increasing the biodistribution of trophic factors for their optimum functioning. More research should focus on developing novel drug-delivery tools to overcome these challenges. There is clear evidence from the clinical studies that early-stage PD patients can benefit from trophic factors-based therapies. Therefore, future clinical trials with trophic factors should be carefully planned in early-stage PD patients with sufficient numbers of remaining DA neuron cell bodies and their fibers. More

attention should be paid to developing an alternative strategy to neurotrophic therapy, for example, neurotrophic factor mimicking small molecules which can target to cure both motor and non-motor symptoms. Importantly, small molecules can penetrate through the BBB, therefore, can be administered to early-stage PD patients soon after diagnosis. Combinational therapy, such as using different trophic factors, should also be considered, which could target multiple pathways. Neurotrophic factors should also be combined with different cell-based therapies to promote the survival of the graft. Last but not least, gene technology methods can improve neurotrophic factors' pharmacokinetic properties.

Due to the high complexity of the human brain, there is a considerable challenge in translating preclinical findings to successful application in clinical trials with PD patients. There are still many open questions, such as how DA neurons die? What drives the disease progression? How does PD pathology differ in different individuals? These questions need urgent answers. Basic research in those areas should be explored to develop an effective therapy for PD. Developing animal models that closely resemble human pathology would allow a more accurate assessment of treatment effects and disease modification in preclinical models. Further, drugs should be tested in multiple animal models of PD. Patient iPS cell-derived human DA neurons and the midbrain organoids should also be considered for the preclinical studies.

Together, neurotrophic factors still hold promise as potential drugs for disease-modifying therapy for PD due to their unique properties. Yet, many challenges remain to be solved, and both preclinical and clinical investigations should be continued for the future of PD treatment.

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Chapter 5

Emerging Nanotechnology for the Treatment and Diagnosis of Parkinson's Disease (PD) and Alzheimer's Disease (AD)



Sumasri Kotha, Manjari Sriparna, Joel Tyson, Amanda Li, Weiwei He,
and Xiaobo Mao

Abstract *Introduction:* Parkinson's disease (PD) and Alzheimer's disease (AD) are neurodegenerative diseases distinguished by the aggregation of pathologic proteins

S. Kotha · X. Mao (✉)

Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

e-mail: xmao4@jhmi.edu

Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

S. Kotha

e-mail: skotha2@jhu.edu

M. Sriparna

Virginia Commonwealth University School of Medicine, Richmond, VA 23223, USA

e-mail: Sriparnam@vcu.edu

J. Tyson

Department of Chemical, Biochemical and Environmental Engineering, University of Maryland Baltimore County, Baltimore, MD, USA

e-mail: jtyson@umbc.edu

A. Li

Washington University School of Medicine, Washington University in St. Louis, St. Louis, MO 63110, USA

e-mail: a.li@wustl.edu

W. He (✉)

Key Laboratory of Micro-Nano Materials for Energy Storage and Conversion of Henan Province, Institute of Surface Micro and Nano Materials, College of Chemical and Materials Engineering, Xuchang University, Xuchang 461000, Henan, P. R. China

e-mail: heweixeicu@gmail.com

X. Mao

Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD 21218, USA

leading to the respective onset of motor dysfunction and cognitive decline, particularly in memory. Emerging evidence showed that these misfolded proteins are prion-like seeds, which can induce endogenous protein aggregation and subsequent cell-to-cell, tissue-to-tissue transmission and propagation. The prion-like protein aggregation and spreading is accompanied by several other cellular phenomena including neurotoxicity, oxidative stress, and neuroinflammation. While traditional therapeutics have been largely unsuccessful in combatting PD and AD and their underlying pathophysiology, the customizability, specificity, and efficiency of nanotechnology presents a novel and promising avenue for the development of successful therapeutics. *Methods:* This chapter was written through a literature review sourced largely from PubMed and ScienceDirect with keywords of *AD*, *PD*, *nanomaterials*, *neuroinflammation*, *biosensing*, *oxidative stress*, and *fibrillization*. Some of this chapter is based on the review “Emerging Nanotechnology for Treatment of Alzheimer’s and Parkinson’s Disease,” published in *Frontiers in Bioengineering and Biotechnology*. *Results:* A thorough review reveals preclinical studies show incredible promise in nanotechnology as an effective combatant of neuroinflammation, protein aggregation, oxidative stress, and neuron death in AD and PD. However, clinical and longitudinal trials are still lacking; further research should be done to establish the safety and long-term side effects and efficacy of nanotechnology-based therapeutics. *Conclusions:* Despite its increasing momentum, research in nanotechnology for the treatment of AD and PD is still in its early stages. While proof of concept is established across several mechanisms of action, further research is necessary to establish clinically relevant information and further exploring regenerative and biosensing applications of nanotechnology. Nevertheless, nanotechnology presents an exciting new avenue for therapeutic development.

Keywords Alzheimer’s disease · Parkinson’s disease · Nanotechnology · Oxidative stress · Protein fibrillization · Biodetection · Regenerative medicine

Introduction

Parkinson’s disease (PD) and Alzheimer’s disease (AD) are the top two most common neurodegenerative diseases characterized by misfolded proteins accumulation. While PD is distinguished by α -synuclein (α -syn) aggregation in inclusions termed Lewy bodies (LB) in dopaminergic neurons, AD is characterized by the accumulation of amyloid- β (A β) plaques and tau neurofibrillary tangles. Studies increasingly support the hypothesis that the spread of pathological α -syn, A β and tau by a prion-like mechanism induce the propagation of proteinopathy from cell-to-cell, causing motor dysfunction in PD and impaired cognition, specifically in memory, in AD [1–9].

Several pathogeneses have been associated with protein aggregation in AD and PD. In AD, A β aggregation is hypothesized to result from the production of reactive oxygen species (ROS) by dysfunctional mitochondria and the consequent dysregulation of metals and metal-oxidation/reduction reactions due to oxidative stress [10, 11]. Tau is known to undergo abnormal hyperphosphorylation, leading to prion-like tau seeds, although the mechanism of this process remains unclear [12–14]. Similarly, in PD, endogenous α -syn misfolds from a random and soluble conformation to an insoluble and oligomeric/fibrillar forms [15]. When localized to the mitochondria, pathogenic α -syn induces mitochondrial fragmentation and decreases membrane potential, eventually causing cell death [16–19]. The specific mechanisms of α -syn pathology and associated effects in PD, while still being investigated, include oxidative stress from the deactivation of the parkin-PARIS complex, microglial hyperactivation, and the internalization of α -syn [5, 20–24]. Biological mechanisms affecting AD and PD thus include misfolded protein aggregation, oxidative stress, inflammation, and cell death via parthanatos and other mechanisms [5, 25]. While novel therapeutics are still in development [26–28], decades of trials with traditional therapeutics have been largely unsuccessful in combatting these processes [29] and methods of disease detection are still in development [30, 31]. With enhanced customizability, ease of production, and appealing physicochemical properties, nanomaterials present a novel avenue to target these mechanisms to improve the understanding of and combat the pathologic progression of AD and PD [32, 33].

With the increasing incorporation of nanotechnologies into biomedical applications, many treatments for neurodegenerative disorders are being explored and are expected to emerge [32]. Nanomaterial formulations have shown the ability to alleviate oxidative stress and inflammation directly [34], and overcome barriers in passage across the blood-brain-barrier (BBB) [35], although the specific mechanism for delivery across the BBB varies and has not been fully elucidated. Nanomaterials also demonstrate improved localized delivery of disease therapeutics by effectively enhancing dosing efficacy of drugs, controlling cargo release profiles, and functionalizing for the specific biological target of interest [36]. This provides an attractive alternative to the off-target effects of several traditional therapeutic drugs. Further, composite nanomaterials are being developed to improve regenerative medicine techniques, encouraging new cell growth in PD and AD, and enhancing biodetection of pathological hallmarks of the diseases [37].

Herein, we will review the current field of nanomaterials for the therapeutic treatment and diagnosis of AD and PD and highlight emerging trends and materials in the field. We will discuss nanomaterial developments in the context of the following major applications: combating the fibrillization process, addressing oxidative stress and mitochondrial damage, targeting of neuroinflammation, aiding cellular regeneration, and aiding theranostic approaches (Fig. 5.1).

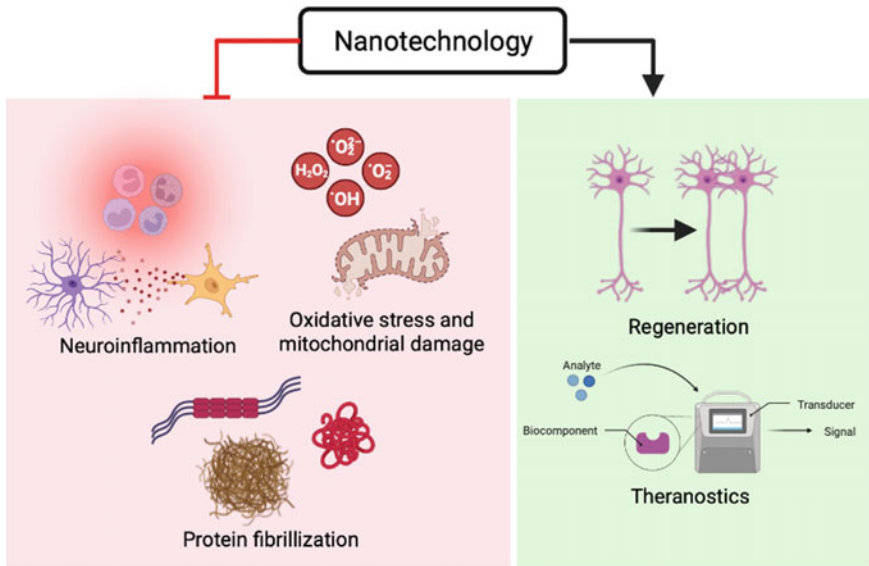


Fig. 5.1 Applications of nanotechnology for the treatment of AD and PD. Created with BioRender.com

Targeting the Fibrillization Process

Targeting α -syn Aggregation in PD

Emerging studies have shown that pathogenic α -syn can induce endogenous α -syn aggregation, and the misfolded α -syn can propagate in a prion-like manner from the cell-to-cell and tissue-to-cell resulting in neuropathology, neuroinflammation, and neurotoxicity [38–40]. Strategies to decrease α -syn aggregation involve targeting misfolded α -syn fibrils themselves or decreasing their gene expression. Here we discuss graphene quantum dots, cerium oxide nanoparticles (NPs), and nanobodies, which bind to various forms of α -syn and disaggregate fibrils directly, as well as a modified exosome that targets the central nervous system to deliver α -syn siRNA and prevent α -syn translation.

Graphene quantum dots (GQDs) are NPs of about 100 nm consisting of layers of graphene. Kim et al. found that GQDs are able to bind specifically with mature α -syn fibrils. In doing so, they can inhibit the formation of α -syn fibrils, disaggregate fibrillated α -syn in a time-dependent manner, and reduce α -syn pathology transmission induced by α -syn pre-formed-fibrils (PFF). Consequently, Kim et al. demonstrated that GQDs confer neuroprotectivity. GQDs can further restore synaptic protein levels, rescue mitochondrial damage, and reduce formation of LBs and neurites after α -syn PFF-induced synaptic function and mitochondrial dysfunction. Moreover, it was demonstrated that GQDs did not display long-term toxicity in vivo or in vitro [41].

Despite their demonstrated efficacy, further investigation is required to uncover their mechanism of action.

Cerium oxide NPs have been demonstrated to most effectively fit into the active site of α -syn and disaggregate α -syn fibrils in vivo in comparison to other nanomaterials, including gold (AuNP) and superparamagnetic, iron-oxide NPs [42, 43]. In a 2020 study, Ruotolo et al. demonstrated CeO₂ NP treatment could significantly reduce α -syn-induced cytotoxicity in a concentration-dependent manner in a yeast model overexpressing human α -syn. Specifically, CeO₂ NPs decreased mitochondrial damage and inhibited the formation of cytoplasmic α -syn inclusions [44].

Exosomes are naturally occurring nanosized vesicles with a lipid bilayer with the added advantage of being already embedded with several adhesive proteins. Exosomes are naturally secreted by monocytes and macrophages, allowing the delivered contents to potentially avoid entrapment in mononuclear phagocytes from the host immune system, and have enhanced delivery of the incorporated drugs to target cells to increase drug therapeutic efficacy [45]. Cooper et al. utilized exosomes modified with rabies virus glycoprotein peptide (RVG) to deliver siRNA to reduce α -syn protein expression. Both wild-type and S129D α -syn transgenic mice expressed lower α -syn mRNA and protein levels following injection with the siRNA RVG-exosome. Additionally, significant reductions in intraneuronal protein aggregates, including in the functionally relevant dopaminergic neurons in the substantia nigra, were observed in S129D mice. Thus, by enhancing the delivery of and blocking expression of pathologic α -syn, the modified RVG-exosome has therapeutic potential for the delay and reversal of α -synucleinopathies [46].

Nanobodies are single-domain antibodies, typically consisting of only the heavy chain of a source antibody [47]. Due to several advantageous properties such as low immunogenicity, high affinity for targets, and low molecular weight, they have been investigated as therapeutic and diagnostic tools in various diseases, including those of the brain [48]. Nanobodies developed for the treatment of PD largely target the formation of α -syn fibrils. These include NbSyn2 and NbSyn87, which target total α -syn and have been shown to inhibit the formation of fibrils in vitro. However, nanobodies targeting all α -syn may disrupt the native function of monomeric α -syn [49]. Thus, it is important to select for nanobodies specific to aggregated fibrils. More recently, a nanobody entitled Nb α -syn01 has been identified to selectively recognize α -syn fibrils over monomers [50]. Given α -syn aggregation is largely intracellular, conjugation of nanobodies to amino acid sequences rich in proline, glutamine, aspartic acid, serine, and threonine (PEST) is being explored for improving nanobody efficacy through increased intracellular solubility [51]. This method has been applied to NbSyn87: NbSyn87PEST intrabodies and has been shown to reduce α -syn aggregation in vivo and in vitro and decrease aggregate cytotoxicity in vitro [48]. Most recently, an α -syn targeting nanobody, PFFNB2, identified by Butler, Liu, & Kumbhar et al. has been identified that can selectively recognize and dissociate α -syn fibrils [52]. PFFNB2 has additionally been shown to prevent the pathologic phosphorylation of serine 129 in α -syn and the spreading of pathologic α -syn to the cortex following injection with α -syn fibrils and is associated with decreased immunoreactivity.

Targeting A β and Tau Aggregation in AD

Like α -syn in PD, tau and A β have been associated with pathology and neurotoxicity in AD. Here we discuss several approaches targeting the aggregation and propagation of these pathologic proteins in AD. These approaches include nanobodies targeting A β and tau, metal NPs and hydrogels functionalized with peptides and other molecules with protein disaggregation, sequestering, or reactive properties.

In an older study, Kogan et al. synthesized soluble AuNPs in the presence of sodium citrate and functionalized them with a peptide to form AuNP-H-Cys-Leu-Pro-Phe-Phe-AspNH₂. When the surfaces of the AuNPs were heated using inductive coupling in a magnetic field, they transmitted their heat to surrounding tissues, disrupting neighboring A β deposits and effectively blocking A β fibril growth in the gel media [53].

Later studies have further utilized AuNPs to mediate protein-based methods of A β aggregation. B-casein (β Cas) proteins have the chaperone-like ability to facilitate enzymatic reactions due to their existence as heterogenous oligomers, lack of tertiary structure, and most importantly, their ability to bind to a variety of partially folded proteins. In binding to these proteins, β Cas proteins can prevent their aggregation [54]. Intracardial delivery of β Cas coated with AuNPs has been shown to control induced A β ₄₂ toxicity in the brains of zebrafish larvae and adults. This ability is lost when β Cas is uncoated, indicating that AuNPs are critical in localizing β Cas to A β ₄₂ regions in the brain [55]. Further, AuNPs have been utilized in polyoxometalate-based nanozymes for a multifaceted approach to AD mitigation [56]. Specifically, Gao et al. functionalized AuNPs in a serine protease-like complex consisting of a polyoxometalate conjugated to an octapeptide motif that is able to target A β fibrilization through protease-like activity, ROS through Cu scavenging, and metal ion accumulation through metal chelation [56]. In 2020, Ma et al. created a similarly peptide-functionalized CuO-based nanozyme. Functionalization with KLVFF modified polyethylene glycol (PEG) with phospholipid allowed the construct to be embedded into an erythrocyte membrane. By binding to A β in blood, the KLVFF motif improved peripheral A β clearance when injected into mice [57]. Recent cellular and mouse model studies show promise that selenium-chondroitin sulfate NPs could be a multifunctional agent for AD treatment, inhibiting both A β aggregation and attenuating the hyperphosphorylation of tau at Ser396 and Ser404 by regulating glycogen synthase kinase 3 [2, 3].

Protein-capped (PC) metal NPs were synthesized by Sonawane et al. (2019) by exposing the fungal species, *F. oxysporum* and *Verticillium* spores, to various metal solutions to create different types of NPs. Aqueous mixtures of ferricyanide or ferrocyanide were used to create magnetite NPs, and Cd²⁺ and SO₄²⁻ were used to create CdS NPs. PC-metal NPs inhibit tau fibrillization by reducing hyperphosphorylation. Sonawane et al.'s study showed the direct effect of PC-metal NPs on tau aggregates, as they were quantifiably inhibited, and mature fibrils readily dissolved [58]. Uncapped CdS NPs are toxic to bacterial and HeLa cells, due to oxidative stress

caused by increased concentrations of ROS [59]. However, capping the NP makes them far more biocompatible and represents a viable therapeutic route.

Vakilinezhad et al. [62] found that nanoformulations of the histone deacetylase (HDAC) inhibitor, nicotinamide, also inhibits tau hyperphosphorylation [60]. Given cell death is a pathological hallmark of AD, a potential cause of AD is hypothesized to be excitotoxicity, in which the overproduction of glutamate leads to cellular apoptosis. HDAC inhibition is hypothesized to decrease glutamate expression. While large doses of nicotinamide may be hepatotoxic [61], Vakilinezhad et al. in 2019 used solid lipid particles (SLN) to provide localized and controlled release of nicotinamide by varying particle size. The authors confirmed that nicotinamide treatment did in fact additionally prevent tau hyperphosphorylation through an enzyme-linked immunosorbent assay (ELISA), marking nicotinamide a viable candidate for AD therapeutic development [62].

Photosensitizing materials have a high affinity for A β and have been shown to impede A β aggregation by generating oxidative stress in response to light [63]. Thus, several nanoassemblies are being investigated as light-activated A β combatants. Fullerene is one such molecule known to inhibit A β aggregation [63]. Ishida et al. utilized hydrophilic moiety hybridization to increase solubility of fullerene while decreasing cytotoxicity. When exposed to ultraviolet (UV), fullerene could denature A β_{42} monomers and oligomers [64]. Carbon based structures have been similarly developed for photochemical targeting of A β . In 2017, Chung et al. reported 10 nm carbon nanodots functionalized with polyethyleneimine able to inhibit the aggregation of A β_{42} monomers using only visible light [65]. Graphitic carbon nitride nanosheets have been used to generate ROS capable of oxidizing A β_{42} and suppressing A β_{42} aggregation using visible light [66]. Aside from visible light, Zhang et al. utilized 655 nm light to inhibit and degrade A β via photodynamic micelle functionalized with light-sensitive chlorine [67]. Recently, iron copper selenide NPs have been shown to prevent amyloidopathy in mouse models via near infrared illumination [68].

Hydrogels composed of collagen, agarose, hyaluronic acid (HA), and PEG have been found to confer neuroprotectivity from A β with specific constitution. Hydrogels made from conjugated HA and curcumin were found to slow down A β aggregation due to opposing forces from the hydrophobicity of curcumin and the electrostatic repulsion of HA [69, 70]. Yau et al. found that the seeding of A β fibril fragments in *N,N'*-methylene-*bis*-acrylamide and *bis*-acryloylcystamine hydrogels successfully sequestered excess A β in solution, preventing pathologic propagation and aggregation [71]. However, these effects are specific to modified hydrogels. In fact, non-functionalized hydrogels have been found to favor A β aggregation due to confinement effects [72].

Several nanobodies targeting A β and tau in AD have been developed. Among these are eight nanobodies constructed by Rutgers et al. (ni3A, ni8B, va2E, va1G, pa4D, pa11E, and pa2H) that can recognize A β . These nanobodies had individual preferences for binding of parenchymal and vascular depositions of A β , indicating conformational differences in brain and blood vessel localized A β that may be of functional and therapeutic relevance [48, 73]. Among forms of A β , A β oligomers

are preferentially associated with neurotoxicity and pathology as opposed to A β fibrils and monomers. Thus, nanobodies such as A4, E1, and V31-1, that have been shown to bind to A β oligomers and block their aggregation have high potential for neuroprotectivity [74–76]. Other nanobodies, such as Asec-1A could be developed to prevent A β induced toxicity by direct degradation of A β [77]. Like α -syn targeting nanobodies for PD, A β targeting nanobodies could also be conjugated with functional peptides and other molecules to facilitate crossing of the BBB and functionality. These include conjugation with A β oligomer binding protein (ABP), which has been shown to correct hippocampal atrophy [78]. While less established, nanobodies targeting tau pathology have also been developed as avenues of AD therapy. This includes nanobody F8-2 which is able to target and bind the C-terminal of tau protein [79]. Further, the nanobody VHH Z60 is able to recognize the PHF6 nucleation sequence and inhibit tau aggregation. Administration of VHH Z60 via a lentiviral vector demonstrates reduced tau spread in the mouse brain [80].

Relieving Oxidative Stress and Mitochondrial Damage

Direct Antioxidant Nanoparticles

This section covers nanomaterials that directly relieve oxidative stress by scavenging ROS and nitric oxide (NO), mimicking natural antioxidant enzymes such as metal oxides and redox nanozymes including catalase, superoxide dismutase (SOD), peroxidase dismutase (POD), and others. Nanozymes are nanomaterials with enzyme-like properties with the advantages of high stability, customizable catalytic activity, and compoundable activity profiles [81]. The function of nanozymes is largely dictated by their composition. For instance, carbon-based nonmetal nanozymes can utilize an aromatic ring that mimics the polyphorin ring in natural enzymes [82]. On the contrary, metal-based nanozymes often mimic the enzymatic activity of natural metalloenzymes through their metal oxide composition. Metal nanozymes mimic peroxidase activity, catalyzing the oxidation of a chromogenic substrate in the presence of hydrogen peroxide [82]. Some metal NP systems can exhibit multiple enzymatic properties. For example, cerium oxide NPs are able to switch between oxidation states and thus display redox activity [83]. Further, metal nanozymes can be customized to optimize and couple activity by combining metal composition. Mimicking either single or multiple nanozymes, the catalytic activity of bimetallic nanozymes, such as the widely used PtCu system, can be altered by the percent composition of each constituent metal [84, 85].

Nanomaterials have been widely demonstrated to be effective in combatting oxidative stress in cellular models of PD. Ruotolo et al. demonstrated the ability of the CeO₂ NPs to decrease α -syn pathology in yeast cells overexpressing human α -syn. Treatment with NPs reduced cytotoxicity by inhibiting the formation of intracellular α -syn inclusions and rescuing mitochondrial damage, including mitochondrial

fragmentation. The rescue of mitochondrial defects was accompanied by a higher number of functioning mitochondria and lower levels of harmful free radicals. These effects of CeO₂ treatment were demonstrated in a dose-dependent manner [44]. Hao et al. applied this property of metal nanozymes to neuronal models of PD. The group demonstrated that treatment with copper-based NPs (particularly Cu₂O and CuO) eliminated ROS in the 1-methyl-4-phenylpyridinium (MPP⁺) neuronal cell model and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. In this study, Cu_xO mimicked the activity of several redox enzymes, including catalase, superoxide dismutase, peroxidase, and glutathione peroxidase to rescue neurons [86]. Singh et al. demonstrated the ability of another metal oxide, Mn₃O₄, nanozymes to exhibit SOD, catalase, and glutathione peroxidase-like activity in the MPP + cellular model of PD [87]. The multipotent activity of these nanozymes allows three different roles in combatting oxidative stress to be filled and could more effectively enhance tolerance to oxidative stress as shown in plant-based models [88]. Liu et al. demonstrated the ability of PtCu NBs to scavenge the standard radical species, 2,2-diphenyl-1-picrylhydrazyl (DPPH) in neuronal cells and mice injected with α -syn PFF. The PtCu nanobodies displayed peroxidase, catalase, and SOD-like activity and significantly prevented the prion-like spread of α -syn in PD [84]. While further study into the optimization of nanozyme treatment to combat PD-associated mechanisms is wanted, the efficacy of these nanozymes in combatting oxidative stress demonstrates redox nanozymes could be potent therapeutics in combatting PD.

The antioxidant activity of metal-based nanozymes in PD models has been mirrored in AD models. Recent studies feature metal oxide nanozymes functionalized with A β -targeting molecules. Ma et al. demonstrated that CuO NPs encapsulated in erythrocyte membranes and functionalized with peptides, PEG, and phospholipids have SOD-like activity and H₂O₂ and superoxide removal capacity in a 3xTg-AD mouse model [57]. Yu et al. demonstrated the efficacy of cerium oxide NPs in the same 3xTg-AD mouse model. Specifically, cerium oxide NPs and retinoic acid (RA) enclosed in MIL-100 (Fe) frameworks derived from iron and trimesic acid were shown to break down in inflammatory environments with H₂O₂. The subsequent release of RA and cerium oxide particles upregulate neurogenesis genes and act as H₂O₂ antioxidants, respectively [89].

Carbon-based nanomaterials are also potent sources of antioxidant nanozyme supports. Carbon-based nanoassemblies can pass through the BBB, including PEG-functionalized carbon clusters, carbon nanotubes, and fullerenes [34]. However, enzymatic activity is highly dependent on carbon nanotube formulations. While Dal Bosco et al. found PEGylated single walled carbon nanotubes induce a delayed antioxidant response in the rat hippocampus with lasting effects on locomotion or memory [90], formulations with different structures have been shown to induce the opposite ROS formation or antioxidant depletion [91].

Indirect Antioxidant Drug Delivery Nanomaterials

Nanomaterials can be utilized to indirectly combat oxidative stress and mitochondrial damage by facilitating the delivery of antioxidant agents. In this section, we cover formulations designed to achieve this, including nanoemulsions, NPs, liposomes, exosomes, and hydrogels. The materials support specific and more efficient delivery of antioxidants through enhanced BBB penetration, bypassing immune detection, and better biological uptake.

While several antioxidant materials have been identified, penetration of the BBB remains a hurdle for the access of these agents to target areas. NPs present a method bypassing BBB impenetrability to allow targeted delivery of antioxidants. Lipoic acid is a molecule naturally present in the mitochondria with powerful anti-inflammatory and antioxidant properties capable of attenuating oxidative stress [92, 93]. The administration of lipoic acid is challenging due to the molecule's short-half life and limited bioavailability caused by hepatic degradation [94, 95]. NPs present avenues for enhancing the yield of intracellular lipoic acid delivery. Piersimoni et al. found that lipoic acid capped gold NPs (GNPs-LA) were biocompatible, capable of easily entering cells, and increased the efficacy of drug-delivery in vitro. GNPs-LA restored physiological conditions in cells by preventing ROS formation and restoring normal mitochondrial function [96]. However, further study is still necessary to elucidate the exact molecular mechanism behind the GNPs-LA system.

Nanoemulsions are a colloidal particulate system that present an additional strategy to improve delivery across the blood brain barrier through a noninvasive and direct delivery of lipophilic drug either through the mucosa intranasally or by improving the solubility and oral bioavailability of lipophilic drugs. Intranasal nanoemulsions are administered directly through the mucosa and cross the BBB. The antioxidant, vitamin E, was loaded in naringenin nanoemulsions coupled with oral levodopa treatment. This nanoemulsion reversed behavior effects and reduced oxidative stress in 6-hydroxydopamine (6-OHDA) induced PD rat model [97].

Oral nanoemulsions are processed in the same way as other orally administered drugs through the intestinal milieu. However, oral nanoemulsions have an advantage of enhancing solubility of the lipophilic drug, as well as the potential to bypass hepatic degradation by utilizing the intestinal lymphatic drug transport system and altering enterocyte-based drug transport. Nanoemulsions loaded with coenzyme Q10 were administered orally to haloperidol challenged rats to attenuate oxidative damage. With spherical droplets of around 60 nm, the CoQ10 loaded nanoemulsion improved oral bioavailability by 1.81 times in comparison to a CoQ10 suspension in a pharmacokinetics study. The nanoemulsion improved behavioral activities, reduced nigrostriatal dopaminergic depletion determined by hematoxylin and eosin staining, and decreased markers of oxidative stress, including glutathione and thiobarbituric acid reactive substances [98]. Both the vitamin E and coenzyme Q10 nanoemulsions were shown to improve behavior effects and reduce oxidative stress in PD rat models, with no signs of ciliotoxicity.

Additional carriers for antioxidants include nanoliposomes and exosomes, which can enhance BBB penetration. Liposomes have a hydrophobic exterior and hydrophilic core that make them a suitable carrier for a diverse array of drugs with potential to enhance BBB penetration. Liposomes as carriers for drugs that reduce alpha-synuclein (α -syn) expression and fibril formation have emerged as potential therapeutics. Antioxidants resveratrol (RES), baicalein, and epigallocatechin gallate (EGCG) have been found to inhibit α -syn aggregation [99–102]. Resveratrol is a polyphenolic antioxidant shown to inhibit α -syn aggregation through autophagy-induced α -syn degradation [99, 100]. RES delivered in liposomes have the potential to have greater bioavailability and success in crossing the BBB due to its hydrophobic shell. Wang et al. demonstrated that liposome loaded RES performed better than free RES in protection from oxidative stress in 6-hydroxydopamine PD rats [103, 104]. After 14 days of oral treatment of resveratrol or resveratrol liposome (20 mg/kg per day), all rats exhibited a decrease in the loss and apoptosis of nigral cells and in the levels of total ROS with resveratrol liposome having more profound effects [103]. Additional nanoliposomes have been shown to be effective at delivering, increasing stability of, and internalizing antioxidants such as baicalein in vitro. Such actions inhibit α -syn fibril formation [102].

Additionally, the effects of one drug, in this case resveratrol, can be compounded when delivered with additional antioxidant PD drugs, such as curcumin or EGCG [105]. EGCG is additionally implicated in inhibiting α -syn aggregation [101]. Kuo, et al. developed complex leptin-conjugated liposomes composed of 1,2-distearoyl-sn-glycero-3-phosphocholine, dihexadecyl phosphate (DHDP), cholesterol and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (PA) to carry RES and EGCG. Leptin-modified liposomes take advantage of the transcytosis across BBB via the Lep receptor as well as the Lep activation of the PI3K/Akt pathway for cell growth [106, 107]. Kuo et al. demonstrated that Lep/RES-EGCG-PA-liposomes crossed the BBB better than non-modified liposomes in SH-SY5Y cells. The PA has specific binding to α -syn, enhancing liposome targeting. Immunofluorescence and Western blot analysis indicated that the RES-EGCG loaded liposomes reduced the apoptosis promoter protein Bcl-2 associated X protein (BAX) and α -syn, while increasing apoptosis-inhibitor Bcl-2, tyrosine hydroxylase (TH), and dopamine transporter expression for neuronal rescue [108]. Overall liposomes take advantage of the lipid bilayer to encapsulate compounds of a variety of chemistries and solubilities to improve the stability of antioxidants and delivery through the BBB.

Similar to liposomes, exosomes can have enhanced delivery of the incorporated antioxidative drugs to target cells. An antioxidant catalase loaded exosome was developed by Haney et al. through five separate methods—incubation at room temperature, permeabilization with saponin, freeze–thaw cycles, sonication, and extrusion. All exosomes ranged in size from 100 to 200 nm. Haney et al. found that reformation of exosomes upon sonication and extrusion, or permeabilization with saponin resulted in high loading efficiency, sustained release, and catalase preservation against protease degradation. These exosomes were readily taken up by neuronal cells in vitro and were substantially detected in PD mouse brains after intranasal administration with significant neuroprotective effects [109].

Hydrogel-nanomaterials have been increasingly explored as a method of improved antioxidant drug delivery, largely through the nasal route. Chitosan gels are a promising medium and have been shown to facilitate the intranasal delivery of the neuroprotectant, piperine, to treat AD [110]. Further, Lai et al. utilized hydrogel NPs to improve the delivery of neuroprotective progesterone in rats [111]. The efficacy of chitosan gels can be attributed to their mucoadhesive properties allowing more efficient, non-invasive drug delivery with a fraction of the oral dosage in rat models [110]. Other hydrogel-nanomaterial composites include xanthan and gellan gum gels containing solubilized RES through nanostructured lipid formations for the nasal administration of AD treatment [112].

Hydrogel formulations have been employed to facilitate the delivery of scarce anti-AD drugs. Chen et al. formulated Timosaponin BII into in-situ gellan gum hydrogels for intranasal administration into mice [113]. Salatin et al. embedded Eudragit RL-100 polymer NPs in a surfactant (F-127) hydrogel to create a thermoreversible delivery system for controlled intranasal delivery of Rivastigmine [114].

Nanotechnology Targeting Inflammation

Nanomaterials for Anti-inflammatory Treatment in PD

Anti-inflammatory drugs have been used to treat both PD and AD, but nanoemulsions and NPs can improve delivery through direct delivery from nasal mucosa or improved BBB penetration.

Lipophilic anti-inflammatory medication, ibuprofen, has neuroprotective effects, and to improve its delivery to the brain, Mandal et al. loaded ibuprofen into sodium hyaluronate based mucoadhesive nanoemulsion (MNEI). As previously discussed, intranasal nanoemulsions can be a safe alternative to traditional oral routes by more directly delivering the product to the brain through nasal mucosa. MPTP mice models were treated with ibuprofen for two weeks as either an intranasal plain drug solution, or with the nanoemulsion. Nanoemulsion delivery of ibuprofen significantly reduced MPTP-mediated dopamine depletion [115]. Higher drug concentrations reached the brain through intranasal administration compared to intravenous and oral routes with minimal nasociliary toxicity. The main challenge with nasal dosing of ibuprofen is mucociliary clearance which can be combatted by incorporation of a bioadhesive carrier, adjustment of the viscosity of the formulation, and using an application of a novel administration device. Additionally, Sanchez et al. [116] created nanospheres loaded with racemic ibuprofen termed dexibuprofen. The nanosphere was a pegylated poly(lactic-co-glycolic acid) (PLGA-PEG) loaded with varying doses of dexibuprofen. In vivo studies demonstrated that amyloid plaques were smaller in size in amyloid precursor protein (APP) overexpression murine models

with significantly lower glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) staining indicative of lower neuroimmunological activity [116].

As mentioned previously, lipoic acid is an organosulfur compound native to the mitochondria with a powerful antioxidant capacity, but limited therapeutic efficacy, due to its short half-life and bioavailability [96]. With the advent of using metals such as gold, nanotechnology demonstrates potential to stabilize otherwise short-lived anti-inflammatory molecules and deliver them to targeted cells. Piersimoni et al. created AuNPs conjugated with lipoic acid. Administration of these NPs to SH-SY5Y cells rescued cell damage caused by high levels of ROS generated from α -syn overexpression [96].

Buildup of cholesterol oxidation products in the brain can occur in individuals with altered cholesterol metabolism and is thought to contribute to neurodegenerative disease. A flavonoid, quercetin, is known to amplify anti-inflammatory properties within SH-SY5Y cells. In experiments trialing a NP with a capsule made of a lipoic acid, there were greater anti-inflammatory effects in neuronal models in comparison to cells pretreated with free quercetin. This is thought to be due to improved permeance through the BBB and increased bioavailability of quercetin to target cells with the NP. The NP delivery of quercetin reduced expression of key neuroinflammatory markers including CD36, β 1-integrin, interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), cyclooxygenase 2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1), and toll-like receptor 4 (TLR4) [117].

Another example is the polyphenol compound curcumin, the progenitor of turmeric. Curcumin is known to have anti-neuroinflammatory properties and decreases the quantity of antioxidants in the body. However, its inability to cross the BBB and rapid metabolism in the blood circulation limit its therapeutic utility. Using nanolipid particles as a delivery modality of curcumin, Ganesan et al. demonstrated diminished inflammation in BV-2 microglial cells stimulated by pro-inflammatory lipopolysaccharides (LPS). Specifically, curcumin loaded solid lipid NPs showed significant inhibition of NO production compared to conventional curcumin administration in a dose-dependent manner. Similarly, the mRNA and proinflammatory cytokine levels of tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin 6 (IL-6) were also reduced in a dose-dependent manner when treated with the nanolipid particle delivery formulation [118]. Similarly, Kundu et al. loaded curcumin into glyceryl monooleate (GMO) NPs. Treatment remarkably inhibited aggregation of α -syn into oligomers and fibrils, enabling a higher density of tyrosine hydroxylase-positive dopaminergic neurons in the substantia nigra of mice [119].

Nanomaterials for Anti-inflammatory Treatment in AD

For the treatment of AD, NPs made from anti-inflammatory molecule quercetin and encapsulated in β -cyclodextrin-dodecylcarbonate were shown to have increased anti-inflammatory effects compared to free quercetin in vitro with the goal of improving

permeation and bioavailability [117]. Additionally, mitochondria-targeted quantum dot nanozymes were designed to switch microglia from proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype, which can mitigate A β aggregate-mediated neurotoxicity. These nanozymes effectively crossed the BBB, escaped from lysosomes, targeted the mitochondria, and prevented spontaneous neuroinflammation through regulation of proinflammatory mediators in vitro and in AD mouse models [120].

Timosaponins are a plant-based compound well known to traditional Chinese medicine. They were initially discovered as a treatment for mediating gliomas through their anti-inflammatory, anti-angiogenesis, and pro-apoptotic activity on a variety of tumor cells [121]. More recently, timosaponin BII has been well-established as an efficient anti-AD agent. However, it has low bioavailability. Chen et al. demonstrated timosaponin BII formulation into a temperature/ion-sensitive in situ hydrogel (ISG) able to be absorbed in the nasal tract. Timosaponin BII treatment increased structural integrity via stabilized F-actin structures in PC12 cells, a cell line classically used to model norepinephrine and dopamine secreting neurons. In vivo studies corroborated the protective capacity of timosaponin BII ISG treatment. Intranasal timosaponin BII delivery in lipopolysaccharide (LPS)-induced models of AD demonstrated lower TNF- α and IL-1 β expression and reduced inducible NO synthase, a marker of AD. Behavioral tests revealed treatment also improved spatial memory and spontaneous behavior in mice. High brain targeting effect of timosaponin BII ISGs was confirmed by in vivo fluorescence imaging. In contrast, in LPS-induced PC12 cells without concurrent Timosaponin hydrogel treatment, the backbone was in disorder and the cell structure outline was not clear [113]. The nasal timosaponin BII dually sensitive ISGs can serve as a promising medication for local prevention of AD. For instance, timosaponin BII ISGs significantly increased the content of M1 choline receptors in the mice hippocampi and specifically ameliorated damage incurred to cornu ammonis (CA1) area of the hippocampus. This may indicate the utility of hydrogels in augmenting the preventive effect on mice with AD induced by scopolamine [122].

Lastly, a modified PLGA NP was conjugated with CD47 extracellular domain via an ROS-responsive phenylborate ester bond, which acts as a “do not eat me” signal. Encased within this particle were CRT (a BBB penetrating peptide) and a microglial modulating agent called necrostatin-1 (Nec-1). Cells with high ROS release signals that inactivate CD47 thereby allowing microglial cells to more efficiently target highly inflammatory cells. Nec-1 is released upon CD47 inactivation. The NP increases the half-life of Nec-1, crucial given its role in modulating microglial activity to states that reduce A β burden, reduce microgliosis/astrocytosis, decrease cytokine production, and minimize oxidative stress in the brains of mice with neurodegenerative disease [123].

Regenerative Medicine Approaches

Nanomaterials for Regenerative Medicine Approaches in PD

Currently, there is no cure for PD or AD, but efforts in regenerative medicine are aimed at promoting neuronal growth and axonal extension to repair cell damage of both diseases. To stimulate guidance of axonal growth and cellular survival, neurotrophic factors can be delivered via nanomaterials to extend their half-life and enhance delivery to the brain. Additionally, scaffolds not only provide a vehicle to deliver neurotrophic factors but are also an ideal physical structure with optimal microenvironment to promote cell growth of host neurons or implanted neurons. PLGA microparticles are one of the most widely used drug delivery biomaterials with biodegradable properties and FDA-approved applications. To address the short half-life of glial cell line-derived neurotrophic factor (GDNF) in vivo and the difficulties in delivery to the brain, Garbayo et al. encapsulated human recombinant glycosylated GDNF into PLGA microparticles created through a water–oil–water emulsion that is injectable and biodegradable. The microparticles were injected into the putamen of Parkinsonian monkeys with severe nigrostriatal degeneration. With a single administration, sustained GDNF levels in the brain were achieved, providing motor improvement and restoring dopaminergic function. Nine months after treatment, a bilateral increase in density of striatal dopaminergic neurons was observed with retrograde transport of GDNF to the substantia nigra [124].

GDNF is not the only neurotrophic factor known to enhance axonal growth, as Lampe et al. encapsulated both GDNF and brain-derived neurotrophic factor (BDNF) in microparticles with different release profiles per neurotrophic factor. Standard and carboxylated PLGA microparticles encapsulated the drugs to generate different release profiles as carboxylating the PLGA modifies the degradation profile to allow more rapid release of its drug. The PLGA microparticles were entrapped within a degradable PEG-based hydrogel. The hydrogels were formed into 7 mm strands (diameter 280 μm), where one 3.5 mm end contained carboxylate PLGA microparticles of 10 ng total GDNF that are released quickly at the caudal end and the other 3.5 mm end contained standard PLGA microparticles of 1 ng total BDNF at the rostral end based on therapeutic ranges of other studies using cultured primary neurons [125–127]. The hydrogel encapsulated microparticles were surgically implanted in rats, and results were compared to those of rats with sham surgeries as the control. BDNF was released slowly over 56 days with a bolus of GDNF around 28 days from the implanted devices. Rats that received implanted devices with both BDNF and GDNF had significantly reduced localized inflammatory microglial response relative to sham surgeries with the temporal release allowing GDNF to enhance initial transplant survival and BDNF to elicit axon extension over a period of months [128]. Hydrogel entrapments of microparticles with different release profiles present a unique temporal specificity in release of neurotrophic factors to aid in both long-term and short-term recovery.

Additional scaffolds loaded with GDNF have been studied in dopaminergic neuron protection and reconstruction in Parkinson disease models. Scaffolds not only provide a vehicle to deliver neurotrophic factors, but are also an ideal physical support with optimal microenvironment to promote regeneration. Ucar et al. loaded GDNF in collagen scaffolds and studied its protective effects on organotypic ex vivo slices modeled by axotomy, rotenone, and 6-ODHA. Collagen was crosslinked with PEG and loaded with GDNF (10 ng per 2 μ L). Placement of the collagen scaffold directly onto the organotypic brain slices yielded protected dopaminergic substantia nigra neurons, with notable enhanced tyrosine hydroxylase expression, indicating growth of dopaminergic neurons [129]. Additionally, collagen scaffolds loaded with GDNF have been compared to other biomaterial scaffolds in promoting reconstruction of the nigrostriatal pathway ex vivo. GDNF or phosphate-buffered saline (PBS) was loaded into collagen hydrogel scaffolds, microspheres, cryogels, and microcontact printing and applied onto brain co-slices. Fiber growth between co-slices was measured via TH staining after three weeks, with results indicating that collagen hydrogels with GDNF slightly promoted TH+ dopaminergic nerve fiber growth. GDNF loaded microspheres embedded within hydrogels did not provide improvement compared to control. However, cryogels alone or loaded with GDNF enhanced TH + fiber growth, along with lines of GDNF immobilized onto membrane inserts via microcontact printing which significantly improved TH+ fiber growth [130].

