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Neuronanotechnology for brain regeneration

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ABSTRACT

Identifying and harnessing regenerative pathways while suppressing the growth-inhibiting processes of the biological response to injury is the central goal of stimulating neurogenesis after central nervous system (CNS) injury. However, due to the complexity of the mature CNS involving a plethora of cellular pathways and extracellular cues, as well as difficulties in accessibility without highly invasive procedures, clinical successes of regenerative medicine for CNS injuries have been extremely limited. Current interventions primarily focus on stabilization and mitigation of further neuronal death rather than direct stimulation of neurogenesis. In the past few decades, nanotechnology has offered substantial innovations to the field of regenerative medicine. Their nanoscale features allow for the fine tuning of biological interactions for enhancing drug delivery and stimulating cellular processes. This review gives an overview of nanotechnology applications in CNS regeneration organized according to cellular and extracellular targets and discuss future directions for the field.

Contents

Abbreviations: NP, Nanoparticle; CNS, Central nervous system; TBI, Traumatic brain injury; PD, Parkinson's disease; BDNF, Brain-derived neurotrophic factor; BBB, Blood brain barrier; EPO, Erythropoietin; NGF, Nerve growth factor; PLGA, Poly(lactic-co-glycolic acid); PEG, Poly(ethylene glycol); AD, Alzheimer's disease; NSC, Neural stem cell; NPC, Neural progenitor cell; PEI, Poly(ethyleneimine); MCAO, Middle cerebral artery occlusion; FUS, Focused ultrasound; PCL, Poly(caprolactone); VEGF, Vascular endothelial growth factor; HA, Hyaluronic acid; CNT, Carbon nanotube; M/Mφ, Microglia/macrophages; ECM, Extracellular matrix; CSPG, Chondroitin sulfate proteoglycans; ChABC, Chondroitinase ABC; MAI, Myelin-associated inhibitor; TNC, Tenascin C; TAT, Transactivator of transcription; SCI, Spinal cord injury; EGF, Epidermal growth factor; NT-3, Neurotrophin-3; GDNF, Glial-derived neurotrophic factor; MSC, Mesenchymal stem cell; PBAE, Poly-beta-amino-esters.

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1. Introduction

Regenerative medicine, the translational discipline of stimulating human cells to regenerate in order to restore healthy biological function in damaged tissues, has advanced rapidly in the few decades since its inception. The field has produced several FDA-approved regenerative therapies currently on the market to address issues such as epidermal and bone injuries via delivery of biologics, pharmaceuticals, scaffolds, and cells [1]. Despite these promising advances, regenerative medicines for treating neurodegenerative disorders and other central nervous system (CNS) injuries have made little progress in clinical translation. The challenges in developing regenerative strategies for nervous system injuries arise from its highly complex nature, as well as the difficulty in accessing damaged nervous system tissues while limiting collateral damage [2]. Regeneration in the brain comprises of not only cellular replacement but also synaptic and functional repair and plasticity. Normal regenerative response following an injury is dictated by the neural and glial cells, the extracellular matrix, the immune system, and interactions between all these components. Manipulation of these systems in conjunction and sequence will be crucial for enhancing normal recovery and promoting repair. Nanotechnology has the potential to provide novel devices and materials to support and stimulate nervous system regeneration and can be leveraged to help manipulate each of these systems (Fig. 1). This review will outline the status of neuroregenerative strategies in the brain, discuss the various nanotechnology platforms being developed in the field, and attempt to provide an outline for potential areas of future growth and research in this field.

2. Current clinical pipeline for neuroregeneration

Currently, clinical strategies for addressing neurological diseases and injuries requiring neuroregeneration have largely focused on ameliorating secondary effects and limiting further cell death rather than directly stimulating cellular regeneration. Examples include traumatic brain injury (TBI), where supportive care and physical therapy are current interventions [3], and Parkinson's disease (PD), where dopamine replacement therapy and symptomatic treatment are mainstays of intervention [4,5]. Table 1 summarizes the ongoing and recently completed clinical trials with therapeutic agents focused on neuroregeneration. Studies on administered hormones and growth factors, in the context of TBI, have largely performed on par with placebos and at worst have exhibited harmful effects. Nonspecific administration of factors at doses necessary to achieve therapeutically relevant levels at the site of brain injury may cause deleterious responses and significant systemic adverse effects, as will be discussed below. Other compounds, such as vitamins and Co-enzyme Q10, have shown limited effects but their anti-oxidant and pleiotropic properties likely lead to amelioration of secondary injuries rather than direct neurogenesis stimulation [6–10]. Administration of stem cells to provide naïve cell populations to differentiate into new neurons are also being explored; however, such therapies have been hampered by challenges in maintaining cell viability, potential tumorigenicity, and regulatory hurdles [11,12]. Taken together, the dearth of strategies to enable neuroregeneration that are in the clinical pipeline or are being clinically translated signify a substantial technological gap that can be addressed with novel nanotechnology mediated approaches.

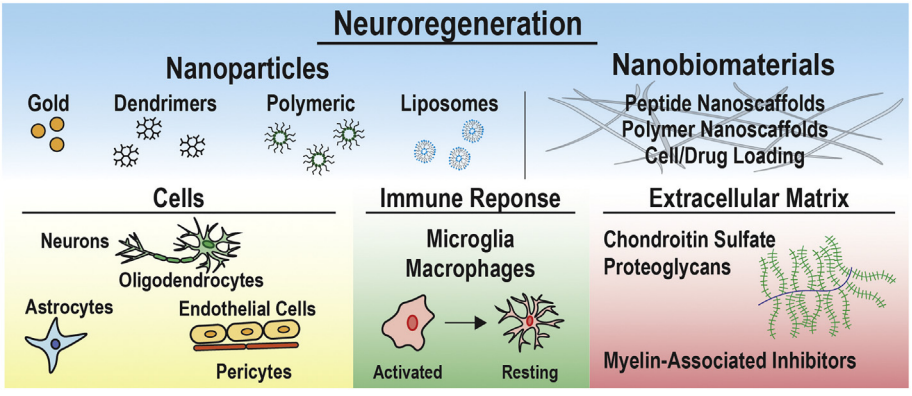

Fig. 1. Schematic of nanotechnology targeting capabilities. Nanotechnology allows for specific delivery of regenerative therapies targeted to cells, the inflammatory environment, and the extracellular matrix.

Table 1
Recent late stage clinical trials focusing on neuroregeneration.

Study ID	Phase	Origin	Intervention	Results/Est. Completion
Traumatic brain injury				
NCT00676104	3	USA	Cerebral hypothermia	No improvements vs. placebo
NCT00987454	3	Australia	Erythropoietin	No improvements vs. placebo
NCT00313716	2,3	USA	Erythropoietin	No improvements vs. placebo
NCT01143064	3	Global	Progesterone	Ineffectual; Mildly harmful
NCT01212679	2	China	Nerve Growth Factor	Not available
NCT00004730	3	USA	Magnesium Sulfate	Worse than placebo
Stroke				
NCT01716481	3	South Korea	Mesenchymal Stem Cells (MSCs)	Not available
NCT01112813	3	Canada	Lithium carbonate	Not available
NCT02881957	3	USA	Vitamin D3	October 2018
NCT02263924	3	Malaysia	Vitamin E	December 2018
NCT02018406	2	South Korea	Erythropoietin and Granulocyte-CSF	Not available
Parkinson's disease				
NCT00180037	3	Germany	Coenzyme Q10 nanodispersion	No improvements vs. placebo
NCT01892176	2,3	Singapore	Coenzyme Q10	Mild improvements in total PD rating scale
NCT03119636	2	China	Neural Precursor Cells	December 2020
NCT03684122	2	Jordan	Umbilical Cord-Derived MSCs	April 2019
Alzheimer's disease				
NCT00876863	2	USA	CERE-110 (NGF gene delivery)	March 2020
NCT03172117	2	South Korea	Umbilical Cord-Derived MSCs	December 2021
NCT03117738	2	USA	Adipose-Derived MSCs	July 2019

3. Nanotechnology approaches manipulating cells for stimulating neuroregeneration

3.1. Nanotechnology approaches for targeting neurons

3.1.1. Nanoparticle delivery of growth factors to promote neurogenesis

Biological factors secreted by neurons and by cells such as microglia, astrocytes, and endothelial cells can regulate neuronal proliferation, migration, survival, and differentiation. The most commonly explored growth factors for these applications are brain-derived neurotrophic factor (BDNF) [13–16], erythropoietin (EPO) [17,18], and nerve growth factor (NGF) [19–22]. Other trophic factors such as glial-derived neurotrophic factor, platelet-derived growth factor, neurotrophin-3, fibroblast growth factor, and others are generally more limited in application and therefore less explored [23–26]. Translation of trophic factors as neurogenic treatments have generally been limited by poor plasma stability, inability to cross the BBB, and significant safety concerns related to off-target growth stimulation [15,16,27–30]. Nanotechnology offers attractive strategies to prolong stability, penetrate the BBB, and selectively deliver these trophic factors to pathologically relevant cells in a targeted manner.

