Central Nervous System Tissue Engineering

Current Considerations and Strategies

Synthesis Lectures on Tissue Engineering

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www.morganclaypool.com

ISBN: 9781608457861 paperback
ISBN: 9781608457878 ebook

DOI 10.2200/S00390ED1V01Y201111TIS008

A Publication in the Morgan & Claypool Publishers series
SYNTHESIS LECTURES ON TISSUE ENGINEERING

Lecture #8
Series Editor: Kyriacos A. Athanasiou and J. Kent Leach, *University of California, Davis*
Series ISSN
Synthesis Lectures on Tissue Engineering
Print 1944-0316 Electronic 1944-0308

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Current Considerations and Strategies

Ashley E. Wilkinson, Aleesha M. McCormick, and Nic D. Leipzig
The University of Akron

SYNTHESIS LECTURES ON TISSUE ENGINEERING #8

ABSTRACT

Combating neural degeneration from injury or disease is extremely difficult in the brain and spinal cord, i.e. central nervous system (CNS). Unlike the peripheral nerves, CNS neurons are bombarded by physical and chemical restrictions that prevent proper healing and restoration of function. The CNS is vital to bodily function, and loss of any part of it can severely and permanently alter a person's quality of life. Tissue engineering could offer much needed solutions to regenerate or replace damaged CNS tissue. This review will discuss current CNS tissue engineering approaches integrating scaffolds, cells and stimulation techniques. Hydrogels are commonly used CNS tissue engineering scaffolds to stimulate and enhance regeneration, but fiber meshes and other porous structures show specific utility depending on application. CNS relevant cell sources have focused on implantation of exogenous cells or stimulation of endogenous populations. Somatic cells of the CNS are rarely utilized for tissue engineering; however, glial cells of the peripheral nervous system (PNS) may be used to myelinate and protect spinal cord damage. Pluripotent and multipotent stem cells offer alternative cell sources due to continuing advancements in identification and differentiation of these cells. Finally, physical, chemical, and electrical guidance cues are extremely important to neural cells, serving important roles in development and adulthood. These guidance cues are being integrated into tissue engineering approaches. Of particular interest is the inclusion of cues to guide stem cells to differentiate into CNS cell types, as well to guide neuron targeting. This review should provide the reader with a broad understanding of CNS tissue engineering challenges and tactics, with the goal of fostering the future development of biologically inspired designs.

KEYWORDS

central nervous system, tissue engineering, spine regeneration, spinal cord injury, brain injury, neurodegenerative disease, nerve guidance, neural stem cells, nerve scaffold, neurotrophic factors

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CHAPTER 1

Introduction

The brain and spinal cord compose the Central Nervous System (CNS), which is the control center of the body. Inputs from muscles, involuntary organs, and senses travel through the nerves of the Peripheral Nervous System (PNS) into the CNS where they are interpreted. Signals may travel within the brain to separate functional areas. Instructions are then sent outward again for voluntary movement and involuntary regulation to complete the endless loop of the nervous system circuitry. The CNS is critical to function of the entire body, which is why incurred injury and disease cripple one's quality of life. In the United States alone approximately 265,000 people are estimated to have spinal cord injuries (SCIs), with over 10,000 new injuries occurring each year [1]. Patients with SCI experience decreased lifespan in addition to life-costs from one to four million dollars, depending on the extent of injury, which is especially disturbing considering the fact that the average age of a spinal cord injured person is 31 [1]. Moreover, traumatic brain injuries (TBIs) occur to over 1.7 million people each year [2]. The devastating physical and psychological effects of CNS damage are felt by both patients and their families. Of SCI individuals experiencing paraplegia or tetraplegia, less than 1% achieve full neurological recovery post treatment [1]. Solutions to recover neurological function are desperately needed for all CNS injuries.

Tissue engineering (TE) in the CNS is extremely difficult because of the intrinsic restrictions and complexity of native CNS tissue. Generally, multi-component approaches are used in attempt to restore natural function to the brain or spinal cord. First and foremost, an understanding of tissue formation and function as well as tissue responses to damage is needed in order to formulate treatments to correct injury and disease in the CNS. Knowledge of native tissue and pathological development will foster improvement of strategies for overcoming damage to the CNS. For the most severe CNS disorders, a complex TE construct involving multiple cues is most likely needed to combat the physical and chemical obstacles of the CNS; the general building blocks of these constructs are physical scaffolding from biomaterials, endogenous or exogenous cells, and stimulatory cues from chemical, mechanical and electrical signals within the construct or on its surface (Fig. 1.1). Within this review, scaffold formation techniques and common biomaterials in CNS TE will be discussed followed by potential cell sources. Subsequent sections will discuss the myriads of stimulatory and guidance techniques currently being employed in CNS strategies. The hope of this review is to give the reader the basic tools for designing or understanding strategies aimed at regenerating the CNS and also for exposure to current approaches. PNS regenerative strategies are often discussed to augment basic understanding and many of these techniques do translate to the CNS. In depth review articles and books are suggested throughout for further reading on particular topics.

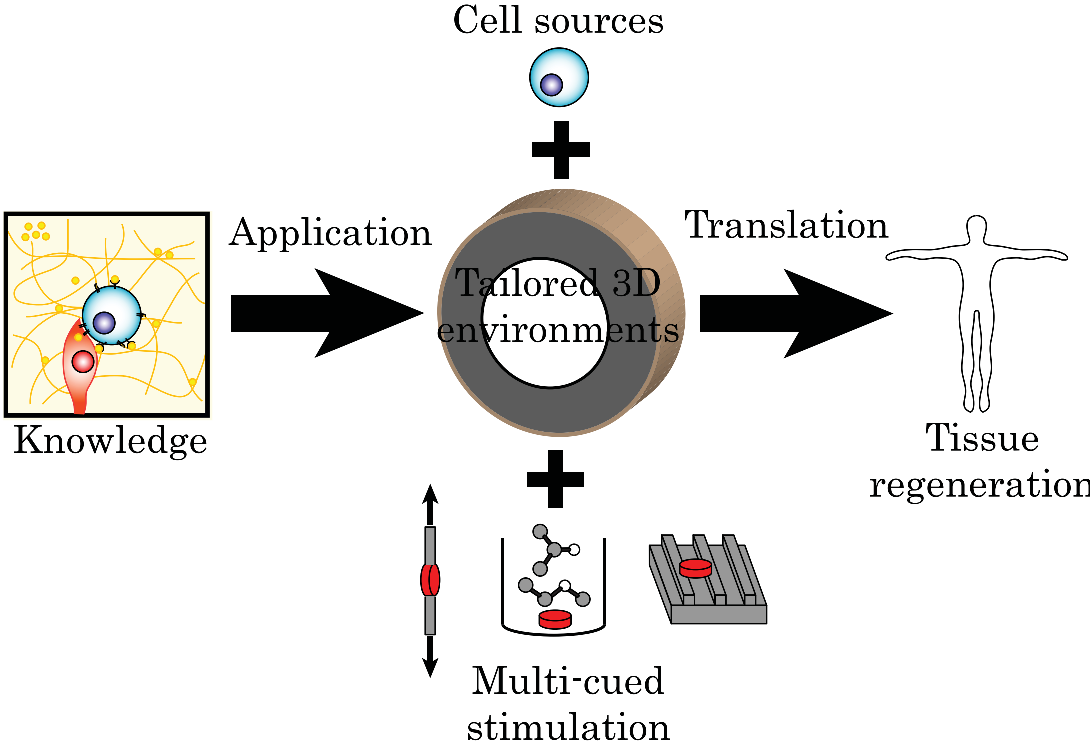


Figure 1.1: General tissue engineering strategy: knowledge of native cell environment is used to combine cell sources with mechanical, chemical, and electrical cues into a tailored tissue engineering construct. This assembly is grown under proper stimulation for translation to host tissue for regeneration.