A composite scaffold with controlled release of GDNF promoted cell survival and axonal growth in vitro and in vivo. The composite scaffold was developed with electrospun poly(L-lactic acid) short nanofibers embedded within thermoresponsive xyloglucan hydrogel and injected into the brain. The Parkinsonian mice injected with the scaffolds had no deleterious effects on host immune response, enhanced survival of ventral midbrain grafts, and reinnervation of the striatum [131].

As hydrogel scaffolds provide favorable environments for neuronal growth and protection from host immune responses, the encapsulation of dopaminergic neurons in scaffolds for transplantation presents improved graft survival in Parkinson's disease. Functionalized hydrogels take advantage of the physical properties of the hydrogel as well as cell adhesion or binding properties of functional groups. Hyaluronic acid (HA)-based hydrogels functionalized with an arginylglycylaspartic acid (RGD) cell adhesion peptide and heparin via click-chemistry provided a scaffold for in vitro maturation of human pluripotent stem cell-derived dopaminergic neural progenitors. RGD peptide promotes cell adhesion and heparin binds to several neurotrophic factors, including GDNF, BDNF, pleiotrophin and fibroblast growth factor, so that the scaffold secures cells and takes advantage of immobilizing exogenous or cell-secreted growth factors [132]. Hydrogel scaffolds can additionally be structured to mimic the nigrostriatal pathway architecture with micro-columns of dopaminergic neurons [133]. This 3D scaffold promoted dopaminergic differentiation and neurite extension, such that five times more hydrogel-encapsulated dopaminergic neurons survived after transplantation in rat striatum compared to unencapsulated neurons harvested from commonly used 2D surfaces [132].

Scaffolds combining the neurotrophic delivery system described in the previous section with implantation of dopamine neurons have been studied as well in creating a more supportive microenvironment [134, 135]. GDNF loaded type I collagen hydrogels significantly increased survival, reinnervation, and functional efficacy of transplanted embryonic day 12 cells extracted from rat ventral mesencephalon in 6-OHDA lesioned hemi-Parkinsonian rats [135]. The hydrogel scaffold can additionally be structured to mimic the nigrostriatal pathway architecture with micro-columns of dopaminergic neurons. Stuzyna et al. created implantable hydrogel micro-columns that were seeded with dopaminergic neuronal aggregates with optimized lumen extracellular matrix, growth factors, and striatal neuron surrogate end targets to restore the lost dopaminergic neurons in the substantia nigra pars compacta and their axonal projections to the striatum. The constructs were microinjected to span the nigrostriatal pathway in rats, where survival of the implanted neurons was observed with maintenance of the axonal projections within the micro-column. Interestingly axonal extension was dependent upon lumen extracellular matrix (ECM), with collagen I and collagen I + laminin providing the longest outgrowth compared to crosslinked collagen, laminin-coated, and empty cores. However, axonal extension was not shown to depend on growth factor enrichment or neuronal end target presence in vitro [133].

Nanomaterials for Regenerative Medicine Approaches in AD

Regenerative medicine approaches to AD have largely employed self-assembling peptides and the stimulation of neurogenesis through neurotrophic factors. Conjugation with scaffolds increases efficacy of such treatments in the restoration of neurons.

Self-assembling peptides have the potential to improve the benefits of neural stem cell (NSC) transplantation to treat AD [37]. In fact, there is reason to believe that the stability of the polymersome membrane was much higher than that of liposomes. Alternating amino acid sequences can be designed to create a nanofibril scaffold that can support neuronal growth and differentiation. One such example is a PLGA-PEG polymersome with a bilayer that self-assembles in the presence of lactoferrin. Fluorescent imaging demonstrated effective transfer of such NPs into the brain when they were loaded with humanin, a peptide known to inhibit A β mediated cell death in AD. Such self-assembling NPs provided increased neuroprotection against overexpression of apoptotic proteins exhibiting neurofibrillary tangle pathology in neurons [136].

Another study by Guohong Cui and colleagues designed a self-assembling peptide modeled after laminin that could undergo spontaneous assembly into well-ordered nanofibers. Neural stem cell transplant under conditions of no peptide addition, peptide alone, or self-assembly peptide demonstrated that the self-assembly peptide in conjunction with NSC transplant most significantly increases neuronal survival, differentiation, and decreases apoptosis of CA1 hippocampal neurons in A β over-expression AD murine models. Additionally, expression of key synaptic modulators such as pre-synaptic protein synapsin-1, were restored. The self-assembly peptide also increased secretion of anti-inflammatory and neurotrophic factors including interleukin-10 (IL-10), BDNF, ciliary neurotrophic factor (CNTF), and insulin-like growth factor 1 (IGF-1) while simultaneously reducing expression of pro-inflammatory factors were decreased, such as TNF- α and IL-1 β [137].

Similarly, retinoic acid within the metal-organic framework was released to upregulate neuronal genes, creating a vital balance for neurogenesis without excessive glial response. Specifically, small interfering RNA (siSOX9) and retinoic acid were loaded into a metal-organic framework responsive to areas with high H₂O₂, a known marker of excess oxidative stress. This NP was able to amplify SOD and CAT key players in the first line antioxidant defense systems of cells, thus prolonging neuronal longevity [89].

Previous studies using RADA16-I and II (repeats of arginine and alanine) as scaffolds in immature mouse cerebellum and rat hippocampus cells showed successful extensive neuron outgrowth. This growth was sustained for more than 4 weeks and resulted in increasing numbers of active synaptic connections. The -IKVAV-penta-peptide, a laminin resembling peptide known to promote neurite sprouting and growth, when combined with -Glu- and A4G3-alkyl residues is another example of using self-assembling peptides in neuronal cell regeneration. In addition, -SKPPGTSS, -PFSSTKT, and RGD motifs combined with the RADA16-1 peptide increased the levels of nestin, β -tubulin, and other neuronal markers indicating increased neuronal regeneration. The self-assembling peptide nanofiber scaffold (SAPNS) composed of RADA16 resulted in reconnection of the injured spinal cord and facilitated axon regeneration and, eventually, locomotor functional recovery in animal models of spinal cord injury and acutely injured rat brain [138].

Theranostics

Antibody Functionalized Nanomaterials

Aside from direct treatment of AD and PD, nanomaterials have been established as agents for the enhancement of biosensors of these diseases, conferring additional biocompatibility, efficiency, and long-term stability to existing agents [139–142]. Coating with antibodies has been harnessed to improve specificity of biomarker detection. Antibody amendments to nanomaterials for detecting AD often target

pathological protein aggregates, including A β and tau protein. Oh et al. developed a carbon nanotube biosensor with proven antibodies derived from *E. coli* outer membrane with auto-displayed Z-domains of protein A. Able to detect A β in human serum at levels of 1 pg/mL, this nanobody may be applicable for early diagnoses of AD. Specificity of binding was enhanced through the use of antibody-binding proteins to coordinate immobilized antibodies [143]. Kurkina et al. developed a graphene-based nanomaterial for similar immunodetection of A β peptide through conjugation of a scalable reduced graphene oxide-based wafer with an anti-A β antibody [144]. Similarly, carbon nanotubes have been developed to detect tau protein using secondary antibodies for tau. Compared to conventional detection strategies, these nanotubes displayed 100 folds increased signal detection and increased specificity [145]. Efficacy of these nanomaterials has been demonstrated in buffers as well as more applicable media, including artificial cerebrospinal fluid and serum. Antibody efficacy in binding and detecting pathological protein aggregates was dependent on conditions (temperature, exposure period, etc.) of incubation, as expected for immunochemistry-based protocols. While less common, Amor-Gutierrez developed an immunosensor for p53, by incorporating immobilized antibodies onto AuNPs to allow specific binding to biotinylated p53. Specificity of binding was demonstrated through work with blood samples from mild cognitive impairment (MCI) and AD patients [140].

This work has been mirrored with α -syn in PD that has been demonstrated to mediate neuroinflammation, neurotoxicity, and the loss of dopamine neurons in patients. In 2010, An et al. reported an Au-doped TiO₂ nanotube microarray with the ability to immobilize primary antibodies for α -syn while retaining their stability and binding bioactivity. Through conjugation with secondary antibodies and glucose oxidase labels, sensitivity to α -syn was enhanced and the signal amplified [146]. Antibody based NPs have become increasingly specific for pathologic forms of α -syn. In 2018, Khatri et al. developed a NP capable of detecting fibrillar α -syn over its monomeric form [147]. In 2020, Tao et al. developed a graphene oxide immunosensor capable of oligomer-specific detection of α -syn in human plasma samples, the pathologic form of the protein [148]. Further, in 2020, Yin et al. designed a specific surface plasmon resonance based immunosensor capable of distinguishing phosphorylated α -syn from total α -syn [149]. Antibody immobilizations on NPs are largely coupled with AuNPs as discussed below. Antibody conjugated NPs of this kind have similar, yet enhanced bioaction in comparison to their nanomaterial counterparts [139].

Metal-Coated Nanomaterials

Metal coatings on NPs for AD and PD biomarker detection have largely been employed to enhance the conductivity, optical ability, and consequently, efficacy of constituent NPs. Among metal coated NPs, the most widely used are AuNPs due to their remarkable electrical and chemical properties, supplemented with excellent catalytic activity, conductivity, and biocompatibility [150–152]. Au coatings can be

combined with several other coatings and modifications to confer specificity in action. For example, several antibody-based NPs used for the detection of A β in AD are supplemented with an Au base. In this context, AuNPs serve as amplifying agents for immunoassays, amplifying the optical signal triggered by antibody-antigen binding. Liu et al. described AuNPs designed for the detection of A β protein based on an electrocatalytic reaction from O₂ to H₂O₂. Utilizing the sensitivity and selectivity of the AuNP surface chemistry, the authors were able to generate a regenerable and sensitive voltametric method for detecting A β peptides in a concentration dependent manner [153]. Carniero et al. developed yet another electrochemical immunosensor for A β , in which the use of AuNPs both allowed structured immobilization of antibodies and improved the electron transfer rate by reducing the electron transfer resistance [154]. For AD, this utility of Au plating has been replicated with antibodies detecting tau protein. While assays for detection of pathological species such as tau exist, detection at low concentrations remains an issue. Thus, Shui et al. employed AuNPs for signal amplification in their aptamer-antibody sandwich assay that utilized differential pulse voltammetry to record the signal response of tau-381 detection [155]. Similarly, AuNPs have been employed in conjugation with functional groups to detect pathological parkers of PD, focusing on α -syn. You et al. reported an interdigitated electrode biodetector with an anti-aptamer- α -syn as the probe, similar to the tau detector nanobody described previously, that utilized AuNP conjugation to enhance its detection limit to the level of 10 pM. The authors also stated that the use of AuNPs in this case reduced NP aggregation, increasing detection efficacy. Given the reliance of this biosensor on current changes upon the binding of α -syn to the aptamer, or “artificial antibody” probes, the authors reported utilizing AuNPs for its enhancement of relevant thermal and optical properties [156]. Similarly, Kumar et al. reported the association of gold nanorods with protein fibrils of the detection of α -syn, illustrating the variety of conjugatory agents in AuNP development [157]. All in all, AuNPs have been employed widely in conjugation with various probes to enhance current and conductivity-based approaches of pathological marker detection, across AD and PD.

In succession to Au coatings, silver coatings are also used in the development of nanobodies for the detection of AD and PD. Despite their significant catalytic enhancement, increased surface area, and high extinction coefficient, silver NPs (AgNPs) do not exhibit as exemplary stability and ease of functionalizability as AuNPs, and thus are not as widely employed for the development of biosensors [140]. In their applications, however, AgNPs can also be conjugated with various agents to allow detection of various relevant elements. Hu et al. reported an AgNP-based colorimetric immunosensor for the detection of A β . Exploiting interactions between Cu²⁺ and A β , this nanobody used the ability of the AgNPs to display a distance-dependent color based on proximity to A β to detect levels in samples. The use of Ag in the place of Au allowed the authors in this case to avoid Au-associated defects such as nonspecific absorption due to binding with amino groups [158]. AgNPs are accordingly often dependent on conjugation with other metals for colorimetric

detection of elements. Xia et al. developed an adamantine (Ad)-labelled AgNP with prion protein (PrP) peptides as the A β specific binding peptide for detection. The AgNPs served as the redox reporters while the Ad-PrP, interacting directly with A β , would unbind to the NP upon binding with A β . Thus, the AgNPs served as a secondary reporter, in which a decrease in their electrochemical signal was indicative of increased amyloid beta [159]. Most recently, You et al. in 2020 described a NP for the detection of A β oligomers, the specific pathologic agents in AD. While the NP was a conjugation with silica NPs, (SiO₂@AgNPs), the use of AgNPs in this case allowed the amplification of a small, highly sensitive electrochemical signal from a sandwich assay to a larger, detectable signal, allowing a limit of detection of 1.22 pg/mL [160]. AgNPs are much less widely described in the context of PD.

In addition to Au and Ag, other metals employed in the development of NP biosensors include iron (Fe) and more recently, metal–organic frameworks, that allow the combination of the properties of organic and mechanical properties to enhance biodetection and employ a wider range of metal composites [140, 161]. Further, metal-based magnetic NPs have been less widely used but are emerging technologies for biosensing. Utilizing their superparamagnetic behavior that may be influenced due to their small shape and size, magnetic NPs can utilize these changes to report biomarkers [139]. For instance, De la Escosura Muniz et al. described a nanobiosensor using porous magnetic microspheres with AuNP tags to detect and amplify a higher quantity of analyte and electrocatalytic label in comparison to non-magnetic microspheres. This signal detection was demonstrated to be specific to A β and apolipoprotein E (ApoE) [162].

Peptide and Oligonucleotide Functionalized Nanomaterials

While antibody conjugation and metal coatings remain the most prevalent applications for nanobody based biosensors, oligonucleotide and peptide coatings have also been used to detect pathological markers. Cheng et al. reported an AuNP-modified indium tin oxide NP able to detect DNA hybridization corresponding to a point mutation in the ApoE gene associated with an increased risk for AD. Utilizing an oligonucleotide addition to the NP, coupled with polymerase chain reaction (PCR), the authors were able to detect ApoE target DNA to the concentration of 10 fM [163]. Similar tools have been developed for the detection of ApoE mutations based on carbon nanomaterials, including a glassy carbon electrode based ratiometric electrochemical nanosensing platform developed by Wu et al. and curcumin-graphene quantum dots with dual electrochemical and fluorescent detection of ApoE4 developed by Mars et al., both of which utilize an immobilized DNA probe to identify the risk gene [164, 165]. While oligonucleotide-based therapies for PD are numerous, development of oligonucleotide biosensors for PD are scarce, likely due to the lack of association of PD with a single genetic mutation like ApoE for AD. Peptide based detection systems have been shown to be very specific and often also focus on detecting pathological protein aggregates in both AD and PD. Plissonneau et al.

developed gadolinium NPs functionalized with peptides LPFFD and KLVFF to allow high specific binding to A β fibrils [166]. As mentioned above, Xie et al. utilized the PrP protein conjugated to AuNPs to similarly detect A β [159]. In PD, Lee et al. reported a peptide-imprinted nanotube that was able to detect higher levels of α -syn in organoid models. Fashioned off a SNCA template molecule for biosensing, the SNCA peptide conjugated nanobody was able to detect α -syn levels in this model within the range of 0.065 pM to 0.65 nM [167].

Conclusion and Outlook

Nanotechnology presents several novel methods for the detection and therapeutic treatment of several pathologic mechanisms induced in PD and AD, including oxidative stress, protein fibrillization, neuroinflammation, and neurotoxicity. Customizability and accessibility of nanomaterials also allows their recruitment for early biodetection of these pathological hallmarks. Despite their great therapeutic potential, current and emerging challenges in NP research include concerns regarding the toxicity of NPs. While NPs can be neurotoxic, largely due to the production of oxidative stress [168], some NP biocompatibility improves with certain modifications. Further research is required to establish the long-term effects of candidate NPs and thoroughly assess NP toxicity prior to therapeutic development. Given the dependence of nanomaterial treatment on physical and chemical properties including size, shape, and composition, additional research on catalytic mechanisms of action depending on its varied properties is necessary to optimize the combination of these factors for treatment efficacy and delivery. Moreover, the biocompatibility and nanobio interactions of nanozymes should be further explored to ensure the safety and efficacy of nanozyme treatment [169, 170]. Altogether, nanomaterials present a rapidly growing field of a novel class of therapies to target neurodegeneration and associated mechanisms in previously untreatable disorders such as PD and AD (Tables 5.1 and 5.2).

Table 5.1 Nanotechnologies for the treatment of PD

Target process	Target molecule/ species	Therapeutic	Mechanism of action
Fibrillization	α -syn fibrils	Graphene quantum nanodots (GNDs) [41]	Inhibit formation, disaggregate fibrils, reduce transmission
		Cerium oxide NPs [42, 43]	Inhibit formation, disaggregate fibrils
		siRNA SVG-exosomes [46]	Inhibit expression of mRNA

(continued)

Table 5.1 (continued)

Target process	Target molecule/species	Therapeutic	Mechanism of action
		Nanobodies—NbSyn2, NbSyn87, Nb α -syn01, NbSyn87PEST [49–51]	Inhibit aggregation
		Nanobodies—PFFNB2 [52]	Disaggregate fibrils, prevent pathologic phosphorylation
Oxidative stress	Reactive oxygen and nitrogen species (RONS)	Antioxidant NPs—Cu ₂ O, CuO, Mn ₃ O ₄ , PtCu [84, 86, 87]	Rescue mitochondrial damage, scavenge free radicals
		Nanoemulsions—CoQ10, Vitamin E [97, 98]	Improve solubility and bioavailability of antioxidants
		Nanoliposomes [103, 104]	Enhanced delivery of antioxidants
		Exosomes [46]	Enhanced delivery of antioxidants
		Lipoic acid AuNPs [96]	Decreased ROS-generated cell damage
Inflammation	N/A	Ibuprofen nanospheres and nanoemulsions [115, 116]	Decreased microglial and astrocyte activation (neuroimmunological activity), reduced dopamine depletion
		Lipoic acid coated quercetin [117]	Increased BBB permeability and bioavailability, decreased neuroinflammatory markers
		Curcumin NPs [118, 119]	Decreased neuroinflammatory markers, reduced TH + dopaminergic neuron depletion
Regeneration	Neurons and axons	PLGA microparticles—GDNF [124]	Dopaminergic neuron restoration
		Hydrogels—GDNF, BDNF [125–128]	Axon extension, reduced inflammatory microglial response
		Scaffolds—GDNF [129, 130]	Physical support for growth, dopaminergic neuron restoration, axon extension

(continued)

Table 5.1 (continued)

Target process	Target molecule/species	Therapeutic	Mechanism of action
		Hydrogel scaffolds [132–135]	Axon extension, dopaminergic neuron restoration
Theranostics	α -syn fibrils	Antibody-coated NPs [146–149]	α -syn specific binding
	α -syn fibrils	Metal-coated NPs—AuNPs [156, 157]	Conjugation with functional groups, increased detection efficiency and conductivity, enhanced electrocatalytic reactions, decreased aggregation
	α -syn fibrils	Peptide functionalized NP—SNCA [167]	α -syn specific binding

Table 5.2 Nanotechnologies for the treatment of AD

Target Process	Target Molecule/Species	Therapeutic	Mechanism of Action
Fibrillization	$A\beta$ fibrils	AuNPs [53, 55]	Disruption of fibril deposits and growth, facilitate delivery of BCAs and functional and proteolytic groups
		CuO nanozyme [57]	Fibril deposit clearance
		Photosensitizing materials—Fullerene [64]	Denature monomers and oligomers
		Photosensitizing materials—carbon nanodots [65]	Inhibit aggregation
		Photosensitizing materials—graphitic carbon nitride nanosheets [66]	$A\beta$ oxidation via ROS generation, inhibit aggregation
		Photosensitizing materials—photodynamic micelles [68]	Degrade $A\beta$
		Hydrogels [69–71]	Slow aggregation via hydrophobicity, sequester excess $A\beta$

(continued)

Table 5.2 (continued)

Target Process	Target Molecule/Species	Therapeutic	Mechanism of Action
		Nanobodies—A4, E1, V31-1 [74–76]	Inhibit aggregation
		Nanobodies—Asec-1A [77]	Disaggregate fibrils
	Tau fibrils	Protein-capped (PC) metal NPs [58]	Inhibit formation, disaggregate fibrils
		Nicotinamide [62]	Inhibit tau hyperphosphorylation
		Nanobodies—F8-2, VHH Z60 [79, 80]	Inhibit aggregation
Oxidative stress	RONS	Antioxidant NPs—CuO, MIL-100 framework encapsulated CeO ₂ and RA [57, 171]	Rescue mitochondrial damage, scavenge free radicals, antioxidant activity
		Carbon-based nanoassemblies [90]	Antioxidant activity
		Nanoparticles—GNPs-LA [96]	Enhanced delivery of antioxidants across the BBB, ROS scavenging, rescue mitochondrial damage
		Hydrogels—chitosan, xanthan, and gellan gum formulations [110, 112]	Enhanced delivery of antioxidants
Inflammation	N/A	Coated quercetin [117]	Decreased neuroimmunological activity
		Quantum dot nanozymes [120]	Regulation of proinflammatory cytokines
		Timosaponin BII ISGs [122]	Decreased neuroinflammatory markers, increased M1 choline receptors
		PLGA NP [123]	Decreased microgliosis and astrogliosis
Regeneration	Neurons and axons	Self-assembling peptides—PLGA-PEG, laminin [136, 137]	Nanofibril scaffold to support neuronal growth + differentiation, decreased apoptosis, increase neurotrophic factor release

(continued)

Table 5.2 (continued)

Target Process	Target Molecule/Species	Therapeutic	Mechanism of Action
		siSOX9 metal organic framework NP [171]	Antioxidant prolonging of neural longevity
		Scaffolds—RADA16-1 and II [138]	Increased synaptic connections, neuronal regeneration, axon extension
Theranostics	A β fibrils	Antibody coated NPs—carbon nanotube, graphene nanomaterials [143, 144]	A β specific binding
	tau fibrils	Antibody coated NPs—carbon nanotubes [145]	Tau specific binding
	p53	Antibody coated NPs [140]	Biotinylated p53 binding
	A β fibrils	Metal-coated NPs—Au coated [153–155]	Immobilization of antibodies, enhanced electrocatalytic reactions
	A β	Metal-coated NPs—Ag coated [166, 167]	Colorimetric biosensing, amplification of electrochemical signals
	A β and ApoE	Metal-coated NPs—magnetic nanoparticles [162]	Sensitive changes in magnetic signal
	ApoE DNA	Oligonucleotide functionalized NPs [163–165]	Binding to mutated patient DNA
	A β fibrils	Peptide functionalized NPs—KLVDD, LPFFD, PrP [159, 166]	A β specific binding

Compliance with Ethical Standards

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Abbreviations

AD	Alzheimer's disease
PD	Parkinson's disease
α -syn	Alpha-synuclein
LB	Lewy body
A β	Beta-amyloid
ROS	Reactive oxidative species
BBB	Blood-brain barrier
NP	Nanoparticle
siRNA	Small interfering ribonucleic acid
GQD	Graphene quantum dots
PFF	Pre-formed fibrils
AuNP	Gold nanoparticle
RVG	Rabies virus glycoprotein
mRNA	Messenger ribonucleic acid
β Cas	Beta-casein
A β 42	Beta-amyloid 42
PEG	Polyethylene glycol
PC	Protein-capped
ELISA	Enzyme-linked immunosorbent assay
HDAC	Histidine deacetylase
SLN	Solid lipid particles
HA	Hyaluronic acid
ABP	A β oligomer binding protein
BBB	Blood-brain barrier
NO	Nitric oxide
SOD	Superoxide dismutase
POD	Peroxidase dismutase
MPP+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin
DPPH	2,2-Diphenyl-1-picrylhydrazyl
RA	Retinoic acid
GNP	Gold nanoparticle
GNPs-LA	Lipoic acid capped gold NPs
6-OHDA	6-Hydroxydopamine
CoQ10	Coenzyme Q10

EGCG	Epigallocatechin gallate
RES	Resveratrol
DHDP	Dihexadecyl phosphate
PA	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphate
BAX	Bcl-2 associated X protein
TH	Tyrosine hydroxylase
MNEI	Mucoadhesive nanoemulsion
APP	Amyloid precursor protein
PLGA-PEG	Pegylated poly(lactic-co-glycolic acid)
GFAP	Glial fibrillary acidic protein
Iba-1	Ionized calcium-binding adapter molecule 1
IL-8	Interleukin-8
MCP-1	Monocyte chemoattractant protein 1
COX-2	Cyclooxygenase 2
mPGES-1	Microsomal prostaglandin E synthase-1
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
GMO	Glycerol monooleate
ISG	In situ hydrogel
LPS	Liposaccharide
CA1	Cornu ammonis
PLGA	Poly(lactic-co-glycolic acid)
GDNF	Glial cell line-derived neurotrophic factor
BDNF	Brain-derived neurotrophic factor
PBS	Phosphate-buffered saline
RGD	Arginylglycylaspartic acid
ECM	Extracellular matrix
NSC	Neural stem cell
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin-10
CNTF	Ciliary neurotrophic factor
siSOX9	Small interfering RNA
SAPNS	Self-assembling peptide nanofiber scaffold
AuNP	Gold nanoparticle
AgNP	Silver nanoparticle
PrP	Prion protein
ApoE	Apolipoprotein E
PCR	Polymerase chain reaction
UV	Ultraviolet

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Chapter 6

Poly(Butyl Cyanoacrylate) Nanoparticles Deliver β -Nerve Growth Factor to the Brain After Traumatic Brain Injury



Yong Lin

Abstract *Introduction* Traumatic brain injury (TBI) is one of the main reasons for disability and death in patients. There is evidence showing exogenous nerve growth factor (NGF) can promote neuron axonal outgrowth and prevent injured neurons from dying. However, as a highly selective barrier, the blood–brain barrier (BBB) hampers most of the therapeutic drugs, including NGF, from entering the central nervous system (CNS) for TBI treatments. Some approaches bypassing the BBB, such as intracerebroventricular and intraparenchymal infusion, can help exogenous NGF to enter the brain, but such invasive routes may cause hemorrhage and infection in the brain. Since poly(butyl cyanoacrylate) (PBCA) nanoparticles can cross the BBB in vivo, these particles are suggested as a drug delivery vector to the CNS. In this chapter, the author reviews TBI treatments and challenges, BBB features, and how PBCA nanoparticles deliver therapeutic drugs such as NGF to cross the BBB for the treatments of CNS diseases including TBI. *Methods* To write this review, the author searched the biomedical literature database on PubMed of the National Library of Medicine and the scholarly literature via Google Scholar. The keywords the author used included TBI, NGF, BBB, drug delivery, PBCA nanoparticles, nanomedicine, and nanotoxicity. *Results* The intravenous NGF delivery to treat CNS diseases including TBI is still limited by the BBB. PBCA nanoparticles can help intravenously injected NGF to penetrate the BBB into the brain parenchyma, preserve its biological activity, and treat TBI and neurodegenerative disease efficiently. The mechanism underlying BBB crossing of PBCA nanoparticles is related to the transcytosis on the barrier mediated by apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLR). Also, PBCA nanoparticles are considered to be of low/no toxicity due to their biodegradation. *Conclusions* PBCA nanoparticles are efficient vectors for the delivery of NGF to the brain and treat TBI in animals.

Y. Lin (✉)

Brain Injury Center, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, 160 Pujian Road, Shanghai 200127, China
e-mail: yonglin1996@hotmail.com

Shanghai Institute of Head Trauma, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, 160 Pujian Road, Shanghai 200127, China

Keyword Central nervous system (CNS) · Traumatic brain injury (TBI) · Nerve growth factor (NGF) · Blood–brain barrier (BBB) · BBB breakdown · Drug delivery · PBCA nanoparticles · Apolipoprotein E (ApoE) · Low-density lipoprotein receptor (LDLR)

Traumatic Brain Injury

Overview

Traumatic brain injury (TBI), one type of brain injury disease, is induced by single or multiple exogenous mechanical force(s). It is a leading cause of morbidity and mortality in people worldwide [76, 97]. A recent model has estimated that there are as many as 64–74 million new cases of TBI happening all over the world each year [44]. Based on the criteria of structural brain imaging, the duration for the loss of consciousness, the duration of altered mental state, and the Glasgow Coma Scale (GCS), TBI severity can be classified as mild, moderate, and severe in the clinic [24, 129]. Among these criteria, GCS, with a range of scores from 3 to 15, is the most typical scale to evaluate the level of consciousness in TBI patients as per the functions of eye-opening, verbal response, and motor response [110, 125, 145].

In general, during the development of TBI, the brain experiences two phases of injury: primary brain injury and subsequent secondary brain injury [53, 120, 126].

Primary Brain Injury

Primary brain injury refers to focal and/or diffuse brain tissue damage at the moment of the mechanical impact. It includes the impact-induced broken blood vessels of the brain as well as damage to the somata and branches of neurons and glial cells. In the clinic, it presents intracranial hemorrhage, the contusion and laceration of the brain tissues, brain structure shifts, and diffuse axonal injury (DAI) [27, 190].

DAI, also known as traumatic axonal injury (TAI) [27], is featured by the widespread, multiple, and small lesions in the white matter composed of the bundles of axons. It is an axonal shearing (tearing) and stretching injury due to the sudden movement and rotation of the brain in the cranial cavity, which is induced by the biomechanical force of rotational acceleration or deceleration [51, 54, 149]. DAI marks a severe status of TBI. Patients with DAI are generally associated with GCS scores lower than eight [121]. A diagnosis of DAI can be made with radiographic imaging technology such as computed tomography (CT) scan and magnetic resonance imaging (MRI). Since primary brain injury occurs suddenly, it is unpredictable [121].

Secondary Brain Injury

Secondary brain injury can be initiated as early as minutes or hours after primary brain injury [66, 83, 167]. It is additional brain destruction as a consequence of a cascade of biochemical and pathophysiological responses to the injury, such as inflammatory responses [148], the breakdown of the blood–brain barrier (BBB) [107], excitotoxicity [70, 168], oxidative stress [30, 50], and mitochondrial dysfunction [122, 124]. These responses will additionally damage the neurons including their axons. The clinical manifestations associated with secondary brain injury include brain ischemia, brain edema, intracranial hemorrhage, increased intracranial pressure, seizures, and others.

Secondary brain injury can gradually develop over a period, such as hours, days, weeks, months, or years [61, 67, 113, 158, 188], which therefore offers a therapeutic window for clinicians to manage this phase and make secondary brain injury become preventable and even reversible. It is more important for TBI patients because secondary brain injury causes more serious and broader damage to the brain than primary brain injury [137, 138]. In general, the earlier treatments for TBI are carried out, the better outcomes can be achieved [5, 116, 153, 174]. The “golden hour” for TBI treatments is believed to be the first 60 min after TBI, for which these patients have better outcomes than those with delayed arrival [45, 174]. However, TBI patients still can benefit from the management within 120 min after the impact [45].

TBI Treatments and Obstacles

The treatments for TBI patients include three therapeutic strategies: surgery, medications, and rehabilitation. Currently, specific medications are not recommended for most mild TBI patients since many are slowly able to return to normal lives. For patients with severe TBI, emergency surgery may be needed to minimize further brain damage by removing intracranial hematoma, repairing depressed skull fracture, draining cerebrospinal fluid to reduce intracranial pressure, and executing craniectomy to relieve swollen brain. The medications are used shortly after TBI to treat TBI symptoms, reduce secondary brain injury, and promote the functional recovery of the injured brain. The rehabilitation therapy may start as early as TBI patients are medically stable, but it may last years or be lifelong.

For TBI patients, the most common route of the medications is systemic administration. However, since the BBB forms a barrier isolating the brain from the blood, intravascular large therapeutic molecules, such as nerve growth factor (NGF), are hindered in passing from the blood into the CNS for TBI treatments. This obstacle indeed limits the potential efficacy of the medications.

BBB Structure and Function

The BBB is a normal physical structure existing between the circulation system and the CNS. It is comprised of endothelial cells, the basement membrane, the foot processes of astrocytes, and tight junctions which seal adjacent endothelial cells [1, 40].

Unlike the endothelial barriers in other systems, the BBB is a relatively impermeable barrier. It blocks most molecules freely crossing para-cellular spaces due to its tight junctions. Some small substances, such as water, small chemical elements like oxygen and carbon dioxide, and small lipophilic molecules, can easily penetrate the BBB via a para-cellular pathway or a transcellular pathway including transcellular lipophilic pathway [2, 40]. On the contrary, most small and large hydrophilic molecules and even large lipophilic molecules cannot simply and passively diffuse through this lipid barrier into the brain parenchyma [63]. Instead, brain essential nutrients must depend on certain specific pathways, such as protein carrier-mediated transport, receptor-mediated transcytosis, and absorptive-mediated transcytosis, on the BBB for penetration. For example, the uptake and transport of water-soluble glucose and amino acids to the brain are mediated by the protein carriers on the endothelial cells of the BBB. In contrast, other molecules, including apolipoproteins (Apos) and insulin, take advantage of their corresponding specific receptors on the BBB, such as low-density lipoprotein receptor (LDLR) and insulin receptor, to get into the brain via a receptor-mediated transcytosis [2, 198]. To maintain an optimal ionic environment for the brain cells, such as the lower concentrations of K^+ and Ca^{2+} and higher levels of Mg^{2+} concentration in the CNS than those in the blood, ion traffics are restrictively controlled by specific ion transporters and channels on the BBB [2].

As a highly selective barrier, the BBB protects the brain from potential insults by the harmful substances in the blood. On the other hand, the complicated impermeable properties of the BBB also limit the therapeutic molecules from crossing the BBB and thereby reduce their treatment efficacy. Thus, scientists have been trying to develop various efficient drug delivery systems to fit the specific features of the BBB. Some promising drug vehicles such as nanoparticles have been made so far.

BBB Opening and TBI Treatments

Therapeutic BBB Opening for CNS Diseases

Given that the structure of the tight junction makes the BBB impermeable to most molecules, including drugs, it has been an attractive therapeutic strategy to increase BBB permeability for CNS disease treatments.

There is evidence showing that the therapeutic opening of the BBB increases certain molecules to cross the barrier [37, 140]. For instance, the 20 s infusion

of hyperosmotic mannitol solution via an internal carotid artery can quickly and reversely permeabilize the BBB and promote intravenously injected methotrexate, an anti-cancer agent and immunosuppressant, to enter the brain of the rat [37]. The mechanism underlying osmotic BBB disruption is believed to be that mannitol shrinks the endothelial cells to open the tight junctions to a width of 40 nm [92, 139]. Another study in the rat has shown after being infused into the internal carotid artery, bradykinin, a peripheral vasodilator acting on endothelial bradykinin β 2 receptor [136, 180], impairs the BBB and makes it permeable to horseradish peroxidase (HRP), a protein with a molecular weight of 44 kDa [140].

In the clinic, therapeutic BBB opening is also applied for the treatment of CNS diseases [4, 59, 141]. Data in patients have shown that the hyperosmotic disruption of the BBB by mannitol helps chemotherapeutic agents to treat human brain tumors and has achieved better outcomes [26, 59]. In addition, the clinical studies, based on patients with Alzheimer's disease and amyotrophic lateral sclerosis, have shown magnetic resonance (MR)-guided focused ultrasound is an efficient and reproducible tool to temporarily and focally open the BBB for drug deliveries to targeted brain areas [4, 141]. The mechanism may be related to the tight junction disruption, caused by the interaction between low-intensity ultrasound energy beams and intravenously injected microbubbles [141].

However, since therapeutic BBB opening may expose the brain to some potentially toxic substances in the blood, such an invasive strategy still has a risk of brain damage even though it temporarily and reversibly disrupts the BBB.

BBB Breakdown After TBI

Studies in the laboratory and the clinic have shown that while TBI causes brain tissue damage, it also disrupts the BBB [154]. In our previous study in the rat, we captured a moment when intravenously perfused Evans blue was leaking from a broken capillary into the brain parenchyma after TBI as evidence of direct mechanical damage to the BBB [107]. Moreover, BBB breakdown also occurs during secondary brain injury. It has been documented that shortly after TBI, activated neutrophils move out of the blood vessels and infiltrate into the injured site by secreting matrix metalloproteinase-9 to disrupt the BBB [184, 189].

There are a variety of reports on the time course of BBB opening following TBI in both the laboratory and the clinic [28]. In the laboratory, for example, a study based on the closed head injury model of mice has shown the elevation in the permeability of the BBB continues to day 4 after the injury [60]. In contrast, a quick opening and shutdown in the BBB are observed in the rat impact acceleration model of TBI, but the rat after lateral cortical impact shows an instantly disrupted BBB, which lasts 4 h [20]. In our previous study in the lateral fluid percussion TBI model of the rat, we found that a significant increase in BBB leakage to a contrast agent Gd-DTPA started 4 h after the injury, which was highly positively consistent with the increased number of the Evans blue-perfused brain capillaries at the injured site [107].

Moreover, the dramatically reduced blood supply at the epicenter is concomitant with the blood clots inside certain cerebral capillaries [107]. These findings indicate that BBB permeability might be related not only to BBB breakdown but also to the blood supply to the BBB-injured capillaries. Indeed, TBI-induced damage to the endothelial cells of the BBB can quickly activate the coagulation cascade [34]. Several studies have reported that intravascular coagulation and reduced blood flow are observed after TBI in animals [152, 164]. Also, the data in patients have shown that the intravascular thrombi play a more dominant role in the reduction in blood flow than cerebral vasospasm post-TBI [151, 186].

In the clinic, studies have also shown an increase in BBB permeability in TBI patients, which lasts days and weeks but may last months and years after TBI [67, 89, 176, 188]. For instance, with dynamic contrast-enhanced MRI, a clinical group has evaluated BBB breakdown in TBI patients by using the values of volume transfer constant (K^{trans}) and normalized permeability index (NPI). The results have shown that BBB permeability is significantly elevated in the lesion and the perilesional and nonlesional areas of the brain approximately 1–11 weeks after TBI. In some individual patients, BBB impairment is also noted approximately 5.5–10.5 months post-injury [188].

Like therapeutic BBB opening, the disrupted BBB after TBI may also provide a therapeutic window for drugs to cross the barrier for TBI treatments. Nevertheless, our previous study in the rat lateral fluid percussion model of TBI showed that although BBB permeability was increased post-injury, BBB opening was not wide enough to allow the large molecular agents, including horse radish peroxidase (HRP) and enhanced green fluorescent protein (EGFP, 29 kDa), to cross the BBB into the brain parenchyma [106]. The degrees of TBI-induced BBB breakdown in animals are affected by a variety of factors, such as the kind of TBI model used, TBI severity generated, impact angle, and time course post-injury [20, 28, 60]. In contrast, BBB breakdown may be more complicated in the clinic due to more unpredictable variables, including various head injuries, patient's age and health condition, and prehospital time. For example, in normal-aged people and patients with Alzheimer's disease, the endothelium degenerates, and the levels of the tight junction proteins are reduced, so BBB permeability is increased [48, 58, 75, 183, 199]. After TBI, greater BBB breakdown is supposed to be seen in these older people and patients than in other healthy people. Thus, it is a big challenge for clinicians to rely on TBI-induced BBB disruption to deliver drugs, especially large molecules, to the CNS.

Disrupted BBB AS a Therapeutic Target in TBI

There is evidence that BBB dysfunction alone is associated with the pathological development of the brain [154, 175]. For instance, a study has shown that BBB disruption initiates a delayed decrease in the volume and neuron number in the local cortex as well as elicits epileptic electronic signals in the rat BBB breakdown model, induced by bile salt sodium deoxycholate [175]. Moreover, BBB breakdown accounts

for the vasogenic edema of the brain after TBI, which peaks within 24 h post-injury [81, 195] It has been proposed that the restoration of disrupted BBB function will reduce the vasogenic edema in the brain post-TBI [47].

While bradykinin, causing BBB opening, plays a key role in vasogenic brain edema, the administration of the antagonists for bradykinin β 2 receptor is shown to decrease the brain edema after TBI [78, 165]. Accordingly, bradykinin β 2 receptor knockout mice present a dramatic reduction in brain edema and a better functional recovery than wild-type mice in a controlled cortical impact model of TBI [178]. In addition, studies have shown that melatonin, a matrix metalloproteinase-9 inhibitor, and tissue inhibitor of metalloproteinase-1 (TIMP-1) protect the BBB from impairment after TBI [9, 171], while exacerbated BBB permeability is seen in TIMP-1 knock-out mice after the brain injury [52].

In the clinic, however, during TBI treatments, most therapeutic strategies focus on normalizing the abnormal changes in the head, such as skull fractures, intracranial hematoma, brain edema, and axonal injury, but less involve the treatment of BBB impairment. The reasons may be due to the following: (1) The disrupted BBB in most patients can gradually recover in time after TBI [154]. (2) BBB opening may offer a therapeutic opportunity for drug delivery to the CNS [2]. (3) There is a lack of specific and effective drugs directly targeting the BBB [154]. (4) The side effects of the drugs limit their usage [8, 85]. For example, an analysis of the clinical trials on acute TBI has shown that the bradykinin β 2 antagonists may reduce intracranial pressure, which is related to brain edema, yet it does not significantly decrease mortality and severe disability in patients [85]. In addition, corticosteroids have been commonly used to treat TBI in the clinic for decades due to their multiple functions, such as anti-inflammation, stabilizing the impaired BBB, and reducing intracranial pressure. However, after reviewing 20 clinical trials with 12,303 participants, a study group found that corticosteroids can cause a significant increase in the number of deaths in acute TBI patients of all severities as compared to the controls without treatment. Thus, corticosteroids are not suggested to be used for TBI treatments [8].

To therapeutically target the disrupted BBB for TBI treatments, the drugs need to be more specifically targeting the BBB and have fewer side effects. Meanwhile, the clinical trials regarding the effects of BBB drugs on TBI treatments need to be more carefully performed and evaluated, e.g., the patients need to be classified into different levels of TBI severity; pre-admission time, time-to-treatment, and time-to-craniotomy are important for the analyses since the golden hour post-trauma is a key factor for mortality [45, 174].

NGF

Overview

NGF is the first identified neurotrophic factor in the neurotrophin family [103]. In addition to NGF, the neurotrophin family also contains brain-derived neurotrophic factor (BDNF) [18], neurotrophin-3 [112], and neurotrophin-4/5 [77, 156]. These members are functionally related proteins with high structural homology [143, 161]. Neurotrophin-6 and neurotrophin-7 have been cloned from teleost fish *Xiphophorus* and carp, respectively, but no homologous sequence of these two neurotrophins is found in mammals [57, 96, 156].