Preventing neuronal death and promoting neuronal proliferation is the primary goal after brain injury, and many nanoparticle-mediated deliveries of growth factors have been evaluated (Table 2). Polymeric nanoparticles targeting brain injury and specifically neurons for targeted delivery of neurotrophic factors have shown promising preclinical results (Fig. 2). BDNF has been the most commonly explored factor for delivery via nanoparticles. In TBI, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles were used to deliver BDNF for promoting neuronal growth after injury [31]. Loading into PLGA nanoparticles for systemic administration improves the short half-life and poor BBB penetration of BDNF, which results in significantly increased BDNF levels in the brain and reversal of cognitive deficits following brain trauma as assessed by passive avoidance paradigm. PLGA has also been used to delivery cerebrolysin, a cocktail of neurotrophic factors including BDNF, glial-derived neurotrophic factor, NGF, and ciliary neurotrophic factor [32]. These nanoparticles exhibited biphasic release of cerebrolysin, resulting in successful prevention of edema after injury with later administration compared to freely administered cerebrolysin [33]. In stroke, polymer nanoparticle delivery of BDNF has similarly exhibited enhanced efficacy. Intravenous administration of polyion complex of BDNF with poly(ethylene glycol) (PEG)-poly(glycolic acid) copolymer increased influx across the BBB, neural uptake, and serum residence time, resulting in greater BDNF levels in the brain and blood [34]. Similarly, PEG-poly(L-glutamic acid) complexes with BDNF improved brain distribution compared to free BDNF administration, resulting in superior neuroprotection with higher dopaminergic neuron counts after LPS challenge [35]. Loading of BDNF in these nanoparticle complexes also conferred improved stability in vivo by decreasing binding to nonspecific proteins while maintaining affinity for BDNF receptors TrkB and p75NTR.

Nanoparticle delivery of EPO has also been explored for improved neuron growth after injury. EPO-loaded PLGA nanoparticles were formed by double emulsion solvent evaporation method and tested in a perinatal hypoxic ischemia model. The PLGA-EPO-nanoparticles when administered by intraperitoneal injection exhibited significantly greater neuroprotective effect, at a ten-fold lower dose than free EPO. Therapy reduced infarct volume significantly and increased brain weights and latencies to fall on rotarod test [36]. In a model of periventricular leukomalacia, intraperitoneal administration of EPO loaded chitosan nanoparticles exhibited neuroprotective effects after injury [37]. EPO saw sustained release from the chitosan nanoparticles over 2 h. Nanoparticle delivered EPO exhibited increased ventricle volume and decreased asymmetry, along with increased neuronal expression of growth associated protein 43 (GAP-43) and improved behavior scoring compared to untreated animals.

NGF delivery via nanoparticles has mostly been explored with other neurogenic factors as part of a combination therapy in vivo or alone in vitro. Iron oxide nanoparticles covalently linked with NGF significantly promoted neurite outgrowth and complexity of neuronal branching, as well as expression of neuronal differentiation markers, compared to NGF alone in vitro [38]. Nanoparticle loading also decreased the degradation rate of NGF. NGF loading on porous silica films achieved sustained release over 26 days in vitro for inducing neurite outgrowth and differentiation of PC12 cells and dorsal root ganglia [39]. PEGylated liposomes were loaded with NGF and surface modified with Cereport and transferrin to improve neuronal uptake and BBB passage [40]. In combination with NSCs isolated from rat embryos, PEG-PLGA nanoparticles loaded with NGF improved disease pathology in Alzheimer's disease (AD) [41]. NSCs were implanted with or without NGF nanoparticles into the hippocampus and basal forebrain. Cotreatment outperformed NSC implantation alone, with significantly improved performance in Morris water maze navigation test, and staining for P75 neurotrophin receptor and synaptophysin synapse marker that was comparable to control mice. Nanoparticle mediated delivery of NGF via systemic delivery has also been shown to ameliorate AD markers. Liposomes composed of cholesterol and cardiolipin, a lipid with affinity for amyloid-β plaques, were surface modified with wheat germ agglutinin for improved BBB penetration [42]. Intravenous administration

Table 2
Nanotechnology for manipulating cells to promote neural regeneration.

Target cell	Platform	Route of administration	Therapy delivered	Model	Effects	Ref.
Delivery of growth factors						
Neuron	PLGA NP	Intravenous	BDNF	TBI	Improved half-life and BBB penetration, reversal of cognitive deficits	31
Neuron	PLGA NP	Intravenous	Cerebrolysin	TBI	Biphasic release, prevention of edema	33
Neuron	Polyion Complex	Intravenous	BDNF	Healthy	Increased neural uptake and BBB penetration	34
Neuron	PEG-PLE NP	Intranasal	BDNF	LPS challenge	Higher dopaminergic neuron counts	35
Neuron	PLGA NP	Intravenous	EPO	Hypoxic Ischemia	Reduced infarct volume, increased brain weights	36
Neuron	Chitosan NP	Intraperitoneal	EPO	Periventricular Leukomalacia	Increased growth factor expression, behavior score	37
Neuron	Liposome	Intravenous	NGF	AD	Increased brain NGF, TrkA activation	42
Delivery of small molecule drugs						
Neuron	PLGA NP	Intravenous	Curcumin	AD	Increased neuronal differentiation	43
Neuron	PLGA NP	Intravenous	Curcumin	AD	Restoration of synapse counts and plaque to healthy levels; improved Y-maze and novel object	44
Neuron	Lipid NP + MSC	Intravenous	Galatamine hydrobromide	AD	Improved memory retrieval, neuron growth markers	45
Neuron	PLGA NP	Intravenous	Ropinirole	PD	Reversion of PD-symptoms	47
Neuron	PEI NP	Intraventricular	Retinoic acid	Healthy	Increased neuron gene expression by precursors	48
Neuron	Lipid NP	Intranasal	GDNF	PD	Improved rotarod performance, increased dopaminergic neuron counts	49
Neuron	Lipid nano-assembly	Intravenous	Adenosine	TBI	Decreased infarct volume, neuron degeneration	50
Neuron	PEG-PLGA NP	Intranasal	NR2B9c	Stroke	Improved plasma stability, reduced infarct volume and improved behavior score	54
Neuron	Liposome	Intravenous	ZL006	Stroke	Decreased infarct volume, TUNEL+ cell counts	56
Oligo-dendrocyte	PLGA NP	Intravenous	Leukemia inhibitory factor	Focal demyelination	Increased myelination	68
Delivery of genetic material						
Neuron	Carbon Nanotubes	Intracranial	Caspase-3 siRNA	Stroke	Reduced neuron apoptosis, improved skill reach test	57
Neuron	Liposome	Intravenous	Caspase-3 siRNA	TBI	Increased caspase-3 knockdown	58
Neuron	PEG-PEI NP	Intracranial	ROCK II siRNA	AD	Improved performance in Morris water maze tasks	59
Neuron	PEG-PEI NP + FUS	Intravenous	GDNF	PD	Increased GDNF expression, neuron counts; improved rotarod performance	62
Oligo-dendrocyte	Exosome	Intravenous	BACE1 siRNA	Healthy	Improved BACE1 knockdown	64
Astrocyte	Chitosan NP	Intravenous	SART3 and hCycT1 siRNA	HIV	Improved knockdown of SART3 and hCycT1 expression	74

of these nanoparticles significantly increased brain levels of NGF, resulting in increased activation of TrkA receptor and ERK proliferative pathway. These promising results indicate that nanoparticle delivery can significantly enhance NGF effects, demonstrating its potential for clinical applications.

3.1.2. Nanoparticle delivery of small molecule drugs and peptides to promote neurogenesis

Nanoparticles have also been used for targeted delivery of drugs and other compounds to neurons after brain injury. PLGA nanoparticles have been used in AD to delivery curcumin, a neuroprotective

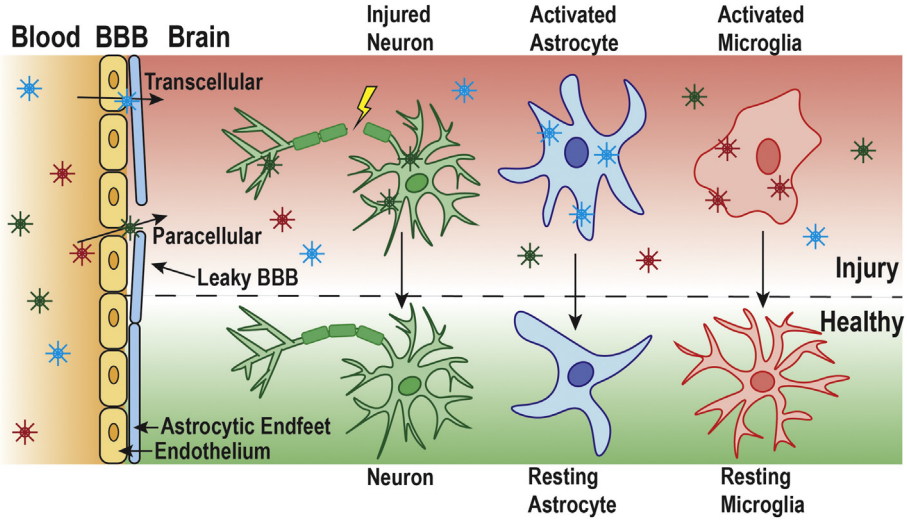

Fig. 2. Schematic of nanoparticle blood brain barrier (BBB) penetration and cell-type specific targeting. Nanoparticles may be designed to permeate the injured BBB via transcellular and paracellular pathways, diffuse through the brain parenchyma, and target specific cell populations using targeting ligands.