CHAPTER 2

Anatomy of the CNS and Progression of Neurological Damage

2.1 ANATOMY AND PHYSIOLOGY OF THE CNS

2.1.1 GROSS ANATOMY

Neurons throughout the body are organized into three main structures: ganglia, nuclei, and lamina [3]. Outside of the CNS, neuronal bodies are grouped together into ganglia; an example of this formation is the dorsal root ganglia (DRG) of sensory cell bodies that form just outside of the spinal column in the PNS. Within the brain, neuronal bodies with a common function are grouped together into nuclei. The bulk of the brain is organized into a layered cortex, including most of the cerebrum and cerebellum. The cerebrum, diencephalon, cerebellum, and brain stem make up the parts of the brain and are all housed within the skull (Fig. 2.1). The cerebral cortex is extremely complex and has a number of functions including, but not limited to, systemic sensory and motor control, speech, recognition and understanding [4]. The spinal cord runs inferior to the brain stem in a columnar form, protected by the vertebrae of the spine. The inner core of the spinal column, with a butterfly like shape, contains the gray matter while the surrounding axons are white matter. Dorsal horn (sensory), ventral horn (motor), intermediate zone and commissural region comprises the gray matter [3]. White matter is made up of the anterior, posterior and lateral columns [3]. Gray matter is largely unmyelinated while myelination provides white matter with its name and color. Nerve fibers enter and exit the spine at each vertebra though holes called Foramen, allowing information to pass to and from the PNS. There are four main groups of spinal nerves that exit at different levels of the spinal cord. Named in descending order down the vertebral column, these are cervical (neck), thoracic (upper back), lumbar (lower back) and sacral (base) nerves. While the PNS is made up of groups of axons termed nerves, in the CNS axons run in groups called tracts that are bound together by the processes of astrocytes, often called 'end-feet' [3]. Descending (efferent) pathways include the pyramidal and extrapyramidal tracts, and ascending (afferent) pathways include the spinothalamic and spinocerebellar tracts as well as the gracile and cuneate fasciculi. Proper bodily function depends on these paths to transmit information between the brain and periphery.

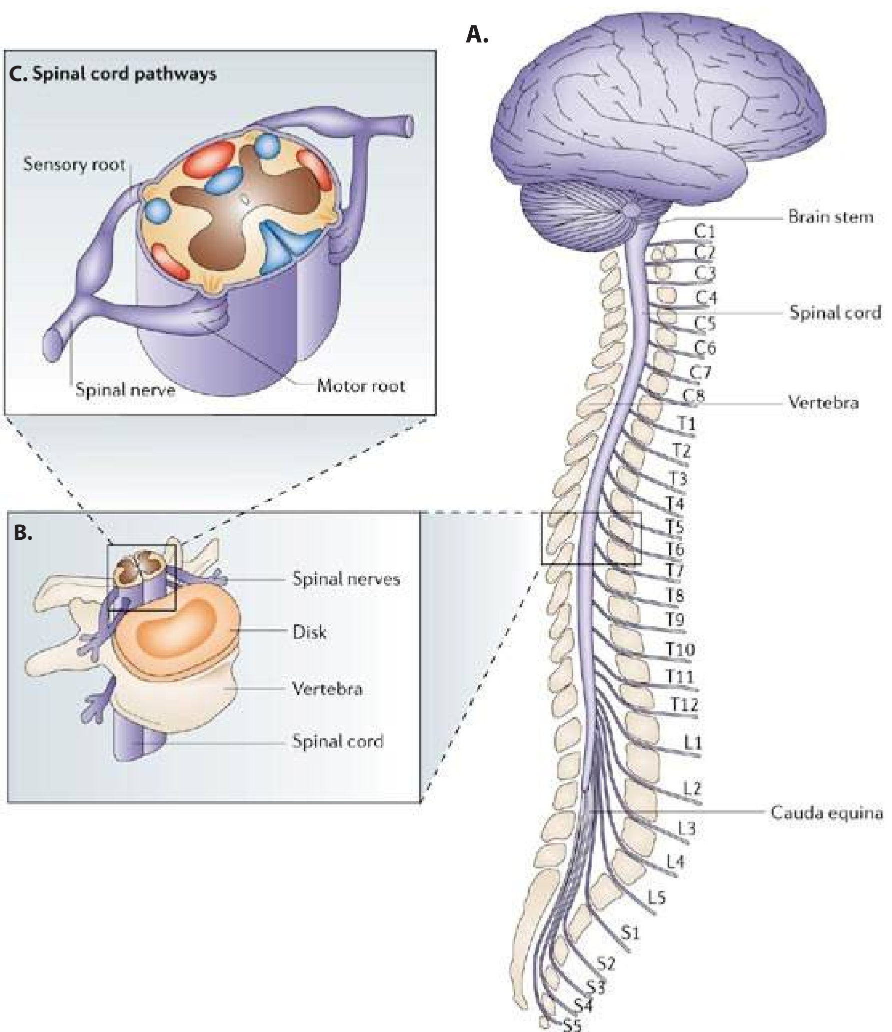


Figure 2.1: (A) Illustration of the CNS displaying the brain and spinal cord. (B) Cross-section of the spinal column showing the spinal cord protected within the vertebrae. (C) The spinal cord is segregated with white matter surrounding gray matter. Spinal roots exit on the ventral side and enter on the dorsal side to and from the PNS. Figure reprinted from [493].

2.1.2 EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is an important component of the CNS, and it accounts for around 20% of the adult brain [5]. More thorough reviews of CNS ECM are described elsewhere; only a few of the common ECM molecules are discussed here [5, 6]. The brain and spinal cord are primarily made up of proteins and proteoglycans - macromolecules with a protein core and glycosaminoglycan (GAG) side chains. GAGs are linear, negatively charged polymers of repeating disaccharide units also present in the extracellular space that help to properly hydrate tissues. Not only does the ECM provide the natural scaffolding for tissue, but it plays an active role in the regulation of diffusion of soluble proteins and in localizing membrane proteins to functional domains. Collagens and laminins are the primary ECM proteins of the CNS, which mainly make-up the basal lamina, and contain specific amino acid sites that interact with cell receptors [5, 7]. Several types of collagens are found in the brain and spinal cord (I, II, IV, XVII, XIX); however, they are not as abundant in the CNS as they are in most other tissues [8]. Integrins are the receptors that cells use to interact with the ECM. These transmembrane glycoproteins are made up of an alpha and a beta subunit that complex to activate a host of signaling pathways within the cell. Integrins provide a link from the ECM to the cytoskeleton. In the CNS, $\beta 1$ integrins are most relevant, binding to ligand sites on laminins, and are critical for proper neuronal migration [6, 7]. A major GAG found in the CNS and important constituent of the brain ECM is hyaluronic acid (HA) [5]. HA is implicated in many cellular functions, including regulating the diffusion of synaptic elements. Specifically, HA rich ECM in the synaptic region of the brain serves to restrict the escape of neurotransmitters and provides a physical barrier preventing the diffusion of the post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor to other areas of the plasma membrane [5]. AMPA receptors are cell ion channels that allow current to pass when activated by bound glutamate and are involved in fast synaptic transmission in the CNS [9]. In this way, HA contributes to maintaining proper synaptic signaling. Chondroitin sulfate proteoglycans (CSPGs) are the most common proteoglycans in the CNS, and include aggrecan, brevican, neurocan, and versican [10]. These CSPGs are termed lecticans due to their lectin-like domain, or a sugar binding domain [11]. Along with heparan sulfate proteoglycans (HSPGs), CSPGs are known to inhibit axon regeneration, but have been implicated in growth factor retention and presentation in healthy CNS tissue [5, 6].