NGF is a protein complex with a molecular weight of 130–140 kDa. It consists of three non-covalently linked subunits of α , β , and γ at a ratio of 2:1:2 [19, 119, 192]. All the biological activity of NGF is derived from the β -subunit, an approximately 27 kDa dimer consisting of two 118 amino acid residues with three intramolecular disulfide bridges. NGF is essential for the development and survival of neurons. There are two receptors for NGF: high-affinity tropomyosin receptor kinase A (TrkA) as well as low-affinity and non-selective p75 neurotrophin receptor (p75NTR). The Trk receptor family contains the receptors Trk A, Trk B, and Trk C, which are for NGF, BDNF and neurotrophin-4/5, and neurotrophin-3, respectively.

NGF Secretion and Function

In peripheral tissues and the peripheral nervous system (PNS), NGF can be produced by various types of cells. These cells include non-neuronal target cells, such as vascular smooth muscle cells and endocrine cells, which are innervated by sympathetic and sensory neurons expressing NGF receptors [38, 161], Schwann cells, which are glial myelinating cells surrounding neurons and derived from neural crest [132], and immune cells, including mast cells and B cells [157, 177]. During development, target-derived NGF is transported from the target cells to the neurons by a mechanism of retrograde flow [55, 130]. The synthesized NGF is secreted from the postsynaptic membrane of a target cell and then binds to its receptor on the presynaptic terminal of an axon. After activating NGF receptor, NGF is retrogradely transported to the body of the neuron via the axon of the neuron.

In the CNS, NGF is mostly synthesized by the neurons, including cholinergic neurons, glutamatergic neurons, and γ -aminobutyric acidergic (GABAergic) neurons [23, 161], while glial cells, such as astrocytes and microglia, also produce NGF [74, 135].

NGF plays an important role in both immature and mature neurons. During ontogeny, NGF is essential to maintain the survival, differentiation, and maturation of the sympathetic neurons and sensory neurons in the PNS [104] as well as the cholinergic neurons in the CNS [72, 82, 105, 150]. Without NGF, these neurons undergo

programmed cell death causing a reduction in cell numbers [36, 86, 133, 169]. It is believed that the mechanisms underlying NGF absence-induced apoptosis are related to the activation of the mitochondrial pathway of the apoptosis, the dysfunction of the transcription factor NF- κ B, and the induction of the heterodimeric activator protein 1 (AP-1) transcription factor c-jun in the sympathetic neurons [91, 99, 111].

In contrast, in adults, the mature neurons become less dependent on NGF for acute survival, unlike the developing and immature neurons. However, NGF is still necessary for maintaining the structures and functions of mature neurons [144, 160]. For instance, NGF withdrawal can gradually cause morphology changes and death in adult neurons over months [144]. In addition, exogenous NGF can modulate the gene expression of the neurons and promote axon regeneration [35, 181, 191], e.g., NGF administration increases the levels of neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, a neurotransmitter and modulator of pain perception, in the mature sensory neurons of dorsal root ganglia (DRG) [109]. In the sympathetic neurons, NGF increases tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) [10, 130]. Pheochromocytoma (PC12) cells are a type of catecholamine cells derived from the adrenal medullary pheochromocytoma of the rat. They have been commonly used as a study model for neuronal differentiation. In vitro studies have shown that NGF elicits the differentiation and process outgrowth of the cultured PC12 cells via a TrK A receptor [108, 191].

NGF and Nervous System Injury

After peripheral nerve injury, the level of NGF is increased [161, 172] or gradually increased after a decrease [101]. In the CNS, an elevation in *Ngf* gene expression is noted after brain injuries including brain ischemia [65, 102, 117] and TBI [56, 131]. A clinical study has shown the level of NGF in cerebrospinal fluid (CSF) is correlated with the severity of TBI in childhood patients [31].

It has been reported that the administration of NGF can promote neuron survival, axonal regeneration, and behavioral functional recovery after injuries in the PNS and the CNS [39, 72, 84, 100]. For example, after brain injury, the cholinergic neurons experience degeneration and death, but NGF treatment can protect the neurons from atrophy and death [68, 93].

In addition, NGF also plays an important role in the protection of neurons against oxidative stress [12, 146]. While NGF depletion induces oxidative stress in PC12 cells, the antioxidant application reduces reactive oxygen species, promotes mitochondrial functional recovery, and maintains cell morphology [12]. Our previous study showed *Ngf* gene expression improved the survival of H₂O₂-damaged PC12 cells [108].

Challenges for TBI Treatment with NGF

For the treatment of brain injuries, there are several routes for the delivery of therapeutic molecules to the brain, including intracerebroventricular (ICV) infusion, intraparenchymal injection/infusion, intravenous injection, and intranasal administration [155, 173].

There is evidence that the ICV infusion is an efficient approach for the delivery of NGF into the brain. For example, a study has shown that after a single injection or continuous infusion into the ventricle, NGF can widely infiltrate into the adjacent brain tissues around the ventricular system in normal adult rats [15]. In an ischemia model of the neonatal rat, the injection of NGF via ICV protects the neurons from ischemic damage [71]. Similarly, clinical studies have shown that ICV infusion of NGF improves the symptoms in two infants with hypoxic-ischemic brain neonatal injury [31, 33]. In the TBI model of the rat, NGF infusion via ICV increases the activity of choline acetyltransferase in the brain [42]. However, the disadvantages of this invasive ICV route include the diffusion of infused NGF is limited at the adjacent brain tissues around the ventricle, especially in the adult brain, and that this approach makes the brain at risk for hemorrhage and infection. In addition, there is a report that the ICV infusion of NGF can cause hypophagia and weight loss in the rat [196].

Intraparenchymal injection/infusion is another efficient route to directly deliver NGF into the brain parenchyma [173]. In our previous study, we directly injected *Ngf* gene carried by a pseudo-lentiviral vector into the dentate gyrus of the hippocampus in the rat after TBI, which then successfully expressed *Ngf* gene in the hippocampus [108]. Nevertheless, this invasive route also might cause hemorrhage and infection.

Intranasal administration is a non-invasive route for drug delivery to the CNS. Both preclinical and clinical studies have shown that the intracranial administration of NGF can protect the brain tissue from injury after TBI [32, 114, 115]. The mechanisms involve bypassing the impermeable BBB [173]. However, the disadvantages of this intranasal drug delivery include the large dose of the drug needed to achieve effectiveness, the low bioavailability of the intranasal drug in the CNS due to its limited absorption in the nasal cavity, the poor permeability for hydrophilic drugs, and the low nose-to-brain delivery for proteins, etc. [7, 173].

In the clinic, for patients, especially for TBI patients, the intravenous injection is the main drug delivery route to the CNS because of its simplicity, safety, and rapidity. However, the biggest challenge for intravenously injected NGF to enter the brain parenchyma is that the highly selective BBB blocks NGF from crossing the barrier due to its large molecular weight. Therefore, the therapeutic effectiveness of NGF for brain injuries, including TBI, is seriously limited through this intravenous route. However, with the development of efficient BBB-crossing drug delivery vectors, such as nanoparticles, BBB impermeability to NGF can be overcome.

Nanoparticles

Nanoparticle Properties and the BBB Penetration

Nanoparticles are ultrafine particles with a nanoscale dimension [69, 147], although some references define the nanoparticles as particles ranging between 1–100 nm in diameter [98, 123, 182]. Multiple properties of nanoparticles, including size, charge, and surface modification, can affect BBB penetration of the nanoparticles [29]. Nevertheless, the nanoparticle sizes play a key role in BBB crossing. Indeed, cells have better uptake for smaller molecules than larger molecules [43, 94].

Several studies have shown that smaller nanoparticles exhibit better BBB penetration than larger nanoparticles [49, 62, 162]. For example, a study has reported that 2 and 24 h after being intravenously injected in mice, 20 nm insulin-coated gold nanoparticles have the highest amount in the brain as compared to 50 and 70 nm ones [22]. Likewise, in another study in mice, 20 nm gold nanoparticles present better outcomes in BBB crossing than 50 and 100 nm nanoparticles [170]. In contrast, the penetration of the nanoparticles with larger sizes, such as 400 and 500 nm, is limited by the BBB [62, 94].

Most studies have used nanoparticles with sizes ranging from 10 to 200 nm for brain drug delivery through the BBB [41, 106, 147]. The reasons are listed as follows: (1) Nanoparticles which are smaller than 5–10 nm are early and rapidly removed by the kidneys in the body [73, 142]. (2) The nanoparticles with a diameter larger than 200 nm can activate the complement system of the host, which causes these particles to be removed from the blood system [73]. (3) The maximal diameter of a formed endocytic vesicle within the cell is 200 nm during the process of clathrin-mediated endocytosis [29], one of the two types of endocytosis on the BBB [16].

However, just because the small nanoparticles are more capable of passing through the BBB than the large ones does not mean the small nanoparticles will deliver more drugs crossing the BBB. Since the small nanoparticles have less surface areas or smaller spaces for encapsulation, the amount of the drug loading on the small nanoparticles is lower than those on the large nanoparticles. Indeed, some studies have also used the nanoparticles with a dimension from 200 to 300 nm as drug delivery systems to the brain [90, 95, 128, 142].

The nanoparticles have broad categories for drug delivery to the CNS. They include polymeric nanoparticles, liposomes, dendrimers, metallic nanoparticles, quantum dots, and nanogels [69]. The polymeric nanoparticles are a core–shell structure with hydrophilic polymers forming an outside shell and the core containing a polymeric matrix. The drugs are either encapsulated in the core or absorbed on the surface of the polymer [6, 46]. One of the commonly used polymeric nanoparticles for drug delivery is poly(*n*-butyl-2-cyanoacrylate) (PBCA) nanoparticles, a type of biodegradable polymers.

PBCA Nanoparticles

Synthesis of PBCA Nanoparticles

PBCA nanoparticles are chemically synthesized from n-butyl-2-cyanoacrylate (NBCA), a liquid monomer, in a polymerization reaction. In the clinic, NBCA is a tissue adhesive used for surgical wound closure [14, 159] and the embolization of arteriovenous malformations [79, 118, 163].

In most of the studies, the mean diameters of the polymeric PBCA nanoparticles generated for drug delivery vary between 100 and 300 nm [25, 90, 95, 128]. Other studies have also included PBCA nanoparticles with sizes less than 100 nm and above 300 nm [142, 185]. In our previous experiment, we produced PBCA nanoparticles with an average size of approximately 150 nm. After loading the large molecules HRP and EGFP, PBCA nanoparticles were increased in size by 15.5% and 25.7%, respectively, which were still less than 200 nm [106].

It has been reported that during the PBCA nanoparticle synthesis, the properties of the nanoparticles are affected by several factors, such as pH values, temperatures, and stabilizers, used in the reaction [21, 200]. For example, under pH 2.5, PBCA nanoparticles show more clear morphology and better separation at the temperature of 65°C than those at 10 °C and 25 °C. In contrast, the optimal condition for the nanoparticle formulation below pH 3 is at 10°C and 25 °C but not 65°C. Moreover, at 25°C, better morphology of the nanoparticles is noted at the pH value of 3 compared with that at the pH 2.5 [21]. However, the significant difference on the size distribution of PBCA nanoparticles was not observed between 2.5 and 3 of pH values in our previous study [187].

Before being used for drug delivery, PBCA nanoparticles will be loaded with therapeutic drugs and then coated by polysorbate-80. Our in vitro study showed β -NGF could be gradually released from the complex of PBCA nanoparticles and β -NGF with polysorbate-8 over at least 7 days [187].

PBCA Nanoparticles as a Drug Delivery Vector for CNS Diseases Including TBI

There is evidence showing PBCA nanoparticles, coated with polysorbate-80, can deliver the drugs with small molecular weights to the normal brain after being systemically injected into mice and rats [11, 13, 128, 197]. Our previous data indicated that PBCA nanoparticles-transported HRP and EGFP could cross the BBB and enter the brain parenchyma in both normal and brain-injured rats [106]. Furthermore, PBCA nanoparticles also can deliver therapeutic drugs to treat CNS diseases [80, 134, 194]. For instance, PBCA nanoparticles-carried dopamine can improve the behavioral deficits in the animal model of Parkinson's disease, which features reduced dopamine levels [80]. The rat model of Alzheimer's disease also benefits from the

anti-Alzheimer's drug rivastigmine intravenously delivered by PBCA nanoparticles [194].

Studies have reported PBCA nanoparticles enable intravenously administered NGF to treat CNS diseases including TBI [95, 187]. For example, 45 min after polysorbate-80 coated PBCA nanoparticles-carried NGF is intravenously injected in the mouse, the highest amount of NGF is detected in the brain. Then, the NGF level is gradually reduced and almost returns to the normal level 24 h post-injection [95]. Moreover, the delivered NGF promotes the recovery of acute amnesia in the rat and improves Parkinsonian symptoms [95]. In our previous study, we found that intravenously administered PBCA nanoparticles could deliver β -NGF into the brain in the rat after TBI [187]. At 4 h after the intravenous injection of PBCA nanoparticles-carried β -NGF, the level of β -NGF was significantly increased in the injured brain compared with that after the injection of β -NGF alone. The elevation in the level of β -NGF continued to 24 h post-injection. To test whether β -NGF delivered into the brain by PBCA nanoparticles still maintains its biological activity after a series of in vitro loading and coating reactions and in vivo blood circulation, we extracted the total proteins, containing β -NGF, from the brains in the groups of both PBCA nanoparticles-carried β -NGF and β -NGF alone. We found that after being added to cultured PC12 cells, the brain proteins from the group of β -NGF delivered by PBCA nanoparticles could dramatically promote the process outgrowth in PC12 cells as compared to that from β -NGF alone. These results indicated that PBCA nanoparticles are not only an efficient drug delivery vector to the brain but also a protective reservoir for the drug to maintain its biological activity. Moreover, β -NGF delivered by PBCA nanoparticles also reduces the mortality in the rats after TBI [187].

It has been reported that ApoE in the blood and its receptor LDLR on the endothelial cells of the BBB are involved in the mechanism underlying BBB penetration of PBCA nanoparticles [64, 87, 127, 193]. After the drug-loaded PBCA nanoparticles, coated with polysorbate-80, are administered in the rats or mice intravenously, polysorbate-80 absorbs ApoE from the bloodstream onto the surface of PBCA nanoparticles. Then ApoE guides the nanoparticles to bind LDLR on the BBB to undergo an LDLR-mediated transcytosis for BBB penetration (as shown in Fig. 6.1). Without either polysorbate-80 or ApoE, PBCA nanoparticles fail to cross the barrier [87, 90].

Biodegradation and Toxicity of PBCA Nanoparticles

PBCA nanoparticles are regarded as biocompatible and biodegradable polymers. The cytotoxicity of PBCA nanoparticles should be related to the given dose of PBCA nanoparticles and how the nanoparticles are biodegraded. There is a study showing that below a therapeutic concentration of 500 μ g/mL, PBCA nanoparticles do not cause cell death and metabolic activity changes in the cells in an in vitro BBB model [88]. Also, an ex vivo experiment has shown that PBCA nanoparticles almost do not affect the release of the multiple inflammatory cytokines in human blood. Moreover,

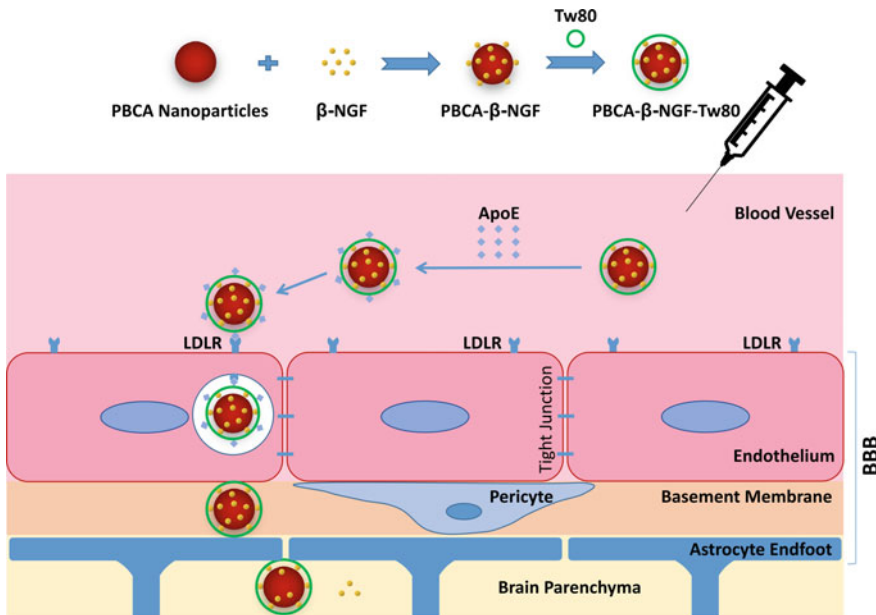


Fig. 6.1 Schematic of the synthesis of β -NGF-carrying PBCA nanoparticles coated with polysorbate-80 (Tween-80, Tw80) and BBB penetration. After being polymerized, PBCA nanoparticles are loaded with β -NGF to form a PBCA- β -NGF complex, which is then coated by Tw80. The synthesized complex of PBCA- β -NGF-Tw80 is intravenously injected into the animals. Tw80 of the PBCA- β -NGF-Tw80 complex absorbs the apolipoprotein E (ApoE) in the blood and then binds to the low-density lipoprotein receptor (LDLR) on the endothelial cells of the BBB. The PBCA- β -NGF-Tw80 complex penetrates the BBB into the brain via LDLR-mediated transcytosis. Finally, the complex gradually releases β -NGF into the brain parenchyma to function

no inflammation or organ damage is noted in the rat after the administration of PBCA nanoparticles [88]. These findings indicate that PBCA nanoparticles have no nanotoxicity under a controlled concentration. In addition, a report has shown that after being taken up by cells, PBCA nanoparticles can be degraded in the cells and release their payload within 24 h [166]. It indicates that unlike metallic nanoparticles [3, 17, 179], PBCA nanoparticles are biodegradable and do not accumulate in cells or organs.

Conclusions

As a neurotrophic factor, NGF plays multiple key roles in protecting the neurons from oxidative stress and death, promoting neurite outgrowth, and improving damaged brain functions. However, due to the blockage of the BBB to NGF, TBI patients hardly

benefit from systemically given NGF via an intravenous, intramuscular, or subcutaneous route. As an effective delivery vector to the brain, PBCA nanoparticles can help NGF to cross the BBB for TBI treatment.

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Disclosure of Interests

The author declares no conflict of interest.

Ethical Approval All procedures in our animal studies were approved by the Shanghai Jiao Tong University School of Medicine and performed following the Guide for Care and Use of Laboratory Animals.

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Chapter 7

The Significance of Biomaterials in Stem Cell-Based Regenerative Medicine



Meina Liu, Kai Pan, Zhikun Guo, and Zongjin Li

Abstract *Introduction:* Stem cell-based therapeutic strategies have shown tremendous potential in attenuating tissue damage and facilitating tissue repair and regeneration. The therapeutic benefits of stem cells are largely dependent on the establishment of a supportive microenvironment that optimizes cell survival, differentiation, and functional integration within host tissues. In this chapter, we will provide insight into the pivotal role of biomaterials in augmenting the therapeutic potential of cellular regenerative medicine and offer guidance for future advancements in this field. *Methods:* This chapter was written with the aid of comprehensive literature reviews found on PubMed and Web of Science Using Materials, Tissue Engineering, and Stem Cells, and websites dedicated to the topic of cell bioengineering. *Results:* Advances in biomaterials and stem cell research continue to lead to amazing discoveries. Nonetheless, it is clear that this area of research holds great promise, especially in elucidating the ability of biomaterials to enhance stem cell-intrinsic characteristics and modulate their immune microenvironment. *Conclusions:* Due to remarkable advancements in various disciplines, such as chemistry, materials science, and aerospace engineering, the amalgamation and advancement of biomaterials has gradually permeated the realm of stem cell therapy. These biomaterials offer dynamic and customized platforms to optimize cell-based regenerative strategies.

Meina Liu and Kai Pan are contributed equally to the present work.

M. Liu

Puyang People's Hospital, Puyang, China

M. Liu · K. Pan · Z. Guo

Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang, China

Henan Key Laboratory of Cardiac Remodeling and Transplantation, Zhengzhou Seventh People's Hospital, Zhengzhou, China

Z. Li (✉)

Nankai University School of Medicine, Nankai University, 94 Weijin Road, Tianjin 300071, China
e-mail: zongjinli@nankai.edu.cn

Tianjin Key Laboratory of Human Development and Reproductive Regulation, Tianjin Central Hospital of Gynecology Obstetrics, Nankai University Affiliated Hospital of Obstetrics and Gynecology, Tianjin, China

Keywords Biomaterial · Hydrogel · Stem cell · Regenerative medicine · Microenvironment

Introduction

Although hailed as a promising approach for treating degenerative injuries, the clinical application of stem cell therapy is impeded by limitations such as low survival and retention rates after cell transplantation [1]. Integration of biomaterials to establish a supportive microenvironment has shown great promise in optimizing the therapeutic effects of stem cells within damaged tissues or organs [2]. The exploration of the tissue microenvironment is revolutionizing our understanding of stem cell therapeutics through the co-transplantation of synthetic or tissue-derived biomaterials [3]. Biomaterials serve as scaffolds that provide structural support to stem cells and assume a pivotal role in tissue repair and regeneration.

The widespread use of biomaterials in the field of cell regenerative medicine is rooted in their multifunctionality as scaffolds, carriers, and templates to facilitate local tissue regeneration and repair, thus enhancing the efficiency and therapeutic efficacy of cell-based interventions [4]. Moreover, biomaterials exhibit favorable biocompatibility and biointeraction properties, enabling their adaptation to host tissues, thus mitigating rejection and minimizing adverse effects, thereby enhancing the reliability and safety of cell regenerative medicine. Consequently, biomaterials have emerged as a key research avenue in the realm of cell regenerative medicine, playing an indispensable role [5].

Enhanced Therapeutic Potential of Stem Cells with Biomaterials

Stem cells possess remarkable potential to differentiate into various cell types, making them promising candidates for treating diseases associated with declining bodily functions. Their ability to evade immune rejection after transplantation has facilitated the development of cell-based therapies [6]. However, the clinical outcomes of cell transplantation therapy are hindered by inherent obstacles [7]. Key limitations include low retention rates of transplanted cells, their limited survival and proliferation in hostile microenvironments, and the detrimental effects of oxidative stress and elevated cytokine levels at the injury site [8, 9]. Moreover, *in vitro* pretreatments may optimize stem cell function [10]. To address these challenges, various strategies have been developed to improve stem cell therapy, focusing on improving cell retention and viability through bioengineering approaches [11]. Researchers have explored different approaches, including the use of biomaterials, growth factors, and tissue-derived extracellular matrix (ECM), to improve the function and survival of

transplanted cells [12]. These strategies offer tunable biophysical and biochemical properties that support the maintenance and differentiation of transplanted cells [13].

Biomaterials serve as versatile platforms for encapsulating, fixing, or capturing cells, enabling their transport and localization to specific target sites [14]. These delivery systems play a crucial role in protecting transplanted cells from immune surveillance, mechanical stress, and shear forces during transplantation, thus improving cell viability and engraftment (Fig. 7.1). Biomaterials can also regulate the behavior of stem cells by incorporating signaling molecules such as growth factors, cytokines, and peptides [15]. These bioactive molecules can be incorporated into biomaterials to precisely control and guide stem cell differentiation, proliferation, and migration. By manipulating the kinetics of release and concentrations of these signals, researchers can orchestrate the desired cellular responses, promoting tissue regeneration and functional restoration [9, 16].

Combining engineered materials with stem cell transplantation has shown promising results in enhancing functional recovery and improving structural integrity. This can be attributed to its ability to stimulate angiogenesis, improve the local microenvironment, and create a favorable environment for tissue regeneration [17].

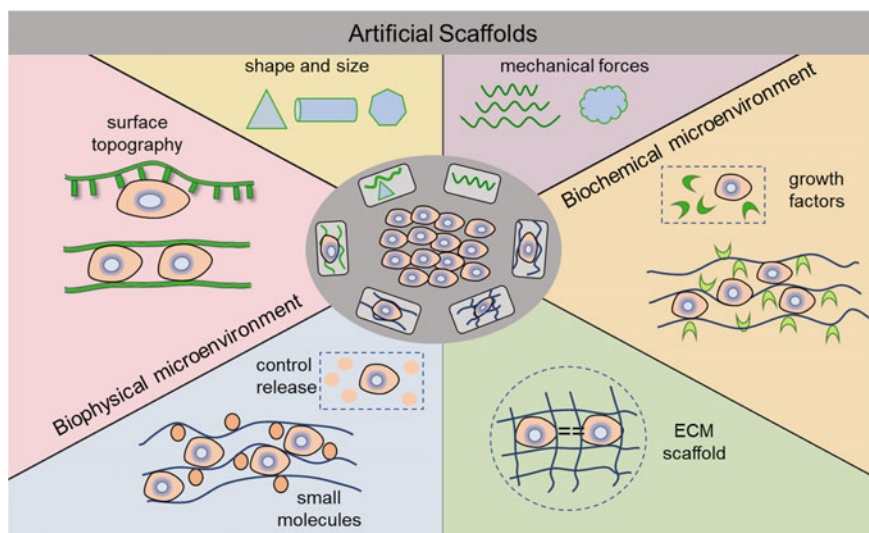
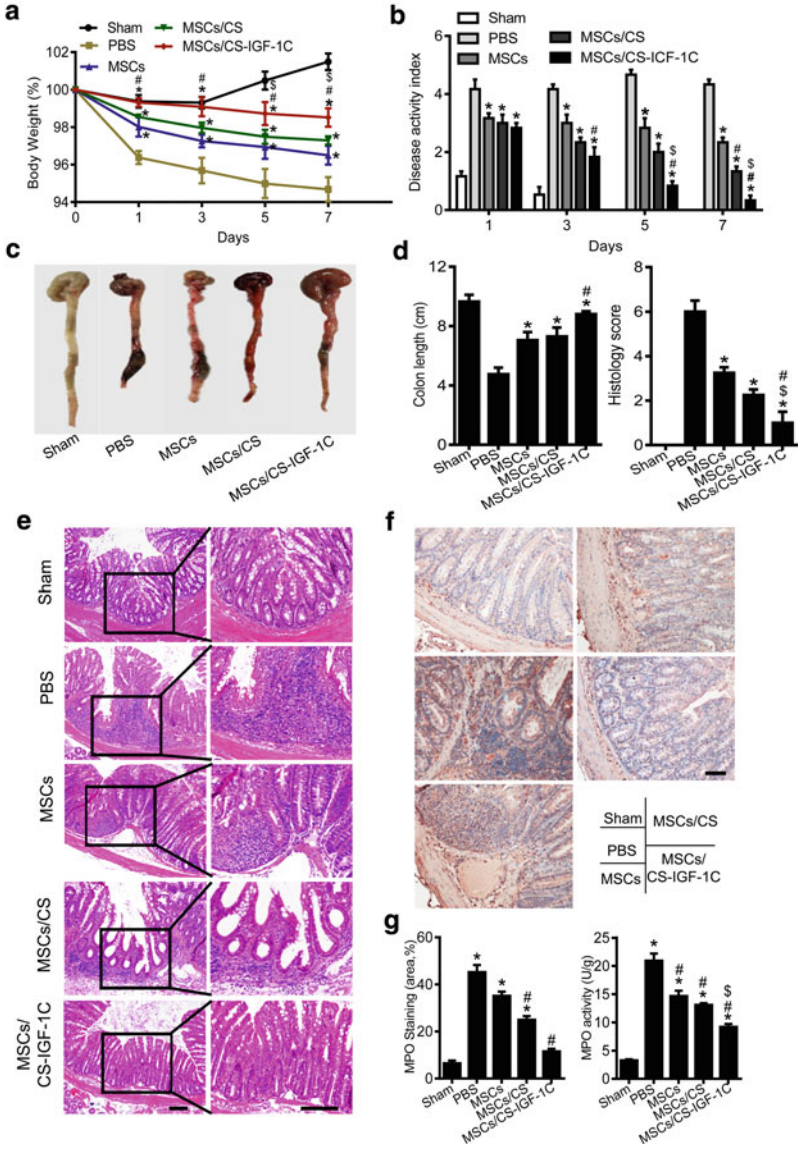


Fig. 7.1 Strategies for artificial scaffolds for cell enhancement. Engineered biomaterials, designed to mimic the biochemical and biophysical properties of the cellular microenvironment, have been widely utilized in stem cell-based therapies to enhance cell maintenance *in vitro* and *in vivo*. These biomaterials incorporate biophysical cues such as surface topography, material shape and size, and mechanical forces, as well as biochemical cues through growth factor conjugation and controlled release of specific small molecules and tissue-derived extracellular matrix scaffolds. The implementation of these strategies, which aim to regulate cell behavior, is crucial in increasing therapeutic effects following cell transplantation. Reproduced under a Creative Commons Attribution 4.0 International License from Stem Cell Res Ther 2021;12(1):583



◀**Fig. 7.2** The therapeutic effect of hP-MSCs facilitated by the CS-IGF-1C hydrogel in a mouse model of colitis. First, the percentage of body weight loss after treatment was evaluated. Second, the Disease Activity Index (DAI) score was calculated on day 7 and included indices for weight loss, bloody stool, and stool consistency. Macroscopic images of colon tissues were taken at day 3 after treatment and the length of a representative inflamed colon was quantified. Histological sections of mouse colons were stained with H&E for evaluation. Immunohistochemical staining for myeloperoxidase (MPO) was performed to assess neutrophil activity in the distal colon and an MPO immunohistochemistry score was determined. The experimental groups included hP-MSCs/CS, where hP-MSCs were co-transplanted with CS hydrogel, and hP-MSCs/CS-IGF-1C, where hP-MSCs were co-transplanted with CS-IGF-1C hydrogel. All experiments were carried out in triplicate and the results are presented as the mean \pm standard deviation (SD). Statistical significance was determined by comparisons against PBS, hP-MSCs, and hP-MSCs/CS groups using appropriate statistical tests (* $P < 0.05$ versus PBS; # $P < 0.05$ versus hP-MSCs; § $P < 0.05$ versus hP-MSCs/CS). Reproduced under a Creative Commons Attribution 4.0 International License from *Theranostics*. 2020;10(17):7697–709

Therefore, the integration of biomaterials and stem cell therapy offers a synergistic effect, promoting tissue repair and regeneration. In a recent study [18], researchers investigated the potential of a chitosan (CS)-based injectable hydrogel with immobilized IGF-1 C domain peptide (CS-IGF-1C) to protect human placenta-derived mesenchymal stem cells (hP-MSCs) and enhance their therapeutic efficacy in promoting colonic recovery. Hydrogels created a microenvironment that facilitated cell survival, increased prostaglandin E2 (PGE2) PGE2 secretion, accelerated macrophage polarization, upregulated interleukin-10 (IL-10) expression, and mitigated inflammation. The study aimed to test the hypothesis that the IGF-1C hydrogel could amplify the therapeutic effects of MSCs in experimental colitis by inducing PGE2-mediated polarization of M2 macrophages. Understanding the immune responses and signaling pathways involved in tissue repair and regeneration is crucial, with particular emphasis on the roles of IL-10 and macrophages in reducing colonic inflammation. To evaluate the therapeutic potential, the CS-IGF-1C hydrogels were co-transplanted with hP-MSCs at the site of 2,4,6-trinitrobenzene sulfonic acid (TNBS) -induced colitis. Furthermore, the study examined whether this co-transplantation approach could ameliorate colitis by promoting the release of PGE2, thus facilitating M2 macrophage polarization. These processes were associated with an increase in IL-10 production, leading to reduced levels of M1 macrophages and pro-inflammatory cytokines (Fig. 7.2). Therefore, the application of biomaterials can greatly enhance the therapeutic effect of stem cells themselves, and improve the microenvironment of in-situ immunity.

Despite the progress made in stem cell therapy and biomaterial-based delivery systems, further comprehensive studies and validation are needed to determine the most optimal materials and strategies for long-term and successful application. Addressing challenges such as low cell retention and engraftment rates, as well as poor long-term maintenance of stem cell function, requires the development of supportive microenvironments that activate or enhance the reparative abilities of transplanted cells [19, 20]. Consequently, ongoing research in this field has great potential for advancing tissue regeneration and expanding the therapeutic options available for various diseases.

In summary, biomaterials form the foundation of regenerative medicine by addressing critical challenges related to cell delivery, survival, integration, and differentiation. They provide a versatile platform for the delivery of therapeutic cells, ensuring their protection and localization to the target site. Additionally, biomaterials create a supportive microenvironment that improves cell survival, integration, and functionality within host tissue. Through the incorporation of bioactive cues, biomaterials also guide stem cell differentiation toward the desired lineages. By addressing these challenges, biomaterials contribute significantly to the success and efficacy of cell-based regenerative therapies, paving the way for the advancement of regenerative medicine as a transformative healthcare approach.

Recapitulating the Regenerative Microenvironment Through 3D Cell Culture

Stem cells residing within three-dimensional (3D) microenvironments, commonly referred to as niches, are surrounded by neighboring cells, components of the extracellular matrix, and crucial cell factors. These microenvironments play a vital role in supporting stem cell function after transplantation [21]. The dynamic nature of these niches influences various fate-determining events, including self-renewal, quiescence, and differentiation, as well as physiological processes such as development, homeostasis, and response to injury [22].

Despite the potential benefits offered by stem cell transplantation, there are challenges associated with the *in vivo* environment that can impede the retention and engraftment of transplanted cells. Transplanted stem cells often face harsh conditions, such as ischemia and hypoxia, leading to increased apoptosis and reduced viability and graft efficiency, ultimately impacting the success of clinical applications [23]. Furthermore, damaged tissues often lack structural integrity, necessitating the use of engineered scaffolds to establish functional artificial niches for stem cell-based therapies. To overcome these challenges, there is a pressing need for advances in regulating stem cell behavior, viability, and retention [24]. Replicating the native microenvironment becomes crucial for directing stem cell fate decisions and controlling cellular behavior. This involves manipulating complex cellular structures, properties, and biochemical and biophysical signals. Therefore, the development of engineered bioactive scaffolds capable of mimicking niche-like 3D microenvironments, with desirable degradation and remodeling properties, holds promise in enhancing the therapeutic efficacy of stem cell-based treatments [25].

A crucial aspect of 3D cell culture is the ability to mimic the structural organization of native tissues. *In vivo*, cells reside in a three-dimensional network of extracellular matrix (ECM), which provides physical support and biochemical signals for cellular activities. Biomaterials, such as hydrogels or scaffolds, can be used to recreate this ECM-like environment in 3D cell culture systems [26]. These biomaterials can be engineered to possess specific mechanical properties, porosity, and

biochemical composition, closely resembling the microenvironment of the native tissue and providing structural support for cell growth.

Advancing stem cell-based therapies requires addressing the challenges associated with the *in vivo* environment. The dynamic nature of 3D microenvironments and their influence on stem cell fate decisions highlight the importance of replicating native niches. By developing engineered bioactive scaffolds that mimic these niche-like microenvironments, researchers can improve stem cell behavior, viability, and retention. Such advances have the potential to significantly improve the therapeutic efficacy of stem cell-based treatments. Three-dimensional (3D) spheroid culture is a valuable tool for studying *in vivo* biology and therapeutic applications of human MSCs [27]. While traditional two-dimensional (2D) plastic adherence is commonly used to define MSCs, it is recognized that 3D non-adherent conditions better recapitulate the native *in vivo* niche of MSCs within tissues and organs. By promoting cell–cell and cell-extracellular matrix interactions, 3D spheroid culture enhances our understanding of the physiological processes and offers significant therapeutic advantages. Continued research and development in this area will contribute to the progress and success of stem cell therapies.

Biomimetic Materials for Cell Enhancement and Immunomodulation

Biomaterials have made significant advancements in clinical applications and serve as a valuable platform for creating artificial niches to control stem cell cultivation and differentiation. Through their composition and structure, biomaterials deliver precise biochemical signals that influence stem cell behavior during tissue repair [28]. Key parameters such as phenotype, chemical composition, and physical properties are essential considerations in guiding stem cell fate. Surface topography and chemical composition have been extensively studied for their impact on cell adhesion, differentiation, migration, and proliferation. Moreover, the integration of scaffolds with favorable properties capable of activating specific biological responses while maintaining their performance is pursued to meet engineering demands [29]. Bionanocomposites, incorporating conductive nanostructures, have emerged as vital tools to improve the mechanical and functional characteristics of synthetic polymers [30].

Enhancement of Stem Cell Function with Nanocomposites

The integration of carbon nanostructures (e.g., nanotubes, graphene, and nanofibers) and metal nanostructures (e.g., gold, silver) into bio-nanocomposites enables the

acquisition of electrical properties [31]. These nanostructures facilitate the development of biofunctional scaffolds with desired characteristics, such as bioactivity, structural integrity, mechanical stability, and electrical conductivity. For example, the incorporation of multi-walled carbon nanotubes (MWCNTs) into a nanocomposite scaffold has shown promising results, enhancing its biodegradability and biological activity [32]. Additionally, factors such as interconnectivity, pore size, shape, and scaffold morphology are critical in stem cell biomaterials, ensuring optimal cell attachment, differentiation, maintenance, migration, and production of bioactive substances.

Bioactive Scaffolds that Mimic Native Niches

Biomaterial scaffolds that mimic the morphology and function of surrounding tissues while enhancing various cell functions are described as artificial scaffolds. These scaffolds provide a conducive environment for stem cell culture, protection, and interaction. Mimicking the native microenvironment is crucial to directing stem cell fate decisions and controlling cellular behavior. Various biomaterials, such as thermosensitive hydrogels and hydrogels based on hyaluronic acid, have been employed as injectable scaffolds for stem cell delivery, improving functional recovery in tissue repair and regeneration [33]. The appropriate morphology and microstructure of biomaterial scaffolds are essential to study cell differentiation and tissue responses.

The integration of stem cells with engineering approaches has emerged as a promising strategy in regenerative and translational medicine, capitalizing on the superior biophysical and biochemical properties of functional materials [34]. Three-dimensional (3D) MSCsl, compared to traditional two-dimensional (2D) culture, have been recognized as an effective culture system that enhances the multidifferentiation potential of MSCs [35, 36]. In this context, thermosensitive hydrogels based on chitosan (CS) have gained significant attention as ideal 3D injectable scaffolds for stem cell delivery in tissue repair and regeneration applications, owing to their ability to undergo a liquid-to-gel transition at room temperature and their excellent biocompatibility. Thermosensitive hydrogels, particularly those incorporating CS, have demonstrated substantial utility in facilitating stem cell delivery for tissue repair and regeneration. For example, CS-coated conduits and neurospheres combined with adipose-derived stem cells (ADSCs) have shown favorable outcomes in promoting functional recovery after sciatic nerve injury [37]. The use of CS-based hydrogel scaffolds improved ADSC delivery for the treatment of burn wounds, leading to significant improvements in neovascularization, wound closure, and scar formation reduction [38]. These examples highlight the efficacy of biocompatible scaffold materials in mitigating detrimental injuries, creating nurturing and protective environments, and augmenting the therapeutic effects of implanted cells.

Biomimetic Materials for Controlled Delivery

Biomimetic materials, acting as scaffolds or carriers, facilitate the controlled delivery of bioactive and therapeutic molecules in tissue engineering. The study of integrin-mediated cell adhesion and activation is crucial for stem cell survival. Natural alginate and self-assembled peptide bioscaffolds provide specific binding sites for cell adhesion, making them excellent choices for hydrogel-based 3D scaffolds [39, 40]. Huang et al. presented a new injectable collagen matrix that was synthesized by chemical conjugation of prostaglandin E2 (PGE2) with the collagen matrix [41]. This PGE2 matrix exhibited a substantial increase in the half-life of PGE2 both in vitro and in vivo. In addition, it demonstrated notable enhancements in tissue perfusion by stimulating angiogenesis and improving microcirculation. Furthermore, the PGE2 matrix exhibited significant potential to promote skeletal muscle regeneration by activating myod1-mediated skeletal muscle production (Fig. 7.3). This finding suggests that the developed PGE2 matrix holds promise as a delivery scaffold for improved tissue vascularization and cell regeneration. These scaffolds enable stem cell differentiation and reduce inflammatory responses after transplantation. They contribute to anchorage-dependent pathways for cell survival and proliferation, improving tissue formation.

Immunomodulation in Stem Cell-Based Therapies

The immunomodulatory roles of innate and adaptive immune cells in tissue repair and regeneration have gained attention. The interplay between immune cells and stem cells is crucial to achieving successful outcomes. Biomaterial surface-adsorbed proteins can alter the state of immune cells by influencing their adhesion, apoptosis, and secretion of cytokines. Combining biomaterials with specific factors, such as prostaglandin E2 (PGE2), can alleviate inflammation and promote desirable immune responses [42, 43]. Similarly, the application of a combination of prostaglandin E2 (PGE2) and chondroitin sulfate (CS) hydrogel in a mouse skin wound healing model has shown enhanced therapeutic effects, providing evidence of improved conditions at the injury site. To guide tissue repair and regeneration, it is crucial to maintain a balanced cytokine and mediator profile during the different stages of wound healing, ensuring that appropriate levels of inflammation, regeneration (angiogenesis) and remodeling (fibrosis) coincide. Furthermore, a study involving mesenchymal stem cells (MSCs) revealed that manipulating specific macrophage immune phenotypes through gelatin/polyethylene glycol-based matrices significantly enhances adipocyte differentiation and subsequently facilitates normal wound healing. By employing cutting-edge developmental engineering strategies, it is possible to construct suitable microenvironments for stem cell-based therapies, protein replacement approaches or gene therapy, offering potential solutions for previously untreatable conditions such

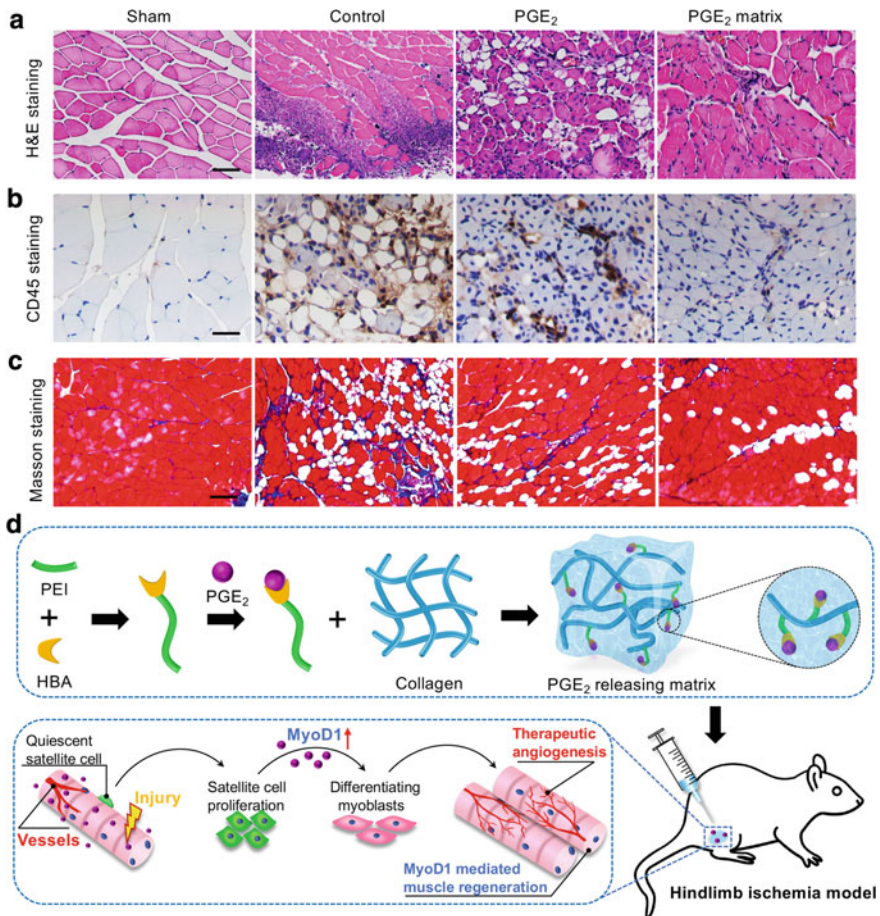


Fig. 7.3 The PGE₂ matrix promoted the recovery of hindlimb in a mouse model of hindlimb ischemia. Representative histological images of muscle tissue treated with different interventions were obtained on day 14. A shows H&E staining and B shows anti-CD45 immunohistochemical staining. C Masson's trichrome staining of muscle sections was performed on day 28. D A schematic diagram illustrating the PGE₂ release matrix was included, highlighting its ability to release PGE₂ with therapeutic potential for the treatment of hindlimb ischemia. The intended therapeutic effects include promoting neovascularization and MyoD1-mediated regeneration of skeletal muscle. Reproduced under a Creative Commons Attribution 4.0 International License from J Nanobiotechnology. 2022;20(1):95

as rare genetic disorders such as epidermolysis bullosa (EB) and bone transplantation [44].