compound with poor brain bioavailability, with the goal of stimulating adult neurogenesis in endogenous NSCs [43]. Via systemic administration, PLGA nanoparticles encapsulating curcumin potently induced neuronal differentiation in hippocampal NSCs via activation of the Wnt/β-catenin pathways. This increased neurogenesis translated to reversed learning and memory impairments in an amyloid-β AD model. For further enhancement, PLGA nanoparticles were loaded with both PQVGHL peptides, an inhibitor of amyloid-β plaque generation, and curcumin, and surface modified with CRTIGPSVC neuron targeting peptide and an iron mimic peptide to target transferrin receptors for BBB penetration [44]. These targeting motifs resulted in increased residence within the brain after systemic administration, with nanoparticles washing out of the rest of the body but remaining in the brain after 24 h. Fully functionalized nanoparticles performed greater than nanoparticles with just one of either therapy or targeting peptide, restoring plaque levels and synapse counts back to near healthy levels. This translated to significant improvements in Y-maze exploration and novel object recognition tasks. Nanoparticles have also been coupled with stem cells to enhance treatment efficacy. Glyceryl behnate lipid nanoparticles were formed with pluronic F127 and Tween 20 surfactants and loaded with galantamine hydrobromide (GH), a potentiating ligand for acetylcholine receptors for stimulating neurotrophic effects [45]. Nanoparticle delivery of GH and mesenchymal stem cell administration demonstrated a synergistic effect, with treated mice outperforming stem cell and free GH cotreatment in Morris water maze memory retrieval tasks. Similar trends were seen with oxidative stress markers, inflammatory markers, and growth associated factors BDNF and BrdU staining, further indicating their synergistic effects.

PLGA nanoparticles similarly improved the delivery of ropinirole, a clinically used non-ergolinic dopaminergic agonist that is administered as a monotherapy or in conjunction with levodopa to treat PD [46]. Ropinirole experiences extensive first-pass hepatic metabolism, has low oral bioavailability, and rapid plasma elimination, which limits its efficacy in vivo. Systemically administered ropinirole loaded PLGA nanoparticles were shown to revert PD-like symptoms of neurodegeneration in preclinical studies and was superior to free ropinirole [47]. Retinoic acid loaded poly(ethyleneimine) (PEI) nanoparticles have also been reported to enhance neuronal differentiation in vivo when administered via intracerebroventricular injection [48]. These retinoic acid containing PEI nanoparticles induced pro-neurogenic gene expression in NSCs specifically within the SVZ at significantly greater levels than freely administered retinoic acid. Lipid nanoparticles have also been explored for treatment in Parkinson's disease (PD). Glial-derived neurotrophic factor encapsulated in nanolipid carriers coated with chitosan that was covalently linked to TAT peptide, was nasally administered in a toxin induced mouse model of PD. The TAT peptide enabled improved cellular internalization and BBB penetration. Mice were assessed with rotarod prior to PD induction and weekly afterward. Mice treated with fully modified nanoparticles performed comparable to control animals in latency to fall and TH-positive fiber counts indicating regeneration and protection of dopaminergic neurons [49]. Adenosine an antiarrhythmic cardiac drug and neurotransmitter with neuroprotective properties, was covalently linked to squalenoyl lipids to form lipid nano-assemblies via nanoprecipitation. Systemic pretreatment of these nano-assemblies prior to middle cerebral artery occlusion (MCAO) in a rat model of stroke resulted in significantly reduced infarct volume and lower levels of neuronal degeneration compared to adenosine alone [50].

The *N*-methyl-D-aspartate (NMDA) receptor found on neurons has also been implicated in neurogenesis [51]. However, due to its role in stimulating progenitor cell proliferation, as well as normal synaptic plasticity, over-inhibition of the receptor yields deleterious side effects [52]. While novel NMDA receptor antagonists with greater specificity and efficacy have been developed [53], nanotechnology has also made improvements via targeted delivery. In a rat model of MCAO stroke, the NMDA receptor inhibitor NR2B9c loaded into PEG-PLGA nanoparticles that were surface modified with wheat germ agglutinin where administered intranasally [54]. Wheat germ agglutinin receptors are abundant in the olfactory epithelium and neurons thereby enhancing intranasal CNS delivery [55]. Intranasal delivery of this nanoparticle resulted in higher NR2B9c levels across multiple brain regions, leading to significantly reduced infarct volume and improved neurological scores [54]. For further improvement, nanoparticles carrying NMDA receptor antagonists may be targeted for more specific cellular delivery. PEGylated liposomes were surface modified with peptides HAIYPRH (T7) for BBB penetration and CLEVSRKNC (SHP), which binds to apoptotic neurons, for specific injury targeting [56]. The addition of the SHP motif resulted in significantly increased nanoparticle signal specific to the ischemic region of the brain, as measured by ex vivo IVIS imaging. Compared to untargeted liposomes, liposomes with T7 and SHP peptides reduced the number of TUNEL positive cells to near sham levels and reduced infarct volume twice as effectively.

3.1.3. Nanotechnology approaches for gene delivery for promoting neurogenesis

Gene silencing strategies have also shown promise with nanoparticles for suppressing the apoptotic environment after injury. Multi-walled carbon nanotubes have been shown to deliver caspase-3 targeting siRNAs. siRNAs internalized in carbon nanotubes enhanced delivery of siRNA in primary neurons and decreased caspase-3 expression and protein levels in neuroblastoma cells compared to freely administered siRNA. Intracranial injection of these nanotubes significantly reduced the number of apoptotic neurons in the peri-injury area and improved “skilled reaching” tests in a rat model of thrombo-embolic stroke compared to free siRNA [57]. Gene silencing strategies have also incorporated neuron targeting ligands to further improve gene silencing. Tandem peptide based nanoparticles have been shown to improve the delivery of siRNAs to the site of injury in TBI and target injured neurons upon systemic administration [58]. The nanoparticle system incorporated siRNAs for caspase-3 apoptotic transcripts with the neuron targeting peptide from rabies virus RVG and the intracellular trafficking peptide transportan. The siRNA nanoparticle complexes were found to target the injured site surrounding the TBI injury, with little in the contralateral hemisphere, and significantly improved decrease in caspase-3 expression compared to free RNA and nontargeted nanoparticles. Cellular uptake was highly specific to neurons, with <5% internalization in astrocytes and microglia [58]. In AD, intracranial injection of PEG-PEI nanoparticles loaded with ROCK II siRNAs to promote neurite outgrowth and synapse formation improved performance in memory tasks compared to untreated and treatment with nanoparticles carrying scrambled siRNAs [59,60]. Gene delivery has been further enhanced with the addition of focused ultrasound (FUS) technologies. PEGylated PEI nanoparticles encapsulating DNA plasmids for reporter genes showed robust distribution in the brain when FUS was applied after intravenous injection. Through a beta actin promoter, transfection was maintained for at least 28 days post administration. Transfection was detected at high levels in both neurons and astrocytes [61]. In a model of PD, plasmid DNA encoding GFP and GDNF were encapsulated into biotinylated liposomes and attached to avidinylated microbubbles. Transfection was improved with FUS application compared to nanoparticles alone and free plasmid DNA. Delivery of GDNF-encoding plasmids with FUS led to increased expression stimulated specifically in neurons, resulting in restoration of dopaminergic neuronal function, increased number of TH+ neurons, and longer latency to fall in rotarod [62].

3.2. Nanotechnology approaches targeting glial cells

3.2.1. Oligodendrocytes

Oligodendrocytes are chiefly in charge of myelination of axons, which has long-term implications for recovery after CNS injury, and it is through this function that they are primarily of therapeutic interest [63]. Due to the complex nature of oligodendrocyte regulation, their contributions to regeneration and mechanisms underlying their injury response are still poorly understood and require further understanding before they can be effectively leveraged for clinical applications. For this reason, there have been few studies in targeting oligodendrocytes directly with nanotechnology although therapies have been shown to demonstrate efficacy by improving oligodendrocyte function and numbers. Exosomes expressing RVG peptide that were loaded with siRNA, have been shown to target neurons, microglia, and oligodendrocytes upon systemic administration and were successful in achieving knockdown of the genes in vitro and in vivo in the brain [64]. These exosomes delivering BACE1 siRNAs achieved significant knockdown of the gene resulting in decreased expression of BACE1 [64], a protein implicated in AD progression via its function as a myelination regulator due to its effect on oligodendrocytes [65]. To improve targeting, superparamagnetic iron oxide nanoparticles (SPIONs) have been explored due to their ability to cross the BBB and, via external magnetic field application, achieve directed regional specificity. However, their translation has been impeded by concerns about toxicity, where exposure to SPIONs has been associated with inflammation, reactive oxygen species (ROS) production, and apoptosis [66]. SPIONs surface modified with PEG for improved biocompatibility did not exhibit inflammatory responses, oxidative stress, cytotoxic effects, or morphological alterations in murine 158 N oligodendrocytes in vitro [67]. In terms of preclinical in vivo efficacy studies, PLGA nanoparticles carrying leukaemia inhibitory factor (LIF) and targeted to NG-2 ligand expressed on oligodendrocyte precursor cells have been explored for increasing myelin repair after CNS focal demyelination. LIF has been recently identified as a myelination promoting factor, and NG-2 is a chondroitin sulfate proteoglycan (CSPG) expressed on the surface of oligodendrocyte precursor cells. LIF was encapsulated within PLGA nanoparticles via double emulsion. These nanoparticles were coated in avidin to enable conjugation of biotinylated anti-NG2 antibody. In vivo, these targeted nanoparticles promoted thicker myelination and increased number of myelinated axons compared to non-targeted nanoparticles and empty targeted nanoparticles [68]. Oligodendrocytes have been inadequately investigated for CNS applications but have the potential to make dramatic impacts in clinical outcomes for treatment of demyelinating disorders such as leukodystrophies and multiple sclerosis, as well as for stimulating their growth factor secretions to promote neurogenesis.