2.1.3 NEURONS

Neurons are the fundamental units of the nervous system that process and transmit information by chemical and electrical signaling. Neurons are regionalized specifically to carry out signaling and can either be efferent, sending information away from the brain; afferent, sending information toward the brain; or interneurons, sending information between functional groups of neurons. The dendrites and soma compile and interpret cues from other cells and the surrounding environment (Fig. 2.2A). The soma serves as the trophic center of the neuron, regulating and producing proteins to be sent to various parts of the neuron [3]. Located here are most of the organelles common to all eukaryotic cells, including the nucleus and endoplasmic reticulum. Most cytoplasmic components are made

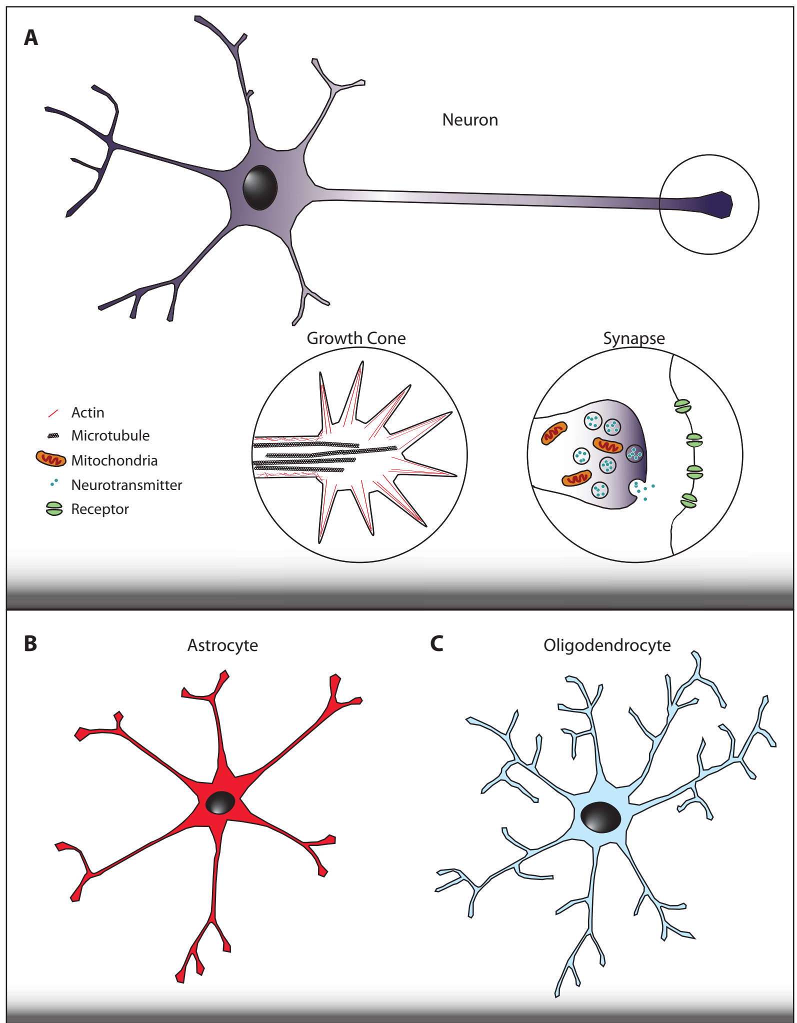


Figure 2.2: *Continued.* Major cell types of the CNS. (A) Different neurons have varied dendrite and axon conformations depending on their specific roles. The typical neuron has a large number of dendrites to gather information and a long axon to transmit the signal to its target. Insets show the terminus of the axon: Left: the growth cone is present during development and regeneration. Microtubules make up the core of the growth cone, while the peripheral lamellipodia and filopodia are comprised of depolymerized and F-actin. Right: once the axon finds its target it forms a synapse full of neurotransmitter vesicles and mitochondria. The neurotransmitters affect target receptors across the synaptic cleft. Illustration of an astrocyte in red (B) and oligodendrocyte in blue (C). Morphologically each cell follows its name; astrocytes are generally star shaped while oligodendrocytes have many branches, allowing them to myelinate more than one neuron at a time.

in the soma and transported to the processes directly or in vesicles. Dendrites sprouting from the soma mainly function to provide space for synapse, or connection to other neurons, and interpret the summation of excitatory and inhibitory signals from other neurons or extracellular space. They take on different formations depending on the type of neuron, but dendrites are thicker than axons and generally have many branches, sometimes even containing protruding spines to enhance synaptic reception area [4]. Although synapses may form on the soma or axon, the majority of synapses are located in the dendrites of a neuron [3].

The axons are the fiber-optic pipeline of the neuron, passing information at high speeds. Electrical signals, or action potentials, travel away from the soma down the axon in an all-or-nothing manner. The cytoskeleton of the axon is made up of intermediate filaments aligned along the axon and some microtubules constructed from tubulin, although not as many as are found in the dendritic processes. Tubulin is a polymer of repeating α and β subunits whose stability is promoted by microtubule-associated proteins (MAPs) and specific nucleotides [4]. Actin microfilaments are found throughout the axon, but are particularly important at the terminal end of the axon, or the growth cone, and will be discussed in greater detail later. The axon can be shorter, as in a satellite neuron, or very long to carry signals great distances. For example, adult human spinal cord axons can reach a length of several feet [12]. Whatever the length, vesicles and proteins need to be transported quickly along the axon to and from the soma. This is accomplished by motor proteins that use ATP hydrolysis to travel along microtubules; kinesin is responsible for soma to terminus (anterograde transport), and dynein carries organelles or vesicles from the axon to the soma (retrograde transport) [4]. Axon transport is extremely important for delivering neurotrophins and neurotransmitters to the terminus, as well as bringing proteins back to the soma for reprocessing. Depending on the molecule or vesicle being relocated, rate of transport in the axon can range from around 1 to 300 mm/d [3].

Axons have developed an evolved way of traveling to their target cell; the terminus of the axon, or the growth cone, interprets growth and directional signaling molecules and guides the axon along its path. Growth cones contain microtubules in their core and actin in their periphery (Fig. 2.2A). Long filaments of actin (F-actin) are found in the spikes protruding from the growth cone (filopodia) and disassembled actin is found in the web like lamellipodia that are just proximal to the filopodia [4, 13]. Actin is extremely important to the dynamic movement of the growth cone. Permissive substrates facilitate focal adhesion attachments of the growth cone to the substrate and stabilization of F-actin keeps the filopodium from being retracted. The lamellipodia follows in tow, advancing the growth cone [13, 14]. New cytoskeleton and membrane must be added to the axon as the growth cone hastens forth; constant transport of membrane components to the terminus as well as protein synthesis within the growth cone itself allows significant advancement even if the cell machinery is distant [4, 15].

Once the growth cone has arrived at its innervating target, a synapse is formed, either chemical or electrical. The chemical synapse includes the pre-synaptic terminal of the axon, the post-synaptic area of the cell being acted upon, and the very small gap (30-40 nm) between the two termed the synaptic cleft [3]. The pre-synaptic area of the neuron is filled with mitochondria and synaptic vesicles

containing neurotransmitters (Fig. 2.2A). When an action potential is generated along the axon it travels all the way to the synapse, leading to an increase in calcium levels that causes neurotransmitters to be exocytosed into the synaptic cleft. These neurotransmitters travel to receptors in the post-synaptic region, diffuse into surrounding tissue, or are endocytosed by nearby astrocytes [3–5]. Receptors activated by neurotransmitters change the permeability of the membrane to specific ions, which will lead to either a depolarization (excitatory response) or hyperpolarization (inhibitory response) of the target cell. Synapses in the dendritic region tend to be excitatory, while those on the soma are usually inhibitory [3]. In electrical synapses, neurons are connected by gap junctions that allow ions to pass directly from one cell to the next without mediation by chemical transmitters. In contrast to chemical synapses, electrical signaling has no delay, but is only excitatory, is not amplified, and may be bidirectional [4].

2.1.4 GLIA AND SUPPORTIVE TISSUE

The main role of astrocytes (Fig. 2.2B) in the CNS is supporting neuronal function by creating and protecting the neuronal microenvironment. There are over one hundred times more astrocytes in the CNS than neurons, and they maintain and mimic neurons directly by absorbing and releasing neurotransmitters into the synapse [3]. Astrocytes protect the CNS by forming the outer glial layer of the brain and enwrapping vasculature to create the blood-brain barrier that is infamous for its extreme selectivity [3]. The structure of the CNS is largely due to the action of astrocytes; they form the outer and inner glial membrane and isolate axons throughout the brain and spinal cord. In the event of injury, astrocytes proliferate and become a glial scar, which is a major blockade for neural TE and will be discussed in greater detail later this chapter.