Immune cells play a crucial role in tissue repair and regeneration processes after implantation or injury, as they secrete cytokines that promote the degradation of the extracellular matrix and debris clearance [45]. Regulation of cytokines and mediators during in vivo wound healing can direct tissue repair and regeneration. In addition,

the properties of biomaterial implants, including physical, chemical, and biological signals, play a crucial role in influencing immune cell responses. The design of appropriate implant conditions can regulate the anti-inflammatory response, facilitating tissue recovery [46, 47]. Modifying the chemical components of biomaterials allows for control of immune cell adhesion and cytokine production. Biomaterials serve as delivery systems and scaffolds to regulate inflammation and maintaining tissue homeostasis [48].

Biomaterials have emerged as versatile tools in stem cell-based therapies, facilitating precise control over stem cell behavior and enhancing their functionality. Advancements in bionanocomposites, bioactive scaffolds, and immunomodulation strategies contribute to the development of biomaterials that mimic native niches and promote stem cell differentiation and proliferation.

Recent Advances in Biomaterials for Stem Cell Therapy

Biomedical materials have gained significant importance in the field of medicine due to their exceptional biocompatibility, biodegradability, and low inflammatory response [49]. These materials are used to detect damaged tissues, repair and enhance human tissues and organs, serving as natural or artificial substitutes for living tissues. While they cannot restore lost parts, biomedical materials can perform, enhance, or replace certain lost functions caused by diseases or injuries, making them invaluable for therapeutic applications [50].

Characteristics of Ideal Biomedical Materials

Ideal biomedical materials should have specific characteristics to optimize therapeutic outcomes. First, they should have good tissue compatibility, ensuring compatibility with surrounding tissues without causing adverse reactions [51]. Second, these materials should have an appropriate three-dimensional structure that mimics natural tissues or the extracellular matrix, providing a suitable environment for cell growth and function [52]. Controlled biodegradability is essential to ensure that materials degrade into harmless small molecules that can be metabolized or absorbed by the body [53]. In addition, favorable cell-growth interfaces are crucial, enabling cells to adhere and proliferate effectively on the surface of the material, thus promoting tissue regeneration [54]. Finally, satisfactory plasticity and mechanical strength are required to withstand physiological forces. Biomedical materials derived from natural sources or resembling natural tissues and extracellular matrix play a crucial role in tissue repair and replacement by maintaining the dynamic processes of the human body [55, 56].

Advancements in Stem Cell Therapy and Engineering Platforms

Research in stem cell therapy has made some encouraging progress, and with many strategies are advancing to the clinical research stage. Stem cells possess regenerative capabilities and can promote tissue-specific regeneration. For example, corneal epithelial stem cell transplantation and mesenchymal stem cell transplantation have shown promising clinical outcomes in the treatment of ocular burns and graft-versus-host disease [57].

In addition, engineering platforms such as biomaterials, growth factors, and extracellular matrix (ECM) have been widely employed in stem cell therapy to provide a favorable microenvironment for stem cells. These engineering platforms can serve as scaffolds or carriers to deliver bioactive agents and therapeutic molecules to promote tissue regeneration [58]. By modulating protein adsorption on the surface of biomaterials, immune and inflammatory responses can be regulated, thereby influencing the fate of stem cells. Furthermore, incorporation of specific components such as chitosan and prostaglandin E2 into hydrogels can alleviate inflammation, promote macrophage polarization towards an anti-inflammatory phenotype, and improve wound healing outcomes [59].

In general, the development of an appropriate microenvironment is crucial for stem cell-based therapies, and the advancement of engineering platforms has facilitated the translation of these therapies. These platforms provide controlled and customizable environments that mimic native tissue or organ microenvironments, enabling researchers to better understand stem cell behavior and optimize their therapeutic potential. Meanwhile, continuous research has found that ideal biomaterials for biomedical applications should have good tissue compatibility, appropriate three-dimensional structures, degradability, favorable cell growth interfaces, and mechanical strength to achieve optimal therapeutic outcomes. It is hoped that these efforts will contribute to improving disease prevention and treatment and will ultimately benefit patients [60].

Hydrogels in Biomedical Applications

Hydrogels, three-dimensional polymers that are physically or chemically cross-linked, offer biocompatible scaffolds with a strong affinity for water. They possess unique characteristics that make them suitable for various biomedical applications. Hydrogels have a hydrophilic porous structure that allows them to absorb and retain water while maintaining structural integrity, allowing the free diffusion of particles within the material [61]. The mechanical properties of hydrogels, such as Young's modulus and atomic force microscopy, play a role in cell migration and proliferation within the scaffold [62]. Hydrogels undergo reversible volume phase transitions or gel-sol transitions in response to external stimuli, including physical (electromagnetic

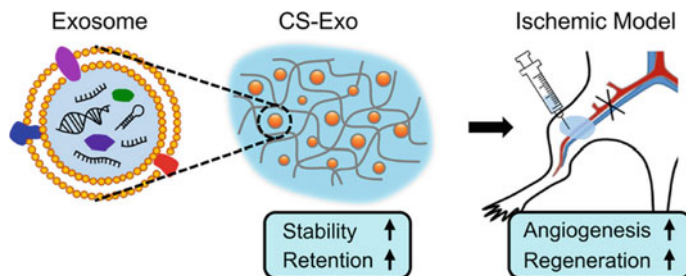


Fig. 7.4 Co-administration of Exo with the injectable hydrogel results in visible retention of Exo in a mouse model of hindlimb ischemia. The chitosan hydrogel effectively preserves the stability of exosomal proteins and microRNAs (miRNAs) under physiological conditions, enhancing the therapeutic potential of Exos. Furthermore, chitosan hydrogel-bound Exos (CSExos) significantly improve the survival rate and angiogenic activity of endothelial cells in the ischemic hindlimb. As a result, CSExos promote accelerated recovery of the ischemic hindlimb. Reproduced with permission from ACS Appl Mater Interfaces. 2018;10(36):30,081–91

field, temperature and pressure) and chemical (pH and ions) factors, allowing precise control over volume changes [63]. The structure of hydrogels, comprising covalent and noncovalent bonds, enhances their adaptability to the microenvironment, mimicking native tissues [64]. Furthermore, hydrogels demonstrate biodegradability, allowing simultaneous degradation to support cell growth in the tissue microenvironment [65]. These unique properties make hydrogels ideal biomaterials for tissue engineering, drug delivery, and cell culture applications.

Hydrogels have been widely applied and continuously modified to enhance various biomedical applications. In tissue engineering, hydrogels serve as scaffolds to provide structural support for cells, mimicking the three-dimensional architecture of natural tissues, and facilitating cell growth and tissue regeneration. In the field of adhesion medicine, hydrogels are utilized to repair and treat tissue adhesion issues by mimicking the properties of adhesive tissues and providing an environment conducive to cell adhesion. Moreover, hydrogels can be employed as cell encapsulation matrices that envelope cells to protect them and provide a suitable environment. This technology finds applications in cell therapy and drug delivery systems, enabling targeted treatments for specific diseases or injuries.

Hydrogels, when combined with processed exosomes, enable sustained local drug delivery, enhancing drug stability and bioavailability and reducing side effects. Mesenchymal stem cell (MSC)-derived exosomes have emerged as promising candidates for cell-free therapy in the treatment of various diseases. However, the effective retention and stability of exosomes *in vivo* after transplantation pose significant challenges for their clinical application. To address this issue, Zhang et al. developed and designed a CS-Exo hydrogel utilizing chitosan as the main component. The integration of exosomes into the chitosan hydrogel resulted in notable enhancements in protein and microRNA stability within exosomes, as well as increased *in vivo* especially in the lower limbs [66]. In particular, in an ischemic model, hydrogel-bound exosomes exhibited significant improvements in endothelial protection and

proangiogenic capacities (Fig. 7.4). The hydrophilicity and cross-linking behavior of hydrogels facilitate effective control of the drug release rate and duration. Combining exosomes with hydrogels allows for sustained local delivery, enhancing the stability and bioavailability of drugs. This approach has shown a significant impact in areas such as angiogenesis, osteogenesis, oncology, immunology, and pain management. Hydrogels can serve as scaffolding materials, providing a suitable growth environment for endothelial cells and promoting neovascularization in angiogenesis. In osteogenesis, hydrogels provide an optimal growth environment for stem cells, enhancing bone tissue regeneration. Hydrogels also enable the controlled release of anticancer drugs in oncology, improving treatment efficacy. In immunology and pain management, hydrogels can control the release of immune factors, modulate immune responses, and alleviate pain.

Hyaluronic Acid (HA) in Biomedical Applications

Hyaluronic acid (HA) is a high molecular weight acidic polysaccharide composed of glucuronic acid and N-acetylglucosamine [67]. It exhibits excellent biocompatibility and mechanical properties and is widely present in animal and human tissues, as well as in the extracellular matrix (ECM). It has excellent biocompatibility and mechanical properties. In the body, HA can undergo self-degradation and promote cell migration and extracellular matrix remodeling by binding to cell surface receptors [68]. HA has unique physicochemical properties, such as high viscoelasticity, plasticity, permeability, and distinct rheological properties, making it a versatile biomedical material with excellent biocompatibility and mechanical properties [69]. It finds application as a moisturizer in cosmetics, a lubricant in ophthalmic surgery, and a scaffold material in tissue engineering. HA is an important component of joint cartilage and synovial fluid, contributing to stress buffering, acting as a filler and diffusion barrier, and scavenging free radicals. Its excellent physicochemical properties have led to its wide use as a scaffold material in the repair of articular cartilage injuries [70]. In recent years, HA has been found to influence adhesion, migration, proliferation, aging, and differentiation of in vitro cultured stem cells. In tissue-engineered cartilage repair, HA serves not only as a carrier scaffold material but also as a regulatory factor to promote the differentiation of mesenchymal stem cells into chondrocytes, adding new ideas to clinical applications [71].

In conclusion, biomedical materials, including hydrogels and hyaluronic acid, offer valuable properties and have diverse applications in medicine. Ongoing research and advancements in these materials continue to improve disease prevention, treatment, and patient outcomes.

Conclusion

Stem cell therapy holds great promise in regenerative medicine for various conditions, including myocardial infarction, peripheral ischemia, skin wounds, colitis, kidney diseases, spinal cord injuries, bone defects/transplants, and ocular burns, due to the paracrine signaling, angiogenesis, and immune modulation effects of bioactive factors secreted by stem cells. The utilization of biomaterials, conjugated growth factors, small molecules, and decellularized extracellular matrix scaffolds (dECM) is critical in creating a favorable microenvironment that enhances the functional recovery of transplanted cells and improves therapeutic outcomes *in vivo*. The integration of bioengineered platforms provides an optimal niche for the survival and retention of transplanted cells. Emphasizing developmental applications and employing engineering strategies in stem cell therapy can further enhance functional recovery and promote the structural integrity of diseased organs.

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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Chapter 8

Endogenous In Situ Tissue Regeneration Using Inductive Bioscaffolds After Acute Brain Injury



Nadine Didwischus, Alena Kisel, and Michel Modo

Abstract *Introduction:* Acute brain injuries lead to severe tissue damage and loss. The brain has a limited ability to repair damaged tissues, but does not spontaneously regenerate lost tissue. To overcome this limitation, engineering strategies can be devised to provide a structural support for brain cells to invade, as well as for these scaffolds to release inductive molecules that stimulate the proliferation, migration and differentiation of brain cells. *Methods:* Inductive bioscaffolds can be derived by the decellularization of tissues to isolate extracellular matrix (ECM). ECM is suitable for brain injections through a thin needle in its liquid form and its cross-linking at body temperature creates a hydrogel. The properties of these natural top-down scaffolds serves as inspiration for synthetic hydrogels that are specifically designed in a bottom-up fashion to invoke the regenerative capacity of the damaged brain. *Results:* ECM-based inductive bioscaffolds have been shown to induce a tissue regeneration response in tissue cavities caused by acute brain injuries. They supply inductive cues to attract immune cells, neural cells, as well as endothelial cells into the tissue cavity, while providing a structural support for cells to migrate and position themselves in a tissue configuration. *Conclusion:* Inductive bioscaffolds provide an exciting engineering platform to overcome the lack of tissue regeneration that hampers recovery after acute brain injuries.

Keywords Bioscaffold · Extracellular matrix · Stroke · Regeneration · Tissue engineering · Stroke · Traumatic brain injury

N. Didwischus · A. Kisel · M. Modo (✉)

Department of Radiology, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: mmm154@pitt.edu

McGowan Institute for Regenerative Medicine, University of Pittsburgh, 3025 East Carson St, Pittsburgh, PA, USA

N. Didwischus

e-mail: NDidwischus@pitt.edu

M. Modo

Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, USA

Introduction

The human brain, with its intricate network of neurons, is unique in its tissue composition and complexity. However, when the brain is damaged due to injury or disease, the regenerative capacity of its neural tissue is limited, leading to potentially devastating consequences for individuals affected by such conditions. Acute/acquired brain injuries are the leading cause of adult disability in industrialized nations with 183 affected individuals per 100,000 population [1]. Traumatic brain injury due to motor, sports or gun accidents is the prevalent cause of acute brain injuries in teens and young adults, whereas stroke is common in, but not limited to, older subjects. This limitation has spurred an effort to explore innovative approaches to restore and regenerate damaged brain tissue. One such promising avenue is the use of inductive bioscaffolds.

Inductive bioscaffolds encompass a range of natural and engineered materials designed to mimic the natural extracellular matrix (ECM) of the brain [2]. The brain's ECM is composed of glycosaminoglycans, proteoglycans, glycoproteins, fibrous proteins, but also proteases and their inhibitors to maintain a state of constant turnover (Fig. 8.1a) [3]. Collagen is the most abundant mammalian glycoprotein and it is highly conserved among species [4]. Collagen has the ability to form large macromolecules through a process called fibrillogenesis [5]. The formation of large collagen macromolecules into a scaffold-like structure enables three-dimensional stress support, which contributes to the overall tissue support function of ECM [6]. Bioscaffolds comprised of natural ECM material have been shown to provoke rapid cell infiltration and scaffold degradation, along with build-up of host-derived neomatrix and constructive tissue remodeling with a minimum of scar tissue [7].

Bioscaffolds possess the ability to provide structural support and deliver bioactive cues [8]. Structural support is required for cells to form a tissue [9]. Bioactive cues include growth factors, cell adhesion molecules, and other signaling molecules that are strategically incorporated into the scaffold. By leveraging these cues, inductive bioscaffolds can create a microenvironment that encourages the infiltration, proliferation and differentiation of endogenous stem cells, as well as blood vessels required to sustain cells with nutrients essential to maintain newly forming tissue. For new tissue to establish itself, the bioscaffold needs to be degraded and replaced with a new tissue appropriate matrix. Mostly, this is achieved by the infiltration of immune cells, highlighting the intricate orchestration of different cellular systems involved in tissue regeneration.

The major advantage of inductive bioscaffolds lies in harnessing the intrinsic regenerative potential of the brain's neural cells, while providing a conducive environment for their growth and differentiation. These scaffolds can be tailored to specific injury sites or diseases, promoting targeted and site-specific regeneration. Furthermore, they can serve as a platform for the delivery of therapeutic agents, such as drugs or genetic materials, to enhance tissue repair and functional recovery. The design and fabrication of inductive bioscaffolds involve a multidisciplinary approach, combining materials science, biology, immunology and engineering principles. Their use for

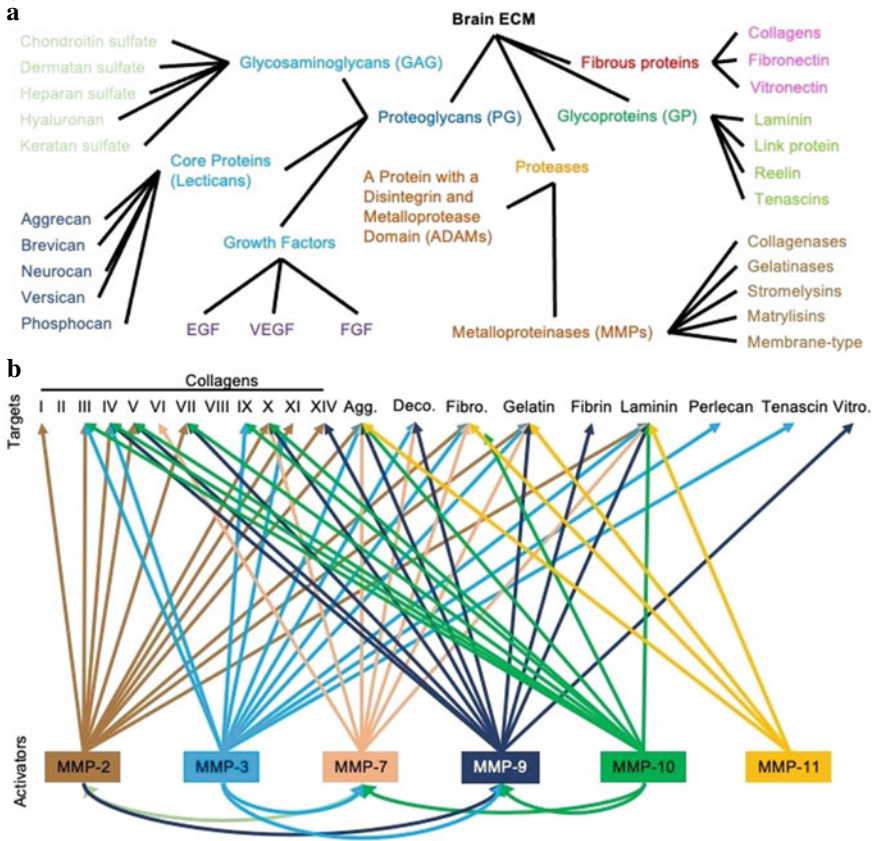


Fig. 8.1 Extracellular Matrix and Matrix Metalloproteinases. **a.** The brain’s extracellular matrix (ECM) consists of a network of different macro molecules. These macro molecules can be differentiated into several macro-molecular classes, such as glycosaminoglycans, glycoproteins and fibrous proteins. **b.** Most ECM macro molecules can be degraded by proteases, such as matrix metalloproteinases (MMPs). MMPs also cross-activate each other creating a cascade of degradation of ECM proteins

regenerative medicine purposes further involve surgery and radiology expertise to deliver these bioscaffolds to the site of injury.

The application of inductive bioscaffolds for brain tissue regeneration is a rapidly evolving field with significant potential for clinical translation. While challenges remain, such as ensuring long-term stability and integration of the scaffold within the brain, the use of inductive bioscaffolds represents a promising strategy to address the limited regenerative capacity of the brain. By leveraging the principles of tissue engineering and harnessing the intrinsic regenerative potential of the body, these innovative scaffolds have the potential to revolutionize the treatment of brain injuries, neurodegenerative disorders, and other neurological conditions.

Why Does the Brain Not Spontaneously Regenerate Lost Tissue?

Neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, or Parkinson's disease are accompanied by a gradual cell death spreading to different brain regions [10, 11]. This neurodegeneration leads to a selective neural loss that results in a shrinking of tissue, rather than a loss of tissue. This cerebral atrophy is usually a longwinded gradual process, whereas traumatic brain injury and cerebral ischemia (e.g., stroke) evoke an acute regionally defined cellular loss that also involves clearing of the extracellular matrix. Two sites of injury are commonly distinguished in acute brain injuries. Firstly, the infarct core is the site of tissue that was directly affected by injury and contains irreversibly damaged tissue and dead cells. This results in a sudden volumetric brain tissue loss that commonly (94% of cases) leads to the formation of a tissue cavity [12, 13]. Secondly, the penumbra is the tissue adjacent to the infarct core, where tissue integrity is not compromised, but cells undergo a lack of nutrients that induces apoptosis. This tissue is potentially salvageable through reperfusion or neuroprotection. Still, a cascade of inflammatory events is triggered in this tissue that results in an extension of the lesion core and eventually the formation of a tissue cavity [14].

Cell death results in the release of damage-associated molecular patterns (DAMPs), which bind to pattern recognition receptors (PRRs) that can activate astrocytes towards a reactive phenotype [15] and induce a pro-inflammatory response by microglia, a type of brain resident immune cells. These activated cells secrete cytokines (e.g. interleukin-1 beta, tumor necrosis factor- α), chemokines, reactive-oxygen species (ROS) and matrix metalloproteases (MMPs) [16, 17]. In consequence to these pro-inflammatory events, adhesion molecules on the cerebral endothelium are upregulated and cause the recruitment of leukocytes from the periphery into the brain [18, 19]. Secreted MMPs proteolytically cleave the ECM that in the core of the lesion produces a tissue cavity void of structural integrity. MMPs are a class of proteases that specifically target ECM proteins (Fig. 8.1b), cleave these and afford their clearance by macrophages [20]. Clearance of ECM, in addition to cellular loss, is a hallmark of tissue cavitation. In contrast, in the penumbral tissue, the ECM is remodeled, but preserved (i.e., tissue structure remains). Unlike in peripheral wound healing [21], changes in the ECM create an inhibitory environment that stymies tissue repair and regeneration [22].

Most notably, remodeling of ECM at the interface between the cavity and penumbra, and reactive astrocytes create a barrier, referred to as glial scar, that seals off the neurotoxic cavity fluids from the remaining viable tissue [23, 24]. This default tissue separation is therefore essential to prevent an enlargement of tissue damage. A diverse multitude of local microenvironments are created after acute brain injury that are characterized by changes in cellular composition, ECM structure, changes in vasculature, as well as the presence and state of inflammatory cells (Fig. 8.2a). One key feature separating viable tissue from the tissue cavity is the glial scar, which is a complex barrier containing reactive astrocytes, oligodendrocyte progenitor cells

(OPCs or NG2 glia), microglia and macrophages, as well as fibroblasts and ECM molecules [25]. One of the major ECM molecules contained in glial scar tissue is chondroitin sulfate proteoglycans (CSPGs), which limit and even prevent axonal re-growth, and also prevent neuronal progenitors from invading the tissue cavity [26–30].

Repair and regeneration in the brain requires neural progenitors to migrate to a site of injury to replace lost neurons along specific pathways to restore lost connections and functions. However, the injured brain often lacks the necessary guidance cues that direct this migration. Without these cues, newly generated neurons struggle to

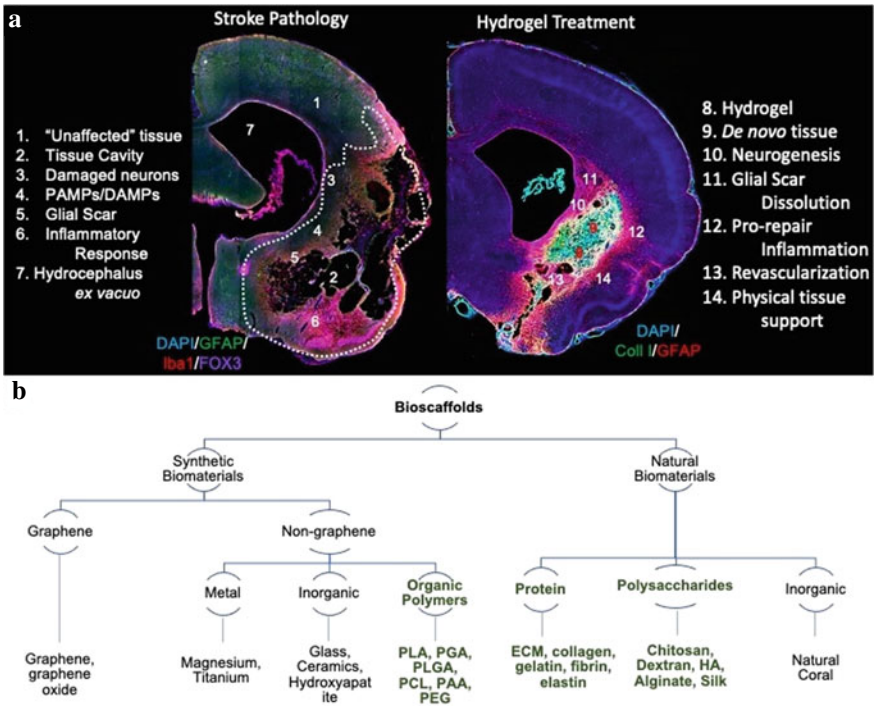


Fig. 8.2 Stroke pathology and bioscaffolds. **a.** In ischemic stroke, a cascade of events creates a multitude of microenvironments that contrast with normal tissue. Inflammatory processes (Iba1) are triggered in the brain to clear apoptotic cells, debris and ECM in the lesion core, which eventually creates a fluid-filled cavity. In penumbral tissues, neurons (Fox3) are lost, astrocytosis (GFAP) occurs, ECM is mostly preserved, and a glial scar matures to seal of the cavity from surrounding tissue. The implantation of an ECM-based hydrogel (Coll I) into the stroke cavity leads to a significant invasion of cells (DAPI) into the bioscaffold, a process that is triggered by ECM inherent signaling molecule and the provision of physical tissue support. As cells invade and degrade the bioscaffold, gradually a new matrix is deposited from brain-derived cells that restore a new tissue in the cavity. **b.** Bioscaffolds are commonly classified into synthetic and natural materials. Only organic polymers, proteins and polysaccharides are typically used to repair soft tissue defects, such as brain tissue (in green lettering). These can also be formulated to be administered through a thin needle to avoid damage to overlying CNS tissue

navigate the complex neural network and integrate properly into existing circuits. The brain also possesses inherent mechanisms that inhibit excessive neural growth. These mechanisms, known as growth inhibitors, prevent uncontrolled and aberrant regeneration, but they also limit the brain's ability to regenerate lost tissue.

Neurons form intricate connections, or synapses, with other neurons to create neural circuits responsible for various functions. The complexity and precise organization of these circuits make it challenging for new neurons to replace damaged or lost ones accurately. Aberrant connectivity and tissue malformations are a common occurrence underlying seizure activity, whereas neoplastic growth is the foundation of brain tumors. Guiding neural cells' differentiation into the specific cell types (astrocytes, oligodendrocytes, and neurons) needed for functional repair is hence a formidable challenge. The brain requires precise orchestration of various signaling molecules and growth factors to direct neural stem cells towards becoming mature and specialized neurons or glial cells. Increasingly these cascades are being decoded in development and applied to the differentiation of neural stem cells *in vitro*, as well as *in vivo*. However, this intricate interplay between different cell types over time in a damaged tissue environment complicates this guidance effort. Without this orchestrated guidance, newly generated cells may not integrate properly into existing neural circuits, which is required to improve behavioral impairments.

The brain has a limited capacity to generate new neural stem cells. The number of neural stem cells available for regeneration decreases significantly as we age, limiting the brain's regenerative potential. Indeed, there is some concern that the aged human brain has mostly lost its ability to generate new neurons. The available pool of cells to contribute to brain tissue repair and regeneration might hence be limited and can be considered the rate limiting step in brain repair.

Understanding these limitations and the underlying mechanisms that hinder spontaneous regeneration in the brain is crucial for developing strategies, such as inductive bioscaffolds, to overcome these obstacles and promote effective brain tissue regeneration.

Can the Brain Repair Itself?

While the brain's spontaneous regenerative capacity is limited, it does possess inherent mechanisms aimed at repairing and adapting to brain damage. These mechanisms are known as neuroplasticity, neurogenesis, and angiogenesis. Neuroplasticity describes the brain's ability to reorganize neuronal circuits and synaptic connections [31], a capacity that is retained into adulthood [32, 33] and forms the basis of most learning processes. It comprises the modification of existing connections and the reassignment of function from one brain region to another (e.g., loss of brain tissue in functional regions after TBI or stroke), which is called functional plasticity and the formation of new neural connections (synaptogenesis). This structural plasticity is essential for functional reorganization of neuronal circuitry and will require the integration of *de novo* tissue with the remaining and already reorganized circuitry [34].

Neuronal plasticity does not require the birth of new cells but can occur due to sprouting of new neurites or axons from existing neurons to create a new neural circuitry. Degradation and deposition of ECM molecules, such CSPG in perineuronal nets by MMPs, are crucial to this process. The process of forming and adapting neural circuitry is the foundation of rehabilitation science. After tissue damage, a high level of synaptogenesis occurs with pruning of inactive synapses, whereas active synapses are preserved. Functional synapses have been integrated into a neural network by a process often referred to as forming a Hebbian synapse. Specifically, activation of two synapses at the same time due to behavioral input forms a functional connection. This behavioral input is provided in rehabilitation through task integration, which then “imprints” on two activated synapses. However, the neuroplasticity of existing neural architecture is limited in the context of tissue repair, mostly due to the sparse local neuronal circuitry and hence severe disabilities often remain after an acute brain injury.

Tissue repair is highly dependent on the availability of new neurons that could enlarge compromised neuronal circuits. The “birth” of new neurons, commonly referred to as neurogenesis, in the adult brain is hence an important process that could extend neuronal circuits by proving novel biological substrates (i.e., neurons). Neurogenesis is thought to be limited to two germinal regions in the adult mammalian brain [35, 36], notably the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) that aligns with the wall of the lateral ventricles. However, low levels of neurogenesis involved in tissue homeostasis have also been observed in other regions. Neurogenesis occurs by the division of neural stem cells (NSCs), which retain an immature phenotype that can continuously produce more NSCs. As NSCs leave their neurogenic niche through migration into surrounding tissues, bioactive cues start to restrict their phenotypic fate. These so-called neural progenitor cells (NPCs) can still give rise to astrocytes, oligodendrocytes and neurons. As NPCs further differentiate, neuroblasts emerge, which eventually will mature into neurons. These neuroblasts migrate from the SVZ towards the olfactory bulbs following the rostral migratory stream (RMS), to then differentiate into mature interneurons [37–39], in a normal tissue function.

In response to a brain injury, NSCs’ proliferation in the SVZ is dramatically increased long-term, followed by migration of NPCs towards the site of injury along blood vessels [40–42]. A process that has been observed even one-year post-injury [43]. The few NPCs that eventually reach the site of injury commonly fail to integrate and form new neuronal circuits [42–45]. Importantly, migrating NPCs do not invade the cavity due to an inability to penetrate the glial scar [46], but also due to a lack of structural support for migration, as well as a lack of guidance cues with the tissue cavity.

It has long been noted that neurogenesis is tightly linked to angiogenesis, the process in which new blood vessels are formed. During brain development, neurogenesis and angiogenesis are intertwined and NPCs use the basement membrane of blood vessels for migration. After a brain injury, tissue hypoxia (i.e., lack or low level of oxygen) increases Hypoxia-inducible factor 1-alpha (HIF-1 α), which in turn activate Vascular endothelial growth factor A (VEGF-A) to stimulate angiogenesis

to restore normoxia (i.e., normal level of oxygen) in a tissue. The proliferation of endothelial cells (ECs) sets of a signaling cascade with NSCs/NPCs that aim to repair damaged tissue [47].

These innate mechanisms of neuroplasticity, neurogenesis and angiogenesis highlight the brain's remarkable capacity to adapt and repair itself to some extent. However, their effectiveness in fully restoring lost brain tissue and functions is limited, especially in cases of severe damage or neurodegenerative conditions. This is where external interventions and therapies, such as inductive bioscaffolds and other regenerative approaches, can play a crucial role in enhancing the brain's regenerative potential and promoting more substantial recovery.

Can Engineering Overcome the Lack of Brain Tissue Regeneration?

The Manifold Properties of Bioscaffold Materials

Bioscaffolds find a broad range of application in fields such as skin wound healing [48, 49], cartilage repair [50], bone regeneration [51], and vascular repair [52], as well as cosmetic procedures [53, 54]. Application in the nervous system are currently limited in the clinic to the treatment of peripheral nerve injuries [55]. Biomaterials harbor a high and diverse potential for therapeutic applications in CNS injury, including in situ tissue regeneration [56]. They have the ability to serve as a scaffold, providing structural support for invading cells, and at the same time can release (depending on the material) signaling molecules, that stimulate the invasion of host cells into the cavity [57]. Bioscaffolds are commonly divided into two classes: 1) synthetic and 2) natural materials (Fig. 8.2b).

Synthetic materials for bioscaffolds are often comprised of organic and/or inorganic polymers, such as polypropylene (PP), polyurethanes (PUR), polyethylene glycol (PEG), but also metals and ceramics have found their applications as bioscaffolding materials, especially in dentistry and orthopedic surgery. These synthetic materials provide more robust reproducibility and can be engineered specifically to meet design characteristics on a chemical level. The lack of biological source material in synthetic bioscaffolds reduces the risk of contamination with microbiota or prions that can exert unwanted effects. However, these materials commonly are biologically inert and bioactive signaling requires these being integrated in its formulation. Specific adhesion proteins and/or growth factors can be incorporated to invoke a particular cell migration, differentiation into selective phenotypes and guide axon re-growth [43]. Without these elements incorporated in the material design process, these bioscaffolds typically do not exert inductive properties. In some cases, the inertness of these materials can lead to a foreign body response by tissue that leads to an encapsulation and failure of therapeutic delivery [58]. A major advantage of these bottom-up materials is that their physiochemical properties can be tightly controlled

to meet design criteria, which makes them well-suited for cell and gene delivery, as well as controlled drug release [59–61].

Natural materials originate from biological tissues that are either of a mammalian or non-mammalian source. Non-mammalian macromolecules are used to create bioscaffolds and can be composed of silk, agarose, alginate, and chitosan [19, 62]. These materials are commonly well tolerated in biological tissues, are cost effective and can be widely sourced. Mammalian sources typically consist of biological tissues that are decellularized, i.e. cells are removed from the tissue with only the ECM and ECM-derived macromolecules (e.g., collagen, fibrin, hyaluronic-acid, and laminin) remaining [63]. A variety of tissues have been used to produce bioscaffolds through decellularization (Fig. 8.3). ECM structure and content is well preserved across organs, although some differences in composition have been noted [64]. Processing of the decellularized tissue variably affects structure and bioactive molecules, which influence their applications. One of the major challenges with these materials is that the complexity of their composition is difficult to characterize [65]. Purified ECM molecules, such as collagen and hyaluronic acid (HA) are therefore widely used as bioscaffold backbones, akin to synthetic bioscaffolds. Collagen achieves physical and chemical cross-linking based on its concentration and hence can produce a hydrogel, whereas HA requires additional cross-linkers to form a hydrogel [66]. ECM, due to its ~ 60% of collagen content, cross-links in a concentration-dependent manner to form hydrogels that adapt to a diverse lesion topology [9]. The major advantage of natural materials as bioscaffolds are that these are cytocompatible, non-toxic, contain bioactive molecules, are simple to harvest and typically do not invoke a foreign body response. ECM-based materials also contain a richness of bioactive molecules that reflect the natural habitat of cells. This richness is difficult to recreate and is the basis of many investigations to determine what specific biological and structural elements are required to elicit the encouraging therapeutic effects observed with these materials. The release of signaling molecules during the degradation of natural scaffolds produces a responsive environment that interacts with stem cells during their differentiation, but also provides chemoattractive cues to recruit host cells into the scaffold [67]. The inductive and response properties are currently difficult to replicate in synthetic materials. A disadvantage of natural materials is that due to differences in decellularization and formulation, they can be highly variable in composition and biological effects [68]. Poor decellularization can also trigger adverse reactions by the host's immune system that can lead to a failure of the scaffold [69]. Top-down natural materials hence currently set the standard for what can be achieved using bioscaffolds in a therapeutic setting, but they often lack the specificity, quality control, scaling and flexibility that synthetic materials provide.

Combinatorial approaches incorporating both synthetic and natural elements to form hybrid bioscaffolds (e.g., ECM coated mesh) are also widely investigated. The aim of these hybrid materials is to achieve a higher reproducibility characteristic of synthetic biomaterials, whilst preserving the excellent biocompatibility of natural biomaterials. In cases where specific design characteristics are required, such as a tightly controlled physical microenvironment influencing stem cell fate [70], these hybrid material are advantageous to natural sources.

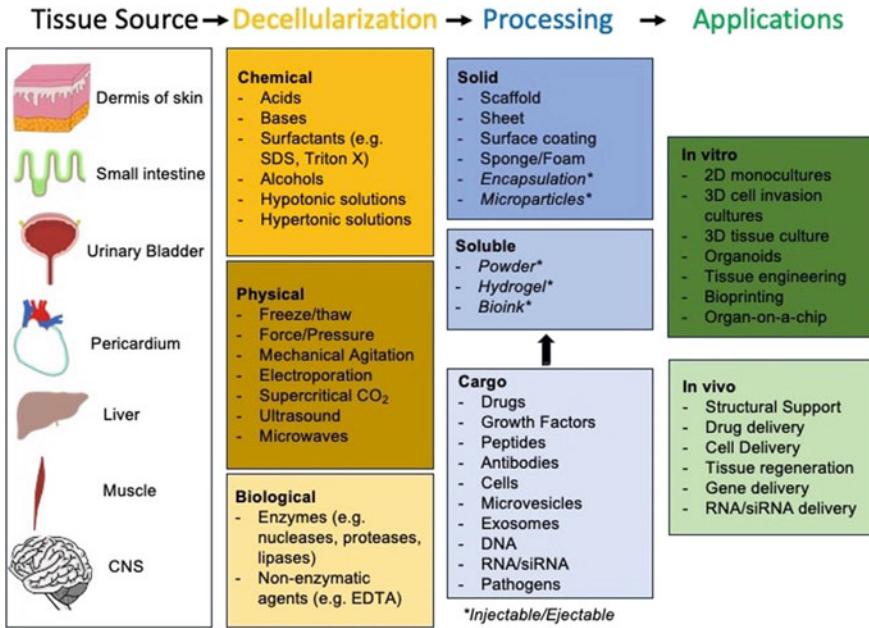


Fig. 8.3 ECM as a bioscaffold source. ECM can be sourced from a variety of tissues. Tissue decellularization is required to selectively retain ECM. ECM can be either used in its native tissue form or it can be further processed. Injectable bioscaffolds (*) typically require formulation as a powder that can be used to produce a hydrogel. This can then be used to encapsulate for instance cells and create microparticles. ECM material can also be used as a vehicle to deliver cargo, in some instances just by mixing both components. These different processes enable a broad spectrum of applications in vitro and in vivo

How to Deliver Bioscaffolds to the Brain?

Enclosure of the brain and spinal cord by the skull and spine, respectively, constitutes a major barrier to treat CNS injuries versus those of the peripheral nervous system (PNS). In the PNS, delivery and/or implantation procedures can be more invasive as organs and/or nerves can usually be exposed by soft tissue dissection (e.g., muscle, fat). In contrast, a tissue cavity in the brain or spinal cord [71] is surrounded by functional tissues that if damaged will lead to additional impairments. A minimally invasive approach is hence required for bioscaffold delivery [9, 72]. Cavity topology and volume are also highly variable, making injectable and shape-conforming materials (e.g. hydrogels, microparticles) preferable over solid scaffolds that require invasive surgical procedures for placement [73, 74]. Pre-gel liquids can be injected through a thin needle (28 Gauge), causing minimal tissue damage, to fill the irregular injury cavity and then polymerize in situ into a stable hydrogel conforming with the tissue cavity [9, 75]. Hydrogel-based microspheres/microparticles can also be administered through a thin needle to fill up tissue cavities. These have the added advantage that

prototypical neurovascular units can be constructed and matured *ex vivo* prior to implantation to provide a faster tissue regeneration in, for instance, a stroke cavity [74].

Hydrogels are advantageous for delivery to the brain due to their elastic and stiffness properties (i.e., rheology) that can be tuned to those of brain tissue. This is achieved by physical or chemical cross-linking of hydrophilic polymers or by self-assembling systems. The brain is a soft tissue when compared to bone, muscle or even fat (Fig. 8.4a). However, in wound healing it is known that the temporary granulation tissue that is filling the tissue void is softer than the mature tissue. The rheological properties of ECM hydrogel are highly dependent on the protein concentration (i.e., collagen content). Lower protein concentrations (2 mg/mL) of urinary bladder matrix-ECM barely form a self-supporting hydrogel and remain within the state of a highly viscous fluid (Fig. 8.4b), whereas high concentrations (8 mg/mL) create much stronger hydrogels, even though their storage and elasticity modulus being lower than mature brain tissue. Weaker hydrogels overall performed better in tissue regeneration compared to stiffer scaffolds [9, 76], reflecting the principle of a granulation tissue being weaker than the mature tissue that is being regenerated.

The gelation properties of different concentrations of ECM are also important to determine how much structural support is provided (Fig. 8.4c). Stiffer hydrogels could for instance provide a more robust and long-lasting structural support that

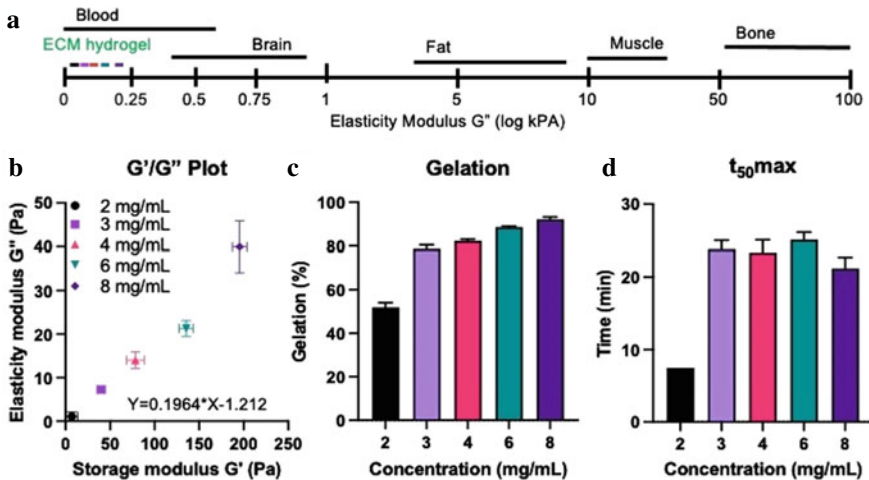


Fig. 8.4 Rheological considerations for hydrogel-based bioscaffolds. **a** Comparison of the elastic modulus for different tissues and its comparison to urinary bladder matrix (UBM)-ECM hydrogels formulated at different protein concentrations. Typically, hydrogels used to repair brain damage are softer than mature tissue. **b** ECM hydrogels exhibit a linear increase in elasticity and stiffness based on their protein concentration. **c** A low protein concentration of 2 mg/mL does not properly gel with > 50% of ECM being washed out. However, even a high 8 mg/mL concentration hydrogel contains a 10% ungelled ECM fraction **d**. ECM protein concentrations > 3 mg/ml show a consistent gelation time that is crucial to time its intracerebral delivery time

prevents tissue and ventricular hypertrophy, especially if this bioscaffold persists and is not readily degraded [76]. Gelation is also key to delivery and retention of the bioscaffold. If the pre-gel liquid gels too fast, it will be extruded in a spaghetti-like fashion and not provide a consistent bioscaffold interface with the damaged brain [74]. Time to 50% maximum gelation is a measure to inform the delivery time for pre-gel to the brain. At gelling concentration this is stable for ECM hydrogels at ~20 min (Fig. 8.4d). Once a bioscaffold is delivered into the cavity and curing, it is important that minimal swelling occurs as this could lead to a rise in intracranial pressure [73, 77].