3.2.2. Astrocytes

The relationship between astrocytes and CNS regeneration after injury is complicated, with their response and presence having been found to be both critical to recovery but also simultaneously highly inhibitory to regeneration due to glial scarring [69,70]. Astrocytes have been reported to secrete a wide range of trophic factors, including NGF, BDNF, fibroblast growth factor, among others, although stimulation of astrocytic growth and trophic factor secretion has not been explored clinically [71]. Interestingly, reactive astrocytes have been found to convert into stem cell-like progenitor states under inflammatory or disease conditions [72,73], raising the possibility of delivering signals to de-differentiate them for potential neuronal regeneration. This requires further confirmation and evaluation before it can be potentially use as a clinical strategy.

Therapeutically, astrocytes have largely remained unexplored as a direct target. Rather, their effects in forming the growth-inhibitive environment due to glial scarring has been addressed, and this concept is explored further in the extracellular matrix discussion. Several astrocyte targeting nanoparticles have been explored in vitro for gene silencing.

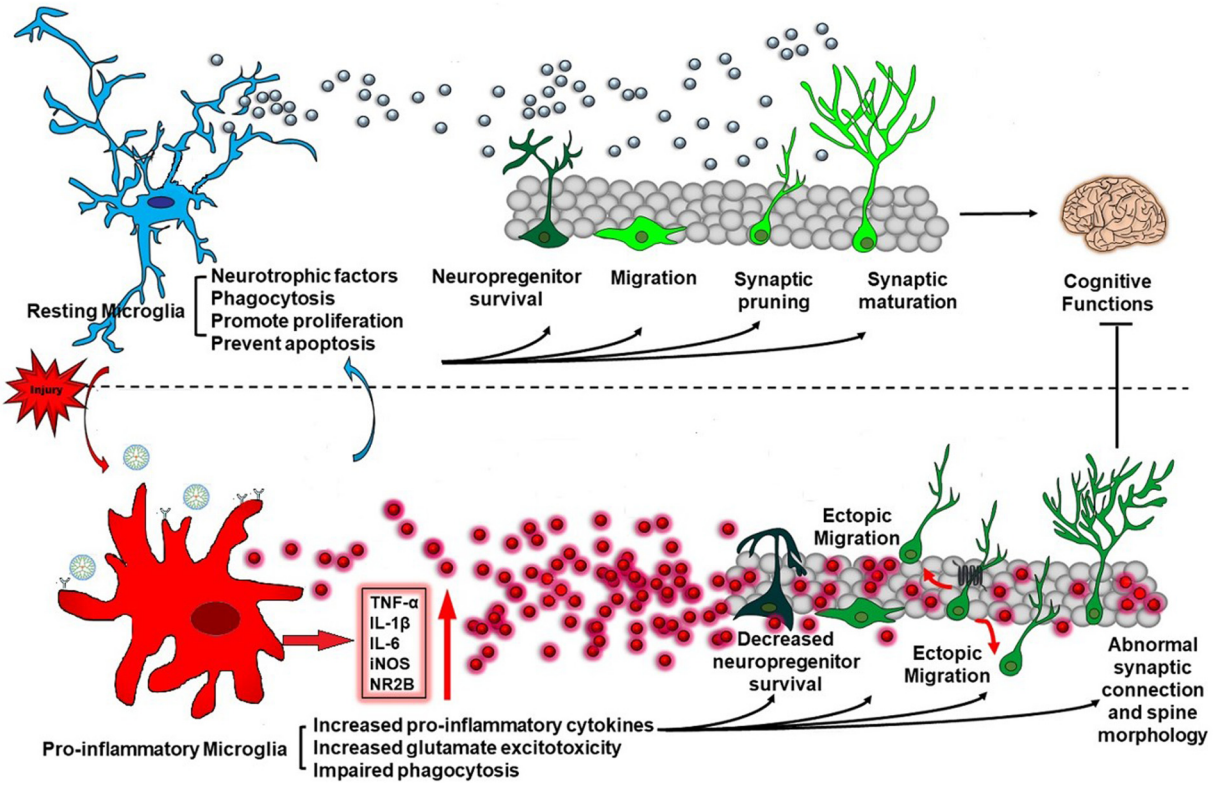

Fig. 3. Inflammatory response and neuronal regeneration. Resting microglia promote neural survival and growth. After injury, resulting inflammation causes microglial activation, which then contributes to growth suppression and neuronal death. Nanoparticle delivery can facilitate the phenotype switch back to a neuroprotective, growth-promoting state.

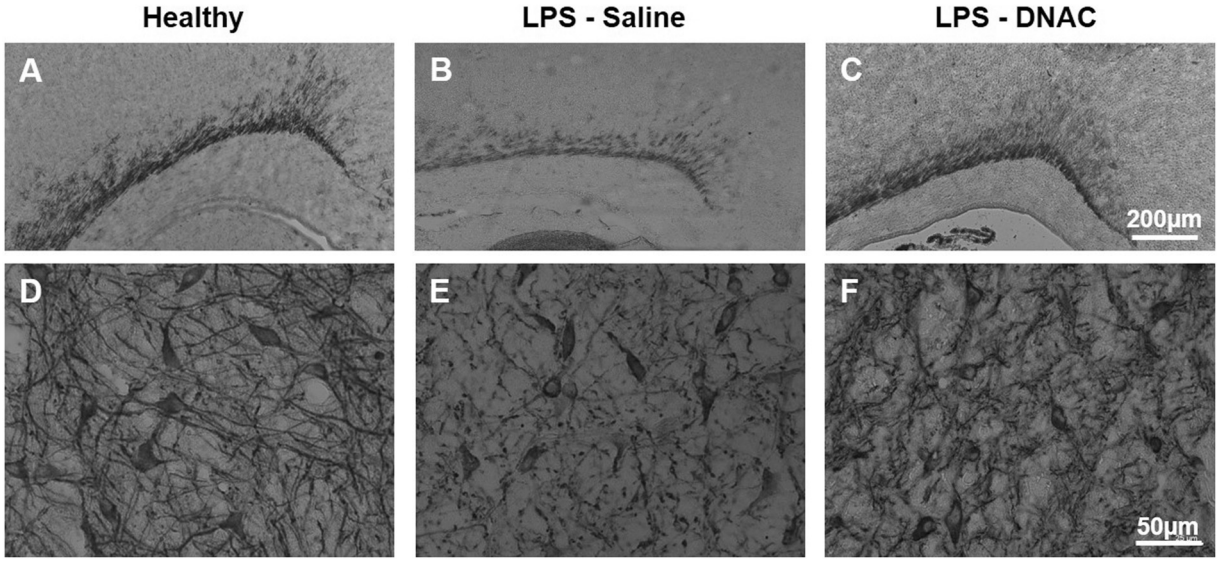

Fig. 4. Dendrimer delivery *N*-acetyl cysteine (DNAC) restores myelination and neuronal growth in a neonatal rabbit model of cerebral palsy induced by intrauterine LPS. Brain sections from healthy control kits (A,D); or saline vehicle treated animals exposed to LPS in utero (B, E) and kits exposed to LPS in utero who were treated with 10 mg/kg of D-NAC on the day of birth (C, F) were sacrificed on day of life 5 and stained with MBP (A,B,C) for myelin or MAP2 (D,E,F) for neurons. MBP staining demonstrated improved myelination in the D-NAC treated animals when compared to saline treated. MAP2 staining showed increased neuronal cells and branching of the dendrites in the D-NAC treated animals when compared to saline treated CP animals.