Oligodendrocytes are the myelin forming cells of the CNS, serving to surround axons in an electron-dense myelin sheath. Processes of oligodendrocytes wrap around a neuron's axons many times, squeezing out most of the cytoplasm. Layers of lipid rich plasma membrane are left, tightly encircling and insulating the axon [4]. Oligodendrocytes have many processes (Fig. 2.2C) and a single oligodendrocyte can myelinate 30-60 axons at once. Myelin is an electrical insulator, whose main purpose is to increase the speed of electrical conduction through the axon while preventing signal loss. Electrical current cannot conduct through the myelinated portions of the axonal membrane, it only occurs at small gaps between myelin, termed nodes of Ranvier, which are several micrometers in length [3, 4]. Not all axons are myelinated, but the ones that are have much faster signal transmission times due to the saltatory conduction of current "jumping" from node to node along the axon. Injury to oligodendrocytes, and subsequent demyelination of axons, has been shown to lead to nervous system degeneration [16, 17]. Similarly, demyelination of axons is the causative factor for the symptoms of multiple sclerosis (MS).

Microglia are the immune cells of the CNS. Microglia originate from monocytes that have been trapped in the CNS during development and evolve into a less active state, or resting state [3]. At any sign of injury or disease these resting microglia proliferate and may become active, expressing class I major histocompatibility complex (MHC). Due to their reactivity, microglia are used in

research to gauge the extent of an insult to the brain or spinal cord by detecting the amount of activated microglia in the area [18–20].

Ependymal cells serve to line the ventricles and central canal of the CNS. These cells, in conjunction with blood vessels in the brain, secrete cerebrospinal fluid (CSF) [3]. Cilia on the surface of ependymal cells circulate CSF in the ventricles. Recently, they have been found to possess plasticity, and have the ability to differentiate into glia of the CNS in response to specific stimuli [21–23].

2.2 LOSS OF NEURAL FUNCTION

Injury response and subsequent nerve regeneration is very different in the CNS and PNS, resulting in contrasting outcomes. Due to differences in the two healing environments, PNS axons are able to reinnervate their targets while CNS axons are inhibited by physical and chemical blockades.

2.2.1 MODEL SYSTEMS AND FUNCTIONAL RECOVERY EVALUATION

Throughout the course of this review, different models of evaluation for TE strategies will be discussed. For understanding the implications of particular situations, a basic knowledge of injury models and evaluation techniques is necessary. Simulating injury in the brain and spinal cord has been standardized to some degree to enable global comparisons of different treatments across research labs. Cut or crush injuries are generally discussed for the spinal cord. Cut injuries are mimicked in the laboratory by surgical removal of all or a columnar portion of the spinal cord, termed complete transection or hemisection, for half of the spinal cord. To simulate a crush injury, the spinal cord is usually exposed and an electromagnetic or weight drop device is used to contuse the tissue. Animal disease models also exist where a key feature of a disease (e.g., demyelination) is simulated genetically for comparison of therapeutic methods.

Once an injury or disease model is created for study, specific methods for gauging deterioration or recovery are used to gather results. "Functional recovery" is a very subjective term and is applied mainly to *in vivo* work but sometimes it can be applied to *in vitro* models as well. *Ex vivo* cell and tissue are analyzed in a number of ways including examination of the cell and tissue anatomy (through histology, immunohistochemistry (IHC), and microscopy) as well as particular DNA, RNA, protein, or receptor expression (via polymerase chain reaction (PCR), microarrays, enzyme-linked immunosorbent assay (ELISA) and patch clamping). In animal models, functional improvement is usually estimated using behavioral observations and a rating score. For motor function, exercise tests and open field walking tests are often used. A popular and systematically defined scoring system is the Basso, Beattie and Bresnahan (BBB) locomotor rating scale [24]. The designers of this scoring system observed rats walking in an open field and assigned points for movements in paw joints, plantar steps, coordination, and limb alignment. In addition, exercise tests are sometimes used to assess motor recovery including swimming, rotor clinging, and narrowing track tests [25]. For sensory evaluation, reflex tests are used; reaction time to a toe pinch or heat application correlate to scoring of the animal's sensory recovery.

2.2.2 AXON RETRACTION AND DEGENERATION

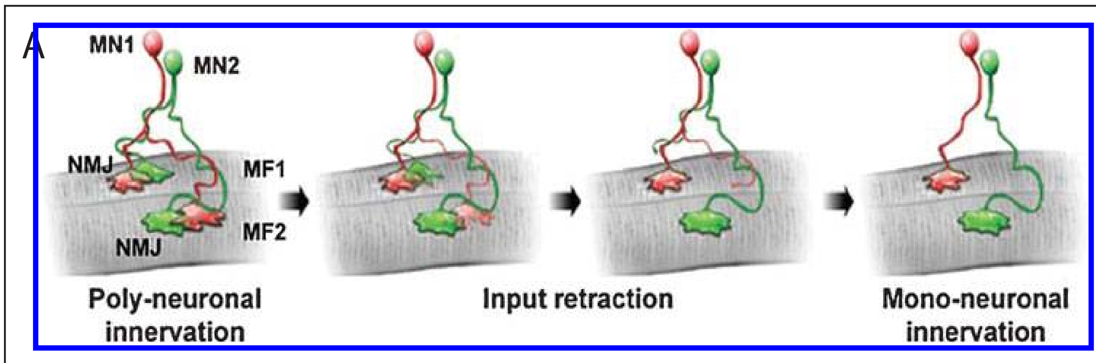
There are two modes through which axons remodel in the body, retraction (small scale pruning of axons and dendrites) and degeneration (large scale elimination of large portion of the primary axon or collateral branches). Retraction and Wallerian degeneration will be covered as well as possible mechanisms for these types of neuronal preservation models. For a more in depth understanding of these occurrences please see [16].

Axon Retraction

Retraction typically concerns small scale maintenance of the nervous system where multiple target innervations are eliminated by local pruning of axonal and dendritic branches. Two primary examples of this phenomenon occur at the primary visual cortex and the neuromuscular junction (NMJ), where a motor neuron axon synapses with muscle fiber motor end plate (Fig. 2.3). As an adult, NMJs are innervated with a single axon; however, during development multiple innervations take place at the same NMJ site. Each axon contends for this synapse and the competition leads to retraction of some axons due to the strengthening of others; finally, a single axon takes its place and vacant, noninnervated post-synaptic sites lose their receptors [26]. Retraction also leads to the highly ordered organization of the mammalian visual system. Retinal ganglion cells (RGCs) travel to the visual information relay center of the brain, the lateral geniculate nucleus (LGN), and neurons are further projected to the primary visual cortex, located in the occipital lobe of the cortex. Higher order neurons are then segregated into specific and complex patterns in this region allowing each eye to receive inputs for visual depth perception. During visual development these neurons overlap and mature connections are made through the pruning and segregation of axonal arbors. These adult, stable connections occur through synaptic plasticity proposed by Hebb's postulate where pre-synaptic efficiency results from the continual stimulation of post-synaptic cells [27].