The rheological properties of scaffolds are also an important consideration for the tissue response to the material. Stiffer inert materials tend to invoke a foreign body response that leads to a fibrotic encapsulation and hence a lack of degradation and integration with existing tissues [78]. Long-lasting materials can cause a chronic pro-inflammatory reaction that precedes or coexists with fibrosis. Bioscaffolds that can be degraded by the host are therefore advantageous. Degradation products from the scaffold should be non-cytotoxic [73, 79, 80]. Bioscaffold degradation also affects the rheological properties of the material, typically reducing its stiffness and elasticity. This in turn will also affect the fate of cells that were delivered in the scaffold or invaded after implantation. Softer materials support more neuronal differentiation, whereas stiffer materials push NSCs into a glial phenotype [81]. In addition to the rheological properties of scaffolds, the topography of hydrogels dramatically affects the NSC attachment and differentiation of neural stem cells [82]. Basic material properties of a bioscaffold hence determine its deliverability to brain tissue, as well as how brain tissue responds to the material itself.

What Is Inductive Signaling?

A key distinguishing feature of inductive bioscaffolds, such as ECM hydrogel, is that in addition to their material properties, they contain and release bioactive molecules that influence cellular behavior [83]. ECM incorporates a wide range of molecules that are naturally present within a tissue at normal physiological concentrations. This is a major advantage over synthetic materials that need to select proteins for inclusion and define its concentration, whilst defining if the protein should be soluble or presented in an immobilized fashion [84]. Non-mammalian natural biomaterials also do not contain these signaling molecules.

One inductive component of native ECM, as well as its formulation as a bioscaffold, are integrins. Integrins facilitate cell adhesion and cell interactions. They interact with the cytoskeleton through receptor binding and impact gene expression, proliferation, and cell survival [85]. Integrins afford the continued migration of cells from native tissue into the bioscaffold, which is a key step to seed bioscaffold in situ to drive their biodegradation and replacement with organ-specific cells. In the CNS, laminin-derived peptides are known to be a motif required by NSCs for migration and invasion [86]. An ECM-derived bioscaffold retains its biological function to

support cellular disposition and movement, which is required to reconstitute a new tissue.

In a tissue, ECM also acts as a growth factor sink which retains these proteins for release upon degradation by MMPs. This process can be seen as a local repair mechanism inherent in tissue, as the release of the proteins is geared towards cell survival and/or angiogenesis in a response to tissue damage. ECM bioscaffolds therefore contain naturally occurring growth factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and tyrosine kinase (Trk), which are known to enhance neuronal survival and differentiation [87–89], as well as vascular endothelial growth factor-A (VEGF-A), associated with the induction of angiogenesis. VEGF-A is known to promote neurogenesis, it exhibits trophic effects on neurons and glia cells, and also supports migration of neuronal cells in the developing CNS [90, 91]. Indeed, angiogenesis and neurogenesis are tightly coupled processes that heavily interact to influence each other [47, 92]. ECM hydrogel is therefore potentially an efficient delivery vehicle for NSCs, as it contains factors aimed at preserving the viability of these cells [93]. Specifically designed delivery vehicles for cell delivery can be derived from this principle [94]. For instance, in a rat spinal cord injury model, fibroblast growth factor (FGF), which supports cell proliferation and survival, was incorporated into a collagen/chitosan hydrogel mix to increase nerve fiber tract regeneration that leads to an improvement of locomotor function [95].

In addition to growth factors, ECM also contains a variety of cytokines, such as monocyte-chemoattractant protein-1 (MCP-1/CCL2), which recruits peripheral macrophages and supports their migration. Macrophages are pivotal for degeneration of the ECM in both the peripheral and central nervous system [96] and are known to be activated by DAMPs (e.g. CSPG) in the event of tissue injury [97, 98]. Fractionation of ECM hydrogels into structural and soluble components reveals distinctive roles involved in regulating macrophage phenotype. Soluble components that occur naturally in ECM, shift macrophages from a pro-inflammatory (M1) towards a pro-repair (M2) phenotype through Notch and P13K/Akt signaling [99]. Regulating the macrophages phenotype upon entering the bioscaffold is considered as a key step in the repair process. Consequently, a considerable effort is geared towards understanding the molecular basis of this process and how this can be exploited to improve the therapeutic applications of inductive bioscaffolds [100, 101], including the brain.

ECM as an Inductive Bioscaffold for Brain Tissue Repair and Regeneration

When it comes to brain tissue repair and regeneration, the unique challenges posed by the complex organization and delicate nature of the brain make it a particularly difficult task. However, ECM-based bioscaffolds have demonstrated promising capabilities in facilitating the repair and regeneration of brain tissue. When the ECM scaffold is implanted into a damaged brain region, it serves as structural support

for cell migration into the lesion cavity, releases bioactive cues that attract cells and impacts their cell fate, but it also serves as a tissue for spatial organization of cells that was lost in the cavitation process. The major advantage of ECM bioscaffolds is that they are essentially no different from naïve ECM [102].

Implantation of ECM hydrogel is dependent on its *in vivo* biodegradation. Stiffer 8 mg/mL UBM-ECM hydrogels did not degrade sufficiently even over a 90-days post-implantation period to support *de novo* tissue formation [76]. Although these hydrogels contained the highest level of inductive proteins, the material properties limited the effects of these bioactive cues, highlighting the importance of both aspects in the regenerative process. It is also emphasizing the importance of adequately conducted *in vivo* studies to verify and refine the design characteristics that are in support of *de novo* tissue formation. *In vivo* studies in rat models of stroke demonstrated that a 4 mg/mL ECM supported the continued invasion of cells (3000–4000 cells/ μ L) over 90 days post-implantation [103].

A 4 mg/mL ECM bioscaffold implanted under an injection-drainage procedure completely filled the lesion cavity at day 1 [9], but gradually was degraded as revealed by the almost complete disappearance (95%) of the scaffold by 90 days post-implantation (Fig. 8.5a) [103]. However, degradation of the ECM hydrogel was also dependent on the time of implantation (*i.e.*, therapeutic window). An efficient biodegradation occurred when implanted 14 days post-stroke, whereas this was significantly attenuated when the bioscaffold was implanted 90 days post-stroke (Fig. 8.5b) [102]. Implantation of ECM bioscaffold 28 days post-stroke exhibited the most efficient biodegradation, with the most significant infiltration of host cells that supported tissue formation in the lesion cavity. Histologically, it was also evident that very few cells invaded the ECM hydrogel when implanted 90 days post-stroke, whereas 28 days post-stroke a very efficient cell invasion was observed. The importance of both the time-dependent biodegradation of ECM hydrogel, as well as the timing of implantation, highlight the spatio-temporal complexity of tissue regeneration.

Biodegradation of ECM in normal tissue, as well as under pathological conditions, is caused by proteases. Foremost of all, MMPs degrade collagen, which is the main structural protein in ECM hydrogels. *In vivo*, however, the molecular mechanisms of degradation are difficult to control. *In vitro* studies provide a more controlled environment and demonstrated that proteolytic degradation through collagen digesting MMP-2, -3 and -9 almost completely degraded UBM-ECM hydrogels within 14 days [104]. However, it was also revealed that a major (~60%) degradation of ECM hydrogel occurs through hydrolysis (*i.e.*, washing out of ungelled proteins or breaking of weak cross-linking). A hydrolysis associated bulk erosion occurs homogeneously throughout the hydrogel with exterior and interior bonds breaking simultaneously [81]. The presence of MMPs in the lesion cavity, as well as the presence of liquid, could hence be a major determinant in the therapeutic time window, with MMP concentrations in a lesion cavity expected to decrease after cavitation is complete. It also stresses the importance of ungelled proteins and how these potentially are washed out of the bioscaffold to permeate into peri-lesional tissues. This would

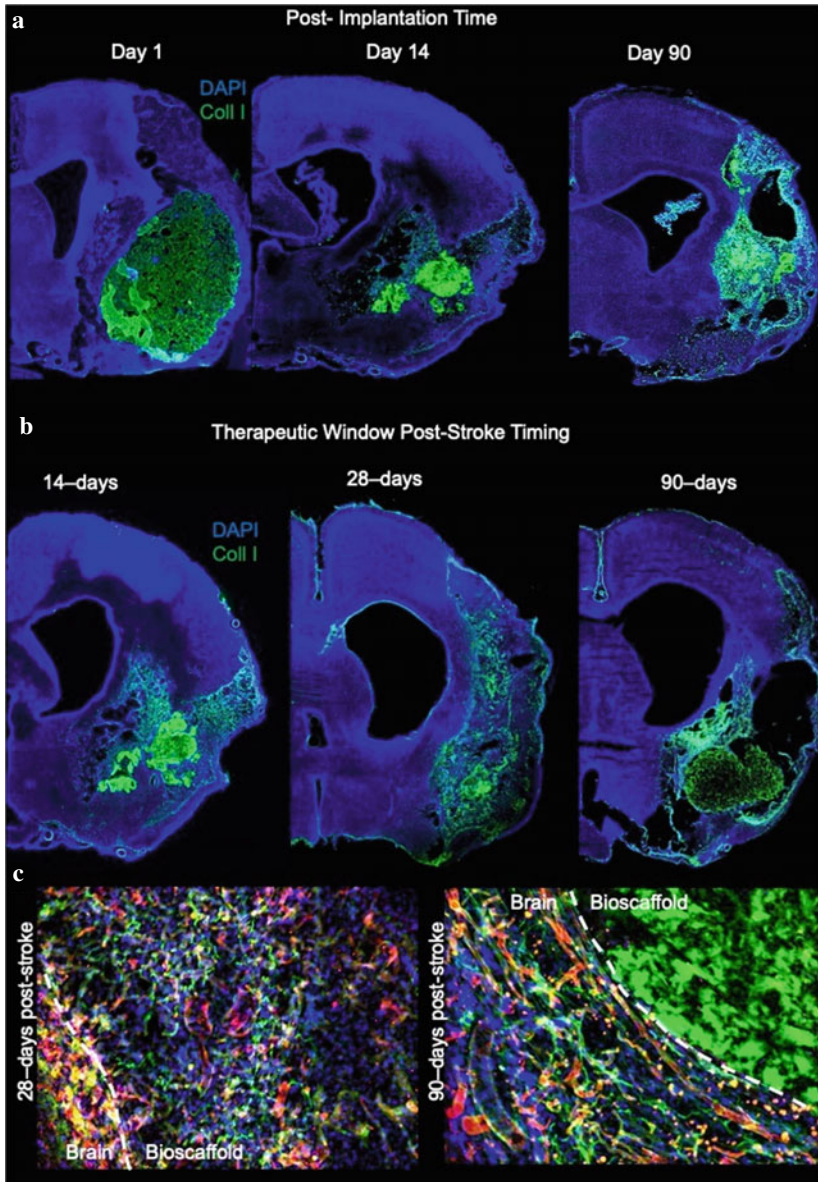


Fig. 8.5 ECM hydrogel for the treatment of stroke. **a.** Histological time course of ECM bioscaffold distribution post-implantation [14 days] in the stroke lesion cavity indicated that there is gradual degradation of the material and its replacement by host cells. **b.** Time course of different post-stroke implantation times indicated that 28-days post-stroke achieved the most efficient degradation and tissue restoration, whereas 90 days post-stroke accounted for steep decline in these parameters. **c.** It is evident that the reduced biodegradation of ECM hydrogel implanted 90 days post-stroke is associated with a very limited cell invasion, whereas the bioscaffold implanted 28-days post-stroke was robustly invaded and replaced by host cells

provide bioactive molecules in tissue to impact its repair but could also be the initiator of the cell recruitment into the bioscaffold.

Cells invade rapidly into the ECM hydrogel. Within about 6 h after implantation (Fig. 8.6a), peripheral immune cells infiltrate through the peri-lesional space first before invading the scaffold (Fig. 8.6b), as revealed by non-invasive time-lapse ^{19}F MRI [105, 106]. Neutrophils are thought to be the first cells to infiltrate, followed by macrophages [106]. Infiltration occurs through migration of blood-borne immune cells through the vascular wall into the brain parenchyma (Fig. 8.6c). Lymphocytes are also expected to infiltrate temporarily between 1–3 days post-implantation. However, macrophages are considered the main effector of biodegradation [107]. Macrophages secrete MMPs to cleave ECM proteins and phagocytose these [108]. In vivo imaging of the biodegradation of gold nanoparticles (AuNPs) labelled collagen-based scaffolds revealed that in the absence of collagenase, no measurable degradation was evident [109]. It is during this degradation process and the associated release of matrix-associated proteins that some macrophages are expected to shift from a M1 pro-inflammatory to a pro-repair M2 phenotype. However, M1 macrophages persist throughout the duration of the degradation process [103] and it currently remains unclear if M2 macrophages also degraded the bioscaffold or if these mainly aid other cells to form, for instance, a neovasculature.

The release of pro-angiogenic factors, such as VEGF-A, from the ECM bioscaffold attracts endothelial cells' invasion and stimulates angiogenesis [110]. Molecules, such as laminin and vitronectin that are present in the basement membrane around blood vessels are prominent proteins in ECM bioscaffolds and support the formation of a neovasculature [111]. Formation of a robust neovasculature is a hallmark of a degrading hydrogel, whereas the lack of a neovasculature is highly associated with a failure of scaffold degradation [103]. The formation of blood vessels in development and regeneration is aided by macrophages and microglia [112]. These are commonly assumed to be M2 pro-repair macrophages [101]. A neovasculature forming in the hydrogel also supports the direct invasion of macrophages into the scaffold rather than their infiltration through the peri-lesion tissue [106], hence providing an acceleration to scaffold degradation. The basement membrane around blood vessels also serves as a migration path for neural progenitor cells [113].

Although there is evidence of neural progenitors invading the bioscaffold within 24 h in a line and file invasion [114], the presence of a vasculature facilitates this process. Indeed, the presence of blood vessels within the ECM has been shown to be a major migratory path for NPCs migration into the bioscaffold [102]. Immature NPCs are the dominant phenotype of brain cells invading the scaffold at 24 h, but gradually there is a shift towards more mature phenotypes, such as neurons, oligodendrocytes, astrocytes [102, 103]. Not only do bioscaffolds hence attract brain cells into the lesion cavity, but they also support the differentiation and survival of mature neural cells required to form brain tissue. Still, the neuronal density in de novo tissue is significantly lower than homologous intact tissue, indicating a deficit in repopulation [102]. It remains unclear if this is an issue of insufficient bioactive cues (i.e., degradation exhausted the available supply) that drive the continued recruitment of NPCs into the bioscaffold or if there is an insufficient neurogenic pool

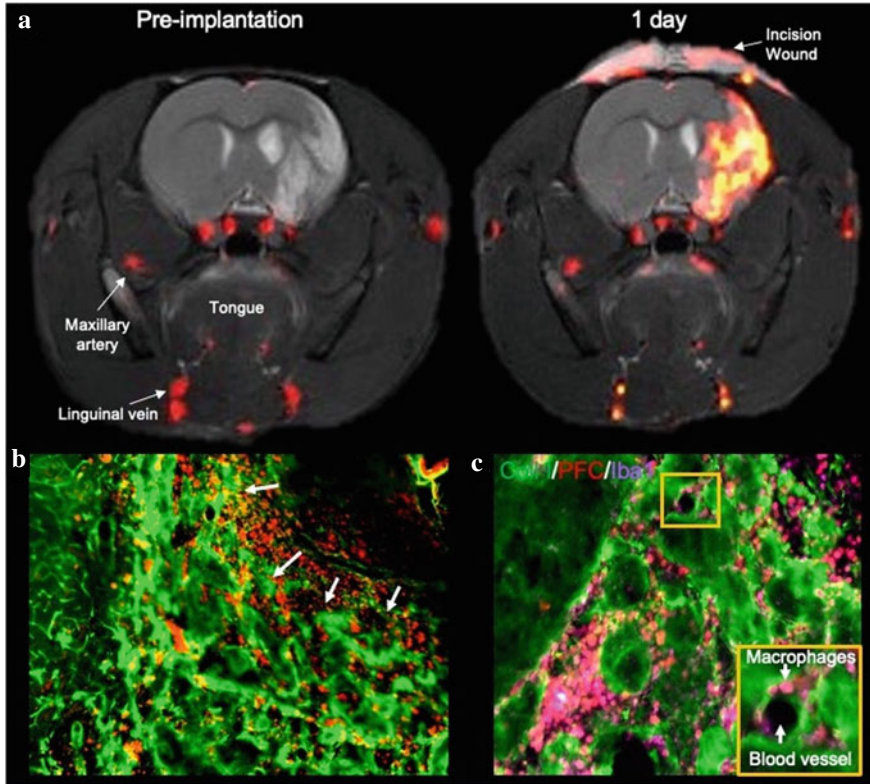


Fig. 8.6 Immune response to ECM hydrogels. **a.** Pre-implantation T₂-weighted and overlaid ¹⁹F MRI scan of a rat brain 14 days after stroke induction. No ¹⁹F signal, indicative of peripheral immune cells is present in brain tissue. However, 1-day post-ECM implantation, ¹⁹F MRI indicates a massive infiltration of peripheral immune cells, affording a spatio-temporal analysis **b.** Immune cells labeled with a ¹⁹F contrast agent (in red) reveal a major invasion of the ECM bioscaffold (collagen I in green). **c.** Infiltration of immune cells, such as macrophages, occurs through blood vessels

to supply sufficient cells. Although neurogenesis is upregulated after tissue damage, the level of repair and regeneration achieved by scaffolds, could potentially decrease the neurogenic signals, hence leading to a decrease in supply of the required NPCs to completely repopulate newly forming brain tissue.

Signs of neuronal maturation within ECM hydrogel are evident with axonal projections, as well as oligodendrocytes being observed [103]. Neurite outgrowth, synaptogenesis, synaptic stabilization and injury-related plasticity are heavily dependent on remodelling of ECM [115]. The local release of neurotrophic factors and other signaling molecules from the bioscaffold, stimulate new neural circuit formation [73], whereas neurons and glia that invaded the scaffold will deposit a neomatrix that underpins the formation of new brain tissue [7]. Identification of the neomatrix in regenerating tissue, however, is challenging [116]. It also remains unclear

if myelination of new axons occurs, or if there is synaptic connectivity that would create new neuronal circuitry within de novo tissue, as well as connectivity with surrounding preserved tissues. It also remains unclear if the forming of new tissue in the cavity impacts the neuronal circuitry in the peri-lesional areas and if the neurogenic stimuli lead to an increase in neurogenesis that benefits tissue repair. Injection of small amounts of ECM hydrogel into the stroke or TBI-damaged tissue has indicated improvements in behavioral outcomes [117, 118], whereas an 8 mg/mL ECM hydrogel in the stroke cavity did not impact behavioral impairments [76].

Currently, these explorative studies are conducted in rodent models of acute brain injury. It therefore remains unclear how these results translate to the more complex gyrencephalic human brain. ECM bioscaffolds have been implanted in > 10 million patients and overall have an excellent safety profile. Although no bioscaffolds have been implanted in patients with acute brain injuries to date, natural scaffolds are extensively used to repair dura matter surrounding brain tissue [119]. Over nine commercially available products (e.g., DuraGen, Durepair, DuraMatric, Lyoplant, Duraform) are available, which are typically composed of collagen I that is sourced from different animal tissues (e.g., bovine Achilles tendon). These natural scaffolds are preferred by surgeons over synthetic products due to their favorable biocompatibility and robust tissue restoration [120]. Delivery of hydrogel to deep seated brain cavities in patients is potentially facilitated through the advent of the Aurora Surgiscope, which can afford an endoscopic visualization of the lesion environment to ensure appropriate placement in a liquid cavity, as well as its ability to deliver and evacuate liquid using a single device [121, 122], as previously described for an injection-drainage delivery of ECM hydrogel into a stroke cavity in rats [9].

Improving upon Nature's Design

Bioscaffolds, such as ECM hydrogel, induce a regenerative effect. However, as illustrated by the insufficient neuronal repopulation, improvements can be envisioned to further enhance the efficacy of these materials [123, 124]. Supplementation or functionalization of bioscaffolds with various bioactive molecules to enhance brain tissue regeneration might be required to achieve optimal restoration of behavioral deficits. For instance, enhancement of vascularization can be achieved by incorporating a VEGF-A or VEGF-A biomimetic peptide (e.g. KLT) into a HA-hydrogel before implantation into a cortical cavity [125, 126]. The functionalized hydrogels achieved a greater angiogenesis, as well as the inhibition of glial scar formation around the cavity. More controlled release of VEGF-A or other angiogenic proteins can also be achieved by incorporating synthetic materials (e.g. PLGA) with a controlled release profile to ensure sufficient delivery over protracted time periods [127]. Combinatorial approaches targeting multiple signaling pathways, such as VEGF-A and BDNF, increase design complexity, but might be required not only to generate new blood

vessels, but also to ensure their survival [128]. Stroke animals injected with the VEGF + BDNF-hydrogels recovered faster with improvements in motor deficits over 28 days after implantation [129]. To direct the restoration of a specific pattern of vasculature, hydrogels can be designed to specifically present integrins to guide the formation of a new and stable vasculature [130].

The formation of new functional neuronal circuits and its support structure of glial cells is incomplete compared to normal functional tissue. Although even damaged and severely depleted neuronal tissues supports behavioral functions, it is evident that improvements in neuronal content can be achieved [102]. Incorporation of stromal cell-derived factor 1 (SDF-1) into bioscaffolds has been shown to improve neural stem cell recruitment after implantation into a rat model of TBI [131–133]. Control over biomechanical and biophysical properties of natural scaffolds can also potentially optimize their pro-neuronal properties. For instance, polyacrylamide (PAA) and N-acryloyl-6-aminocaproic acid (ACA) copolymer (PAA-ACA) produce soft hydrogels that increased neuronal maturation and differentiation of NPCs. A combination of a lower hydrogel stiffness with a smaller topography further enhanced neurite branching and elongation, emphasizing the importance of a combination of several different biophysical cues to achieve a maximum therapeutic effect [82]. A further dimension often ignored is that integration of neurons into circuitry is enhanced by electrical stimulation. The incorporation of electricity conductive polymers (i.e. electroceuticals) can hence improve outcome [134]. The complexity of interactions between biomechanical, biophysical and signaling cues is immense and poses a significant challenge to achieve optimization. Moreover, *in vitro* testing of single factors might be misleading as *in vivo* multiple factors interact in a responsive fashion to achieve tissue restoration. *In vivo* testing of design concepts is hence paramount.

This complexity is further increased by incorporating other biologicals, such as stem cells. Not only does the scaffold reciprocally interact with host tissue, but so do transplanted cells, as well as scaffolds and stem cells influencing each other. Two paradigms can be distinguished, one that aims to support exogenous cells during the delivery process through a thin needle (i.e. reducing shear stress, providing a supportive cell environment upon implantation) [135, 136] versus those aimed at reconstructing a new tissue in the cavity [71, 93, 137]. Designed hyaluronic-based hydrogels containing BDNF and a cross-linking peptide that is sensitive to MMP can control the rate and type of degradation of the scaffold to support bone marrow-derived human mesenchymal stem cells (hMSC) delivered, for instance, into a model of spinal cord injury [138]. These engineered scaffold systems can potentially go beyond their natural properties to address specific shortcomings in an application to exert greater control over the regenerative environment.

A more complete control over the regenerative tissue environment is achieved by the implantation of pre-fabricated tissue units, such as organoids [139, 140] or microspheres containing neurovascular units [74]. Conceptually, this approach aims to assemble all elements required for a tissue to form (i.e., ECM, neural stem cells, endothelial cells) prior to implantation (Fig. 8.7a). A major advantage is that these can be configured *ex vivo* to express positional specifications for particular brain regions. Forming of a primitive vasculature (i.e., endothelial cell tubes) and maturation of neural stem cells into partially differentiated neurons would allow a much more rapid restoration of tissue. Importantly, it should be avoided that these constructs have already extended axons that could be damaged during the implantation process and potentially lead to apoptosis of implanted neurons [141]. Implantation of these pre-fabricated neurovascular units is not trivial, especially to achieve a homogeneous distribution through the cavity and is best achieved by embedding these in ECM hydrogel, avoiding the floating or settling of microspheres (Fig. 8.7b) [74]. This achieves also a much more efficient re-seeding of the tissue cavity and perilesional tissues with neural stem cells, as these will migrate through the ECM scaffold holding in place individual microspheres (Fig. 8.7c). The presence of endothelial cells also achieves better survival and rapidly initiates the creation of a neovasculature (Fig. 8.7d). However, ECs are highly immunogenic if these are not derived from the same subject. Immunosuppression is hence required or NSCs and ECs have to be autologous iPS cell-derived to avoid an immunological mismatch. NSCs have a low immunogenicity even in a pro-inflammatory environment [142, 143]. These tissue building blocks are complex but are potentially a very sophisticated bottom-up approach to rapidly reconstruct lost brain tissue.

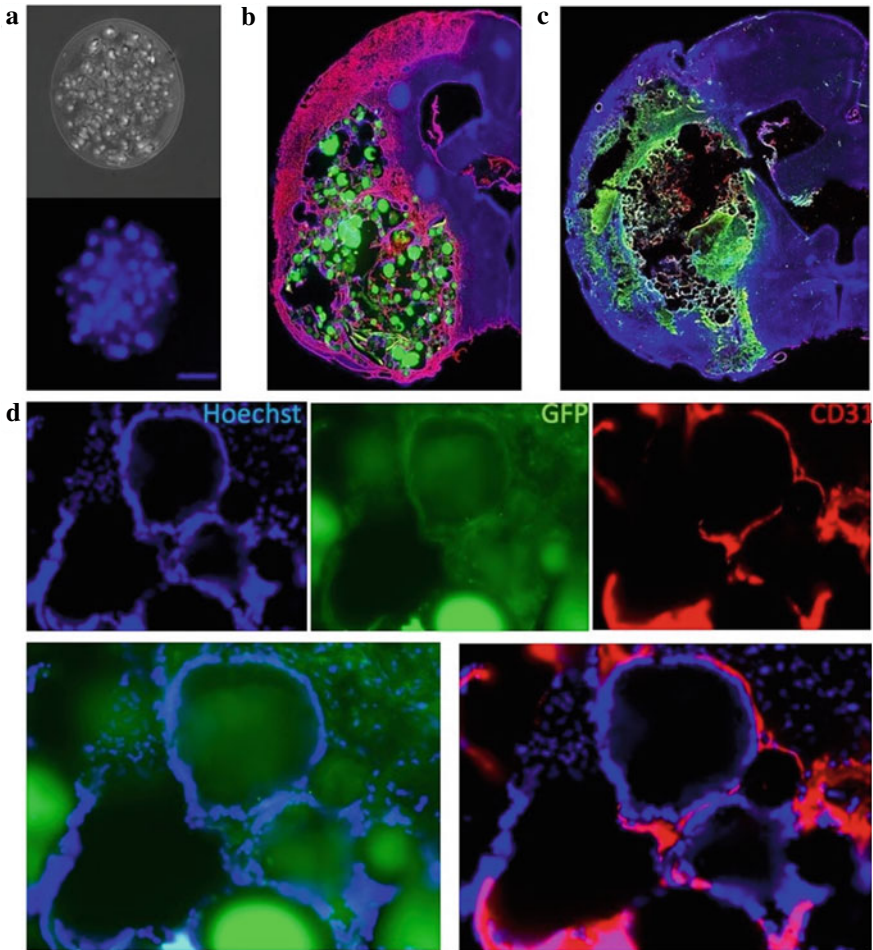


Fig. 8.7 Implantation of pre-fabricated neurovascular units. **a.** Prefabricated microspheres containing neural stem cells (NSCs) and endothelial cells. **b.** Blank microspheres (green) mixed in ECM hydrogel prior to implantation in the stroke cavity illustrate a homogenous distribution. **c.** Implantation of prefabricated units afforded the efficient delivery of green fluorescent protein tagged NSCs throughout the tissue cavity and peri-lesional tissues. Migration of these cells was facilitated by the ECM hydrogel in-between microspheres **d.** Endothelial cells implanted in MMP degradable microspheres also invaded the surrounding ECM hydrogel and created a neovasculature that will be required to sustain a newly forming tissue

Conclusions

The goal in tissue regeneration is to rebuild the architecture of the lost tissue and recover its functionality. Inductive bioscaffolds hold great promise for regenerating brain tissue by providing a supportive environment that actively stimulates and guides

processes to create a new tissue [116]. Despite great strides having been accomplished, functional tissue regeneration has not yet been conclusively demonstrated. An ongoing effort is hence required to determine the required design characteristics and optimize the properties of these inductive scaffolds. Gradually this process will deliver improvements in tissue restoration and bring us closer to a future where brain tissue regeneration is feasible, offering new hope for individuals affected by acute brain injuries [57].

Ethics Statement The authors declare no conflict of interest or financial relationships pertaining to the materials reviewed in this chapter.

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Chapter 9

Alginate Nanofiber Scaffolds for Amyotrophic Lateral Sclerosis



Davis M. Maulding, Julia Bielanim, Parker Cole, Yang Tian, Mahsa Saeedi,
Hari S. Sharma, Aruna Sharma, and Ryan Tian

Abstract *Introduction:* Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disorder that causes the loss of upper and lower motor neurons which leads to muscle atrophy and loss of voluntary movement. Currently, treatments approved by the FDA for ALS are expensive and have little benefits, emphasizing the need for more affordable and effective treatment options. This chapter aims to determine the potential of alginate nanofibers cross-linked with calcium, barium and strontium to deliver methylene blue as a treatment for ALS. *Methods:* This chapter was written with the help of Julia Bielanim's thesis work and multiple literature reviews found on Web of Science discussing ALS and alginate nanofiber scaffolds for drug delivery. *Results:* Alginate has been shown to successfully encapsulate different ALS therapies including stem cells and other neurotrophic factors. These therapies have been found to promote the survival of motor neurons and improve motor function in animal models of ALS. Additionally, we have shown that alginate nanofibers cross-linked with strontium had the highest loading efficiency at 59.9% and loaded the most moles of methylene blue per mg of nanofiber at 2.9 nmol/mg when compared to barium and calcium cross-linked nanofibers. The barium alginate nanofibers had the highest structural integrity followed by strontium then calcium. *Conclusion:* Overall, the use of alginate nanofibers to treat ALS has been shown to be a promising approach for the field of neurodegenerative medicine. The unique properties of these nanofibers such as biocompatibility, biodegradability, and similarities to the human tissue extracellular matrix make them an ideal drug delivery method to treat ALS. Additionally, their ability to enhance the survival of motor neurons, provide a scaffold for cell therapies and regulate immune response make them promising biomaterials for any motor neuron disease. Although there is still much research to be done, the potential of alginate nanofibers for the treatment of ALS is exciting and can advance treatment methods for this disease.

D. M. Maulding · J. Bielanim · P. Cole · Y. Tian · M. Saeedi · H. S. Sharma · A. Sharma ·
R. Tian (✉)
(ARSC)-Arts & Sciences. (CHBC)-Chemistry & Biochemistry, University of Arkansas,
Fayetteville, AR, USA
e-mail: rtian@uark.edu

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Abbreviations

ALS	Amyotrophic lateral sclerosis
MND	Motor neuron disease
SOD1	Superoxide dismutase type 1
TARDBP	TAR DNA binding protein
TDP43	TAR DNA binding protein 43
FUS	Fused in sarcoma
OPTN	Optineurin
VCP	Valosin-containing protein
UBQLN2	Ubiquilin 2
C9ORF72	Chromosome 9 open reading frame 72
PFN1	Profilin 1
hnRNP	Heterogeneous ribonucleoproteins
CNS	Central nervous system
ATP	Adenosine triphosphate
3NT	3-Nitrotyrosine
AGEs	Advanced glycation end products
VEGF	Vascular endothelial growth factor
bFGF	Basic fibroblast growth factor
BMPs	Bone morphogenetic proteins
PBS	Phosphate buffered saline
SEM	Scanning electron microscopy
MRI	Magnetic resonance imaging
LIF	Leukemia inhibitory factor

Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disorder a type of motor neuron diseases (MNDs) [1], characterized by progressive degeneration, dysfunction, and demise of motor neurons [2]. ALS represents the most prevalent form of MNDs, alongside progressive bulbar palsy, primary lateral sclerosis, and Kennedy's disease, in this classification [3]. MNDs are classified based on their etiology, categorized as either inherent or sporadic [2]. In ALS, approximately 5–10% of cases have a hereditary component, while the majority, around 90%, are considered sporadic in nature [4].

Usually, MNDs are classified according to the specific motor neurons that are predominately affected. These diseases can impact upper motor neurons, lower motor

neurons, or both [2]. Classical ALS involves the degeneration of both upper and lower motor neurons. Upper motor neurons, located in the cerebral cortex, serve to activate interneurons and lower motor neurons, which, in turn, directly control muscle contraction and relaxation [2]. The third category for MNDs is based on the muscle groups where the symptoms first occur. In ALS, symptoms typically start in the limbs or bulbar region [5]. Limb onset ALS is more common accounting for about 66% of cases, and presents with muscle weakness, stiffness, decreased stability when standing, and involuntary muscle twitches [1]. Conversely, bulbar onset ALS affects the muscles in the head and neck, leading to difficulties in chewing, slurred speech, and respiratory impairment. As the disease progresses, it progressively involves all muscles essential for moving, eating, speaking, and breathing [1].

Additionally, cognitive or behavioral changes are observed in about 50% of ALS patients during the advanced stages of the disease [6]. Notably, a prevalent behavioral symptom is the loss of sympathy. Ultimately, ALS patients succumb to respiratory failure typically within 2–4 years from the onset of symptoms [7]. Presently, no definitive cure or effective treatment for ALS exists. Management primarily revolves around alleviating symptoms, enhancing the quality of life, and providing comprehensive support to patients and their caregivers. Ongoing research endeavors aim to identify potential therapies and interventions that can slow down disease progression and improve the outcomes for patients affected by ALS.

Prevalence and Incidence of ALS

Globally, ALS affects approximately 4 individuals per 100,000 population, with an annual incidence rate of around 2 cases per 100,000 [8]. Sexual dimorphism is observed as there are higher prevalence and incidence rates in males when compared to females. Plausible explanations for these disparities include differential exposure to toxins and variations in the biological responses to these toxins [9]. It is also possible that the mechanisms underlying nervous system repair may differ between genders and result in differences observed in ALS incidence and prevalence [9]. However, the precise etiological factors driving these discrepancies remain unclear. Additionally, the typical age of ALS onset occurs between 60 and 70 years old [8].

Geographical location also plays a role in ALS epidemiology. In the United States, ALS exhibits slightly higher prevalence rates compared to other regions worldwide, with an estimated prevalence of approximately 5 cases per 100,000 individuals [10]. In contrast, South Asia displays lower prevalence and incidence rates, although disease onset tends to occur at an earlier age. Intriguingly, South Asia demonstrates prolonged survival times following ALS diagnosis [8]. This discrepancy in ALS rates between developed and developing regions may be attributed, in part, to the variations in average age across these populations [8]. Furthermore, smoking has been identified as a potential risk factor for ALS development. Wang et al. reported a 9% increase in risk per 10 years of smoking and a 10% increase in risk for individuals consuming 10 cigarettes a day. The detrimental effects of smoking on motor

neurons, such as direct neurotoxicity and increased oxidative stress, may contribute to the heightened ALS risk associated with tobacco use [11].

Various other factors have been linked to ALS incidence and prevalence. Notably, occupations characterized by higher socioeconomic status, such as mathematics, architecture, engineering, law, and education, exhibit increased ALS disease mortality rates [12]. Professions involving intense physical activity, including American football players, Italian soccer players, and United States military veterans, also demonstrate higher ALS prevalence rates [13–15]. For instance, American football players exhibit a four-fold increase in ALS prevalence, likely due to the rigorous physical demands and elevated risk of head injuries [13]. However, a comparative study analyzing ALS mortality rates among road cyclists, professional basketball players, and professional soccer players revealed that only soccer players displayed significantly elevated mortality rates. This discrepancy suggests that factors beyond physical activity contribute to the observed ALS risk in soccer players, potentially including chemicals exposures, such as fertilizers, and an increased incidence of head injuries [15].

Projections shown in Fig. 9.1 indicate a substantial increase in ALS cases over the next two decades, highlighting the urgent need for further research and intervention. ALS not only carries a high mortality rate but also imposes a significant economic burden. The economic impact arises from frequent hospital admissions, rehabilitation, medication costs, medical testing, and reduced workforce productivity [16]. The annual cost per ALS patient is estimated to be near \$69,475 in the United States, \$59,018 in Spain, and \$47,092 in Germany [16–18]. These costs escalate with disease progression, adversely affecting the quality of life for both patients and caregivers, underscoring the profound impact of ALS on individuals and society.

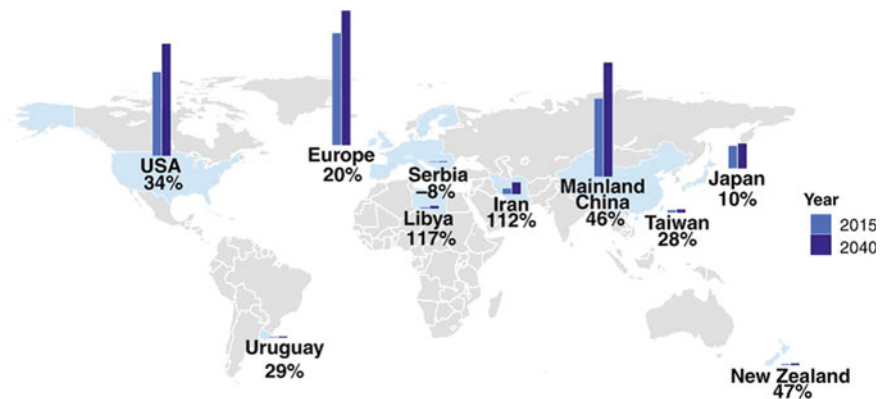


Fig. 9.1 Projected increase in the number of individuals with ALS from 2015 to 2040 [17]

Etiology of ALS

Considerable progress has been made in understanding the genetic etiology of ALS by identifying underlying genes. These genes include SOD1, TARDBP, FUS, OPTN, VCP, UBQLN2, C9ORF72 and PFN1 [19]. In combination, these genes help explain 75% of inherited cases and 11% of sporadic cases. A comprehensive understanding of the genetic etiology of ALS provides fundamental insights into the cellular mechanisms resulting in neuron degeneration. The relationship between superoxide dismutase 1 (SOD1) and ALS was first discovered in 1993 as SOD1 mutations are associated with ALS [20]. However, ALS developed from SOD1 mutations is thought to be distinct from other types of ALS as it lacks TDP43 and/or FUS pathology which are present in nearly every other case of ALS [19].

Abnormal aggregation of TDP43 is a pathological feature in over 90% of ALS cases. Here the protein that typically localizes to the nucleus for transcription misfolds and aggregates in the cytosol [21]. The ubiquitination and hyperphosphorylation of the protein lead to amyloid-like aggregation, increased hydrophobicity, and confinement of crucial cellular components [21]. Mutations to TARDBP (the gene encoding TDP43) account for 4% of familial cases while the fused in sarcoma (FUS) mutation also accounts for 4% of familial cases [21, 22]. Both proteins are structurally close to the family of heterogeneous ribonucleoproteins (hnRNP) and can directly bind RNA and DNA which alters RNA processing [23]. These proteins have a shared pathology of misfolding and incorrect localization to the cytosol which further emphasizes the role abnormal RNA metabolism plays in motor neuron degeneration.

Other mutations linked with ALS include optineurin (OPTN), valosin-containing protein (VCP), ubiquitin 2 (UBQLN2), Chromosome 9 Open Reading Frame 72 (C9ORF72) and sequestosome 1 (SQSTM1) mutations [19, 24]. Mutations in UBQLN2 for both inherited and sporadic cases implicate proteasome degradation of ubiquitinated proteins as a possible contributor to the pathology [25]. A large intronic hexanucleotide repeat expansion in C9ORF72 accounts for 40% of familial and 7% of sporadic ALS cases for people of European ancestry [19]. Additionally, RNA foci are observed in patients with C9ORF72 mutations which implicates the disruption of RNA metabolism through sequestration of RNA binding proteins and other RNA species [26].

Overall, the similarities in genetic mutations between familial and sporadic ALS has led to the view that separating ALS into inherited ALS and sporadic ALS is outdated. Inherited and sporadic ALS share many mechanisms of neurodegeneration including mitochondrial dysfunction, axonal transport, toxic protein aggregation, impaired protein degradation, prion-like spreading, excitotoxicity, oxidative stress, hypermetabolism, inflammation and RNA metabolism defects [27]. This evidence indicates that ALS is a clinically and genetically heterogeneous motor neuron disease with multiple underlying pathophysiological mechanisms.

Methods of Pathogenesis

The complexity of ALS has led to a variety of different treatments, but apparently no generally accepted treatment is able to reverse neural damage and cure the disease. Since its discovery in 1869, many studies have been conducted to understand more about ALS. These studies have led to more than 60 molecules and 30 genes being associated with the pathogenesis of ALS [19]. While the exact cause of ALS is still unknown, continued clinical investigations have led to the proposal of several methods of pathogenesis. Four of the major methods of pathogenesis include excitotoxicity, mitochondrial dysfunction, oxidative stress and neuroinflammation.

Excitotoxicity is one of the prominent mechanisms associated with the pathogenesis of ALS. Neuronal excitotoxicity refers to an excessive stimulation of glutamate receptors which is associated with the release of ions into the cell resulting in calcium overload. This can be neurotoxic and activates enzymes that degrade proteins, membranes and nucleic acids which results in cell injury or death [28]. This is due to the role glutamate has in the transmission of nerve impulses and the regulation of synaptic plasticity as it is the primary excitatory neurotransmitter in the central nervous system [29]. Since this mechanism relies on the overstimulation of glutamate receptors, inhibiting the glutamate receptors can minimize neuronal death.

The inflammation of neurons has been shown to be associated with neuron death, even during the pre-symptomatic stage of ALS. Neuroinflammation is characterized by the activation of microglia and astrocytes, T lymphocyte and monocyte infiltration and the overproduction of inflammatory cytokines [30]. Zhao et al. demonstrated a positive relationship between motor neuron degeneration and the levels of microglial activation [31]. An increase in other immune system cells, including mast cells, is associated with nerve loss in neuromuscular junctions which is commonly found in muscles of ALS patients [32]. During early stages of ALS anti-inflammatory and neuroprotective factors are present but inflammation becomes proinflammatory and neurotoxic as the disease progresses. As motor neurons are injured through progression of ALS, these cells release distress signals. This induces an inflammatory response produced by surrounding glial cells and other immune cells in the central nervous system (CNS). Overall, the balance between these pro- and anti-inflammatory factors affects the rate the disease progresses as well as survival times for ALS patients [33].