In the context of HIV therapy, chitosan nanoparticles have been used to deliver siRNA across the BBB to inhibit HIV replication in astrocytes [74]. Nanoparticles were modified with two targeting antibodies targeting the transferrin receptor for enhancing BBB passage and bradykinin B2 receptor (B2R) for astrocytic specificity. They were loaded with two siRNA sequences targeting SART3, which is required for viral transcription, and hCycT1, a transcription factor. B2R antibody enhanced astrocytic uptake in vitro and resulted in significant knockdown of SART3 and hCycT1 mRNA expression and protein levels. More directly, polymer-siRNA complexes were evaluated in vitro to inhibit CSPG production in reactive astrocytes. Cyclized cationic polymer consisting of 2-dimethylaminoethyl methacrylate and ethylene glycol dimethacrylate was used to condense siRNA into nanoparticle complexes. siRNA targeting XT-1, an enzyme critical to formation of CSPGs, were delivered as an alternative to ChABC therapy. Nanoparticle delivery significantly reduced mRNA expression of XT-1 and increased neurite length [75]. These studies indicate that delivering therapeutics to astrocytes to ameliorate their contribution to CSPG formation is a potential regenerative strategy. Moreover, these results demonstrate the viability of delivering genetic material targeted to astrocytes, making them attractive targets for inducing sustained growth factor secretions at the site of brain injury.

3.3. Neuroimmune modulation using nanotechnology

3.3.1. Microglia, macrophages, and monocytes

As mediators of the inflammatory response after injury in the brain, microglia and macrophages (M/Mφ) have been reported to confer both beneficial and negative effects after injury, likely dependent on the timing and magnitude of activation. In the acute phase after injury, M/Mφ have neuroprotective effects to clear toxic debris and promote growth, while the persistent neuroinflammation eventually creates a growth inhibitory environment consistent with the pathological disease state that prevents recovery and may cause additional tissue damage (Fig. 3) [76–78]. Therefore, interventions that seek to modulate the M/Mφ activation profile post-injury must be carefully designed to prevent neuroinflammation-induced damage while allowing the natural growth promoting inflammatory response to take place. M/Mφ also directly influence regeneration by secreting trophic factors into the injured site and surrounding tissue. Activated M/Mφ have been shown to secrete BDNF, glial-derived neurotrophic factor, NGF, neurotrophin-3, and the neuroprotective cytokine TGFβ [76,79,80].

Our group has worked extensively in the field of targeted delivery to M/Mφ in neurodegenerative disorders. We have shown that hydroxyl-terminated polyamidoamine (PAMAM) dendrimers have the intrinsic ability to localize specifically to activated M/Mφ in the brain upon systemic administration while exhibiting minimal residence in healthy brain tissue and peripheral organs [81–85]. Additionally, we have demonstrated in many neurodegenerative models that systemic dendrimer delivery of *N*-acetyl cysteine (D-NAC) to activated M/Mφ exhibits significantly greater therapeutic effect than freely administered *N*-acetyl cysteine (NAC) [86–88]. In the context of brain regeneration, we have shown that D-NAC treatment can decrease pro-inflammatory cytokines to enable regeneration and growth with improved myelination and increase in neuronal counts, comparable to healthy rabbits [86] (Fig. 4). Given the high specificity and uptake of these dendrimers in M/Mφ

Table 3
Nanotechnology for Immune Modulation to promote neural regeneration.

Target cell type	Nano-platform	Route of administration	Therapy delivered	Model	Effects	Ref.
Microglia	PAMAM dendrimer	Intravenous	NAC	Cerebral Palsy	Decreased inflammation, increased neuron counts and myelination, functional recovery	86
Microglia	PAMAM dendrimer	Oral	NAC	Necrotizing Enterocolitis	Decreased inflammation, increased myelination and brain volume	87
Microglia	PEG-PLA lipid NP	Intravenous	C3 siRNA	Ischemic Reperfusion	Decreased microglial activation, C4 expression, infarct area	90
Microglia	PEG-lipid NP	Intravenous	NBP	Ischemia	Decreased inflammation, reduced infarct volume, improved neurological score	91

[89], we believe that they are prime candidates to achieve immunemodulation or stimulation of growth factor secretion in activated M/Mφ to promote brain regeneration.

In addition to our work, other groups have also had successes in targeting microglia in neurodegenerative disorders to suppress the inflammatory response and create a more growth conducive environment (Table 3). PEG-PLA lipid emulsions loaded with siRNA targeting complement protein 3 (C3) expression conferred neuroprotection from neuronal toxicity after ischemic reperfusion injury [90]. After injury, activated microglia deposit C3 onto neurons to mark them for complement-mediated removal. Treatment with nanoparticle loaded siRNAs resulted in significantly reduced C3 positive neurons and increased number of surviving neurons compared to naked siRNA in vitro. In vivo, nanoparticle delivery achieved ten-fold greater levels of siRNA in the brain compared to naked siRNA after intravenous injection, resulting in significantly decreased microglial activation, C3 expression, immune cell recruitment, infarct area, and ultimately significantly improved neurological scores. Active microglial localization via targeting ligands has also been explored. PEG-lipid nanoparticles were prepared by solvent diffusion and encapsulated3-n-butylphthalid (NBP), an anti-inflammatory that activates NFκB for stimulating cell growth and proliferation [91]. Nanoparticles were surface conjugated with Fas ligand, which had been previously shown to be upregulated in microglia after ischemic injury to the brain [92]. NBP itself exhibited minimal brain penetration while lipid nanoparticles showed significant signal in the brain and Fas targeted nanoparticles localized specifically to the ischemic injured region of the brain. Fas targeted nanoparticles localized specifically to activated microglia in the injured region and resulted in significant improvements to neurological deficit scores and infarct recovery compared to free NBP [91]. Taken together, these studies indicate that targeting activated microglia after brain injury is a promising clinical strategy for stimulating neurogenesis.

3.3.2. Other immune cells of relevance to CNS regeneration

There is increasing recognition of other immune cells such as B cells, T cells, and mast cells in CNS disorders including in stroke, TBI, autoimmune disorders such as MS, myasthenia gravis, neuromyelitis optica, etc. [93–98]. Although nanotherapies have been extensively explored in immune targeting in cancer [99–103], these approaches have not been explored adequately for neurologic disorders, providing a novel area for potential exploration in the future.

3.4. Other cell types as targets for nanotechnology stimulation of brain regeneration

3.4.1. Endothelial cells

Endothelial cells have been explored extensively as targets to increase BBB penetration of nanoparticle delivered compounds. Targeting ligands such as vascular cell adhesion molecule 1, angiopep-2, transferrin, and TAT peptide have all been used to target the endothelial layer of the BBB to improve brain penetration in other disease models [104–108]. Vascular endothelial growth factor (VEGF) delivery to endothelial cells to repair the BBB has been explored. VEGF expression is detrimentally upregulated after brain injury, and its inhibition has been shown to decrease glial scar formation, BBB leakage, and cell apoptosis [109–113]. VEGF delivery to endothelial cells after brain injury has seen inconsistent results, most likely due to timing of administration. In the early stages following injury, VEGF can exacerbate damage while afterwards, angiogenesis and BBB repair is critically regulated by VEGF. Nanoparticles can enhance VEGF delivery to endothelial cells [114], but such strategies will need to be carefully designed and timed so as to elicit a favorable, rather than negative, response. Endothelial cells have also been shown to secrete EPO to stimulate axonal sprouting [16], which has not yet been explored clinically but may yield a novel potential target for promoting neurogenesis.

3.4.2. Pericytes

Pericytes are largely responsible for angiogenesis and vascular homeostasis in the mature brain, as well as neuronal maintenance, and their loss results in impaired CNS function [115]. After CNS injury, pericytes are known to secrete trophic factors such as VEGF, fibroblast growth factor, BDNF, NFG, and glial-derived growth factor [116]. Targeting pericytes to promote regeneration has been limited to stimulating vascularization and angiogenesis and thus far are a potential unexplored nanotherapeutic target.

3.4.3. Ependymal cells

Upon injury, ependymal cells in the forebrain can act as a source of neural progenitor cells for replacement of lost neurons [117,118]. Under normal conditions, ependymal cells are quiescent but after injury differentiate into neuronal and glial cell types. In vitro ependymal migration and proliferation has been shown to be stimulated primarily by epidermal growth factor [119]. Accessing and manipulating this ependymal cell reservoir for neural regeneration could constitute a novel therapeutic strategy for nanotechnology.

4. Nanotechnology strategies for promoting neuroregeneration by modulating the extracellular environment

The extracellular matrix (ECM) plays a critical role in mediating neuroregeneration and can likewise be harnessed for therapeutic effects (Fig. 1). Foundational studies have shown the ECM's role in neurogenesis and its pathological changes under CNS injury, revealing a promising target for nanotechnology to precisely manipulate the physical and chemical cues that promote regeneration (Table 4) [120].