Mechanisms of Axon Retraction

The mechanisms of retraction are currently poorly understood; as such, the basic definition of retraction is a change in cell shape, manipulation of the cytoskeleton, signaling pathways, or environmental cues. As described above, cytoskeletal components such as microtubules and actin are involved in growth cone migration. Axon retraction is a dynamic process involving interaction between these factors. Retraction of neurons results when microtubule polymerization is inhibited; whereas, retraction does not occur with blocked reduction in ATP microtubule assembly [28]. Inhibition of dynein on intact microtubules can lead to axon retraction; however, this does not occur when microfilaments are depleted [29]. Therefore, during retraction it is believed that motor molecules counterbalance changes in microfilaments. Cytoskeletal regulation and changes result from intercellular signaling cascades. Inhibition of rho-associated protein kinase (ROCK) blocks Ras homolog gene family, member A (RhoA) downstream signaling activation on axonal and dendritic retraction in hippocampal neurons [30]. Inhibitory guidance molecules (discussed in more detail in Chapter 5) located in the extracellular environment initiate RhoA signaling cascades, and are therefore thought



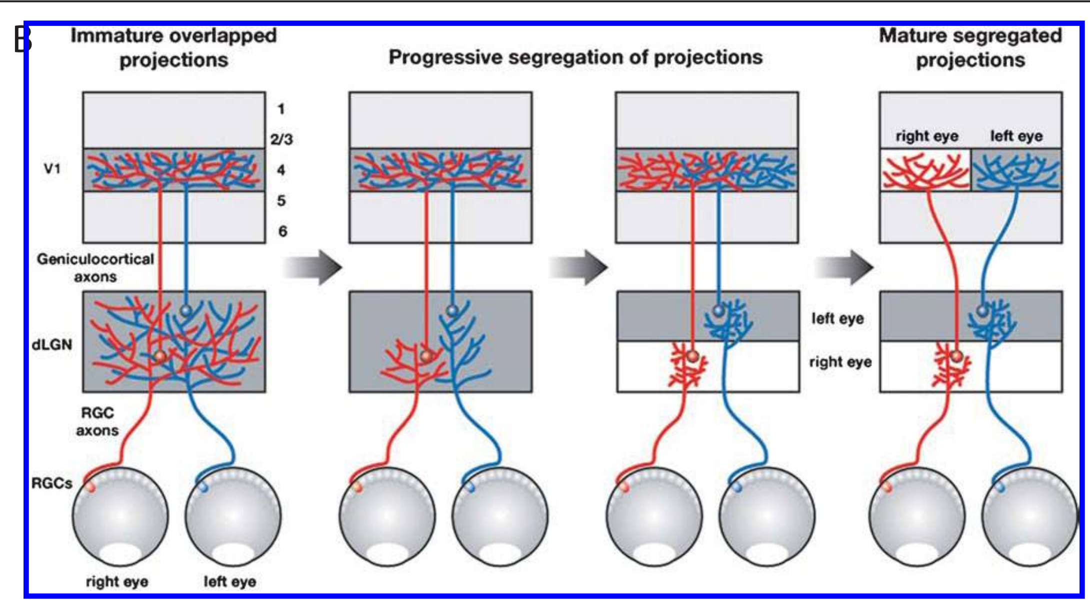


Figure 2.3: Two common axonal retraction examples during development. (A) Input signals from a motor neuron (MN) to a muscle fiber (MF) creates a neuromuscular junction (NMJ). During development, multiple synapses are formed (left). As MNs undergo synaptic plasticity, weaker neurons retract as a single neuron is victorious, resulting in a mature, adult NMJ (right). (B) Retinal ganglion cells (RGCs) are separated into specific layers of the dorsal lateral geniculate nucleus (dLGN). Geniculocortical axons are projected from this region and segregated into eye-specific columns in layer 4 of the primary visual cortex (V1). These synapses mature (from left to right) via competition from overlapping neurons. Small-scale elimination and retraction cause these overlapping monocular inputs to become isolated into specific regions of the dLGN and V1, producing a mature visual system. Images reprinted from [16].

to be key players in axon retraction [31, 32]. Even though many of the causative initiators of axon retraction remain unclear, it plays a significant role in neuronal development and the establishment of functional connections. Uncovering and fully understanding these mechanisms more clearly could result in better models and functional recovery outcomes.

Wallerian Degeneration and Slow Wallerian Degeneration

For Wallerian degeneration following injury, a latent period precedes rapid progression into active cytoskeletal breakdown, membrane blebbing, and axon fragmentation (Fig. 2.4) [16, 33–36]. Upon injury, degeneration occurs in 3-4 d; however, in lower vertebrates and invertebrates the axon segment can last for a period of more than ten times longer before degeneration proceeds [37]. Although Wallerian degeneration occurs after a cut or crush injury, it is also similar to axon degeneration observed in the later stages of some neurodegenerative diseases [16, 33]. Study of this response is invaluable for understanding axonal degeneration due to this commonality. PNS, CNS, and explanted nerves exhibit Wallerian degeneration, allowing for controllable Wallerian degeneration initiation *in vitro* and *in vivo* and thus the creation of valuable research models.

The discovery of the Wld^S mutant mouse by Lunn *et al.* in 1850 accelerated the study of Wallerian degeneration mechanisms and allowed for better understanding of some nervous system diseases [35, 36]. Lunn and associates originally observed that a special strain of mice was able to propagate action potentials for over two weeks following sciatic nerve transection, whereas, after injury, wild-type mice only carry action potentials for 1.5 d [38]. This delay was correlated to axon degeneration and facilitated further study of physical and molecular events that occur following nerve injury. Subsequent studies revealed that Wld^S axons degrade in a more gradual atrophic process whereas wild-type axon degeneration is self-regulated by pre-existing machinery in the axon, similar in manner but not in mechanism to apoptosis [16, 34, 39, 40]. The wide-held belief is that Wld^S mice have the ability to either defer or completely suppress Wallerian degeneration since injured axons die by different mechanisms in these mutants. Obstructed axonal degradation of Wld^S mice is dependent and a sole property of neurons themselves, requiring zero assistance from the neural system or glia of the mutant strain [41, 42]. Found mainly in the nucleus, Wld^S is a fusion protein containing nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), a specific segment of ubiquitin conjugation factor E4 B (Ube4b), and a unique 18 amino acid sequence joining the two [35, 43]. The question remains as to whether Wld^S protein acts from within the nucleus by recruiting and directing other pathways or if low concentrations within the axon are sufficient to interfere with degeneration. There is controversy over whether slowing degeneration is beneficial to injury recovery. Several studies have shown that in cases where degeneration was slowed using Wld^S mice; regeneration was also delayed and often muted after axotomy [44, 45]. While attempting to compare developmental pruning to axonal injury, Martin *et al.* found that regenerating axons in developing zebrafish avoided persistent fragments from induced axotomy [46]. The group speculated that slow clearance of degenerating axons may be detrimental to innervation.