Oxidative stress is another mechanism that has been associated with the pathogenesis for ALS. The overproduction of reactive oxygen species can cause an imbalance in the produced and accumulated oxygen species resulting in oxidative stress. This stress can break down cell tissue and cause damage to DNA which can cause inflammation. This was first considered in the pathology of ALS due to the discovery of the mutated SOD1 [34]. SOD1 is a Cu–Zn metalloprotein that converts the superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and is primarily located in the cytosol but also found in the nucleus, peroxisomes, and mitochondria. This protein is responsible for the antioxidant defense of the cell and regulating cellular respiration and energy metabolism [35]. While the pathogenesis is still not fully understood, it

is theorized that mutated SOD1 can result in the aggregation of cytotoxic proteins which may reduce enzymatic function. Since SOD1 helps the clearance of reactive oxygen species, a reduced enzymatic activity can potentially increase the degree of oxidative stress [34]. Additionally, oxidative stress causes TDP43 and FUS to be delocalized from the nucleus to the cytoplasm and increases the aggregation of both proteins [36].

Mitochondria dysfunction is a common feature among neurodegenerative diseases, including ALS. The mitochondria is responsible for generating energy and maintaining the function of neurons. Dysfunctional mitochondria have been observed in both humans and animals with ALS which is why it is considered for the ALS pathology. Several studies have shown that patients with ALS have displayed reduced mitochondrial function through decreased ATP production and impaired mitochondrial respiration [37, 38]. Additionally, changes in mitochondria morphology, including fragmentation and reduced quantity, have been observed in the spinal cord of ALS patients [39]. These studies have suggested that dysfunctional mitochondria may contribute to the injury and death of motor neurons in ALS patients. The exact role mitochondria dysfunction has is still not fully understood but one proposed mechanism is that misfolded proteins accumulate in the mitochondria and lead to oxidative stress and damage to the mitochondrial DNA which may lead to further dysfunction and neuron loss [40].

Current Therapeutics for ALS

While there still is no cure for ALS, different therapies and medications have been introduced to slow the progression of the disease. These therapies and medications have been developed through the understanding of the different methods of pathogenesis. Since excitotoxicity occurs when the glutamate receptors are overstimulated, any method to reduce this excessive stimulation can help slow the progression of ALS. This idea led to clinical trials of different drugs and the FDA approval of riluzole to help treat ALS [34]. Riluzole modulates the glutamate receptors indirectly by modifying the sodium channel current and inhibiting the release of glutamate [41]. This drug is used by approximately 50–75% of people with ALS and is associated with a 35% reduction in mortality [42].

Due to neuroinflammation being a common condition in ALS patients, several compounds with anti-inflammatory properties have been used to observe their effect on ALS patients [34]. Of these compounds, masitinib has shown promising benefits during clinical trials. Masitinib is a highly selective tyrosine kinase inhibitor that has increased the life span in SOD1 model mice through the prevention of neuroinflammation. In the SOD1 mice, masitinib prevented axonal pathology, mast cell and neutrophil infiltration and secondary demyelination [31]. Additionally, combining masitinib and riluzole treatment has reduced the functional decline of ALS patients by 27% [43].

One FDA approved drug that is currently used to combat oxidative stress in ALS patients is edaravone. Edaravone is considered a free radical scavenger that has been proven to eliminate lipid peroxides and hydroxyl radicals which cause damage to neurons [44]. This drug is shown to decrease oxidative stress as it causes a decrease in 3-nitrotyrosine (3NT) levels. 3NT has been considered a marker of oxidative stress as ALS patients have displayed increased levels of 3NT. This biomarker is formed from the nitration of tyrosine which results in neurodegeneration [45]. While edaravone has been shown to decrease oxidative stress and delay disease progression, it has not displayed the ability to reverse neuronal damage [46]. Since this drug helps with oxidative stress, it can also improve mitochondrial dysfunction. Other mitochondrial targeted therapies include MitoQ, a mitochondria targeted antioxidant, and rapamycin which promotes mitochondrial biogenesis and function [47].

Alginate Nanoscaffolds in Programmable Drug Release

Alginate nanofiber scaffolds are an ideal material to use for drug delivery to motor neurons that have been affected by ALS. This is due to alginates biocompatibility, low toxicity, low cost, and ability to undergo simple modifications. Additionally, alginate can achieve different bioactivities such as anti-tumor properties, counteraction of oxidation, regulation of immune response, reduction of inflammation and neuroprotection [48]. Alginate scaffolds of nanowires that form nanopores resemble that of natural bioscaffolds in the extracellular matrix in human tissues. The nanoscale bioscaffolds possess a high surface area to volume ratio and an ample diffusion path for loading and releasing drugs [49].

Alginate is a naturally occurring anionic polymer often obtained from brown seaweed. The alginate is extracted from Phaeophyceae, commonly referred to as brown algae, but other sources have been used to make alginate from two genera, *Pseudomonas* and *Azotobacter* [50]. Polymeric alginate nanofibers can be extracted from brown algae and are excellent candidates for regenerative medicine because they can be modified to better match the extracellular matrix's structure, surface chemical composition, and mechanical properties. Additionally, alginate nanofibers are similar to glycosaminoglycan, a proteoglycan found in the extracellular matrix of humans. Glycosaminoglycan has many roles in cell signal processing including regulation of cell growth, proliferation, promotion of cell adhesion, anticoagulation, and wound repair [51]. This resemblance allows alginate nanofibers to provide mechanical support and regulate cellular activities [52].

The composition of the alginate depends on the natural source which has different ratios of two sterically different repeating subunits. One subunit is 1,4- α -L-guluronate, commonly referred to as the G subunit, and the other subunit is 1,4- β -D-mannuronate, regarded as an M subunit [52]. These subunits are linked together by β -1,4-glycosidic bonds. These subunits can be cross-linked using cations, but only the

G subunit is thought to contribute to the intermolecular cross-linking. The composition sequence, G subunit length, and molecular weight are all highly influential factors that influence the physical properties of the alginate scaffolds [50].

The molecular weight of alginate nanofibers can be modified by changing the composition of G and M subunits. This changes the physical properties of alginate nanofibers as a higher molecular weight can result in greater mechanical strength. However, increasing the molecular weight also leads to an undesired high viscosity and low bioavailability [50]. To overcome this disadvantage, alginates can be degraded into oligosaccharides which increases the absorption of drugs and biocompatibility [48]. Alginate is typically oxidized using sodium periodate to increase bioavailability. The sodium periodate oxidation cleaves the carbon-carbon bond from the cis-diol group from one of the subunits which alters the chair conformation, and the alginate backbone is degraded. The degree of oxidation has a large influence on the degradation of the fibers into oligosaccharides [50].

Alginate can be cross-linked using divalent cations which can increase the biodegradability, as cross-linked alginate scaffolds release divalent ions, while also incorporating cations present in biological systems. Ionic cross-linking is the most common method to cross-link alginate [50]. The degree of cross-linking with alginate is dependent on the charge density of the cation (i.e., charges-to-radius ratio) and the number of G subunits.

Alginate is an attractive biodegradable material for the potential treatment of ALS. The antioxidant, immunoregulatory, anti-inflammatory and neuroprotective properties of alginate are great properties to battle the complex pathogenesis of ALS previously discussed. Several studies have demonstrated alginate's abilities to inhibit oxidative stress-induced neurotoxicity and inhibit the formation of amyloid β in rat models diagnosed with Alzheimer's [53]. When amyloid β is deposited in the brain, amyloid plaques are formed which are misfolded proteins that form in spaces between nerve cells, causing neurotoxicity. Alginate has also shown its immunoregulation properties through increased cytokine activity. Additionally, multiple studies have shown the ability alginate has to activate macrophages and the host immune system [54]. This ability to influence the immune system allows alginate to suppress the activation of microglia and reduce proinflammatory mediators. The neuroprotective properties of alginate are from its ability to inhibit oxidative stress and regulate the immune system. In, alginate is an antiglycation agent [48]. Glycation is a chemical process where abnormal binding occurs between sugars in the blood and cells with proteins, lipids, and nucleic acids [55]. The process of glycation results in advanced glycation end products (AGEs) which participate in the pathogenesis of neurodegenerative disorders and can cause abnormal brain protein binding, oxidative stress, and loss of neurons [48]. Overall, the biological properties of alginate make it ideal to combine with a drug delivery system to help combat ALS.

Alginate Nanoscaffold Programmable Release Kinetics

Due to their versatility, oligosaccharides derived from alginate can be used to regulate the kinetics of drug release. Self-assembled alginate nanofibers are nanoporous which allows for the rapid diffusion of small molecules, including low molecular weight drugs [50]. The varying release kinetics for different alginate nanofibers allows these scaffolds to be utilized as a practical vehicle to deliver different types of drugs. A better understanding of these kinetics helps determine if different alginate nanoscaffolds can maintain drug levels in the targeted tissue within the therapeutic time-window without requiring multiple doses [49]. Whenever single large doses are administered, the dose initially exceeds the minimum toxic concentration then rapidly drops below the minimum effective concentration. This makes a drug delivery system capable of a sustained release above the minimum effective concentration but below the minimum toxic concentration more preferred than a single large dose or multiple small doses [49]. Providing the drug in one extended release helps avoid fluctuations of drug concentration and reduces risk to the patient by minimizing invasive delivery mechanisms. Since sustained release and time-programmable release at a low dosing frequency is preferred, where zero-order drug release profiles (i.e. kinetics) are often used. This means the drug will ideally be released at a constant rate.

As a biodegradable polymer, alginate's ability to deliver drugs is accomplished and modified through three different mechanisms. These include diffusion-controlled release, solvent-controlled release, and degradation-controlled release. For drug release, the drug encapsulated in alginate polymer particles (full of interconnected nanopores) is released via drug diffusion and the polymer network degradation. The initial release is due to the dissolution of drugs deposited on the surface and a gradual delivery of drugs from the alginate particles' surface and subsurface. Thereafter, swelling and erosion cause the drug molecules deeper in the nanopores to be released to the medium. Finally, degradation of the alginate nanoscaffold allows the remaining drugs to be released. This degradation process is dependent on cross-linking density, porosity, and the release medium [56]. Since mammals do not have the enzyme alginate, alginate itself is not able to be degraded in humans. This problem is eliminated as the surface erosion and destruction of the cross-linked structure results in the degradation of the polymer in biofluids [50]. For alginate nanofibers, the polymer degrades faster due to the short distance water is required to travel to reach the interior of the fiber [49]. These degradable polymeric drug delivery systems are ideal because they are easily removed from the body without causing other side effect issues.

Methods of Alginate Nanoscaffold Synthesis

There are several methods to synthesize alginate nanofibers, in which the wet spinning is a commonly used ultra low-cost method. The typical procedure for this synthesis first requires the preparation of a sodium alginate solution which is used as the prepolymer [57]. This solution is extruded into a cross-linking bath containing cross-linking agent, such as CaCl_2 , at various concentrations to serve as a coagulation solution. This cross-linking bath is usually under constant stirring during the gradual addition of the sodium alginate solution. The diameter of these nanofibers can be varied widely by controlling the polymer composition and the rate that sodium alginate is added to the cross-linking bath. Additionally, different cross-linking agents and their concentration influence the mechanical strength, drug release and the scaffold's pore size. This technique has many advantages including ultra low-cost, biosafe, biodegradable, quick and simple synthesis, and high yield [57].

In addition to wet spinning, electrospinning is another method to synthesize alginate nanofibers. For this method, a high voltage is applied to an alginate solution containing the cross-linking agent. This solution is injected into a spinneret, forming a charged jet that is extruded onto a collector plate where nanofibers are solidified as shown in Fig. 9.2b [58]. These nanofibers have a large surface to volume ratio, an interconnected network structure, and a malleable porosity. This can be modified by controlling the solution properties, setup parameters including the voltage used, and ambient conditions such as temperature and humidity [58]. Alginate nanofibers synthesized using this method have been applied to different fields such as bioremediation, tissue engineering, drug delivery and even sensors.

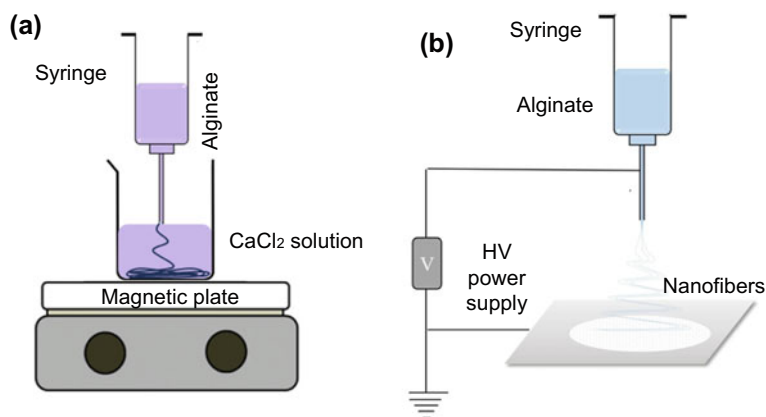


Fig. 9.2 Schematics of methods to synthesize Alginate nanoscaffold by **a** controlled gelification using CaCl_2 solution and **b** electrospinning

Current Applications

Alginate has shown promising applications for efficient biological drug release due to its adaptability and beneficial properties. Various alginate technologies have been used to regenerate different tissues and have been shown to successfully regenerate blood vessels, bones, cartilages, muscles, nerves, pancreas, and liver [50, 59, 60]. These technologies have also been used to increase neovascularization in tissue with restricted blood flow. This has been accomplished through the localized and sustained release of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to the ischemic tissue [50]. Alginate cross-linked with calcium cations has displayed the ability to sustainably release dibutylryl cyclic adenosine monophosphate and stromal cell-derived F-I to heal wounds [59]. Alginate can also be used to effectively deliver bone morphogenetic proteins (BMPs) to improve osteogenic differentiation of bone-marrow derived cells [61].

With the promising effects of alginate nanofibers on other tissues of the mammalian body, further research has been done to understand the ability for alginate nanofibers to assist the repair of the central and peripheral nervous systems. When alginate fibers are incorporated into spinal cord lesions in rats, axonal growth can be directed [62]. Alginate gels can serve as a scaffold to restore gaps in cat sciatic nerves while also promoting outgrowth of regenerated axons [63]. Overall, alginate has shown it can be used as an effective method of drug delivery to help tissue regeneration and repair the central and peripheral nervous systems.

Methylene Blue

Methylene blue, discovered in 1876 by Heinrich Caro, is a tricyclic phenothiazine. Under physiological conditions, methylene blue is a cation to interact with the anionic alginate nanofibers. An extensive amount of research on methylene blue has also shown the drug's ability of controlled release to treat diseases including depression, enzymopenic hereditary methemoglobinemia, Ifosfamide-induced neurotoxicity, vasoplegic adrenaline resistant shock and Alzheimer's disease [64]. Methylene blue can treat Alzheimer's disease as it inhibits the aggregation of tau proteins and A β peptides which are both common events for Alzheimer's patients. Additionally, methylene blue inhibits the aggregation of TDP43 which is a protein that aggregates in 97% of ALS patients [21, 64]. While the benefits from methylene blue are consistent, the mechanisms of these therapeutic effects are still not entirely concluded. It can be from to the ability to inhibit aggregation of different proteins or the antioxidant effects from interacting with the electron transport chain [64].

Methylene blue is ultra low-cost, can permeate the blood-brain barrier and has approximately 73% bioavailability [64]. These properties and its displayed ability to prevent the aggregation of TDP43 proteins indicate methylene blue may have a

promising role in the treatment of ALS. Additionally, methylene blue shares structural and dimensional similarities with current drugs used to treat ALS including riluzole and edaravone. This makes it ideal to use the highly affordable methylene blue to better understand how alginate nanofibers can effectively be used as a drug delivery vehicle to treat ALS.

Loading, Release and Degradation of Alginate Nanoscaffolds with Methylene Blue

We have conducted some experiments to understand more about alginate's ability to load and release methylene blue to further improve treatment methods for ALS [65]. This work was conducted by analyzing different alginate nanofibers synthesized via the wet spinning method with different parameters including the cross-linking agent and the concentration of the cross-linking agent. All nanofibers were analyzed with scanning electron microscopy (SEM) to confirm the nanoporous and amorphous structure. These selected nanofibers were analyzed with different concentrations of methylene blue solutions. Strontium chloride (SrCl_2), barium chloride (BaCl_2) and calcium chloride (CaCl_2) were used as the cross-linking agents in the cross-linking baths with concentrations of 50, 100, 200, 300, and 400 mM. These alginate nanofibers produced were loaded with methylene blue at 12.2 nM, 48.8 nM, and 97.5 nM in phosphate buffered saline (PBS). Approximately 30 mg of the cross-linked alginate nanofibers were added to the different concentrations of methylene blue for 18 h under constant agitation. After the nanofibers have been loaded with the methylene blue, they are washed and then transferred to a PBS solution for studying the release kinetics. Both the loading and releasing efficiencies were determined by using UV-Vis spectroscopy to measure the concentration of methylene blue in the sample. For the release studies, UV-Vis measurements were performed on the PBS solution every 30 min for 3 h.

The highest loading efficiency (59.9%) for methylene blue was observed for 300 mM strontium cations cross-linked alginate and 48.8 nM methylene blue as this nanofiber absorbed the highest proportion of methylene blue present in solution. Most strontium cross-linked alginate nanofibers outperformed the barium and calcium counterparts in respect to loading efficiency as shown in Fig. 9.1.

Additionally, barium cross-linked nanofibers had a greater loading efficiency than calcium cross-linked nanofibers. The data may imply that 300 mM cross-linked nanofibers have a maximum molar capacity near the methylene blue concentration of 48.8 nM as the loading efficiency was halved on doubling the concentration as shown in Fig. 9.3. It is important to notice that different modes of delivery, intrathecal administration versus typical intravenous and oral administration, may change the optimal drug dose.

The release studies do not follow any specific trends to indicate that the concentration of cross-linked agents has an influence on the release of methylene blue.

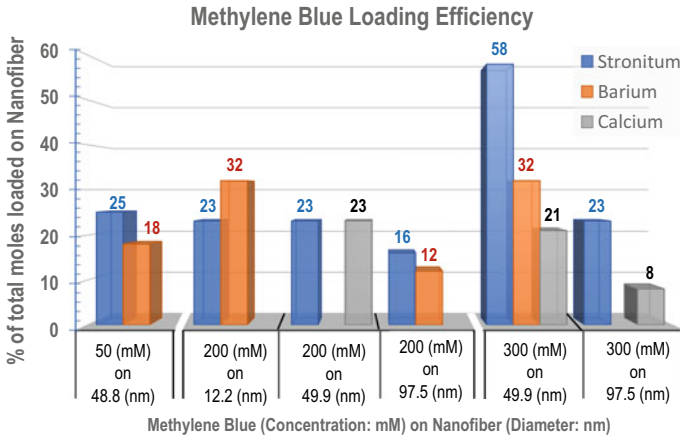
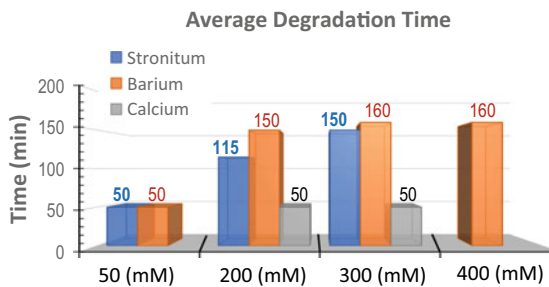


Fig. 9.3 Methylene blue (concentration: mM) on nanofiber (diameter: nm)

However, barium linked alginate nanofibers displayed the most ideal release profile, showing that barium can also be an ideal cross-linking agent for the delivery of methylene blue, especially where barium cations’ toxicity is not a large issue.

To further examine this claim, degradation micro profiles (or kinetics) must be determined. Ideally, the alginate nanofibers should degrade slowly to retain their integrity long enough to allow for a programmable and sustained release of the loaded drug. As the concentration of the cross-linking agent increases, the degradation time slightly increases, which is useful. Additionally, barium linked nanofibers had the longest average degradation time and calcium linked nanofibers had the shortest degradation time as shown in Fig. 9.4. It has been shown that as the size of the cross-linking agent increases, the degradation time increases. This implies that the biocompatible strontium can be better than the biocompatible calcium and not that of biocompatible barium in cross-linking alginate into nanofibers for delivering methylene blue.

Fig. 9.4 Ionic crosslinking cations concentration (mM)



Potential Treatment of ALS

Contrasting many studies done to show alginate's excellent drug delivery capabilities to regenerate tissue and repair the central nervous system, many fewer studies have been reported on using alginate nanoscaffolds to treat ALS. Kalkowski et al. have synthesized alginate-based hydrogels for cell transplantation, particularly stem cells, to help treat neurodegenerative diseases including ALS [66]. These hydrogels can provide a microenvironment for cells to protect the encapsulated cells from destruction during injection. Additionally, this lab integrated manganese (II) cations to allow for non-invasive magnetic resonance imaging (MRI) to monitor the hydrogel activity after injection. The Araszkiwicz lab has further improved this work by analyzing and optimizing the properties of the alginate hydrogels [67]. These studies have shown that alginate hydrogels can be used as a supporting matrix for therapeutic cells to help treat ALS.

While these labs have loaded cells into the alginate hydrogels to help treat ALS, another lab has attempted to use alginate rods to treat this disease. Feeney et al. used calcium alginate rods to deliver leukemia inhibitor factors (LIFs) to the central nervous system to be used as an ALS therapy. The LIFs were successfully transported from the calcium alginate rods; however, the LIFs were found to have no beneficial effects for SOD1 mice [68].

Alginate Nanowires Drug Delivery with *in vivo* Models

Our teamwork is currently engaged in a new collaboration with the University of Arkansas Medical School to investigate the kinetics and efficacy of alginate nanowires in facilitating the delivery of drugs across the blood brain barrier (BBB). While more research is needed to study how alginate nanowires can be used to treat neurodegenerative diseases, there is plenty of evidence to display the ability for alginate to deliver drugs across the BBB. The BBB presents a difficult challenge in drug delivery to the brain, as it restricts the entry of many therapeutics. Alginate nanowires can offer a solution to this as their high surface area and porous structure offer a platform for encapsulating drugs and potentially passing through the BBB. It has been shown that the use of alginate nanowires can significantly improve the delivery of drugs across the BBB. Mamo et al. have shown that probucol can be encapsulated in alginate nano-capsules to be delivered across the BBB. When encapsulated, the plasma and brain concentration of probucol increased significantly in mice compared to nonencapsulated probucol [69]. Additionally, the tunable mechanical properties of alginate nanowires allow them to be functionalized with ligands which can result in targeted delivery. While this technique has had little application to delivery across the BBB, ligands have been chemically coupled to the alginate backbone to promote and regulate cellular interactions. Lee et al. has shown that peptides including the sequence of arginine-glycine-aspartic acid can be used to promote the

adhesion and growth of cells [50]. Lastly, alginate formulated nanoparticles have been reported as successful delivery vehicles across the BBB. Alginate-cholesterol micelles coated with lactoferrin have been shown to deliver a neuroprotective steroid to the brain in mice [70]. Furthermore, doxorubicin-alginate nanocomplexes with chitosan matrices showed enhanced uptake into the brain of rabbits [71]. Overall alginate nanowires show promising applications for delivering drugs across the BBB due to their biocompatibility, tunable mechanical properties, and ultra-low cost.

Conclusion

The utilization of alginate nanofiber-based bioscaffolds as a therapeutic strategy for treating ALS presents a promising avenue in the realm of neurodegenerative nanomedicine. The distinctive characteristics exhibited by these nanofibers, including biocompatibility, biodegradability, and resemblance to the extracellular matrix of human tissue, render them highly suitable as a drug delivery system for ALS treatment. Moreover, their capacity to enhance motor neuron survival can modulate immune responses and position them as compelling bionanomaterials for various motor neuron disorders. Although further extensive *in vitro* and *in vivo* investigations are warranted, the potential application of alginate nanofiber scaffolds in ALS therapeutics holds considerable promise and holds the potential to revolutionize treatment modalities for this debilitating condition.

Compliance with ethical standards

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Conflict of Interests All authors declare they have no conflict of interest.

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Chapter 10

Developing High-Fidelity In Vitro Models of Traumatic Brain Injury to Test Therapeutic Biomaterials



Christopher Adams, Bushra Kabiri, Raja Haseeb Basit, Jessica Wiseman, and Divya Maitreyi Chari

Abstract *Introduction:* Traumatic brain injury (TBI) can cause significant disability and is a leading cause of death amongst under 40s. There is a clinical need to develop new and effective therapies for TBI but this represents a significant challenge given the complexity of the injury and pathological barriers to regeneration. Development of combinatorial therapeutic biomaterials, capable of achieving multiple clinical goals such as promoting nerve growth, disrupting scarring tissue and modulating neuroinflammation holds promise to augment repair. Fine tuning material properties during developmental screening to optimise efficacy requires complex and pathology mimetic models of TBI. Animal models are not appropriate for initial optimisation given their expense, requirement for infrastructure and expertise and associated ethical hurdles, requiring appropriate alternatives to simulate TBI. Our goal in this chapter is to review the current state of the art in modelling TBI using in vitro model systems which replicate complex pathological processes of TBI, can be interfaced with biomaterials, provide detailed histological and functional readouts, whilst offering ease of use in the laboratory. *Methods:* Searches of literature in PubMed and Web of Science were conducted using combinations of “traumatic brain injury”, “neurological injury”, “in vitro model”, “3D model”, “stem cells”, “organoids”, “organotypic”, “biomaterials”, “nanomaterials” and “tissue engineering”. Primary

C. Adams (✉)

Neural Tissue Engineering Group, School of Life Sciences, Keele University, Staffordshire, UK
e-mail: c.adams@keele.ac.uk

R. H. Basit

Bradford Royal Infirmary, Bradford, West Yorkshire, UK

J. Wiseman

Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield, UK
e-mail: jessica.wiseman@sheffield.ac.uk

B. Kabiri · D. M. Chari

Neural Tissue Engineering Group, School of Medicine, Keele University, Staffordshire, UK
e-mail: b.kabiri@keele.ac.uk

D. M. Chari

e-mail: d.chari@keele.ac.uk

research articles, literature reviews and book chapters were used to inform the writing of the article. *Results*: Many in vitro models of TBI exist, ranging in complexity from monocellular cultures to 3D self-assembling organoids and 3D organotypic slices. Each system has advantages and disadvantages for testing therapeutic biomaterials in TBI. The primary drawbacks of the most common models are lack of complex modelling of pathology (especially the neuroimmune component), limited capacity for biomaterial interfacing and limited functional readouts. However, we identify promising in vitro models and new strategies to address these challenges. *Conclusions*: Novel advances in in vitro TBI modelling can allow for a translational pipeline of therapeutic biomaterial development in pathology simulating, yet facile benchtop systems.

Keywords Traumatic brain injury · Neurological injury · In vitro model · 3D model · Stem cells · Organoids · Organotypic · Biomaterials · Nanomaterials · Tissue engineering

Introduction

Clinical biomaterials are of high interest to regenerative neurology as they have the potential to modulate multiple pathological processes in sites of neurological injury. For example, biomaterials can integrate into sites of injury, provide topographical guidance for regenerating tissue, modulate pathological responses and allow controlled and targeted delivery of therapeutic factors. Accordingly, they are under investigation in multiple neurological diseases and injuries [55, 126]. Penetrating traumatic brain injury (pTBI) is of particular interest given the clinical severity of the injury, the need for combinatorial treatments and the generation of a cystic cavity within which therapeutic biomaterials can be implanted. Several novel biomaterials are currently in development to treat pTBI [83, 182]. Animal models have long been the gold standard for testing new TBI therapies. However, these models are not suitable for exploratory testing and development of biomaterials due to ethical issues, expense and requirement for extensive technical training, specialist infrastructure and staff. There has been a global drive in the past decade to develop high fidelity in vitro models of pTBI within which biomaterial properties can be optimised. In this chapter, we will explore pTBI pathology, highlight new therapeutic biomaterial development, outline the need and ideal parameters for an in vitro test bed and discuss some of the recent advances in this field.

pTBI Has a Profound Socioeconomic Effect

pTBI is defined as head injury that occurs when a foreign object traversing the skull causes direct parenchymal injury. It has the worst prognostic outcomes of all

TBIs and is the commonest cause of death in under 40 s in the UK population as of 2015 [37]. Typical mechanisms of injury include road traffic accidents, sports related injuries, abuse/assault cases and, less commonly in the UK, gun-shot injuries [107]. Generally, patients die before reaching a hospital with initial mortality rates of up to 70% reported [61, 74, 140]. The morbidity of patients that survive but typically do not recover fully to pre-injury functionality represents a significant socioeconomic burden to society. For example, TBI is estimated to cost the UK around £15 billion per year, which includes providing treatment and ongoing care and the subsequent loss of earnings of patients [145]. There are also substantial social impacts on the patients and their support networks, with head injuries doubling the risk of developing mental health problems compared to the rest of the population [145]. Mainstay clinical management focusses on supportive measures, and occasionally decompressive surgery that aims to preserve life, but does little to mitigate the long-term clinical sequelae of severe injury. Currently, with the brain's limited internal capacity for repair and lack of approved targeted neuroregenerative therapy administered in early injury, there is a great clinical urgency for more research in this area. A major challenge for experimental research is to simulate the complex pathophysiology and refractory nature of the injury.

The Pathophysiology of pTBI Is Complex with Many Barriers to Regeneration

Description of pTBI pathology is commonly split into primary and secondary injury processes. Primary injury occurs within the first few seconds to minutes of injury and is attributed to the direct impact of the object entering the brain [135]. This can be described depending on the characteristics, velocity and pathway of a foreign object travelling through the brain. Penetration results in shearing, rotation, compression, lacerations and ricocheting (typically in gunshot wounds) forces. This causes haemorrhage and haematomas, contusions, vascular injuries, disruption of the blood brain barrier (BBB) and focal or diffuse axonal injury [193]. Axonal shearing usually results in permanent loss of the associated neuronal circuitry and focal neurological deficits. The impact can also cause crush injury and necrosis of cells at the lesion edge [158]. High velocity injuries will also trigger wave forces that cyclically stretch and compress the surrounding tissue, forming cavitations and oedema. The irreversible nature of primary injury means prevention is key, through public health education and safety training to prevent common mechanisms of injury (falls, road traffic accidents, gunshot wounds etc.) [107]. Secondary injury is described as the expanded, longer-term effects of the initial injury. It is the result of complex inflammatory cascades, excitotoxicity, mitochondrial dysfunction and oxidative stress which leads to chronic pathology.

Excitotoxicity is the accumulation of high levels of neurotransmitters causing cytotoxic overactivation of their associated receptors, usually due to high glutamate levels [14], but also high aspartate [32] and gamma-aminobutyric acid (GABA) [34, 139, 186]. This eventually results in loss of neuronal membrane integrity, necrosis, oedema and BBB damage [9, 207]. Excitotoxicity triggers rises in intracellular calcium through many mechanisms [11, 28, 45], and affects both neurons and glia [54]. N-methyl D-aspartate receptors (NMDARs), an ionic glutamate receptor, are the primary glutamate receptors to mediate intracellular influx of calcium [71, 195] and explains the beneficial effects of NMDAR antagonists, including MK-801 (Phillips et al. 1998, [32], memantine [208] and ketamine [198, 197]) for treating excitotoxicity associated injuries. Once an intracellular rise in calcium occurs, catabolic intracellular cascades mediated by lipid peroxidases, proteases and phospholipases, result in early neuronal swelling, gradual neuronal disintegration and eventually irreversible neuronal injury [33]. Mitochondrial dysfunction is one of the main pathways leading to cell death and is triggered by intracellular rises in calcium, as mitochondria harbour many Ca^{2+} -sensitive enzymes triggered in excitotoxicity [67]. Once this occurs, apoptosis, necrosis, and necroptosis are triggered through caspase-dependent and independent pathways [31, 56].

Oxidative stress is another primary pathophysiological mechanism of brain injury [50]. The effect of TBI on cerebral circulation by either directly tearing arteries, via primary injury, or through inflammatory changes in the microvascular environment, via secondary injury cascades, results in hypoxia and oxidative stress. Reperfusion injury, defined as the paradoxical rise in cellular injury after blood circulation is restored, also further increases free radical and reactive oxygen species (ROS) production. Free oxygen, nitrite and superoxide radicals (e.g., the formation of peroxynitrite from nitric oxide) further impair cerebrovascular function [38, 153]. Together, they collectively tip the oxidant-antioxidant balance and further exacerbate the pro-inflammatory state.

At a cellular level, the surrounding chemotactic debris [116], pro-inflammatory cascades and cytokines trigger the mobilisation of immunocompetent cells, microglia, astrocytes and peripheral immune cells [162] to the lesion site [166]. Astrogliosis, the process of astrocytes and microglia forming a glial scar, begins as astrocytes ‘palisading’ towards the injury. Here, astrocytes extend long, fibrillary processes perpendicular to the injury border [25, 216]. The release of myelin fragments exposes inhibitory molecules such as neurite outgrowth inhibitors (NOGO) which have been shown to limit axonal repair and neuroregeneration at the injury site in the long-term [24, 68, 99]. Microglia adopt hyperreactive, amoeboid morphologies and trigger a positive feedback cycle exacerbating the anti-repair and pro-inflammatory state [41, 43]. Collectively, this translates clinically to the initial parenchymal oedema, cerebral vasospasm and raised intracranial pressures causing high initial mortality levels.

In the longer-term, astrogliosis, triggered in early injury, results in glial scar formation, made up of hypertrophic astrocytes, hyperreactive microglia and oligodendrocyte precursor cells (OPCs) [2] that limits axonal regeneration across the injury site—a critical barrier to neurorepair. Once inflammation triggers astrogliosis and

long-term glial scarring, targeted neurotherapy options become of limited efficacy [171, 203]. Hence modulating the early anti-repair, pro-inflammatory response in a time-specific window after injury is critical to reduce long-term functional disability.

Biomaterials as a Pro-Regenerative Therapy for pTBI

The complex pathophysiology of pTBI necessitates multifaceted and combinatorial therapies; combinations of biomaterials, nanoparticles and stem cell transplantation show significant promise. Several biomaterials have been proposed as suitable therapeutic candidates and are both naturally and synthetically derived. Hydrogels, 3D polymeric scaffold materials, polymers and nanomaterials are the main categories of biomaterials that are commonly tested in neural tissue and will be explored here. Many studies also commonly use different biomaterials in combination to create 'hybrid materials', such as combining naturally and synthetically derived scaffolds in hydrogel form, or functionalising nanomaterials using naturally derived polymers (Table 10.1).

Hydrogels

Hydrogels are some of the most common biomaterials tested in in vitro neural studies. They are cross-linked, water-enriched macromolecular networks that can be derived from natural polymers such as collagen [101, 179], gelatin [89, 212], hyaluronic acid [63], chitosan [3] and alginates [149, 217]) and synthetic polymers, such as polyethylene glycol [21]. The macroscopic, moisture-rich 3D architecture of hydrogels means they are ideal for biological interfacing, and can also be used to deliver cell therapies, creating a 'live biomaterial' for implantation. The primary advantage of hydrogels is the ability to inject the material as a liquid which then gels in situ, to fill the injury cavity. This facilitates less invasive delivery than implants, whilst still allowing formation of a scaffold for promoting tissue regeneration. Further, delivery can be directly into the parenchyma bypassing the BBB for direct interaction with surrounding neurological cells [29, 36, 82, 137]. A similar effect has been shown with delivery of neurotrophic factors [85, 202]. Mechanical properties such as compressive modulus, porosity and biodegradation can be altered, usually by altering the density and nature of crosslinks. The ideal hydrogel compressive modulus has been shown to vary depending on cell type, with neurons typically preferring softer surfaces (0.1–1.0 kPa) [53, 109] and astrocytes and oligodendrocytes preferring stiffer surfaces (0.5–10 kPa) [62]. This modulus may also vary depending on the length of time after transplantation, as biodegradation can lead to decreased structural integrity of the material, making it slowly more conducive to neuronal growth and infiltration over time [77]. Biodegradation can therefore be controlled, not just to synergise with the natural proliferation and infiltration of endogenous cells, but also to be slowly replaced by the body's endogenous extracellular matrix (ECM), which is an important long-term goal. Biodegradation should also be slow enough to mitigate the long-term neuroinflammatory effects after injury. Synthetic oligopeptides

Table 10.1 Various types of biomaterials and their application in repairing pTBI

Biomaterial	Description	Advantages	Disadvantages	References
<p><i>Naturally derived 3D scaffold biomaterials</i></p> <p>Collagen (<i>rodent, marine and bovine based</i>)</p>	<p>Key naturally occurring structural protein</p> <p>Usually type I fibrillary collagen forming major component of connective tissue</p> <p>Can be used as 2D, 3D scaffold or hydrogel</p> <p>Frequently combined with other polymers (e.g., Collagen-PGA tubes, collagen-laminin bio-copolymers)</p>	<p><i>All collagen types</i></p> <ul style="list-style-type: none"> • Abundant • Easily sourced • Extensively researched • Non-toxic, low immunogenicity • Degradation products non-toxic • Biomodulatory effects (natural cell binding sites, guides natural neural growth and proliferation) • Can be magnetically aligned for directed axonal growth <p><i>Marine</i></p> <ul style="list-style-type: none"> • Low antigenicity • High levels of cellular adhesion • High biodegradability • Theoretical decreased risk of cross-species disease transmission (zoonosis) <p><i>Bovine</i></p> <ul style="list-style-type: none"> • Some products (e.g., DuraGen™) FDA approved for clinical testing and use in neural tissue • Clinically already widely used as dural graft 	<ul style="list-style-type: none"> • Low mechanical and tensile strength • Batch to batch variability • Risk of immune reactivity (cross species implantation) • Sterilisation decreases integrity and/or biomodulatory effects 	<p>[16, 17, 88, 90, 100, 120, 179, 206, 210, 214]</p>

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
Chitosan	<p>Linear polysaccharide derived from chitin, abundant polysaccharide typically sourced from crustaceans and shellfish, also from insects and fungi</p> <p>Gel forming capabilities</p> <p>Can be processed into sponges, membranes, beads, scaffolds, nanofibers</p>	<ul style="list-style-type: none"> • High adsorption capacity • High biodegradability • Non-cytotoxic, low-immunogenicity • Antibacterial, antifungal, anti-tumour and anti-inflammatory properties • Immunoadjuvant, anti-thrombogenic and anti-cholesteremic agent • Often used to enhance biocompatibility with other synthetic polymers with better mechanical properties • Can be used as 3D printing bioink for neural constructs 	<ul style="list-style-type: none"> • Poor mechanical properties when moist • Low solubility at pH > 7 • Cationic polymer (risk of neurotoxicity) 	<p>[3, 59, 75, 183, 198, 197]</p>
Gelatin	<p>Denatured linear protein obtained by acidic or alkaline hydrolysis of collagen</p>	<ul style="list-style-type: none"> • Long history of safe use in pharmaceuticals, cosmetics, food products • Low cost, high availability • High biocompatibility and biodegradability • Less antigenic than collagen • Chemically modifiable for increased cell adhesion and proliferation • Absorbs water, can be used to form hydrogels 	<ul style="list-style-type: none"> • Poor printability • Batch variability • Lack of thermostability • Poor mechanical stability • Linear, need to crosslink, attempting to crosslink can lead to cytotoxic byproducts • Complex crosslinking process difficult to scale up 	<p>[59, 82, 212]</p>

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
Elastin; elastin-like polypeptides (ELPs)	ECM protein that provides elasticity to tissue and organs, consists mainly of tropoelastin and microfibrils ELPs; a synthetic protein-like material based on repetition of conserved motifs found in native tropoelastin, more commonly used to enhance biocompatibility and stability	<ul style="list-style-type: none"> Inherent biological recognition properties, presence of receptor-binding ligands Sensitive to proteolytic destruction (biodegradable) ELPs: <ul style="list-style-type: none"> Greater control in targeted drug/neurotrophin delivery Can be used to obtain microfibers, nanofibers, hydrogels, scaffolds ELP hydrogels can be used as biotink for 3D printed scaffolds 	<ul style="list-style-type: none"> Low mechanical/tensile strength Batch variability Immunogenicity ELPs: <ul style="list-style-type: none"> High polydispersity Production is a long, complex process, high cost 	[79, 130, 133, 159]
Hyaluronic acid	Glycosaminoglycan found in ECM, crucial role as lubricant	<ul style="list-style-type: none"> Biodegradable, biocompatible, natural so inherent biological recognition properties Supports neurite outgrowth, differentiation and proliferation Mechanical properties induce differentiation of neural progenitors Can be used to form nanoparticles, hydrogels, granular hydrogels or microgels Combinable with other biodegradable synthetic polymers (PLGA, poly-L-lysine) Can decrease inflammatory response to electroconductive polymers Dynamic cross links with shear-thinning and self-healing capabilities allows for injectability, where removal of the force allows cross-links to reform the HA materials 	<ul style="list-style-type: none"> Cells not as adherent to surface of hydrogels, commonly blended/cross-linked with other materials to improve cell adherence 	[118, 130, 167, 172, 174, 196]

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
Alginate	Naturally occurring anionic biopolymer obtained from brown seaweed, can also be obtained from bacteria Can form hydrogels	<ul style="list-style-type: none"> • Biocompatible, low toxicity, low cost • Drug and protein delivery, wound healing, cell transplantation • Relatively high stiffness 	<ul style="list-style-type: none"> • Natural presence of impurities (e.g., heavy metals, endotoxins, proteins etc.) • Needs to be purified in multi-step extraction procedure • Limited long-term stability of cross-linked alginate gels as they can be dissolved by surrounding media 	[108, 112, 149, 180, 198, 197]
Keratin	Polypeptide with intermolecular bonding of disulphide cysteine amino acid	<ul style="list-style-type: none"> • Biodegradable, biocompatibility and non-immunogenicity • Good cellular adhesion and proliferation • Can be formed into hydrogels and scaffolds 	<ul style="list-style-type: none"> • Limited data in the context of neural tissue engineering 	[46, 155, 218]

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
<p><i>Synthetic polymeric scaffolds</i></p> <p>Poly(lactic acid (PLA), polyglycolic acid (PGA), Poly-lactoglycolic acid (PLGA)</p>	<p>Thermoplastic polymers, polyester links of lactic or glycolic acid respectively</p> <p>Can be used to generate scaffolds or multi-channel conduits, microparticles</p>	<ul style="list-style-type: none"> • Can be absorbed or hydrolysed in vivo, biodegradable • Successfully shown to support neuronal growth and proliferation, as well as NSC differentiation and maturation • PLA/PGA can be easily combined to form PLGA for superior mechanical properties • Synthetic, therefore lower risk of immunogenicity • Can be combined with natural polymers, e.g., gelatin • Specific ratio of PLA:PGA in PGLA can be altered for specialised functions e.g. drug delivery microparticles • PLGA microspheres shown effective at transporting molecules across the BBB 	<ul style="list-style-type: none"> • PLA scaffolds can be structurally unstable, and easily shatter and crumble • PGA mechanical strength tends to fall months after implantation • Non-biological material, lack of biomodulatory effects seen in naturally occurring polymers 	<p>[137, 205]</p>

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
Poly-ethylene glycol (PEG)	Poly-ethylene glycol polymer, well suited for hydrogel formation due to hydrophilic properties	<ul style="list-style-type: none"> • Highly biocompatible, hydrophilic • Biochemically inert, versatile • Non-immunogenic, resistant to protein absorption, non-toxic • Shown to improve neural cell survival, proliferation, differentiation • PEG hydrogels closely matched stiffness of native brain parenchyma 	<ul style="list-style-type: none"> • Biologically inactive (synthetic), hence typically used in combination with other polymers 	[21, 130, 172, 209]
Poly-caprolactone (PCL)	Synthetic polyester polymer of caprolactone, semicrystalline polymer	<ul style="list-style-type: none"> • Controllable hydrolytic degradability rate • Hydrophobic, slower degradation rate than other synthetic polymers (24–36 months), useful for long term implants/drug delivery • Non-toxic • Can be combined with other polymers (altering degradation rate etc.) • Can be developed to form calcium phosphate gradient, can lead to spatial gradients in mechanical properties, altering cell proliferation • Versatile processing, can be 3D printed, heat moulded, good shape memory • Good solubility in organic solvents • Can be electrospun to generate sheets, tubes, sponges, parallel oriented fibres 	<ul style="list-style-type: none"> • Cell adhesion typically poor (due to hydrophobic properties) • Needs coating with another molecule (e.g., collagen) to promote cell attachment 	[130, 136, 205]

(continued)

Table 10.1 (continued)

Biomaterial	Description	Advantages	Disadvantages	References
Polypyrrole (PPy)	Organic polymer of pyrrole monomer, commonly used conductive polymer for neural tissue engineering Usually used in combination with other synthetic and non-synthetic polymers (PPy-PLA, PPy-PLGA, PPy-PCL, PPy-HA etc.) to enhance biocompatibility	<ul style="list-style-type: none"> Electrically conductive material PPy-PLA: enhances neural adhesion, alignment and elongation PPy-PCL: supports cell proliferation Can be electrospun into nanofibers, cells can be thus electrically stimulated to encourage topographical neurite growth Can be combined with hyaluronic acid to construct electroconductive 3D hydrogels 	<ul style="list-style-type: none"> On its own, poor biocompatibility and cell adhesion 	[167, 211, 200]
<i>Nanomaterials</i>				
Carbon nanotubes (CNTs)	Allotropes of carbon with cylindrical structures Can be single walled nanotubes (SWCNTs) or multi-walled (MWCNTs)	<ul style="list-style-type: none"> High thermal conductivity and stability Optimal mechanical and electrical properties Biocompatible and conductive SWCNTs modulate and stimulate neurite growth MWCNTs have greater stability, have been applied to 3D printing scaffolds for nerve regeneration or targeted drug delivery devices 	<ul style="list-style-type: none"> Generally non-biodegradable (can be useful instead as long-term implants) In vivo long-term accumulation risk not established 	[22, 84, 127, 138, 167]
Graphene	Allotrope of carbon as 2D layers in a hexagonal lattice Can form 3D scaffolds, foams, graphene nanogrids Typically chemically functionalised further to enhance biocompatibility	<ul style="list-style-type: none"> Bactericidal, antiviral, highly biocompatible with generally low cell toxicity Electrically conductive (enabling stimulation, differentiation and proliferation of NSCs) 3D scaffold can encapsulate other nanoparticles (e.g., gold) encouraging neuronal differentiation and axonal growth and alignment 	<ul style="list-style-type: none"> Reports of 2D graphene causing matrix cellular toxicity (3D scaffold foams reported more effective, biocompatible) Higher toxicity in smaller forms 	[5, 6, 114, 174]

have been made into polymers using crosslinkers that can be targeted by endogenous hydrolysing enzymes, such as matrix metalloproteases (MMPs), collagenases, or plasmin [106, 199].