4.1. Nanotechnology for creating artificial ECM to support neuronal growth

4.1.1. Polymer and peptide nanoscaffolds

Nanoscaffolds are produced to mimic the ECM as much as possible in terms of architectural and functional properties, which not only provides mechanical support, but also plays a key role in the regulation of cellular behavior such as adhesion, proliferation, differentiation, and migration (Fig. 5) [19,121]. Scaffolds composed of peptides have been explored due to their non-immunogenicity and non-toxicity of their building blocks upon degradation. A self-assembled peptide nanoscaffold composed of RADA16-I peptide sequence nanofibers was applied in a hamster model of midbrain injury induced vision loss. The peptide scaffold solution was injected into the brain and self-assembled at the lesion site, resulting in axonal bridging across the lesion, optical tract regeneration, and most importantly enabled functional return of vision [122]. However, peptide scaffolds are more easily degraded, generally fail to penetrate the BBB, and provide less mechanical stability, and as a result, efforts have moved to composite nanoscaffolds combining peptide and polymer nanofibers to gain both benefits. A combined PLGA-RADA16-I-BMHP1 nanoscaffold exhibited significantly increased neural developmental markers by Schwann cells compared to PLGA scaffold alone [123]. LENK peptide nanofibers were coated with GCPQ polymer, which enables escape from liver uptake and avoidance of enzyme degradation, resulting in longer circulation time [124]. This polymer coating over peptide nanofibers improved brain penetration and plasma half-life while decreasing liver uptake. Different methods of combining polymers and peptides have also been explored. Polycaprolactone (PCL)-PLGA nanofibers were combined with Ac-FAQ peptides in four different ways: core polymer fiber with peptide shell, peptide fiber with polymer shell, blended nanofibers, and heat annealed fibers [125]. All compositions presented full integration into the host CNS tissue, but polymer fiber with peptide shell configuration exhibited the greatest in vivo response, minimizing host inflammatory response while increasing astrocytic presence and collagen IV expression for an environment at the injury site conducive to regeneration.

Table 4
Nanotechnology for ECM Manipulation to promote neuroregeneration.

Nano-platform	Route of administration	Therapy delivered	Model	Effects	Ref.
Mimicking ECM					
Peptide scaffold	Intracranial	N/A	Midbrain Damage	Axonal bridging across lesion, optical tract regeneration, functional recovery	122
PLGA-peptide scaffold	Systemic	N/A	Healthy	Increased brain penetration, plasma half-life	124
PCL-PLGA scaffold	Intracranial	N/A	Healthy	Decreased inflammation, increased host integration	125
PLA scaffold	Subcutaneous	Liraglutide	AD	Decreased plaque formation, increased neuron proliferation	127
HA hydrogel	Intracranial	NPCs	Stroke	Increased neovascularization, NPC viability; decreased inflammatory response	128
HA hydrogel	Intracranial	VEGF	Stroke	Increased vascularization, axon growth; Decreased glial scarring; improved behavioral outcomes	129
HA hydrogel	Intracranial	N/A	Brain lesions	Integration with host tissue; increased axon growth	130
Carbon nanotubes	Intraventricular	N/A	Stroke	Reduced apoptotic marker expression, infarct volume; improved rotarod performance	136
Manipulating ECM					
Agarose hydrogel	Intracranial	ChABC	Healthy	Sustained release, reduced enzymatic degradation	148
Collagen scaffold	Intracranial	BMSCs	TBI	Decreased NogoA expression, increased axonal regeneration	152
PLGA NP	Intravenous	NEP1–40	Stroke	Reduced infarct area; improved survival	153
Collagen scaffold	Intracranial	NgR	TBI	Limited glial scar formation, stimulated migration of NSCs to lesion	154
PLGA-PEG NP	Intravenous	N/A	Cancer	Increased survival	157
Multi-functional nano-systems					
HAMC hydrogel + PLGA NP	Intrathecal	Anti-NogoA Ab + NT-3	SCI	Increased neurofilament density, functional recovery	159
Fibrin scaffold	Intrathecal	NT-3, PDNF, ChABC	SCI	ChABC is anti-synergistic; without ChABC, improved neuronal regeneration and reduced macrophage infiltration	160
Collagen scaffold	Intrathecal	NEP1–40, EphA4LBD, PlexinB1LBD, BDNF, NT-3	SCI	Reduced cavity volume, reduced macrophage infiltration, increased axonal regeneration	161
HA hydrogel	Intrathecal	Anti-NgR Ab + VEGF or BDNF	SCI	Improved motor function, decreased glial scar formation	162
Hydrogel + PLGA NP	Epicortical	EGF, EPO	Stroke	Increased NSC proliferation; reduced apoptosis, inflammation	166
Chitosan spheroid + Iron oxide NP	Intrathecal	MSCs	SCI	Increased BDNF expression; improved nerve regeneration and activity	167
Silk scaffold + Gold NP	Intrathecal	Schwann cells	SCI	Improved muscle activity	168
HA hydrogel + PBAE NP	Intracranial	Neurogenin-2	TBI	Increased neuronal differentiation; improved survival	169

Polymer scaffolds can also be enhanced with growth factors for enhanced effect. PCL nanoscaffold was optimized with BDNF to sustain release over 21 days [126], resulting in increased synaptic density and neuronal survival in vitro. For AD, PLA nanofibers were loaded with liraglutide (LG), a glucagon-like peptide analog and implanted subcutaneously at the base of the head in the APP/PS1 AD mouse model. Compared to daily LG injections, the implanted nanoscaffold significantly decreased amyloid-β plaques and increased DCX-positive novel neurons, assessed 4 weeks after implantation [127]. However, while polymer scaffolds are generally mechanically stronger than peptide scaffolds, their degradation products can be toxic. Based on these results, moving forward, composite peptide polymer nanoscaffolds enhanced with drugs or growth factors should be explored for the greatest therapeutic effect to combine the advantageous features of both platforms.

4.1.2. Hydrogels

Hydrogels have also been applied for neuroregeneration, namely in stroke by providing a substrate within the brain cavity for promoting growth of endogenous or implanted cells. A hydrogel composed of hyaluronic acid (HA), heparin, and collagen used to implant neural progenitor cells into the infarct in a mouse model of stroke exhibited significantly improved NPC survival after implantation. NPCs implanted within the hydrogel saw two-fold increase in counts 2 weeks after implantation compared to freely implanted cells. Transplanted cells were found to improve neovascularization and decrease microglia infiltration when loaded within the hydrogel [128]. Interestingly, extracellular vesicles derived from stem cells have been shown to be as effective as stem cells themselves in improving rotarod, tightrope, and corner behavioral tests as well as neurogenic markers without any signs of adverse immune responses in a pre-clinical stroke model. Loading stem cell-derived extracellular vesicles into hydrogels to promote neurogenesis may avoid the logistical and safety concerns associated with stem cell implantation. Hydrogels can be further improved by loading or surface-decorating with biological motifs to improve cellular infiltration and neuronal growth. An HA hydrogel carrying angiogenic factors exhibited improved outcomes for stroke compared to the hydrogel alone in a MCAO mouse model of stroke [129]. In this model, mice were implanted with hydrogels five days after injury. When coated with high densities of VEGF, these hydrogels provided sustained release of VEGF into the infarct, resulting in decreased glial scarring and improved

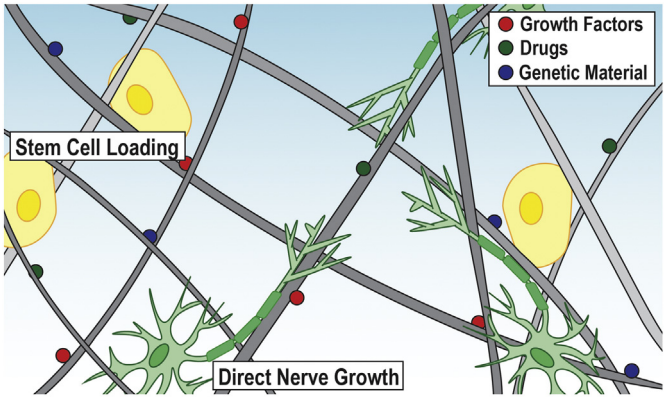

Fig. 5. Schematic of nanoscaffold capabilities in directing neuronal growth, loading stem cells and other cell types, and sustained release of growth factors, drugs, and genetic materials.

vascularization. This increased vascularization was found to promote axonogenesis as shown by increased levels of NF200 in the infarct and peri-infarct regions, resulting in novel functional networks. Ultimately, these improvements led to enhanced behavioral outcomes in stroke recovery, including cylinder test for forelimb dexterity, grid test for hindlimb, and pasta tests for forepaw. In all assessments, hydrogels surface-decorated with VEGF significantly outperformed hydrogel alone, as well as hydrogel with soluble VEGF treatment [129]. In this case, neovascularization stimulated by VEGF promoted axonal growth into the hydrogels, while hydrogels alone saw minimal neuronal infiltration. This is consistent with previous findings with ECM-derived hydrogels, where cell infiltrate was largely limited to the periphery of the gel [130]. Another strategy to enhance deep cellular infiltration into hydrogels is to introduce biological motifs such as peptides. HA hydrogels modified with IKVAV peptides for promotion of neurite growth showed efficacy in rat brain lesions [131]. These hydrogels were fully integrated with the host tissue, with infiltration of native tissue elements and improved neurofibril regeneration through the hydrogel.