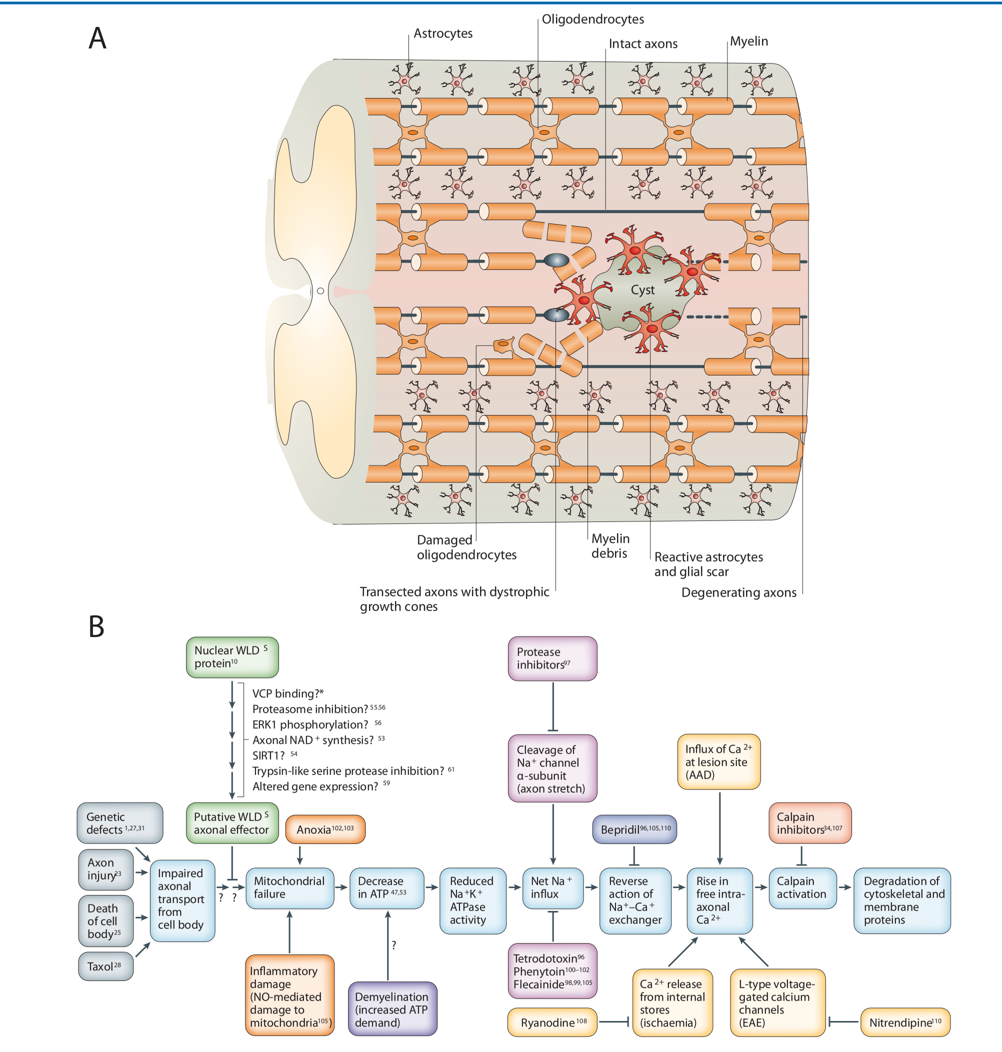


Figure 2.4: *Continues.*

Figure 2.4: *Continued.* (A) CNS injury response is extremely restrictive. Damage incurred in the spinal cord is followed by fragmentation of axons and myelin, with limited clearance. Reactive astrocytes form a glial scar, preventing the axons from resynapsis. (B) Axon degeneration pathways showing the scope and complexity of Wallerian degeneration. AAD, acute axon degeneration; EAE, experimental autoimmune encephalomyelitis; ERK-1, extracellular signal regulated kinase 1; NO, nitric oxide; SIRT 1, silent information regulator; VCP, valosin-containing protein; WLDS, slow Wallerian degeneration protein. Images reprinted from [57] and [33].

Mechanisms of Wallerian Degeneration

The complete molecular pathway for Wallerian degeneration is still unknown, despite the recent identification of several key players within the axon that are associated with the process (Fig. 2.4B). Axonal transport failure and microtubule dissociation are believed to be one of the earliest initiators of Wallerian degeneration [16, 33, 47]. Once the axon is lesioned, transport is limited or completely cut-off. A lag phase follows in which the distal axon is separated from the proximal portion, yet is still capable of conducting action potentials. Once the latent period is over, a catastrophic and active breakdown of the distal segment takes place via innate machinery within the axon. One culprit implicated in axonal degradation is the ubiquitin-proteasome system (UPS) [16, 33, 46, 47]. Ubiquitin acts to tag proteins for destruction via proteasomes, enzymes that denature proteins by peptide bond scission. This system is present in most cells and is important to many biological processes. Recent studies have shown that pharmacological and genetic inhibition of the UPS can significantly increase the lag time after axotomy, but only when administered before a lesion is made [46, 47]. The need for priming of UPS inhibition suggests its involvement in the early events of Wallerian degeneration. Additionally, a rise in intracellular calcium ion (Ca^{2+}) concentration is necessary for the progression of Wallerian degeneration and subsequent regeneration. Increased Ca^{2+} promotes cyclic adenosine monophosphate (cAMP) activity and neurofilament breakdown by calpain, a calcium dependent protease [47, 48]. Axon degeneration is often compared to apoptosis, or programmed cell death, because of similarities in the way each works on the cell including targeted ubiquitination [33–35]. Since axon degradation is performed by activation of similar cell proteins to apoptosis, studies have attempted to uncover similar pathways for the two processes. So far, research has revealed that the mechanisms of each are independent; moreover, when distal portions of injured axons were subject to nerve growth factor (NGF) deprivation, UPS inhibition resulted in delayed axon degeneration and only inhibition of apoptosis saved both the soma and axon of deprived neurons [47].

2.2.3 NEURODEGENERATIVE DISEASES

Wallerian-like degeneration has been observed in many neurodegenerative diseases, including Charcot-Marie-Tooth, MS, amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's, Huntington's, and prion diseases, such that axonal transport is disrupted without transection or crush

injury [16, 33–35, 49, 50]. The base cause of these diseases often occurs because of improper or missing axonal transport protein function, frequently implicating either microtubules or antero and retrograde motor proteins. Proteins and organelles become trapped in varicosities, or the minor axonal swellings, leading them to become spheroid formations (major swelling) [33]. Aberrant swelling and spheroid formation within the axon from loss of transport has been linked to initiation of Wallerian degeneration in disease models [33, 51, 52]. After spheroid formation, the axon degrades in a strikingly similar fashion to traditional Wallerian degeneration [51, 52]. Much of the focus of therapeutic Wallerian degeneration in neurodegenerative diseases has shifted to axonal survival (or sparing) rather than strictly neuronal survival and the prevention of apoptosis. Administration of Wld^S protein, or portions of it, could potentially act as a therapeutic agent to delay degeneration as shown in models of Parkinson's and motor neuron disease [16, 49, 53–55]. In the case of diseases, slowing degeneration may have significant benefits as opposed to injury where it could inhibit regeneration.

2.2.4 ROLE OF GLIA IN DEGENERATION AND REGENERATION OF CNS AXONS

Owing to different extracellular milieu, CNS neurons have a more difficult time regenerating than in the PNS. Schwann cells (SCs) are an important component of PNS regeneration, and act with macrophages to breakdown myelin and form new sheaths to guide the axon back to its target [56]. SC sheaths, termed bands of Büngner, are important for isolating the axon and growth cone from the damaged environment. Response to disease and injury in the CNS is quite different, since the guiding glial tubes that protect axons from surrounding environment are not present. Oligodendrocytes do not clear inhibitory myelin debris in the CNS, and astrocytes form a glial scar that permanently blocks passage of regenerating growth cones (Fig. 2.4A) [56, 57]. Microglia in the CNS are not nearly as efficient at clearing axon fragments as SCs and macrophages in the PNS, which can be detrimental to the nerve stump attempting to regenerate.

2.2.5 OLIGODENDROCYTES AND MYELIN ASSOCIATED INHIBITORS

Some membrane proteins, as well as fragmented and intact myelin from oligodendrocytes, are capable of inhibiting neurite outgrowth of CNS neurons. The Nogo family of membrane proteins is known to inhibit axon growth, and two specific inhibitory domains have been identified in Nogo-A that can both be detected on the extracellular surface of oligodendrocytes [58]. A recent study used gene silencing to knockdown Nogo receptors in transplanted neural stem cells (NSCs), which led to increased functional recovery in rats with TBI over cells transplanted without the receptor silencing [59]. The Nogo family of proteins exhibits complex interactions with axons and is discussed in further detail in Chapter 5 [14, 60]. Myelin-associated glycoprotein (MAG) has been shown to not only inhibit neurite outgrowth, but to initiate growth cone collapse [61]. Although MAG has so far proved detrimental to axon regeneration, it is known to encourage embryonic neurite outgrowth and has been implicated in maintaining and encouraging healthy, myelinated axons [62].