Polymeric implants

Other types of protein scaffolds that can be wet or dry, have also been trialled as biomaterials for neural interfacing after injury. These polymeric implants have similar advantages to hydrogels for transplantation into injury. They are malleable/mouldable to injury sites, support endogenous infiltration/proliferation and/or can house exogenous biological therapies (cell transplants, antibodies etc.) [23]. Their 3D microarchitecture aims to mimic lost ECM, while being biodegradable to slowly be replaced with host's ECM. They can be made from similar materials as hydrogels. Frequently, several polymers can be combined to form co-polymers such as poly-lactic-co-glycolic acid (PGLA) or poly- ϵ -caprolactone-co-ethyl-ethylene phosphate (PCLEEP), PCL-PGLA etc. These materials can be formed through electrospinning, freeze-drying, water-emulsion, 3D printing, microfluidic fabrication, and can be adapted to different mechanical and physical parameters.

A key advance in polymeric scaffold design is the development of electrically conductive polymers [134, 173] that can potentially restore the electrical activity lost within injury sites [211]. They have been shown to enhance the migration or proliferation of neural cells to facilitate the reconstruction of lost neuronal circuitry post-injury [82]. Notably, their conductivity can also be used for controlled, electrostimulation mediated release of encapsulated neurotherapeutic molecules [95]. These includes materials such as polypyrrole (PPy) or polyaniline (PANi) [187]. The key disadvantage with conductive polymers is their enhanced stability limits their ability to degrade in vivo, which may potentially cause further inflammation or immunogenic reactions. Hence these polymers are frequently crosslinked with other biodegradable natural or synthetic polymers to augment biological compatibility.

Nanomaterials

Nanoparticles have also shown great promise for neurotherapy following injury, with various types and formulations tested. They can be formulated for specific biomolecule encapsulation and targeted delivery to injury sites. Nanoparticles are also often incorporated into 3D scaffold materials or hydrogels to enhance their functionality [8, 22], or have been used to engineer cell transplant populations to increase their therapeutic efficacy [49]. Their adaptability has many advantages in a wide range of the therapeutic applications, depending on the characteristics of the nanoparticle. Specific nanomaterials that have been developed for neurological injury include carbon nanotubes (CNTs) and graphene.

CNTs have the benefits of being biocompatible, electrically and thermally conductive with optimal mechanical properties. They are made exclusively of carbon atoms forming sheets which wrap into small tubes, either single-walled (SWCNTs) or multi-walled (MWCNTs). Pure CNTs are typically insoluble in water, however this has been resolved by chemical functionalisation of their surfaces, leading to increased solubility and application in biological environments. Studies have demonstrated that

CNTs can enhance neuronal electrical signalling [121], decrease astrocyte proliferation and reduce microglial density, mitigating glial scar formation and encouraging regeneration [22, 129]. These findings are attributed to their surface conductivity and molecular interactions, resulting in adsorption of endogenous proteins mediating cell adhesion. Neuronal adherence has also been shown to be susceptible to changes in the surface roughness using electron microscopy analyses, where neuronal growth cones preferred surface roughness which matched the diameter of the neuronal processes [175].

Like the previously mentioned biomaterials, CNTs can also be combined with other polymers, forming mixtures to create a desired blend of mechanical, biological and electric characteristics. They have also been demonstrated to be biocompatible with stem cells offering combined multifactorial therapy [127]. One disadvantage to their use is that they are non-biodegradable. However, this can be used as an advantage when designing implants for long term recovery, providing molecular, electric and topographical cues for longer term regeneration.

Graphene is an alternative carbon-based allotrope composed of a hexagonal 2D lattice of carbon atoms. It is one of the thinnest, strongest and lightest materials known. It is highly thermally and electrically conductive and known for antibacterial and anti-inflammatory activity. Graphene can be fabricated into sheets, foam, substrates and nanogrids, and has been widely used in *in vitro* studies [5, 6, 114, 174]. The disadvantages to graphene include being highly susceptible to an oxidative environment and presence of jagged edges that can pierce cell membranes. Graphene is generally considered a more unstable allotrope, with insufficient active sites, limiting its interaction with other biomolecules. Typically, for this reason, graphene is chemically functionalised by adding an oxide or amine group. Collectively, these functionalised forms of graphene are called graphene-based materials (GBMs) which are stronger and more uniform, although more expensive to produce. Toxicity has also been reported with GBM use, although this is affected by various factors, including size, shape, impurities, functionalisation groups and combination with other therapies (e.g., other nanoparticles). The consensus is that smaller forms of GBMs are associated with high toxicity levels [39]. Finally, there is a lack of *in vivo* studies demonstrating efficacy and patterns of accumulation, degradation and/or excretion of the nanoparticles from the body.

Requirements for Alternatives to *in vivo* TBI Models for Developmental Testing of Biomaterial Based Therapies

Clearly there are a wide range of therapeutic biomaterials under development, all with the capability to modulate different aspects of neuropathology. Optimising biomaterial mode of action therefore requires complex neurological injury models to provide multifaceted, mechanistic readouts. Live animal, *in vivo* models of pTBI provide some of the most useful scientific data on the efficacy and applicability of a

neuroregenerative therapy. These models typically involve either large animals, such as monkeys, pigs, sheep etc., or small animals, such as rats, mice and other rodents, to evaluate the efficacy, applicability and side-effects of introduced biomaterials, either in living or post-mortem tissue. These studies provide valuable research data, relevant to human neuropathology. For instance, features such as an intact, functional circulatory system and BBB are difficult to replicate *ex vivo*, but significantly influence the delivery, mechanism of action and efficacy of a proposed neurotherapy. However, live adult animal models have considerable drawbacks with respect to developmental testing of therapeutic biomaterials. For example, significant costs are associated with housing and rearing animals and in training research staff in animal handling and surgical skills. Further, several animals are typically required for statistical analysis, with the numbers of animals required increasing exponentially with the number of biomaterial variables to be tested. Animal experiments are also low throughput given the time needed to rear the animal, administer an injury and therapeutic biomaterial, monitor behavioural changes and analyse large volumes of tissue post-experiment. The current scientific consensus is to limit the use of animals, with a focus on the 3Rs (replacement, reduction and refinement). Therefore, researchers should aim to reduce animal usage and limit the suffering and distress associated with *in vivo* models. Overall, these models are poor options in the *initial* testing of an emerging neurotherapy and may be considered a later option, if a therapy has sufficient *in vitro* evidence to warrant further testing.

Outlining Important Parameters for In Vitro pTBI Modelling to Test Therapeutic Biomaterials

Given the complexity of pTBI and combinatorial nature of biomaterial mediated repair, there is a key requirement in the field to develop *in vitro* models of pTBI with greater relevance to *in vivo* injury for development and testing. Ideally, such models would be simple to use but with high predictability for clinical use. A summary of the ideal properties of a pTBI model for testing therapeutic biomaterials is shown in Fig. 10.1 and will be discussed further in this section.

Of high importance is the model's relevance to *in vivo* and human pTBI. For this, we believe models should replicate major pathological processes of pTBI such as limited nerve fibre growth at injury sites, astrogliosis and glial scarring and neuroinflammation. Whilst relatively simple cellular systems can model these processes in isolation, few model systems are described which can model them *simultaneously*. Further, very few *in vitro* models contain the neural immune component—the microglia. This is a major omission given the role of microglia in modulation of nervous tissue responses to injury and implanted/injected biomaterials.

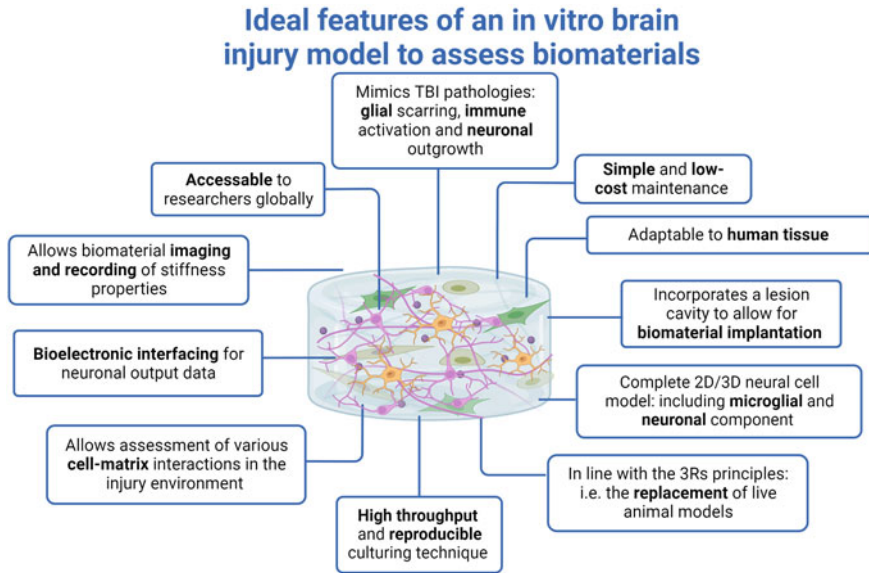


Fig. 10.1 Schematic highlighting ideal features of an in vitro neurotrauma model to test the utility of biomaterial therapeutics

Alongside replicability of injury/bioimplant modulation, the model should be compatible with standard and advanced analytical procedures. Immunocytochemical evaluation of in vitro systems is widely reported. Advanced imaging techniques, including time-lapse microscopy, should also be able to be used to investigate real time cellular responses to biomaterials, offering advantages over in vivo systems where this is challenging. Interfacing in vitro injury models with bioelectronics allows for the understanding of electrophysiological characteristics before and after injury, which is critical for functional readouts post treatment. There has been little focus on this aspect to date. When recapitulating brain physiology and the mechanisms of injury within in vitro models, it is vital to understand the maturation of neuronal networks and the developmental changes in electrophysiological activity. To achieve this, multi electrode arrays (MEAs) have been developed in two-dimensional (2D) and three-dimensional (3D) designs for the monitoring of neuronal firing activity. These systems record culture wide dynamics by recording changes in extracellular ionic concentrations generated by endogenous action potentials and provide a potential system to achieve this.

Finally, any in vitro model generated should be scalable, high throughput and adaptable by neuroregenerative laboratories globally. Whilst there will always be a trade-off between simplicity and replicability, new approaches may mean enhanced orders of replicability can be achieved by using relatively simple techniques. For widespread use, an in vitro model should also be cost-effective. Costs associated with in vitro culture are not always obvious, but include funding requirements for animal

facilities, initial cost of commercially available cells, licencing and management costs for animal and particularly human cells and the expense of specialised media.

Current In Vitro Models of pTBI and the Extent to Which They Meet Idealised Criteria

To address these challenges researchers have developed in vitro models of the brain which range in complexity from the simplest, 2D monolayer cell cultures to the highly complex 3D organotypic slice or organoid cultures. Different in vitro models have specific associated features which determine their research utility i.e., pathological relevance, technical difficulty and the ease of maintenance. An overview of in vitro models for modelling central nervous system (CNS) tissue, pathology and testing of therapeutic biomaterials is highlighted in Table 10.2. In this section, we will discuss these different systems in detail and how they compare with an idealised pTBI model.

Cell Lines and Primary Cell Monocultures

Immortalised single cell monocultures represent the simplest end of the spectrum, in relation to cost and convenience, versus in vivo models. Monocultures of single cell types are often bought commercially and used frequently. These cell lines are derived from cancer biopsies or are artificially immortalised. They have fewer ethical concerns, are easy to set up and maintain, require minimal training and are cost effective. The downside is their over-reductionist and simplistic nature which make them poor models of pTBI. They are neoplastic, showing a high variability in genetic makeup and expression over time and may thus exhibit abnormal physiological responses to injury. At best, they represent the behaviour of a single cell type, lacking the complex inter-cellular behaviours and interactions seen in the pathophysiology of pTBI. Moreover, studies have also shown frequent cross-contamination with cell lines procured from sources not always validated. Thus, evidence from these studies has limited therapeutic applicability.

Primary single cell monocultures have the benefit of being sourced from live animals, typically small rodents, so are considered more physiologically representative than cell lines. Classically for TBI modelling, monocultures of cortical neurons are established by plating cells from the dissociated cortex of embryonic rodents. Typically, this culture produces highly pure neuronal cultures, with less than 5% glial cell populations [115], in which an injury can be induced. With an adjustment to culturing techniques, individual glial cells can also be isolated and plated out into glial monocultures. Here, the method of culture is adapted from [128] who initially outlined a method for the bulk production of primary glial cells, to be separated

Table 10.2 In vitro systems for modelling brain tissue, traumatic injury mechanisms and biomaterial interventions

	Description	Advantages	Disadvantages	References
<p><i>In vitro models</i></p> <p>3D 'organotypic' slices</p>	<p>Ex vivo brain tissue slices</p>	<ul style="list-style-type: none"> • Retains in vivo cytoarchitecture • Retains major brain cell types (including microglia) • Ease of mechanical manipulation • Moderate difficulty to induce mechanical injury • Can be interfaced with biomaterials • Displays complex injury responses • Adaptable for excitotoxicity/hypoxia studies 	<ul style="list-style-type: none"> • Moderate throughput • Can be technically difficult to isolate and maintain slices • Requires more animals versus other in vitro models 	<p>[13, 26, 103, 146, 190]</p>

(continued)

Table 10.2 (continued)

	Description	Advantages	Disadvantages	References
3D organoids	Stem-cell derived self-organising suspension cultures forming brain-like spheroids (iPSC origin)	<ul style="list-style-type: none"> • Cytoarchitecture recapitulates developing tissues • Can be human/patient specific • Closely simulate in vivo cellular communication • Adaptable for excitotoxicity studies • Ideal for disease and development studies 	<ul style="list-style-type: none"> • Moderate throughput • Little uniformity between aggregates • Largely immature in cellular development • Long culture periods • Few injury systems reported • Lack vascular and immune components • Problematic for mechanical manipulation due to free floating nature • Spheroid centres can become hypoxic due to lack of nutrient access • Difficult cellular analysis • Complicated for biomaterial interfacing 	[20, 94, 143, 152, 165, 169]
Brain-on-a-chip	Microfluidic culture systems of 3D iPSC derived cultures	<ul style="list-style-type: none"> • Tissue-like physiology • Perfusion system of 3D tissue • Adaptable for disease/toxicity mechanisms • Axonal strain injury attempted 	<ul style="list-style-type: none"> • Low throughput • Scalability limitations • Lack immune and vascular components • Lack cellular maturity • Difficulty with mechanical injury induction • Difficulty with biomaterial interfacing 	[12, 42]

(continued)

Table 10.2 (continued)

	Description	Advantages	Disadvantages	References
3D hydrogel constructs	Cells encapsulated within a 3D matrix	<ul style="list-style-type: none"> • 3D architecture resembling tissue-like environment • Physiologically relevant cellular morphology • Simple maintenance • Moderate to high-throughput • Moderate technical difficulty for injury mechanisms • Biomaterial interfacing feasible 	<ul style="list-style-type: none"> • Difficult analysis of 3D environment • Can lack complexity if not multicellular constructs e.g., lack immune component if cells are NSC derived • Not currently documented with all the major cell types of primary brain cell dissociates • Lack vascular component (but feasible with tissue engineered blood vessels) 	[10, 78, 151]
2D cultures	Single or multicellular neural cultures grown as a monolayer	<ul style="list-style-type: none"> • Can encompass major brain cell types (including microglia and neurons) • Simple injury mechanisms • High throughput • Low technical difficulty • Simple maintenance and analysis • Biomaterial interfacing feasible 	<ul style="list-style-type: none"> • 2D environment • Cells undergo artificial responses to adapt to the flat, stiff substrate • Lack vascular component 	[16, 17, 58, 66]

(continued)

Table 10.2 (continued)

	Description	Advantages	Disadvantages	References
<i>Cell source</i>				
Primary multicellular models	Complex multicellular cultures of brain dissociates	<ul style="list-style-type: none"> • Can encompass all major brain cell types (including microglia and neurons) • High throughput • Low technical difficulty • Simple maintenance and analysis 	<ul style="list-style-type: none"> • Lack vascular component 	[14–18, 66]
Primary NSC cultures	Cultures of differentiated stem cells isolated from neurogenic regions e.g., subventricular zone (SVZ)	<ul style="list-style-type: none"> • High throughput • Low technical difficulty • Multicellular cultures 	<ul style="list-style-type: none"> • Lack immune component • Moderate length differentiation protocols • Preferential differentiation to astrocytes • Lack vascular components 	[49, 58, 124, 178, 192]

(continued)

Table 10.2 (continued)

Description	Advantages	Disadvantages	References
<p>Induced pluripotent stem cells (iPSCs)</p> <p>Stem cells genetically reprogrammed from adult cells</p>	<ul style="list-style-type: none"> • Indefinite propagation • Can be of human origin • Patient specific (retain genetic identity) • Beneficial for patient specific disease modelling 	<ul style="list-style-type: none"> • Moderate technical difficulty • Moderate throughput (long differentiation protocols) • Differ genetically/phenotypically from endogenous counterparts – altered morphology • Heterogeneity of cells • Resistant to cell death • Risk of mycoplasma contamination • Biomaterial-injury interface not reported • Lack immune and vascular components 	<p>[19, 70, 143, 152, 164, 165, 169, 98, 147, 189]</p>
<p>Primary pure cell cultures</p> <p>Primary cultures from brain dissociates; purified through sequential shaking or specific media components</p>	<ul style="list-style-type: none"> • High throughput • Low technical difficulty • Useful to study specific cell responses 	<ul style="list-style-type: none"> • Overly simplistic model of the brain • Absence of multicellular interactions • Lack vascular and immune component (if not ‘pure’ microglial cultures) 	<p>[30, 60, 40]</p>

(continued)

Table 10.2 (continued)

	Description	Advantages	Disadvantages	References
Cell lines	Immortalised cell lines	<ul style="list-style-type: none"> • Indefinite propagation • High throughput • Facile • Can be of human origin 	<ul style="list-style-type: none"> • Genetically and phenotypically differ from endogenous counterparts • High risk of mycoplasma contamination • Cellular heterogeneity • Resistant to cell death • Lack immune and vascular components 	[27, 65, 184]

The table is split into culture model systems and cell sources with advantages and disadvantages to each system described. Adapted and updated from [18]

out into individual cell type populations. Postnatal rodents are euthanised, cortical tissue is extracted and dissociated before being grown onto 2D plastic or glass. Cells mature to show a typical stratification with astrocytes forming a base layer upon which oligodendroglia and microglia form, allowing for easy isolation of specific cell types using a shaker. Injury induction in the 2D single cell cultures is also facile, using a pipette tip or needle to create an injury, making this a high throughput choice [58]. However, such systems can only provide data on single cell responses to injury and biomaterial intervention, which are not likely to be reflective of cellular responses in a complex, pathological environment.

Primary Defined Co-culture Models of pTBI

Primary monocultures of neural cells are easy to establish but do not recapitulate the intercellular interactions which occur in complex sites of neurotrauma. A relatively simple way to address this is to combine monocultures to form more complex in vitro cellular systems. Indeed, initial co-culture models were developed with combinations including astrocyte-microglia [92] and astrocyte-neurons [66]. Such systems were able to simulate key pathological neural responses seen within pTBI, for example palisading astrocytic glial scarring responses to trauma [15]. In addition, these systems have been employed to investigate intercellular differences in biomaterial handling, demonstrating that microglia out-compete other cells paired in the co-culture for uptake of therapeutic nanoparticles [91].

Co-culture systems provide a useful platform for the study of individual cellular responses to bio/nanomaterials, including complex intercellular interactions. Therapeutic testing for functional recovery in pTBI is often centred around targeting neurons/OPCs (regeneration) or attenuation of glial scarring (reactive astro/microgliosis) to create a repair- conducive environment. For such readouts, co-cultures suffice as a facile, repeatable and dependable strategy to study high throughput interactions. However, one drawback is that setting up defined co-culture systems requires multiple culture steps to establish two separate cell types with subsequent mixing at a later point, adding complexity and time to the procedure.

Primary Multicellular Models of pTBI

To address the above issues, complex in vitro systems derived from dissociated cortices have been developed which contain multiple, relevant neural cell types. Using similar protocols to those originally dissociating embryonic cortical tissue, we have shown that two multicellular systems for modelling pTBI can be established from post-natal mice that address many limitations associated with previous models. Using post-natal mice confers additional advantages as cultures do not require sacrifice of the mother. The first system is a mixed glial/polyglial culture system where

the stratified mixed glial cultures from [128] are plated directly onto glass coverslips. Here, astrocytes, oligodendrocytes and microglia are present in reproducible ratios. We demonstrated that a reliable scratch injury could be introduced into the cultures with simulation of key pathological responses (including reactive astrocytic scarring and microgliosis- amoeboid microglia infiltration of lesions). Further, a clinical grade collagen bioscaffold, DuraGen Plus, was interfaced into the lesion core and showed microglial infiltration and astrocytic invasion of the material [16, 17]. This model is particularly suited to studying glial responses to therapeutic biomaterials (including glial scarring around materials, glial based material remodelling and microglia-based material breakdown) in injury environments.

We have since evolved the model to include the neuronal population alongside multiple glial cells, through a simple switch in growth medium. Using similar techniques to the mixed glial system, an injury was established and pathological processes were shown to be replicated along with limited nerve fibre growth into the lesion cavity. We have also tested therapeutic biomaterials within this model, indicating high and dominant nanoparticle uptake by microglia in comparison to all other cell populations, when nanoparticles are delivered to the injury site (Fig. 10.2). In addition, straightforward interfacing of the model with MEA systems can provide global, network electrophysiological data to be generated at sites of injury and regeneration [204]. We are currently scaling the mixed neuronal injury model to 3D by encapsulation in soft, collagen hydrogels.

Stem Cell Derived Models of TBI

In vitro TBI models based on the use of stem cells have been extensively reported (Table 10.2). For TBI modelling, stem cells are differentiated into target neural cell populations (e.g., neurons, astrocytes or mixed cultures) prior to induction of injury. Broadly speaking, the advantages of stem cell models are the ability to expand the parent cell to increase experimental replicates, the capability to generate multiple neural cell types simultaneously, the potential to control cell phenotypes (e.g., specific differentiation into cortical neurons) and the ability to replicate more immature injuries e.g., foetal or young adult. Further, with the advent of cerebral organoid, brain-on-a-chip and 3D scaffold based culture, there is now added capability to replicate 3D neuroanatomical areas of the brain using differentiated stem cells.

Numerous sources and classes of stem cells exist and have been reported. To simplify this section, we will refer to three different classes—tissue specific, embryonic and induced pluripotent stem cells. Tissue specific stem cells are found within niches in foetal to adult neural tissue e.g., the subventricular zone, which maintains a population of neural stem cells (NSCs) throughout life in mammals. These stem cells are already committed to a neural fate. Therefore, there are short, simple and cost-effective differentiation protocols to generate neural cells. Robust protocols for NSC derivation from rodents are available, and the culture and differentiation of these cells is extensively characterised within the literature [215]. We have

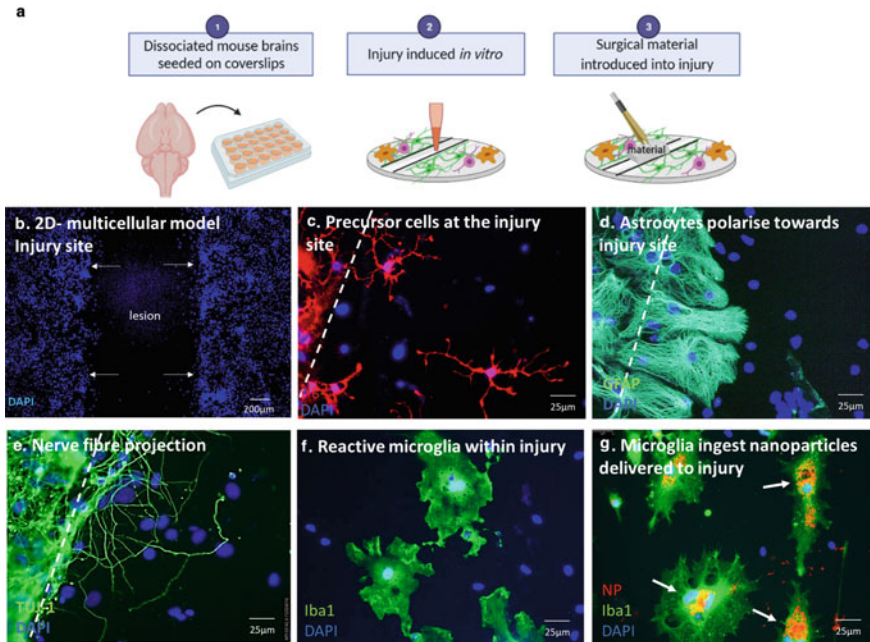


Fig. 10.2 Development of complex 2D multicellular models of pTBI. **a** Schematic demonstrating general protocol for establishing multicellular models of neurotrauma from dissociated cortex tissue. In summary, (1) cortical tissue is dissected and enzymatically dissociated. Once the dissociated culture is plated it matures into multicellular cultures with a “mixed glial culture” containing glial cells (astrocytes, oligodendrocyte lineage cells and microglia) or a “mixed neuronal culture” containing glial cells and neurons together. (2) An injury can be induced through scratching with a pipette tip with (3) subsequent biomaterial introduction and investigation into pathological responses. **b–g** Representative images from the mixed neuronal culture showing **b** reproducible injury induction, **c** progenitor cell migration at injury borders, **d** astrocyte hypertrophy and polarisation towards the injury site, **e** nerve fibre projection into the lesion and **f** reactive, amoeboid microglia within the lesion. Biomaterials can be delivered to the injury with **g** demonstrating nanoparticle (NP) uptake by microglia in the injury. Unpublished data from the NTEK group

previously shown differentiated populations of NSCs generate all major neural cell types (astrocytes, neurons and oligodendrocytes) simultaneously and can be used to test biomaterial function. For example, we demonstrated astrocytes out-compete neurons and oligodendrocytes for cellular uptake of nanoparticles in these multicellular cultures, which may be driven by enhanced levels of membrane activity [47, 48]. Elegant studies from the Erlandsson laboratory have shown the utility of differentiating NSCs to model TBI. Here, NSCs derived from embryonic mice cortices were differentiated for seven days to generate mixed cell cultures. Scratch injuries were induced with a scalpel and the authors were able to show astrocyte and neuron growth towards sites of injury, autophagy of dying cells by astrocytes and enhanced levels of S100 β and glial fibrillary acidic protein (GFAP, markers of astrogliosis) secretion into the medium in injured slices [124, 178]. Our group has adapted these

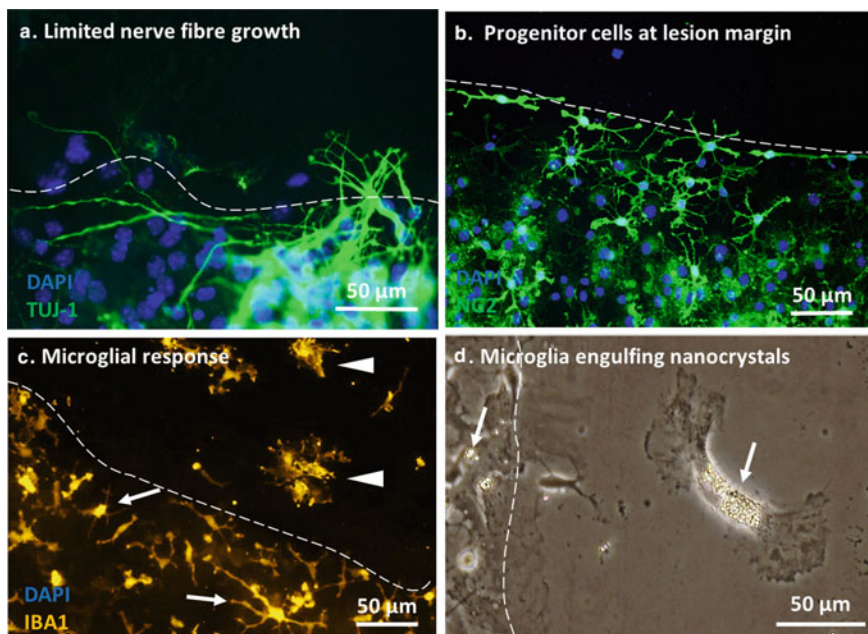


Fig. 10.3 pTBI modelling using differentiated populations of NSCs. Representative images showing **a** poor nerve fibre growth into the lesion cavity, **b** progenitor cell accumulation at injury margins, **c** microglial activation and invasion of injury sites, note amoeboid microglia in injury cavity (arrowheads) versus ramified microglia in cell dense areas (arrows). **d** Biomaterials can be introduced into the injury site. In this example, nanocrystal growth factor carriers appear to be ingested by invading microglia (arrow). Unpublished data from the NTEK group

systems to NSCs derived from post-natal mice to test biomaterials in sites of TBI. Our preliminary data indicate that injured differentiated NSC cultures demonstrate poor nerve fibre growth around injury sites, progenitor cell accumulation at injury margins and microglia invading the injury (Fig. 10.3, unpublished data).

In order to generate human-relevant neuromimetic models, NSC derivation from human foetal tissue is also well established [122]. For more mature models, adult rodent NSC derivation has been reported [4, 87]. Despite these potential advantages, primary NSC-based models of neural tissue injury are rarely reported. An alternative is to use an earlier precursor to generate NSCs. Embryonic stem cells are derived from the inner cell mass of a human blastocyst, normally donated during in vitro fertilisation processes. Many hESC lines are now commercially available, with protocols available to differentiate them into NSCs, then into specific neurons and glial cells. Ordinarily, this process still requires embryo use, raising ethical concerns [194].

These concerns have, in part, driven the development of human induced pluripotent stem cells (hiPSCs)—somatic cells reverted to an embryonic stem cell like state through introduction of transcription factors or proteins into the cells. Crucially, hiPSCs avoid embryo use yet are human stem cells capable of generating neural cells.

These systems can develop into the three main cell types of the CNS (neurons, astrocytes and oligodendrocytes) under appropriate culture conditions. Further detailed characterisation has indicated establishment of different cortical neuronal subpopulations within a hiPSC preparation [143], showing different cellular responses can potentially be investigated. Electrophysiological recordings have demonstrated that neurons mature over time, with mature, burst action potential signatures found after 8–10 weeks [70]. hiPSCs can also be derived in a patient specific manner, for potential testing of personalised therapeutic approaches. There is a recent surge in the use of hiPSCs to model TBI. Sherman et al. [164] demonstrated hiPSCs grown as 2D monolayers in adapted 96 well plates generated human neurons. The adapted plates allow for a stretch injury to be administered in a controlled manner to the 2D cultures. After injury, cell death and axonal degradation were observed mimicking some aspects of TBI pathology.

Perhaps the most exciting development in terms of hiPSCs are their ability to self-aggregate into 3D CNS organoids. These organoids have been shown to mimic embryonic brain development which results in the formation of a “mini brain” displaying multiple regions of neural tissue comparable to that of the human brain [150]. Myelination has also been detected within the structures [143] which may allow investigation into myelin pathology following TBI. Using this system, Ramirez et al. [152] demonstrated organoids with cortical layering could be achieved. Using a controlled cortical impact on the structures, astrogliosis was initiated, with astrocytes elevating GFAP expression and becoming hypertrophic. Further, apoptosis and neuronal damage was elevated in the model. Using a similar system, Shi et al. [165], also demonstrated astrogliosis and cell death in an impact-based model for mild TBI, indicating such approaches can be reproducibly adapted across laboratories. Shoemaker et al. [169] showed that compression of organoid-like constructs also resulted in suppressed electrophysiological activity and secretion of neurofilament light chain, a candidate biomarker for indicating neurotrauma, into the culture medium.

Whilst highly promising, some drawbacks exist with the use of hiPSCs. The protocols are time-consuming and result in significant inter-culture variability. For example, Ramirez et al. [152], applied their injury after 220 days in culture. Even so, these are relatively immature cultures, more akin to foetal or neo-natal ages, meaning relevance to older tissue may be limited. Further, hiPSCs do not form microglia in these self-patterned systems. The immune component is of critical importance when assessing biomaterial implantation. There has been mechanical disruption induced by high-intensity focused ultrasound [105] and controlled cortical impact devices [94, 152]. However, a clear injury cavity was not defined therefore not readily adaptable for biomaterial implantation. Finally, mechanical manipulation of organoids is likely to be technically challenging given their size (<1mm in diameter) and free-floating nature. Some solutions are being developed with protocols now available for hiPSC derived microglia, which could be incorporated into the system [156]. One study even raises the possibility of microglia developing innately within organoids, cultured under conditions which permit some mesodermal lineage cell differentiation [141]. We have not found any examples of hiPSC-based testing of therapeutic biomaterials; therefore, this appears an attractive area for research.

Organotypic Slice Models of pTBI

Organotypic slice (OTS) cultures represent a transitional system that preserves the original tissue, and its inherent structural architecture, combining the features of in vivo and in vitro models [204]. Whole organs are dissected, e.g., the brain, and the tissue is cut into slices using a tissue vibratome (without any cellular dissociation), and thickness of slices vary depending on experimental need [72]. There are two methods of achieving organotypic culture. The first developed was the roller-tube culture, where slices are embedded into a collagen/plasma matrix on glass coverslips and then placed into culture tubes with growth medium which undergo a continuous slow rotating action. This rotation permits the oxygenation of the slice by generating an alternating liquid–gas interface. Via this method, slices can be cultured for several weeks [72]. The second technique (and now the most common technique for neural OTS culture), involves a semi-porous membrane supporting the tissue slice above the growth medium creating an air-medium interface. The medium below the membrane allows for appropriate nutrient absorption, facilitated via capillary action and oxygenation is permitted from above the slice [73, 177]. An advantage of this interface membrane method is that the medium does not submerge the OTS permitting therapeutic agents, materials, stimuli or mechanical injury to be applied directly to the slice at any stage of culture. Before simulating an impact injury upon these slices, time is required after initial culture to allow for slices to adapt to the culturing conditions, attach to the membrane and mature. This period of time can vary; some groups advise 12–14 days [1] others suggest less than a week or four days for sufficient attachment/stabilisation [102, 181]. There have been several established brain OTS models derived from various species including mouse, rats, rabbits [160] and human [93].

Ex vivo tissues in culture retain their native tissue cytoarchitecture and structures, including white and grey matter, neuronal tracts and the central canal. In addition, they continue to develop and mature within culture. In terms of mechanisms for modelling injury, manipulation of the slices is more straightforward with no requirement for the invasive procedures or ethical issues associated with in vivo TBI models. Various types of injury can be introduced in the slices; ischemic injury (involves a depletion of oxygen) [110], injury through over exposure of glutamate [163], LPS-induced neuroinflammation [123], or traumatic injury (involving damage from an external force). Traumatic external force mechanisms include: (i) weight drop mechanisms mimicking contusion injuries [1, 102, 144], (ii) stretch mechanisms [146, 131, 132], (iii) a compressed-air driven shock tube [26], (iv) focal injury mechanisms induced by dropping a metal stylus under stereomicroscopic control [103] or, (v) through a transecting mechanism where a doubled bladed scalpel is used to create an injury cavity within the organotypic tissue [69, 201, 188]. The primary advantage of transecting lesion injury models over the other models mentioned here, is the generation of an injury cavity which is ideal for the implantation of biomaterials that can fill the lesion space.

Implantation of biomaterials into in vivo models of TBI can be complicated as it includes an intricate secondary procedure after the initial injury with risks of infections and arduous physiological monitoring. In contrast, implantation into organotypic slices is much simpler due to ease of mechanical manipulation within this in vitro setting. Further, slices are easily stained and imaged with standard imaging apparatus. Therefore, OTS offer a moderate throughput platform to simultaneously monitor parameters of neural regeneration (i.e., nerve fibre outgrowth, glial scar formation, remyelination, and immune cell activation) in response to various injury mechanism and therapeutic biomaterial implantation. A general protocol for this process is shown in Fig. 10.4, alongside preliminary data from our laboratory which indicates the multiple pathological parameters which can be investigated at the material-tissue interface.

It is important to note that most successful organotypic slice models are generated with immature tissue from early postnatal animals, due to greater plasticity than adult tissue [52]. The majority of traumatic injuries to the CNS will occur at adult ages so younger tissue is a limitation when considering translational therapy assessment. However, brain OTS cultures have been successfully maintained for weeks to several months to increase tissue age before injury [125, 142, 181].

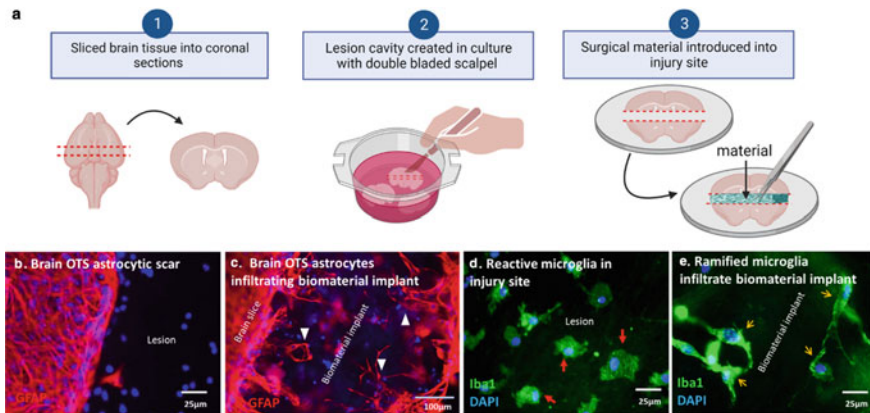


Fig. 10.4 Development of mouse organotypic slice models of pTBI. **a** Generalised schematic for organotypic slice modelling of pTBI and biomaterial implantation. In summary, (1) the appropriate brain region is dissected and sliced using a tissue chopper; (2) These slices can be maintained on culture inserts for months, with traumatic injury induced via a transecting lesion; (3) Lesioning creates an injury cavity within which biomaterials can be implanted/injected with monitoring of complex pathological responses. **b** Representative immunofluorescence image indicating upregulation of GFAP at the injury margin with clearly defined injury border. **c** Representative fluorescence image showing astrocyte processes infiltrate a bioimplant inserted into the lesion, disrupting the injury border. **d** Representative image showing reactive microglia with amoeboid morphologies invading the injury site. **e** In contrast, exploratory microglia invade a bioimplant inserted into the lesion, with a morphology depicting a transitional state from amoeboid to ramified. Unpublished data from NTEK group. *OTS* Organotypic slice

In recent years, there has been increasing focus on human organotypic tissue cultures. However, human organotypic TBI models are extremely rare and an efficient and accessible, well characterised human injury model is yet to be developed. In 2014, a group developed a brain OTS model that facilitated in vitro culture of human adult brain tissue for up to 3–4 weeks. This tissue was obtained during surgeries in patients with focal epilepsies [44]. Another group was able to establish a long-term adult human brain slice culture in 2019 [161]. Lin et al. [117] achieved the first human spinal injury/TBI organotypic model through culture of human foetal brain stem and spinal cord slices. This tissue was harvested from pregnancy donors within the first trimester and a weight drop injury mechanism was performed on transverse slices [117]. In 2022, our Keele Neural Tissue Engineering (NTEK) group developed a benchtop brain injury model using resected donor tissue from patients with Chiari malformation. Viable tissue was maintained for two weeks with all major neural cell types detected. After induction of a traumatic transecting injury into the slices, some cardinal pathological features were identified demonstrating potential for biomaterial assessment and development [188]. Our group has also developed a human foetal spinal cord injury (SCI) slice model with a direct comparison to an established mouse SCI slice model. Here, a surgical grade biomaterial was successfully implanted into the lesion site with promising readouts (unpublished data).