4.1.3. Carbon nano-surfaces

The electrically active nature of carbon nanomaterials confers favorable growth environment for cells, and therefore these structures have been extensively applied to tissue regeneration. Electrospun carbon nanofibers coupled with externally applied biphasic electric field significantly improved neural proliferation and differentiation compared to non-conductive polyacroylonitrile scaffolds [132]. Similarly, 3D scaffolds composed of rolled graphene oxide sheets improved cell proliferation with electrical stimulation and accelerated differentiation of human NSCs into mature neurons [133]. 3D printing has also been employed to construct carbon nanostructures. Carbon nanotubes were incorporated with poly(ethylene glycol) diacrylate (PEGDA) polymer to form a nanoscaffold created with stereolithography 3D printing. This technique allows for tunable composition and porosity. This scaffold resulted in greater NSC proliferation and neuronal differentiation compared to PEGDA polymer scaffold alone, and further neuronal maturity when coupled with electrical stimulation in vitro. Carbon nanotubes (CNT) have also been incorporated into other nanoscaffolds to confer electrical conductivity for improved growth stimulation. Carbon nanotubes aligned within chitosan nanoscaffolds resulted in increased mechanical strength compared to chitosan alone [134], as well as highly electrically active properties. These properties result in enhanced neuronal viability and directed axonal growth along the carbon nanotube alignment direction. When combined with collagen hydrogels, carbon nanotubes similarly confer electrical conductivity [135]. The addition of carbon nanotubes and external electrical stimulation improved neurite outgrowth synergistically. These results demonstrate the potential applicability of using carbon nanostructures as substrates to facilitate ex vivo production of nerve grafts, but more work is required to understand if these favorable properties can be realized clinically in vivo. One such in vivo application involved single walled carbon nanotubes modified to have amine surface functionality. Pretreatment via intraventricular administration 1 week prior to MCAO stroke and injury resulted in significantly reduced infarct areas compared to saline treated mice, from 90% of hemisphere area down to only 20%. CNT treated mice also saw reductions in apoptotic markers via TUNEL staining and protein levels for p53 and Bax. These resulted in improved latency to fall times on rotarod tests at 14 days post-injury, that was comparable to pre-injury levels, indicating robust functional recovery [136].

4.1.4. Nano-patterned surfaces

Nanopatterned structures have generally been used to facilitate nerve growth for in vitro research applications but are gaining more attention as a means of stimulating and directing nerve growth for in vivo transplantation [137,138]. PLGA surfaces created with solvent assisted capillary force lithography were coated with 3,4-dihyroxy-*l*-phenylalanine to immobilize poly-L-lysine and fibronectin on the surface [139]. Neurite growth was observed along the patterned grooves on the surface via contact guidance, leading to enhanced focal adhesion, skeletal protein reorganization, and neuronal differentiation of human NSCs, an effect that was further enhanced with the administration of NGF. Polystyrene surfaces with nanopore array pattern to mimic ECM structure were created via nano-injection molding process [140]. Nanopatterned substrates yielded greater cell adhesion, neurite growth, and expression of neural markers of stem cells compared to flat surfaces. They can also be enhanced with factors to further promote cell growth in addition to topographical stimulation. PLGA films with patterned surfaces were formed by dry phase inversion and loaded with NGF [141]. NGF exhibited rapid release from the film on the timescale of several hours, facilitating directional neurite outgrowth.

4.2. Nanotechnology strategies to manipulate native ECM after injury

4.2.1. Chondroitin sulfate proteoglycans

Chondroitin sulfate proteoglycans (CSPGs) are found on the ECM surrounding all cells and form perineuronal networks (PNNs) by linking with hyaluronan and linker proteins Crtl1 and Bral2 [142]. Injury causes increased formation of CSPGs, leading to inhibited axonal outgrowth and converting of growth cones into the dystrophic state [143]. CSPGs are digested by the enzyme chondroitinase ABC (ChABC), which is expressed to moderate the growth inhibitory activity of CSPGs, allowing for limited plasticity necessary in mature neurons. ChABC treatment has been shown to promote axonal sprouting and CSPG digestion in preclinical models of injury, resulting in functional recovery [144,145]. The improved plasticity from ChABC treatment allows for the formation of bypass networks via connections between damaged and undamaged axons in the injury site. However, its translation has been impeded by its poor in vivo properties. ChABC is rapidly degraded and highly non-diffusive in tissue.

ChABC treatment has shown promise for promoting regeneration but its clinical translation held back by its poor stability, rapid clearance, and limited diffusivity in tissue [142]. Logistically, its poor thermal stability has limited its clinical application because it rapidly loses activity at body temperature, room temperature, and in refrigeration [146]. Efforts have been made to address its thermal stability via stabilizing agents and chemical modifications for improved stability and efficacy [144,146]. In terms of nanotechnology-mediated stabilization, ChABC has been encapsulated in porous silicon nanoparticles [147]. Silica particles were electrochemically etched and incubated with ChABC to form immobilized ChABC within 180 nm porous silica nanoparticles. While free ChABC loses activity rapidly at all temperatures, immobilized ChABC in silica nanoparticles retained activity at greater levels and for over 2× longer at all conditions tested, enabling ChABC supply transport and processing with less loss in activity.

To improve its in vivo properties, ChABC has been explored as an enhancement on biomaterials to further regenerative properties. ChABC was encapsulated in the pores of an agarose-carbomer hydrogel for sustained delivery [148]. The hydrogel was injectable and gelled upon irradiation in vivo, and ChABC was homogeneously entrapped in its pores after gelation after simply mixing ChABC PBS solution with the hydrogel polymer solution. Hydrogel loading enabled sustained release over ~7 days, and enzymatic assays confirmed that activity of ChABC was retained up to at least 7 days. ChABCs have also been shown to have synergistic effects with myelin-associated inhibitors, discussed in the following section. Codelivery of ChABC and NEP1–40, a peptide that inhibits NgR1, was achieved via microparticle enhanced fibrin scaffolds [149]. NEP1–40 was loaded into PLGA microspheres while ChABC was loaded into lipid microtubes, and the two components were then embedded into the fibrin nanoscaffolds. In vitro release was sustained

for one week for ChABC and two weeks for NEP1–40. Additionally, in vitro treatment of embryonic DRGs demonstrate the synergistic effects of the codelivery, with both factors achieving significantly greater neurite extension than individual ChABC or NEP1–40. However, in the design of combination therapies, while synergistic effects may sometimes be seen, further understanding of the interplay between each component is needed to inform therapy design.

4.2.2. Myelin-associated inhibitors

Remyelination of damaged axons and removal of myelin debris after injury is crucial for functional recovery. Myelin-associated inhibitors (MAIs) are present in the mature nervous system to prevent unnecessary remodeling of established networks. However, this natural inhibitory mechanism becomes problematic after injury when growth promotion is desired. Nogo-A is the most commonly studied MAI and is highly expressed in mature neurons to control structural plasticity. Myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OgMG) are other known MAIs. All three of these MAIs bind to the same receptor, Nogo receptor 1 (NgR1), making it an attractive therapeutic target. Anti-Nogo-A treatment has been shown to improve peripheral nerve recovery and innervation through the lesion, and knockdown of NgR1 results in increased axonal sprouting and motor function [150,151].

Delivery of MAIs via implantable or injectable scaffolds can achieve local sustained delivery while removing the need for high doses and invasive repeated intrathecal administration. Inhibition of NogoA has also been shown via delivery of bone marrow stromal cells in collagen scaffolds [152]. Human bone marrow stromal cells (BMSCs) were loaded into collagen scaffolds and applied to the lesion cavity of rats after TBI. Cell delivery within the scaffold was superior to cells alone, with significant knockdown of NogoA expression in olidodendrocytes after TBI, as well as enhanced axonal regeneration. In stroke, PLGA nanoparticles were loaded with NEP1–40 via double emulsion solvent evaporation and subsequently surface decorated with chlorotoxin, which targets MMP-2 overexpressed in the ischemic brain microenvironment, and lexiscan, which improves BBB penetration [153]. Nanoparticles exhibited specific targeting to the ischemic region upon systemic administration, with nanoparticles decorated with both motifs exhibiting the greatest specificity. Compared to freely administered NEP1–40, these nanoparticles significantly improved reductions in infarct area and increased survival.

Alternatively, MAI inhibiting strategies have also targeted their receptor, NgR. The delivery of soluble free NgR to scavenge MAIs rather than inhibiting endogenous NgR has also shown efficacy. Collagen scaffolds were covalently linked with a small amount of soluble NgR (sNgR) prior to crosslinking, as well as incorporated with sNgR via incubation with sNgR after crosslinking to create dual-phase release profile [154]. Injection into the lesion site after penetrating brain injury one week after injury showed that the scaffold integrated with viable surrounding brain tissue, limited glial scar formation, and stimulated the migration of neural progenitor cells to the lesion border, although direct regeneration was not found.

4.2.3. Tenascin C

Tenascin C (TNC) is a glycoprotein that plays both growth promoting and inhibiting roles dependent on the presence of $\alpha1\beta9$ integrin receptor [155]. In its presence, TNC promotes axonal sprouting and formation of growth cones while in its absence, TNC limits axonal outgrowth. In the mature brain, $\alpha1\beta9$ integrin receptor exists at low levels, and TNC's function is to limit axonal sprouting to form neuroanatomical regional subdivisions. After injury, TNC is upregulated and deposited at the site of injury, where it resides in the lesion site and contributes to the suppression of regeneration [156]. Therapeutic strategies involving TNC and/or $\alpha1\beta9$ integrin receptor must be carefully designed to maintain their balance such that growth promotion is achieved, and nanotechnology can provide this type of precise delivery and release.