2.2.6 ASTROCYTE ACTIVATION AND GLIAL SCARRING IN THE CNS

Membrane proteins from oligodendrocytes and myelin are not the only source of inhibition at CNS injury sites. Microglia and astrocytes are recruited to damaged CNS areas, and while some astrocytes may support axons, injury stimulates a reactive phenotype characterized by cell hypertrophy. In response to injury, engorged astrocytes upregulate glial fibrillary acidic protein (GFAP) and vimentin expression in response to cytokines and growth factors released by microglia and other immune cells [63]. Vimentin and GFAP have both been shown to negatively affect axon regeneration [64]. Reactive astrocytes also upregulate CSPG expression, which inhibits regeneration at high concentrations. At the site of injury the concentration of CSPGs is very high but decreases as distance from the center of the insult increases [65]. The mechanism of inhibition from CSPGs is still not completely clear; some evidence suggests the GAG side-chains are to blame while other research points to the protein core. Treatment of injured sites with chondroitinase ABC (enzyme that cleaves GAGs from proteoglycans) decreases inhibition and could thus be used therapeutically to aid regeneration [57]. In the event of injury, the permeability of the blood-brain barrier and blood-spinal cord barrier is affected, resulting in infiltration of macrophages and cytokines that induce an inflammatory response [66–68]. Ultimately, astrocytes play a protective role in reestablishing these barriers and preventing the spread of injury; however, the effect is a highly inhibitory environment, caused by glial scar formation and release of neurodestructive molecules, resulting in failed neuron resynapsis and thus permanent loss of CNS function [69].

CHAPTER 3

Biomaterials for Scaffold Preparation

3.1 DEFINITION OF BIOMATERIAL AND REQUIREMENTS FOR NEURAL TE SCAFFOLDS

Most TE approaches (Fig. 1.1) begin with a biomaterial scaffold of natural or synthetic origin to provide structure for cells while preventing cavitation caused by massive tissue loss. Although the exact make-up of an ideal CNS TE construct is rarely agreed upon, some general requirements are widely accepted. A desirable scaffold is [70, 71]:

- biodegradable with the ability to release therapeutic agents if necessary
- mechanically similar to target host tissue
- easy to manufacture and process
- adhesive for cells or can easily be modified to be cell adhesive
- biocompatible or elicits an appropriate host response minimizing inflammatory and immune reactions

The ECM is extremely important for normal cell behavior and tissue function [72]. A common approach in TE scaffold design is to mimic the natural environment to facilitate full regeneration of damaged tissue. Often this includes using different materials to recreate the ECM. Surface interactions of biomaterials with both endogenous and exogenous tissue are extremely important due to the dependence of cell adhesion and migration to cellular functions. Figure 3.1 exhibits the different reactions of CNS host tissue to the same material with varied surface charge [73]. Chemical or physical surface modifications can be used as a strategy to remedy the unfavorable tissue interactions of some materials.

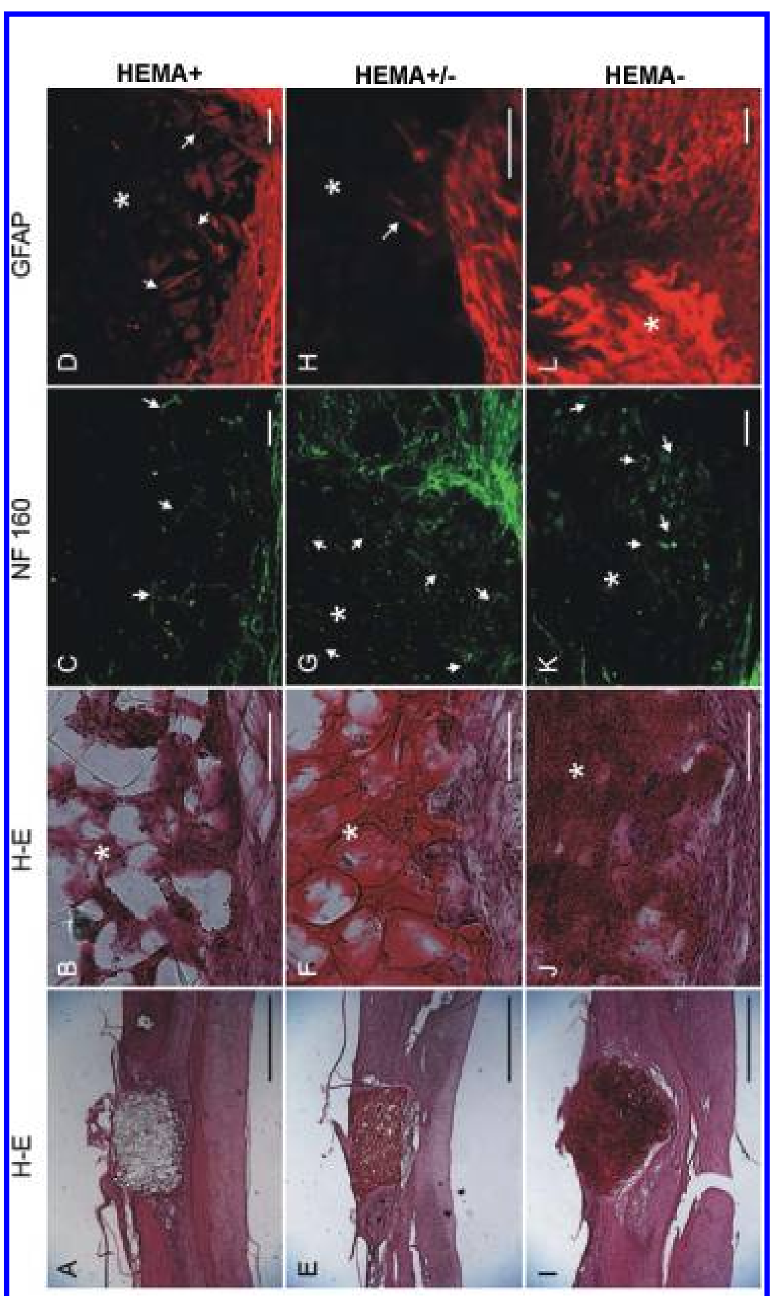


Figure 3.1: *Continues.*

Figure 3.1: *Continued.* Image of pHEMA gels implanted into spinal lesions, illustrating the importance of surface chemistry on tissue ingrowth. Gels with negatively, mixed, or positively charged surface were tested. Tissue penetration is shown in A, B, E, F, I, and J by hematoxylin-eosin stained sections. C, G, and K sections are stained for neurofilament and display the increased axon infiltration in the scaffold on the mixed charge surface. In D, H, and L sections, GFAP staining shows astrocytic activation of the positively charged pHEMA scaffold. Scale bar A, E, I = 1 mm; B, F, J = 50 μm; C, D, G, H, K, L = 100 μm. Image reprinted from [73].

An introduction to materials and common modifications will accompany specific material examples and subsequent chapters will carry these topics forward. Materials discussed here can be found in Table 3.1; in depth examination of these biomaterials and others can be found in review papers dedicated to biomaterials [70, 74–77].

3.1.1 BIODEGRADABLE SCAFFOLDS

Non-degradable biomaterials are used in neural TE; however, utilizing a material that can degrade over time and be completely replaced by natural tissue is typically the preferred approach. Most non-degradable materials offer control of synthesis and less complex design considerations, but at the cost of becoming a permanent fixture in the body [76]. Degradable scaffolds eradicate the necessity for surgical removal and allow for complete reinstallation of host function. In addition, when materials are designed to be degraded and eradicated from the body, they do not induce lasting immune or inflammatory responses. In the body, implanted materials can chemically degrade by enzymatic mechanisms or by hydrolysis. Cell interactions with the surrounding matrix are important for homeostasis, allowing for endogenous or exogenous cell migration throughout the area during regeneration [72]. The polymer on its own must be harmless to the body; in addition, its monomers should be nontoxic during the length of degradation time without eliciting any response preventing it from fulfilling its purpose. This often requires preventing significant alteration to the local cellular environment (*e.g.*, pH, osmolarity, microglial or astrocytic activation). For example, poly(lactic-co-glycolic acid) (PLGA) degrades into lactic acid and glycolic acid, which can decrease the local tissue pH, encouraging inflammatory responses [74]. Slowed degradation could circumvent pH changes, as long as degradation products are removed from the local environment quickly.