Although these human OTS studies show proof of concept, the clinical availability of human brain tissue that is not pathological, post-mortem or foetal limits their biological utility and scale-up potential. Additionally, due to the ambiguity of human OTS cultures and the extensive use of rodents for in vivo therapeutic testing, rodent organotypic modelling systems may be the most suitable replacement at this point for screening novel pharmacological tools. Although these current rodent TBI OTS cultures offer significant benefits particularly in studying biomaterial implantation, there is still a need to develop robust human specific model systems. This could aid with overcoming the lack of clinical translation that may be associated with cross-species differences [76].

Advanced Approaches for Modelling Brain Injury In Vitro

2D in vitro monolayer models such as those in culture wells can be beneficial in high throughput drug screening but fail to model the intercellular-matrix intricacies and complexities of cellular signalling that 3D models can offer. Modelling systems which employ human cells (in particular, hiPSCs) such as microfluidic bionetworks, brain-on-a-chip and 3D brain organoid systems have gained considerable popularity lately. Microfluidic systems can mitigate the challenges of long-term maintenance and environmental control presented with some 2D and 3D cultures. Recently, there has been a large focus on combining hiPSCs with microfluidic systems to generate brain-on-a-chip models which resemble a tissue-like physiology [12]. These microfluidic systems are chips that support miniature engineered 2D or 3D cellular constructs, integrating a cellular scaffolding with mechanical, biochemical

and topographical cues and a controlled flow of air, blood and nutrients to create physiological conditions and recapitulate specific organs. These chips offer precise control, manipulation and real-time analysis of the tissue through a system of interconnected channels in a microfluidic device [12]. In recent years, there have been significant technological advancements in the generation of human-specific brain models mimicking complex pathophysiological responses. There are three broad categories of neural systems in microfluidic chips including: microfluidic, compartmentalised and hydrogels chips [35]. These systems are more commonly used to understand neurophysiological mechanisms or to model neurodegenerative disorders and diseases rather than modelling traumatic injury. However, some injury mechanisms have been studied. For example, Hosie et al. [81] studied TBI related glutamate excitotoxicity in a compartmentalised neural chip which isolated soma and axons. A stretch strain injury model has been developed by Dolle et al. [42], where a uniaxial strain can be applied through a specific microfluidic device. The model was designed to be capable of applying a range of mechanical strains with the ability to strain individual axons or bundles of axons through the control of microchannels. Siddique and Thakor developed a model to manually induce neural injury within a compartmentalised device and administer isolated treatments in a biomimetic 3D environment [170]. Ghannad-rezaie et al. generated a microfluidic CNS device that enabled live imaging of the neural response to axonal injury in *Drosophila* larvae that was induced via application of a UV laser for 5 s [64]. Other axotomy models within the microfluidic system have used pinching of the nerve fibres in situ with forceps to generate injury, vacuum-assisted axotomy [157] and chemical injury [185].

Although neural microfluidic devices can be useful platforms for studying neural injuries within a relatively complex model of neural anatomy compared to other *in vitro* cellular models, they still lack the cellular microenvironment niches that arise from larger 3D tissue structures. Additionally, these models are predominantly employed for the study of pathomechanisms or drug development purposes rather than interactions with biomaterials. These systems are particularly beneficial in developing personalised medicine, as brain-on-chip devices can be produced using patient specific iPSC cells to generate the neurons. However, with regards to biomaterial development as an implantation therapy, microfluidic devices are not yet suitable for this application.

As an alternative, engineered 3D neural tissues, such as cortical cells embedded in hydrogels or seeded in polymer scaffolds, can be generated with animal or human cells. Hydrogels can be modulated to match the endogenous tissue stiffness of the brain which can change depending on area (3–10 kPa) [96] and upon injury. Prager et al. [148] demonstrated an efficient clinical method to match collagen hydrogel stiffness to the measurable stiffness of an injured canine spinal cord using ultrasound elastography. This would be useful for generating an efficient 3D modelling platform and for matching bioimplant stiffness with target tissue. Liaudanskaya et al. [115], reported a controlled impact study on a 3D cortical neuronal culture, using a controlled impact device similar to that used in *in vivo* models. Others involve calcium dependant injury and oxygen/glucose deprivation injury on 3D constructs of primary human NSCs [192] or the use of a compression device on cortical neuronal

3D hydrogels [13]. These 3D brain-like culture systems can mimic many aspects of in vivo responses, providing evidence that they can be used to study the molecular, cellular, and functional dynamics of TBI, opening up new possibilities for discovery of therapeutics. However, for biomaterial study, a specific lesion cavity within these cellular-hydrogel models must be generated for implantation and further assessment within an injury environment. While there are several injury mechanisms investigated within 3D constructs, a 3D neural brain-like tissue model that studies penetrating injury and biomaterial implantation has not yet been reported.

Future Directions to Enhance the Translational Utility of pTBI Models

Whilst substantial progress has been made in developing 3D models of traumatic injuries, there is still potential to develop more advanced systems for biomaterial testing specifically: (i) introduction of other non-neural but key cellular and tissue components in pathological response and biomaterial handling; (ii) spatial precision in mimicking the cytoarchitecture of the nervous system; (iii) reproducible scale up for high-throughput testing and (iv) developing 3D analytical tools to examine spatiotemporal cellular responses throughout the model. Cutting edge technologies are now being developed which could address some of these issues and further advance in vitro modelling of pTBI processes.

Brain microvasculature and the BBB are key physiological features in the response to neurological insult and injury processes, yet most models lack these. Introduction of vasculature into 3D systems, not only more faithfully replicates native tissue, but also allows perfusion of the tissue with nutrients, enhancing longevity. Further, there is the potential to add circulating immune cells which are vital in pathological progression post-TBI. Studies are starting to demonstrate that vasculature can be introduced in 3D culture systems (both organoid and scaffold-based) using a number of methodologies. Co-culture of pericytes, endothelial cells and astrocytes in a 3D spheroid was demonstrated to spontaneously produce neurovasculature-like structures [191]. Tubular structures can also be templated into 3D scaffolds and seeded with endothelial cells. The surface of the tubes then mimics the surface of the vasculature with the capability to grow neural cells around these tubes [57]. Templating structures into 3D scaffolds requires subsequent seeding of cells into different areas of the scaffold. As an alternative, advances in 3D bioprinting facilitate bio fabrication of complex cellular structures, including vessel structures for vascularisation [57].

Building on 3D printing of vasculature, there is a lot of excitement around 3D printing to engineer constructs matching the cytoarchitecture and physicochemical properties of the nervous system. 3D printing allows for precise spatial control over single and/or multiple polymer arrangements in the same structure. Several scaffold stiffnesses can be printed to match the physical properties of target tissue, and different ECM proteins (or mimics) can be used to match tissue-specific chemical

properties [119]. Polymers can be rapidly and reproducibly printed for scalable, high throughput production of scaffolds. Some studies have shown that neural cells can be bio printed with high viability [119, 154, 213]. Encapsulation of patterned growth factors in constructs has also allowed control over neural cell differentiation in 3D bioprinted scaffolds [86]. The area is still in its infancy with respect to developing neural tissue models, especially neurological injury models, but appears a highly promising area for future research.

As mentioned in “[Outlining Important Parameters for In Vitro pTBI Modelling to Test Therapeutic Biomaterials](#)” section, electrophysiological data provides functional readouts after biomaterial implantation. However, most recording techniques are complex (single cell patch clamping) or developed for 2D systems (MEAs) limiting their utility for high-throughput 3D model systems. A more traditional technique of electrical readout is calcium imaging, but this is limited by optical penetration into 3D structures and field of view for large scale cultures. New bioelectronic platforms are needed for the study of multicellular CNS models and injury-induced pathologies. These must capture network wide neuronal activity within dense mixed cultures of neurons and glia in 2D or 3D formats, to support the assessment of injury development and functional readouts of post injury regeneration after biomaterial implantation. The application of MEAs is considered an important paradigm for detailed mapping of functional damage and time-dependent evolution of injury [104], so we believe this will be essential for assessing recovery of function. There are now reports of 3D MEAs, with electrodes positioned throughout tissue-engineered brain structures [168, 176]. Cutting edge developments in soft, nanoelectronics are demonstrating mesh electrodes that can be integrated with organoid systems for dual recording and stimulation [111]. One example shows a 3D organoid forming around an electrode mesh for generation of recording traces throughout the depth of a hiPSC derived cardiac organoid [113]. In addition, Heidemann et al. [80] successfully cultured organotypic spinal cord slices on MEAs for at least three weeks by using the roller-tube method. In these experiments, two slices were cultured adjacent to each other. Connections were formed between the two separate organotypic spinal cords which lead to synchronised bursts between the slices. These connections were then severed and this disrupted the synchronised signal. Over time they observed the return of synchronisation of electrophysiological activity as the slices regenerated and re-connected. Kang and Morrison also developed an advanced MEA system which allowed measurement of electrophysiological activity before and after mechanical deformation (stretch injury) of living organotypic hippocampal brain slice cultures at tissue strains and strain rates relevant to traumatic brain injury [97]. A novel challenge is to combine such systems with 3D pTBI models to allow real-time, functional data generation in the most complex in vitro TBI models currently available.

Advanced proteomic techniques are also now being applied to biomaterial testing. We have employed an unbiased, SWATH (Sequential Window acquisition of All Theoretical mass spectra) mass spectrometry approach to evaluate safety of nanoparticle mediated genetic engineering of neural cell populations. We found only a few proteins demonstrated altered expression in engineered compared to non-engineered

neural stem cells, indicating the safety of the procedures [51]. If adapted to in vitro neural models, such techniques could provide global and unbiased assessments of pathological processes, proteomic disturbances upon application of biomaterials and insights into mechanisms underpinning any regeneration observed upon biomaterial implantation.

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Chapter 11

Challenges and Future Perspectives of Using Bioactive Scaffolds in Brain Repair



Rodrigo Ramos-Zúñiga, Carlos Isaac Ramírez-Bañales,
and María Fernanda Guerrero-Islas

Abstract *Introduction:* Scientific and technological developments have allowed the transfer of knowledge to the translational vision of materials, under an increasingly diverse scenario of their natural and synthetic components. One of the contemporary challenges is to move beyond the structural concept of scaffolds and turn them into bioactive scaffolds, which means an advance in the possibility of forming vehicles for different compounds that can generate biological effects through a wide range of active principal components. *Methods:* A systematic review of articles was conducted using the PRISMA system in Web of Science using various keywords and a 10-year timespan. A total of 995 articles were obtained using key words and boolean operators. Finally we included 112 articles of interest. *Results:* These scaffolds are expected to have neuroprotective and anti-inflammatory effects and modulate the processes involved in tissue repair and neuroregeneration. It is necessary to more clearly define molecular mechanisms of binding, adhesion, and release of biologically proactive molecules to exert regenerative actions in the tissue matrix and target cells. The reproducibility and accuracy in the design of the different scaffolds according to the materials composing them, as well as the minimization of adverse and collateral effects, is a future challenge and is still in progress. *Conclusion:* New trends define the future of biomaterials beyond their structural perspective. Natural and synthetic components have been developed, integrating active principals that can act biologically through a wide range of substances, drugs, genes, biological factors and stem cells for the development of new scaffolds. The expectation of a dual effect with both structural and biological kinetics also poses challenges linked to precision regenerative medicine and acceptable risk/benefit balance in its application in humans.

Keywords Bioactive compounds · Biomaterials · Regenerative medicine · Scaffolds · Tissue engineering

R. Ramos-Zúñiga (✉) · C. I. Ramírez-Bañales · M. F. Guerrero-Islas
Institute of Translational Neurosciences, Department of Neurosciences, CUCS Universidad de Guadalajara, Sierra Mojada 950, Col. Independencia, CP 44340 Guadalajara, Mexico
e-mail: rodrigo.ramos@academicos.udg.mx

Introduction

Technological advancements have helped in developing alternative techniques for the treatment of acute injuries that affect the structure and function of the central nervous system. The underlying mechanisms of these techniques attempt to achieve the therapeutic objective through endovascular techniques or direct insertion while considering the condition of the blood–brain barrier. These techniques promote limitation of the damage and induction of regenerative mechanisms through various molecular mechanisms.

There are several challenges associated with regenerative medicine, involving the confirmation of whether it can alter the natural course of the disease in patients with traumatic, vascular, infectious, inflammatory, immune/autoimmune, or neurodegenerative diseases [15, 17, 25, 33, 39, 42, 57, 78, 109] (Fig. 11.1).

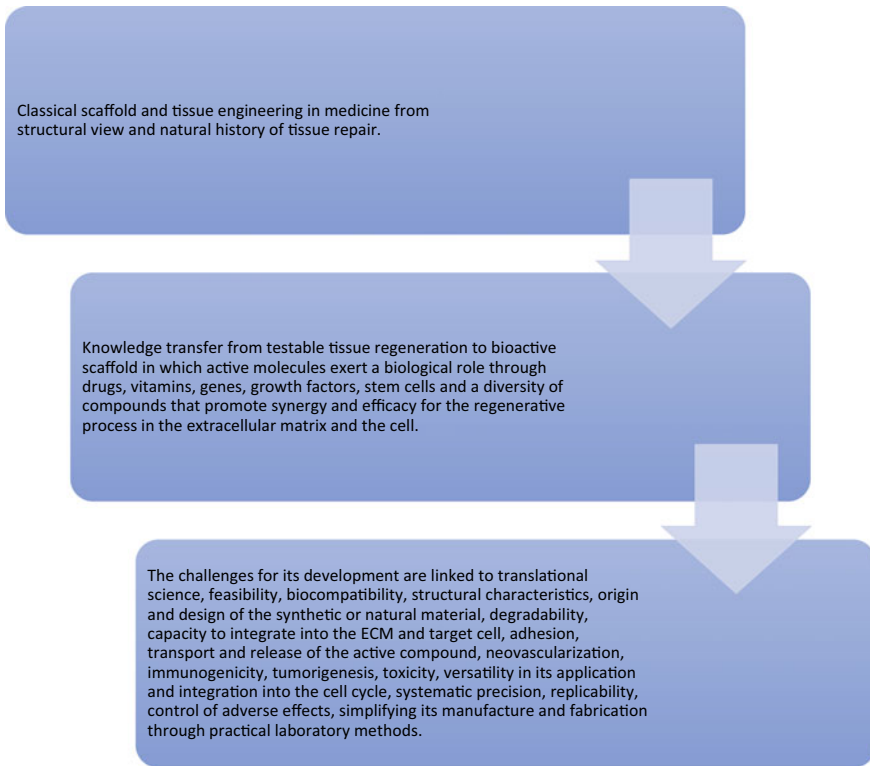


Fig. 11.1 Bioactive scaffolds can be of different structural configurations, and one of their features includes the characteristic of carrying mature cells, stem cells, drugs, vitamins, and growth factors, among other biomolecules, which may contribute to a dual effect: structural role and functional activity from active biological principal with various actions to favor regenerative processes, but also with challenges to be solved [47, 57]

The variation in the structure of biomaterials imparts various characteristics owing to their biocompatibility. Studies are being conducted to counteract the challenges regarding the ability of these materials to adapt to a microenvironment and promote cellular differentiation, migration, and adjustment mechanisms in the cellular microenvironment of the host tissue. The efficacy of these materials is defined by their ability to function as structural scaffolds, which may facilitate the regenerative processes of the injured environment themselves, and directly act via a potential active substance [6, 19, 26, 84, 96].

Nonetheless, technological development has resulted in the alteration of these parameters, as the molecular mechanisms of active substances acting alone and those acting in combination with other substances have been identified that help them to have a more direct role in the activation of cellular repair which may eventually foster a functional recovery strategy.

Currently, to operationally define the characteristics of bioactive materials, it is crucial to determine whether the structure of bioactive materials contain different compounds, such as enzymes, ceramic materials, drugs, antibiotics, vitamins, growth factors, or stem cells, and if they can generate potentiation in single or conjugated forms, to promote active responses in the host tissue [2, 5, 12–14, 45, 49, 59, 91].

It has been indicated that bioactive scaffolds may first promote specific molecular signals to induce tissue regeneration based on their affinity to the scaffold and then subsequently to be released into the tissue. Therefore, it is essential to address the current challenges of bioactive scaffolds and their potential application in humans while not only focusing on the designing of new tissue bioengineering strategies based on innovative technological advancements but also considering the challenge of its translational application, as short-term clinical trials may solve specific problems with no collateral effects. In other words, a virtuous, safe, and efficient model can be developed by studying the interaction between the host and corresponding bioactive compound [91, 98, 104, 111].

The development of new compounds from the conventional structural composition to particles or molecules is a major challenge in the advancement of what we now call nanotechnology. The credit for coining this term was first given to physician N. Taniguchi, although its generic aspects have been previously assessed in R. Feynman's works in 1965 [32]. Therefore, the field of bioactive scaffolds is closely linked to the scientific principle features of colloid interfaces, materials and surface engineering, and quantum physics. These previous studies gave rise to the development of micelles and liposomes, such as lipid nanovehicles, and peptides and proteins, like nanoparticles [44, 88, 89, 90].

This was followed by the introduction of other materials and development of carbon platforms, which promoted the use of a wide range of synthetic and natural materials to create the different scaffold designs.

Methodology

A systematic review of articles was conducted using the PRISMA system in Web of Science using various keywords and a 10-year timespan. A total of 995 articles were obtained. The titles and abstracts of these articles were read to select those that included relevant and updated information related to this chapter’s core theme, and 877 articles from the selection were excluded. The remaining 118 articles were read in their entirety, except for 2 articles that were not fully accessible. Finally, four articles were excluded owing to their lack of relevance (Fig. 11.2).

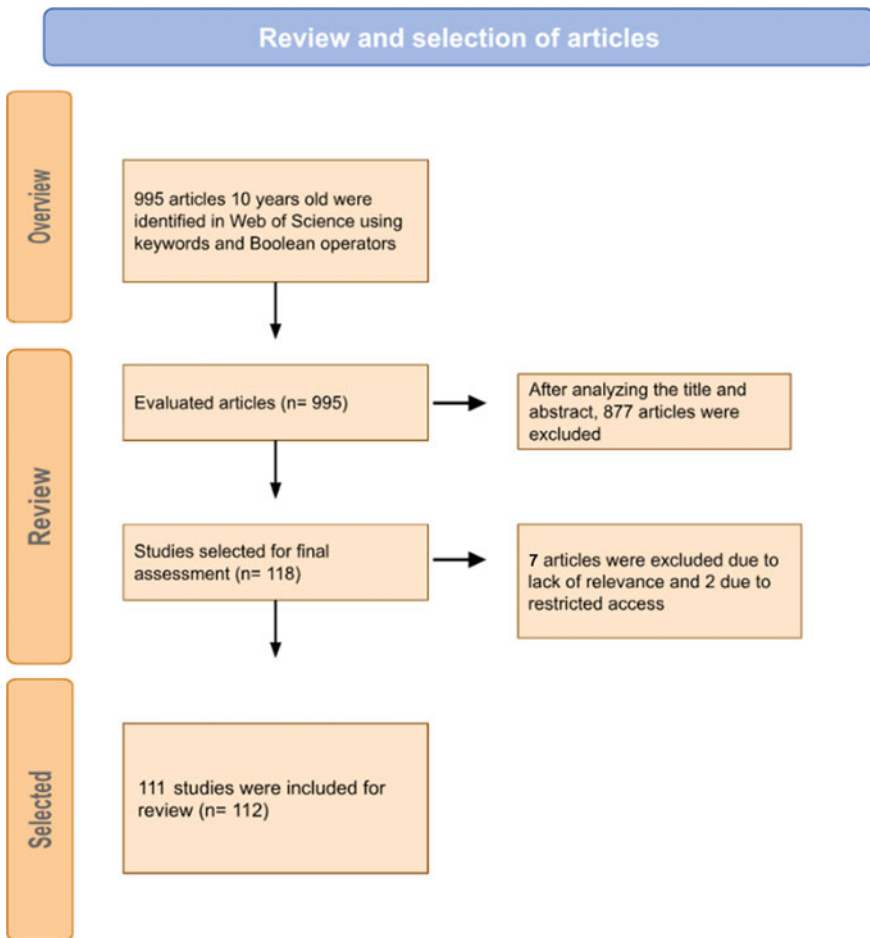


Fig. 11.2 Using keyword and Boolean operators, 995 published papers in the last 10 years were extracted from the Web of Science and finally reviewed 112 (PRISMA review system)

State of Play and Substrate for Action in Brain Repair

Modern medicine has identified a series of processes that, at the molecular level, are crucial in identifying the common elements of neurodegenerative diseases. Consequently, the cascade of events that trigger mechanisms involved in acute stages of diseases is considered to subsequently perpetuate the disease to chronic stages. This may represent the mode of action of several compounds that have been developed owing to the proposed technological innovation and advancement in the field of materials science, genomics knowledge, molecular biology, and are currently considered the new pharmacological strategies by design and personalized medicine [10, 11, 22, 51, 54–56, 65, 73, 80, 95, 97].

Likewise, it is worth considering the role that has been historically represented by the blood–brain barrier in the central nervous system. It plays a role in the peculiar arrangement of astrocytes and formation of their microstructural connections with the cerebral circulation, which has consolidated the idea of protection against certain toxic elements that might gain access to the brain through the blood circulation. This structural feature also limits the use of drugs targeting the central nervous system, as these therapeutic substances have high molecular weight and solubility characteristics that do not allow for their passage into the neural tissue.

It is pertinent to mention that the immunological system at the level of the brain extends beyond the role of microglia if we consider the findings regarding a lymphatic membrane recently found in the subarachnoid space, which affects the interaction between the brain tissue and the body's circulation [53].

In light of these events, some of the most significant elements that normally characterize neurodegenerative processes are as follows: inflammation, genomic alterations (DNA and RNA), altered mitochondrial energy homeostasis (includes oxidative stress), cytoskeletal abnormalities, altered protein formation, synthesis and structural stability (proteostasis), abnormal sympathetic neural network, generation of abnormal proteins (Tau-protein), and neuronal death due to acute cytotoxic mechanisms or apoptosis [99].

The Role of Bioactive Scaffolds in Regenerative Processes in Brain

One of the basic starting points is to unify the criteria and operational definitions in the concepts of bioactive scaffolds. [106] Part of this leads to a first variable: (1) to define the basic reactive behavior of tissue repair in the face of different molecular mechanisms of injury and (2) to identify the components involved in a regenerative process mediated by specific inducers or promoters of tissue regeneration [52].

This requires knowledge of the mechanisms involved in the injury, and the molecular strategies that can modify the natural course of these processes, in a limited scenario for neuronal regeneration. In the first example, it is possible to delimit

the extent of the reactive process, reduce scarring and avoid the proliferation of glial fibrosis. But in the second case it is required to demonstrate the influence and the nanomolecular mechanism that induces a regenerative process, precisely because it goes beyond a conventional scar reaction and has functional objectives [16, 24, 36, 67, 68, 109].

In the case of design, three key elements are involved, such as the (1) characteristics of the material, its biocompatibility with the tissue area of the host, design, degradation, and biosafety to avoid immunogenicity and toxicity. (2) The second mechanism depends on the capacity and versatility to conjugate in different patterns and materials with bioactive principals that can demonstrate a direct effect on the extracellular matrix and on the same cells of the receptor tissue through drugs, growth factors, stem cells, exosomes, microspheres, or genes. And finally, (3) in the evidence of this regenerative process that specifically shows cell regeneration, neovascularization and colonization of new tissue with host tissue characteristics that result from a synergy between the structural scaffold and the bioactive component [52, 70] (Table 11.1).

BDNF brain-derived neurotrophic factor, *bFGF* basic fibroblastic grow factor, *BMHPI* bone marrow homing peptide, *BMSCs* bone marrow mesenchymal stem cells, *CGRP* peptidergic sensory fibres, *ChAT* cholinergic alpha motor neurons, *CNS* central nervous system, *CPI* metalloproteinase 2 cleaved sequences, *GPQG + IASQ*, *ECM* Extracellular Matrix, *hEnSCs* Human endometrial-derived stromal cells, *IKVAV* C16V2A2E, *iPSC-NSCs* induced pluripotent stem cells-derived neural stem cells, *LN* Laminin, *NSC* neural stem cell, *NGF- β* nerve growth factor β , *NT-3* neurotrophin-3, *PA* peptide amphiphile, *PCL* Polycaprolactone, *PDL* poly-Dlysine, *PLA* poly-L/DL lactic acid, *P(TMC-CL)* poly(trimethylene carbonate-co- ϵ -caprolactone), *RADA16-FGL* AcN-RADARADARADARADAGGEVYVVAENQQGKSKA-CONH₂, *SCI* spinal cord injury, *SC-NSCs* spinal cord-derived neural stem cells, *VEFG* vascular endothelial growth factor, *VKIVA* C16V2A2E4 scramble sequence, *3-D* three-dimensional, *5-HT* serotonergic neurons of the raphespinal tract.

Bioactive scaffolds are a promising therapeutic strategy because they can be used as cell and bioactive compound vehicles that promote the host tissue response and protection, emulating the ECM of the brain with bioactive molecular activity. The use of different active principals and stem-cell based therapies can also promote regeneration and neuroprotection. We present a list of the main studies and approaches for CNS obtained from the survey of the literature (Modified and updated from [70]).

Table 11.1 Bioactive biomaterials in the central nervous system

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
Scaffold and cellular based hydrogels	Growth factors BDNF, bFGF, VEGF	In vitro studies	Neurodegenerative diseases	Signal cellular pathways	Not available yet. Clinical long-term results	Collins et al. [16]
BioScaffold and stem cells in trauma	Mesenchymal stem cells	Animal models	Spinal cord and brain trauma	Pathophysiology of injury repair	Viability to clinical strategies. Risk of tumourigenesis, ethical issues, immunogenicity	Köhli et al. [36]
Bioactive scaffold membranes	Regenerative activity vs tissue repair	Mouse model	Vascular brain ictus. Angiogenic factors	Advances in neuroregeneration in stroke	Degradability of the scaffold	Modo et al. [52]
Bioscaffold and neural stem cells	Neurogenesis process through growth factors in stem cells niches	Mouse and in vitro models	Growth factors in vivo. Brain injury	Active repair and neurogenesis and differentiation promotion	Long term effect. Not yet available in humans	Zheng et al. [109]
Scaffolds evolution	Stem Cells, gene therapy and growth factors	In vitro and mouse models	Oriented to neurorepair and neurogenesis. Brain injury	Diversity of choices in neurorepair	The level of functional response	Zamproni et al. [106]
Mesenchymal stem cell-derived exosomes	Neurogenesis and cognitive function recovery	Mouse model	Alzheimer's disease	Production of neurotrophins Neuroregeneration and functional recovery	No clinical studies yet to quantify the efficacy of this approach	Reza-Zaldivar et al. [74]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
IKVAV-PA, VKIVA-PA self-assembled nanofibres	Neural transdifferentiation of human BMSCs	Human BMSCs culture on IKVAV-PA and VKIVA-PA gels and laminin PDL-coated surfaces	Traumatic injuries in the central nervous system	<ul style="list-style-type: none"> Induction of neuroectodermal lineage commitment Self-assemble into supramolecular nanofibres 	<p>Significant decrease in viability without spreading on the VKIVA-PA gel</p> <p>No clinical studies yet to quantify the efficacy of this approach</p>	Ji et al. [30]
Fibrous scaffolds and hydrogel matrices	Promote cell survive	In vivo 3D microenvironments	Ischemic stroke	<ul style="list-style-type: none"> Emulation of ECMs 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Tang and Lampe [85]
NT-3/fibronin complex	Decreased IBA-1 positive cells	Canine spinal cord	SCI	<ul style="list-style-type: none"> Inhibits inflammation Enhances nerve fibre regeneration Improves motor function 	<ul style="list-style-type: none"> No clinical studies yet to determine the optimal concentration of NT-3 used for humans 	Li et al. [41]
Injectable hydrogels in combination with stem cells	Stem cell adhesion, proliferation	In situ	Ischemic stroke	Site-specific stem cell biomolecule delivery	Lack of control, nonuniform cell distribution and low cell penetration capacity	Gopalakrishnan et al. [25]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
Chitlac-THICK	Neural network reconstruction	Primary cultures of hippocampal neurons from rat pups	CNS injured systems	<ul style="list-style-type: none"> Promote neuronal growth, differentiation, maturation and formation of synapses 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Medelin et al. [48]
PCL/iPSC-NSCs	Differentiation into neural stem cells	Rat spinal cords	SCI regeneration Cell transplantation	<ul style="list-style-type: none"> Could promote motor function 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Zhou et al. [110]
Fungal metabolites	<ul style="list-style-type: none"> Scaffolds for the design and synthesis of new multi-target ligands 	<ul style="list-style-type: none"> In vitro 	Alzheimer's Disease	<ul style="list-style-type: none"> Antioxidant and anti-amyloidogenic activity 	<ul style="list-style-type: none"> No clinical or animal models studies yet to quantify the efficacy of this approach 	Piemontese et al. [62]
P(TMC-CL)/Ibuprofen	<ul style="list-style-type: none"> Decrease on RhoA activation Favors CD163 + cell recruitment Increases βIII-tubulin and GFAP positive cells 	Dorsal hemisection rat SCI sub-cultured on ND7/23 cells	SCI	<ul style="list-style-type: none"> Contributes to resolution of the inflammatory process Supports axonal growth (βIII-tubulin cells) in the lesion periphery and cellular infiltration 	<ul style="list-style-type: none"> The folding of an electrospun fibrous fabric impaired nerve regeneration 	Pires et al. [63]
Cell interaction with extracellular matrix IKVAV peptide	Neuronal differentiation	In vivo	Traumatic brain injury	Promote axonal regeneration	Not effect on astrocyte proliferation	Sahab et al. [77]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
Tenascin-C mimetic peptide amphiphile nanofibre gel	Neurite outgrowth	P19 embryonal carcinoma cells	<ul style="list-style-type: none"> Spinal cord injuries Repopulation of dysfunctional areas of the brain 	<ul style="list-style-type: none"> Self assembled nanofibre Transition from a solution to gel upon exposure to physiological divalent cations 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Berns et al. [9]
Injectable non-toxic silanized-hydroxypropyl methylcellulose hydrogel	Neuronal differentiation of grafted cells	3D environment	Neuronal damage	Secretion of tissue repair factors of stem cells	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Kandalam et al. [31]
(RADA)4-IKVAV-CP1-MVG	Neurite outgrowth	In vitro PC-12 cells	Neuroinflammatory and neurodegenerative conditions	<ul style="list-style-type: none"> Neuroprotection Neurogenesis 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Koss et al. [37]
3D multichannel/LN silk fibroin	Stimulates growth, development, and the extension of primary hippocampal neurons	Culture of primary hippocampal neurons in vitro, to then implant them in a SCI of rats	SCI regeneration	<ul style="list-style-type: none"> Promotes axonal extension Mediates cell migration Neuronal adhesion promotion Stimulates blood capillary formation 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Zhang et al. [107]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
(RADA)4-GG-BMHP1	<ul style="list-style-type: none"> • Axon regeneration and lamination • Less reactive astrocytes (GFAP expression) and gliosis • Motor neuron recovery 	hEnSCs implanted into rats	Chronic SCI	<ul style="list-style-type: none"> • Improvement in motor function • Neural differentiation • Higher myelination 	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	Tavakol et al. [86]
RADA16-FGL	Promoted SC-NSC proliferation and migration into the 3-D scaffold	SC-NSCs from neonatal rats	SCI regeneration Tissue engineering	<ul style="list-style-type: none"> • Self-assemble into nanofibrous structure and support SC-NSC adhesion and survival 	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	Wang et al. [93]
Radially aligned electrospun PLA70/30 fibres release L-lactate	3D organization and supportive function of radial glia embryonic neural stem cells	Radial scaffolds implanted into the postnatal mouse brain	Traumatic injuries in the central nervous system	<ul style="list-style-type: none"> • Reactivation of embryonic neurogenic and angiogenic programs • Maintenance of pools of neuronal and glial progenitor cells 	<ul style="list-style-type: none"> • Specific scaffolding topology • Risk of induction of neuroglial tumours 	Alvarez et al. [4]
Human mesenchymal stem cells	Neuronal differentiation	In vitro	Brain damage	Produce neuronal progenitor and mature neural cell markers	<ul style="list-style-type: none"> • No clinical studies yet to quantify the efficacy of this approach 	Okolicsanyi et al. [58]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
Hydrogel	Neuronal differentiations	In vitro	Injury environment and scarring	Greater axonal infiltration, astrocyte migration and myelinated axon growth	Determine the most relevant biological features, and size scale and determine how to create similar architectures within biomaterials	Khaing et al. [34]
Transplantation of stem cell in the urinary bladder matrix	Cell differentiation	In rats	Traumatic brain injury	Reduce neuron tissue loss Repair damaged neural network	Previous studies have shown a significant neuronal loss	Wang et al. [94]
NT-3/Chitosan	• Axon regeneration	Spinal cord of rats	SCI regeneration	• Restoration of locomotor function	• No clinical studies yet to quantify the efficacy of this approach	Wang et al. [94]
Electrospun PCL-Spirulina nanofibre mat	Improves the growth and metabolic activity of primary astrocytes	Rat primary astrocytes exposed to Spirulina extract	CNS injured systems	• Alleviated astrocyte metabolism without cytotoxicity • Could reduce inflammation induced by gliosis	• Spirulina extract is rarely released from the PCL-Spirulina mat to the media within 24 h	Kim et al. [35]

(continued)

Table 11.1 (continued)

Biomaterial	Bioactive Effect	Experimental Model	Translational perspective	Advantages	Disadvantages	References/ Author
Extracellular matrix	Neuronal differentiation as well as neuronal outgrowth	PC-12 cells	Injury of the CNS	Rich in axonal growth inducer proteins	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Mammadov et al. [46]
Nerve Growth Factor Coated-poly(2-hydroxyethyl methacrylate) Hydrogel	Enhancing of neuron survival and sprouting	Dorsal root ganglion cultures	Interfacing SNC electronic devices	<ul style="list-style-type: none"> Controlled local neurotrophin delivery 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Jhaveri et al. [29]
3D aragonite coralline matrices	Neuronal tissue restoration	Hippocampal (dentate gyrus) neurons and astrocytes of rat pups	Neuronal tissue development in 3D	<ul style="list-style-type: none"> Proliferation of neuronal, astrocytes and other glial cells Support mesenchymal stem cells differentiation into osteoblasts 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Peretz et al. [60]
NT-3 from fibrin-HBDS scaffolds	<ul style="list-style-type: none"> Enhances the sprouting of CGRP, ChAT, 5-HT of the raphe spinal tract 	Spinal cord of adult rats	SCI regeneration	<ul style="list-style-type: none"> Neuronal fibre sprouting Reduction of glial scar formation 	<ul style="list-style-type: none"> Motor function does not improve 	Taylor et al. [87]
IKVAV-PA	Promote neurite sprouting and to direct neurite growth	Murine neural progenitor cells	Neuroinflammatory and neurodegenerative conditions	<ul style="list-style-type: none"> Rapid differentiation into neurons 	<ul style="list-style-type: none"> No clinical studies yet to quantify the efficacy of this approach 	Silva et al. [82]

Strategies for Using Bioactive Scaffolds in the Central Nervous System

Recently, studies have reported the development of new materials with varying structural configurations that can act as scaffolds. One such study reported the use of graphene as a scaffold for neuroregeneration, especially for the optic nerve and spinal cord, including an effect linked to its ability to join other molecules that may have biological activity. Nonetheless, further studies to obtain toxicity data are needed [3, 7, 18].

In the Table 11.1 this review the state of art perspectives of the future directions of bioactive scaffolds related with recent research, advantages and disadvantages in an analytic comparison. The main target in biomimetic materials is the creation of microenvironment with mechanical properties, but also using signalling molecules that promote the cell migration, proliferation and differentiation through biocompatible scaffolds.

There are still challenges to be solved in bioactive scaffolds, and they are related to the affinity of the molecule and its integration to the biomaterial, and on the other hand with the capacity to release them in the specific host site and the way in which the activity that delimits the damage and induces the regenerative capacity is generated.

One of the most relevant issues is that they exhibit a good diffusion through extracellular matrix because the bioactive scaffolds should be suitable to the local physiology, adapt to pH, temperature and biodegradable conditions. The nano-scaffolds is an example of the excellent delivery of bioactive compounds. Usually the polymers required complex synthesis and potential assembly with toxic co-solvents it is therefore necessary to consider these factors in their design, because of the potential effects on their ability to achieve a controlled release of bioactive compounds.

Another example of a material that provides structural configuration to the scaffold itself involves using collagen to induce peripheral nerve regeneration, which may also be supplemented by carbon nanotubes [23, 72]. Such materials can also be developed using *in vivo* electrospinning, which generates nanofibers with the ability of cell supplementation on a 2D surface; however, their restricted structural strength and rapid dissolution in aqueous media are major drawbacks [20, 54].

The various structural configurations have led to the creation of nanogels that are capable of crossing the blood–brain barrier and act as vehicles for drugs such as methotrexate, antiretrovirals, or T lymphocytes for antitumor management. However, their vulnerability is still a limiting factor [91]. Specifically, strategies are employed to achieve scaffolds with transport capacity that can improve the pathology of diseases associated with the central nervous system, thereby favoring pathways that induce cell differentiation from progenitor cells [1].

Various structural configurations have been used in different acute and chronic degenerative pathologies, and their potential application to treat traumatic brain injury, which combines scaffold-stem cells and a suitable drug, has been described ([45, 64], Zibara et al. 2018). Eventually, a single scaffold may contain different bioactive compounds [84, 102].

Beyond its role as a vehicle, the application of nanomaterials has contributed to the consolidation of the strategy to gain access to the nervous system. The combination of these nanomaterials is highly diverse in terms of their source of origin and molecular design, which facilitates their blending with specific drugs to manage neurodegenerative diseases and stroke (e.g., tPA-plasminogen activating factor, antioxidants, and neuroprotective exosomes) [95]. The most successful models that have been reported are those that are biodegradable, hydrophobic, and similar to the extracellular matrix. Therefore, many studies have used the electrospinning technique in combination with bioactive factors [83].

This technological and innovative diversity also includes the incorporation of 3D techniques for fabrication of biomaterials that consider certain variables to increase their efficiency and compatibility with the recipient tissue (geometry, structural configuration, porosity, 5% water retention, etc. [8, 28].

Another possibility is using nanocarbon- or graphene-based scaffolds, adding some type of protein that can also be directly implanted to the posterior brain tissue by surgery [3, 7, 18, 23, 27, 38, 72, 81, 105, 108]. For instance, Paclitaxel/sponge evolves to its gel form that can be directly applied in situ after the resection of brain gliomas, and it incorporates an antibody called aCD47 into it. Although the results in this report have been promising in mice, further evaluation is required in controlled clinical trials to establish their efficiency in humans [92].

All these studies have been preceded by preclinical experiments, including cell culture studies that specifically follow the interaction between synthetic and natural materials and their behavior with specific cell groups. Among these, a recent study based on neural stem cells of mesenchymal or hematopoietic origin stands out as it focused on directing them toward a particular cell lineage and identifying their behavior as they interacted with the various scaffold materials. [15, 44, 80].

Technology used for 3D printing has also contributed to the generation of more customized designs for materials and spaces for ceramics, cells, or biological factors that make up highly functional scaffolds in the technological perspective of cellular regeneration.

Side effects such as tumorigenicity and its relationship to immunogenicity conditions are an issue that still requires attention, particularly with the use of stem cells and some growth factors, and long-term follow-up is necessary.

Future Scenarios

The area to be used as the recipient site represents a basic condition for the scaffold's design and characteristics, as well as the anatomical and recipient area to be considered for a scaffold with structural or barrier physical effect, or to be applied as a vehicle for different compounds or particles that may contain an active principal or promote a specific biological activity.

Some factors must be considered while fabricating scaffolds, such as mechanical properties, physicochemical characteristics, solubility, hydrophilicity, load, and

biocompatibility. On the other hand, design of the scaffold depends on the following factors: 3D printing, size, porosity, degradation, biodegradation kinetics, strength, and design options such as gel, sponge, and mesh. These are some of the most relevant factors to preserve its efficacy and resolution capacity in terms of biological behavior such as cellular adhesion, vascularization, chemical surface, porosity, and biodegradation.

One of the advantages of polymers is their wide use as materials owing to their versatility in terms of design and the possibility of introducing active particles. Materials of natural origin are noteworthy, as they are made up of proteins such as silk, collagen, gelatin, fibrinogen, actin, or keratin, whereas polysaccharides include chitosan, cellulose, alginate, dextran, chitin, glycosaminoglycan, hyaluronic acid, and agarose. It is worth mentioning that it has been recently reported in the case of polynucleotides, thus generating the possibility of conducting gene therapy [50, 71, 75, 100, 101].

These alternatives focus on applications more specifically related to connective and hard tissue (bones), different organs and tissue, and drug vehicles, including soft tissue or implants.

Feasible synthetic materials are classified into degradable, including polyesters, polyorthoesters, polylactones, polycarbonates, and polyanhydrides, and non-degradable, such as polyether, polysiloxanes, polyethyleneimine, polyethylene, methacrylate, and acrylic.

Finally, the application of cells, tissue designed for the scaffold, growth factors, and a series of stem cells is a trend that should be assessed for its long-term results. The use of fibroblasts, albumin, extracellular matrix, and growth factors (vascular endothelial, transforming, and epidermal tissue, and fibroblasts) has been reported, and, in the case of stem cells, this trend has been reported for adipose tissue, mesenchyme, bone marrow, and endothelial tissue [21, 31, 57, 77, 90, 109].

The current challenges faced by bioactive scaffolds are those associated with their physicochemical, biocompatibility, and degradation properties, which affect their interaction to achieve a standardized effect in the extracellular matrix, which helps in creating designs focused on specific cellular targets that can be generated using bioactive molecules [40, 66].

It would also be relevant to define the mechanisms that improve its interaction with the host tissue, avoiding the inflammatory reaction, necrosis, and loss of adhesion between the active molecule and the scaffold that may result in failure of its accessibility to the target tissue, and alter its efficacy both in the ECM and in the cell itself with bioactive molecules [19, 31].

Another challenge involves achieving greater diversity in the design and manufacturing of these bioactive platforms in terms of the wide range of recipient tissue based on the spatial, biomechanical, and functional conditions. On the other hand, the reproducibility and accuracy in the design of the different scaffolds according to the materials composing them, as well as the minimization of adverse and collateral effects, is still pending [103].

As for production, the principle of efficacy should be prioritized while reducing the risk from the perspective of precision medicine and regenerative effect in such a way

that production processes are simplified, and research should be conducted to improve the production of greater volumes of materials, so that it can be made available to the majority of the population, in accordance with the principle of translational science [28, 56, 61, 69, 73, 76, 79, 102].

“The key in the utility of the scaffolds should be able to present the correct number of epitopes with high affinity for binding with the bioactive compound and at the same time, maintain the mechanical and physical properties for its biocompatibility in the surrounding host tissue. The same issue is also found for the case of the kinetics of the receptors in the cell membrane or intracytoplasmic domain. Neurotropic factors have been the most promising signal molecules for this effect, but the reproducibility, predictability, and safety of the results in the therapeutic target have yet to be thoroughly elucidated” [69].

Conclusions

Bioactive scaffolds are the consequence of the technological innovation that resulted in the advancement of the biomedical field in regenerative medicine. Diverse scaffolds have been designed using new materials and strategies. Scaffolds containing different bioactive compounds that can serve different purposes are currently under development. These scaffolds are expected to have neuroprotective and anti-inflammatory effects and modulate the processes involved in neurodegeneration. The challenge also includes proving and achieving higher accuracy of tissue regeneration, beyond its structural functions as a mechanical scaffold.

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