Thus far, TNC has been used as a target for cancer therapy, and these efforts demonstrate that in addition to a therapeutic target, tenascin C may be used in brain injury therapies as a means to specifically target the injury site. In glioblastoma, PLGA-PEG nanoparticles were modified with a new peptide sequence targeting TNC to improve tumor specific targeting [157]. FHK peptide sequences have shown high affinity for TNC and was conjugated to the nanoparticle surface via maleimide-thiol coupling. A sequence targeting neuropilin-1, a vascular and tumor cell surface marker, was also incorporated to enhance tumor targeting. Targeted nanoparticles exhibited significantly greater accumulation in the tumor than nontargeted or single targeted nanoparticles. Paclitaxel loaded targeted nanoparticles significantly increased survival compared to controls, Taxol, and single targeted nanoparticles. Similarly, PEG liposomes sporting FH peptide for TNC targeting significantly improved the tumor growth inhibition of Navitoclax [158]. Studies such as these that TNC may be used as both a targeting ligand to target brain injury and, in the context of brain regeneration, a potential therapeutic target to promote neural proliferation and growth.

5. Multifunctional nanosystems

Due to the highly complex nature of neural regeneration discussed above, strategies integrating multiple therapeutic targets in both cells and the ECM constitute a more holistic approach that may yield greater therapeutic efficacy. Nanotechnology approaches can be combined and designed to address multiple disease pathologies in a single nanosystem.

5.1. Combining cell and ECM modulating agents

Simultaneous manipulation of cells to promote regeneration and the ECM to create a regeneration friendly environment can be combined for further enhanced neural regeneration. PLGA nanoparticles encapsulating NT-3 were embedded in hyaluronic acid methyl cellulose hydrogels carrying anti-NogoA antibodies to create a dual delivery platform [159]. Loading into this hydrogel nanoscaffold enables the sustained release of both components over 5 and 40 days for Anti-NogoA and NT-3, respectively. In vitro efficacy exhibits stimulated neurite outgrowth that is sustained for at least 58 days. Intrathecal injection of the hydrogel system increases neurofilament density in the lesion area. A synergistic effect is demonstrated, with dual delivery resulting in greater NogoA inhibition than anti-NogoA alone. These effects result in improvements to functional recovery as measured by ladderwalk test and BBB motor scoring, with dual delivery outperforming individual delivery [159].

In a study administering a scaffold containing progenitor motor neurons (pMN), growth factors, and MAIs, the full cocktail unexpectedly decreased efficacy. Fibrin scaffolds were incorporated with growth factors NT-3 and platelet-derived growth factor, as well as lipid microtubes containing ChABC and pMNs. pMN viability was not affected by the codelivery of other factors in vitro. However, in vivo viability of pMNs implanted in fibrin scaffolds containing the growth factors and ChABC was significantly reduced and increased macrophages infiltration without improving neuronal regeneration. Scaffolds delivering only pMNs and growth factors performed the best, suggesting the incorporation of ChABC has may elicit an adverse immune response leading to inhibitory effects [160].

In contrast to ChABC, combination delivery of MAI inhibitors and growth factors exhibited synergistic effects for greater regenerative improvements in the peripheral nervous system, and these promising results can inform the strategies for CNS regeneration [161]. Inhibitors NEP1–40, EphA4LBD, and PlexinB1LBD to inhibit Nogo, ephrin B3, and sema 4D, respectively, were incorporated into the fibers of a collagen scaffold along with BDNF and NT-3. In addition to scaffold administration, cAMP, which has previously been shown to activate pro-growth genes, was also injected in the peri-lesion site. In a rat model of spinal cord injury (SCI), delivery of these factors exhibited strong synergistic effects, with the addition of each component further improving

regeneration. The full combination therapy exhibited the greatest efficacy, with significantly reduced cavity volume, minimal infiltration of macrophages, and significantly increased axon counts. Newly formed neurons were found regenerated in the lesion area with greater number and thicker myelin sheaths, indicating strong inhibition of MAIs. These regenerative effects resulted in significantly improved BBB scoring approaching that of healthy rats, underscoring the promise such strategies may show in CNS injury applications.

Anti-NgR antibodies immobilized in HA hydrogels have shown efficacy in SCI. HA modified with poly-L-lysine formed nanofibers that oriented axially to form a hydrogel. Anti-NgR antibodies are incorporated into the scaffold nanofibers after simply incubating in phosphate buffer and $NaIO_4$ mixed with the hydrogel for 24 h. For further improved efficacy, PLGA microspheres were prepared with VEGF or BDNF to stimulate growth and similarly incorporated into the hydrogel. VEGF or BDNF were slowly released from the microspheres over two weeks, and delivery with the hydrogel achieved significantly greater protein levels in spinal cord tissue than freely administered microspheres. Enhanced hydrogels resulted in greater BBB scores in rats with SCI than the hydrogel alone. The anti-NgR antibodies significantly decreased the glial scar formation and deposition of CSPGs after injury. Microphotographs demonstrated neurofilament extension across the lesion site with enhanced HA hydrogel application [162]. Taken together, these results indicate that simultaneous, multi-functional nanotechnology platforms targeting multiple aspects of the inhibitory ECM environment can lead to neuronal regeneration in the peripheral nervous system and can be applied to regeneration for brain injuries and neurodegenerative diseases.

5.2. Nanoscaffold-mediated nanoparticle delivery

Nanoparticles embedded in scaffold structures have been used to enhance the delivery of drugs and biologics. Electrospun PCL-gelatin nanoscaffold decorated with gold nanoparticles encouraged longer outgrowth of neurites and fewer branches compared to PCL-gelatin alone in vitro [163]. Similarly, 3-D printed hydrogels via stereolithography loaded with PLGA nanoparticles containing bovine serum albumin and NGF significantly improved neurite growth and directional extension compared to hydrogel alone [164]. Gold nanoparticles can be further modified with peptides to promote cellular internalization and biocompatibility [165]. Sequential delivery of compounds via polymeric nanoparticles loaded into hydrogels have been shown to increase proliferation of neural stem and progenitor cells, reduce cell death from the stroke injury, and attenuate the inflammatory response around the infarct in vivo [166]. Epidermal growth factor was conjugated to PEG and encapsulated into PLGA nanoparticles, which were then further encapsulated in microparticles containing EPO. These particles were dispersed in a HAMC hydrogel. This noninvasive epicortical delivery platform exhibited sustained, sequential release in vivo over three weeks for significantly enhanced therapeutic effect compared to freely administered particles and drug compounds. The limitation of such a platform is diffusion of nanoparticles through brain tissue, which was partially addressed with PEGylation and can be further optimized. In a model of peripheral nerve damage, mesenchymal stem cells (MSCs) labelled with iron oxide nanoparticles for in vivo tracking embedded in chitosan spheroids resulted in improved nerve regeneration and activity [167]. MSCs isolated from rat adipose tissue embedded in chitosan spheroids exhibited enhanced expression of neural-associated genes and improved labelling by iron oxide nanoparticles. Embedded MSCs also exhibited greater transfection with BDNF in spheroids vs. single cell suspensions for improved expression of neurotrophic factors. In vivo, this resulted in greater nerve regeneration and activity. Silk nanoscaffolds loaded with gold nanoparticles have also been reported to improve nerve regeneration in peripheral nerve injury [168]. Loading with nanoparticles did not affect structural integrity, as the silk nanocomposites withstood implantation and processing without physical damage or deformation. Animals implanted with the silk nanocomposites performed superior to silk nanoscaffold alone as measured by compound muscle action potential and sciatic function index. Additionally, loading with both gold nanoparticles and Schwann cells created a synergistic effect. Long term implantation of the nanocomposites for up to 18 months resulted in recovery of normal nerve structure and myelination without adverse inflammatory response. For brain regeneration specifically, neural stem cells loaded in HA hydrogels and transfected with poly-beta amino esters (PBAE) nanoparticles delivering neurogenin-2 resulted in enhanced neuronal differentiation and improved post-transplant survival in a rat model of TBI [169]. These results indicate that loading nanoparticles into nanoscaffolds or hydrogels for sustained release of growth factors has the potential to significantly improve neuronal regeneration in the brain for enhanced clinical outcomes.

6. Conclusions and future directions

Since the inception of the field of regenerative medicine, great strides have been made to stimulate tissue regeneration for improving patient outcomes. Given the incredibly challenging nature of the CNS, successes in neural regeneration have been more limited. Few regenerative strategies for CNS injuries have achieved FDA approval for use in patients, and there is a lack of promising technologies upcoming in the clinical pipelines. Nanotechnology can provide the precise and robust stimulation of regeneration in CNS injuries needed, and preclinical studies have shown promising results. Due to the complexity of the nervous system and the multiple components involved in regeneration, single agents may be insufficient to address and promote regeneration following CNS injury. Multi-functional strategies that combine cellular stimulation and ECM manipulation, potentially along with gene and drug nanoparticle delivery, may be necessary to facilitate regeneration. Additionally, there remain several cellular targets that, while playing critical roles in neural regeneration, have been largely unexplored as therapeutic targets. Further understanding of these cells, including astrocytes, pericytes, ependymal cells, and T cells, may reveal mechanisms which can be effectively leveraged to induce neural proliferation and growth. Nanotechnology approaches that integrate all relevant components of neurogenesis will produce the most promising therapies for the future of brain regeneration after injury.

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