Most naturally derived biomaterials, such as proteins or polysaccharides, can be degraded by enzymes, and do not have toxic byproducts since specific elimination mechanisms exist in the body. In particular, proteolytic enzymes are integral to the processes of tissue remodeling and formation where migrating cells require active control of the ECM. Matrix metalloproteinases (MMPs) have been identified as important proteases in cell migration and ECM remodeling [78, 79]. The study of MMP proteolysis has lead to the identification of specific peptide sequences or substrates for each MMP [80–82]. These peptide substrates are short ($<$ 7 amino acids) and have been incorporated into polymeric crosslinkers in biomaterial scaffolds [78, 79, 83, 84]. The MMP-1, or collagenase, cleavable sequence Ala-Pro-Gly-↓-Leu (↓ for cleavage site) has been incorporated into

Table 3.1: Common biomaterials used in CNS tissue engineering.

Material	Chemical Formula	Source	Degradable	Form	References
Agarose		Red algae	No	Preformed gel, injectable gel, electrospun mesh	[97, 115-116, 498]
Alginate		Brown algae	Dissolution at neutral pH	Preformed gel, electrospun copolymer	[75-76, 118-120, 129]
Chitosan		Crustaceans	Enzymatic	Preformed gel, injectable gel, electrospun copolymer	[94, 121, 123, 129-130]
Collagen		Animals, humans	Enzymatic	Preformed gel, injectable gel, electrospun mesh	[74, 76, 106-107, 111-112]
HA		Animals, humans	Enzymatic	Preformed gel, electrospun mesh	[75-76, 91-92, 99, 109, 114]
Matrigel		Mouse sarcoma	Enzymatic	Preformed gel, injectable gel, electrospun mesh	[75-76, 133]
Methyl-cellulose	R=H or CH3	Plants	Enzymatic	Preformed gel, injectable gel	[71, 75, 91, 99]
PCL		Synthetic	Hydrolytic	Electrospun mesh	[105]
PEG		Synthetic	No	Preformed gel	[75-76, 134-135]
pHEMA		Synthetic	No	Preformed gel	[73, 136]
PLGA		Synthetic	Hydrolytic	Preformed gel Electrospun mesh, drug delivery	[91, 105, 135]
PPy		Synthetic	No	Film, particles Preformed ge	[40-45]
SAP		Synthetic	Enzymatic or hydrolytic	Injectable gel	[76, 137-138]

the backbone of photopolymerizable poly(ethylene glycol) (PEG) allowing MMP-1 mediated cell migration through the hydrogel scaffold [78, 79]. The MMP-2, or gelatinase, peptide substrate Pro-Val-Gly-↓-Leu-Ile-Gly (PVGLIG) has been used as a linker for dextran and methotrexate to enable the creation of MMP-2 activated drug delivery microparticles [84]. The PVGLIG peptide has also been included in larger self assembling peptides for the creation of MMP-2 sensitive self-assembled hydrogels. The gelatinases (MMP-2 and 9) are important in neural development and differentiation [85]. MMP-2 has been shown to be particularly important in postnatal development and in migration [86] and axon outgrowth [87]. In the native CNS environment, MMPs facilitate migration and differentiation; thus, CNS tailored biomaterial scaffolds should allow for material remodeling to promote differentiation, migration and cell process extension.

In contrast to enzymatic degradation, hydrolytic degradation occurs at hydrolytically labile bonds, such as esters, orthoesters, and anhydrides. Due to the patient-to-patient variation in autogenous enzymes, hydrolytic degradation of materials is more predictable over time and location, while holding standard throughout different populations of patients, as compared to enzymatic degradation [88, 89]. Polymers degrade via bulk or surface erosion mechanisms. Bulk deterioration of hydrolytically degraded polymers occurs over the whole volume due to faster water penetration as compared to hydrolytic cleavage of bonds at the surface [70]. Surface erosion of a polymer occurs when water does not penetrate easily and bonds are cleaved before water can reach the inner mass of the material. This type of degradation is characterized by mass loss at the surface that penetrates inward over time. Most often, surface erosion leads to a more linear release of any encapsulated agents included in the scaffold [70, 90]. The mechanism and rate of degradation are typically selected depending on the application. Enzymatically degradable materials are incorporated more often into TE constructs and hydrolytically cleavable materials are typically used to achieve stable release profiles in drug delivery applications. An example of a scaffold with both types of degradation is examined later, where an enzyme susceptible scaffold is impregnated with a nanoparticle delivery system with the potential to release therapeutic drugs or growth factors [91].

3.2 SCAFFOLD CREATION STRATEGIES

3.2.1 HYDROGELS

Hydrogels are a very popular choice for neural TE scaffolds due to their characteristic similarity to CNS tissue. Hydrogels are polymer networks typically composed of 1-5 wt% polymers that swell with water and can have a low mechanical stiffness, similar to native soft tissues [70, 71]. Mechanical, as well as surface, properties have been shown to be important for neural cell adhesion, migration and survival, as well as the differentiation of stem cells [92–95]. To control the degree of crosslinking, and indirectly the mechanical properties, the polymer can be chemically crosslinked, photopolymerized, or irradiated either in a dry state or in solution [70, 75]. Photoinitiation of crosslinking in gels is the preferred approach to fill lesioned cavities of irregular shape and size, especially in neural TE where reduced chemical linking is required for soft gel formation. Alternatively, preformed gels can use any crosslinking method and be shaped accordingly. Swelling behavior of a hydrogel

influences properties such as nutrient/waste diffusion, surface properties, optical properties, and mechanical properties [70]. Diffusion of nutrients and waste products throughout the scaffold is essential for cell maintenance within the scaffold and for encouraging the ingrowth of homologous cells and tissues when the construct has been incorporated into the body. One way to control swelling behavior is through environmentally sensitive hydrogels. The most common environmental cues are pH and temperature [70]. Researchers have utilized environmentally sensitive hydrogels that exhibit complexing in the presence of a stimulant to initiate physical crosslinking from inter or intrapolymer interactions. For this reason, thermogelling polymers have become popular because they can be injected, and crosslink into place at body temperature, obviating the need for invasive surgical implantation [91, 96–99]. Overall, hydrogels are the most common form of CNS scaffolds, and have been widely employed to fill nerve guidance tubes for PNS constructs [100, 101].

3.2.2 ELECTROSPUN FIBERS

Electrospinning has been adapted for a variety of regenerative medicine applications, including neural TE due to its ease of use, versatility in fiber thickness and composition, and characteristic similarity to ECM on the nanoscale [102, 103]. The relatively simple set-up for electrospinning is shown in Figure 3.2, which consists of a voltage generator, a spinneret, and a grounded collecting plate. The process requires that a voltage is applied to a liquid droplet at the tip of the spinneret causing a jet of fluid to travel to a collecting plate [102, 104]. As the voltage is applied to the droplet, it works against surface tension initiating the formation of a Taylor cone, where a stream of liquid is ejected from the droplet towards the collecting plate. A pump is typically used to keep the polymer solution at a constant flow rate to the end of the spinneret, and is the simplest parameter that can be adjusted to control fiber size. In addition to feeding rate, the collection gap, electric field strength, polymer, and solvent can be varied to adjust the fiber diameter. Electrospinning on a normal collecting plate yields a mesh of randomly aligned fibers; however, further modifications of the collecting apparatus can be made to create parallel fibers. Rotating mandrels and drums, oscillating frames, metal frames, and a pair of electrodes with an insulating gap have been used to acquire specifically aligned electrospun fibers (Fig. 3.2) [104]. Researchers have also demonstrated ways to tune electrospun fiber morphology and composition by altering the collecting method. One distinct method used is a collecting media, as opposed to the traditional collecting plate, which allows for porous fibers or for the incorporation of different therapeutic agents into distinct fiber regions [104]. Electrospinning offers a relatively easy and cheap technique to create highly tunable TE scaffolds. Specific examples of electrospinning for neural TE will be discussed later; however, it should be noted that hydrogels are still more common than electrospinning for the formation of CNS constructs and newer hybrid approaches of the two offer advantages of both techniques [105].

3.3 CURRENT BIOMATERIALS IN CNS TE

There are many different biomaterials that have been used in CNS regeneration, and those discussed here are not an exhaustive list. The following discussion should serve as an introduction that is

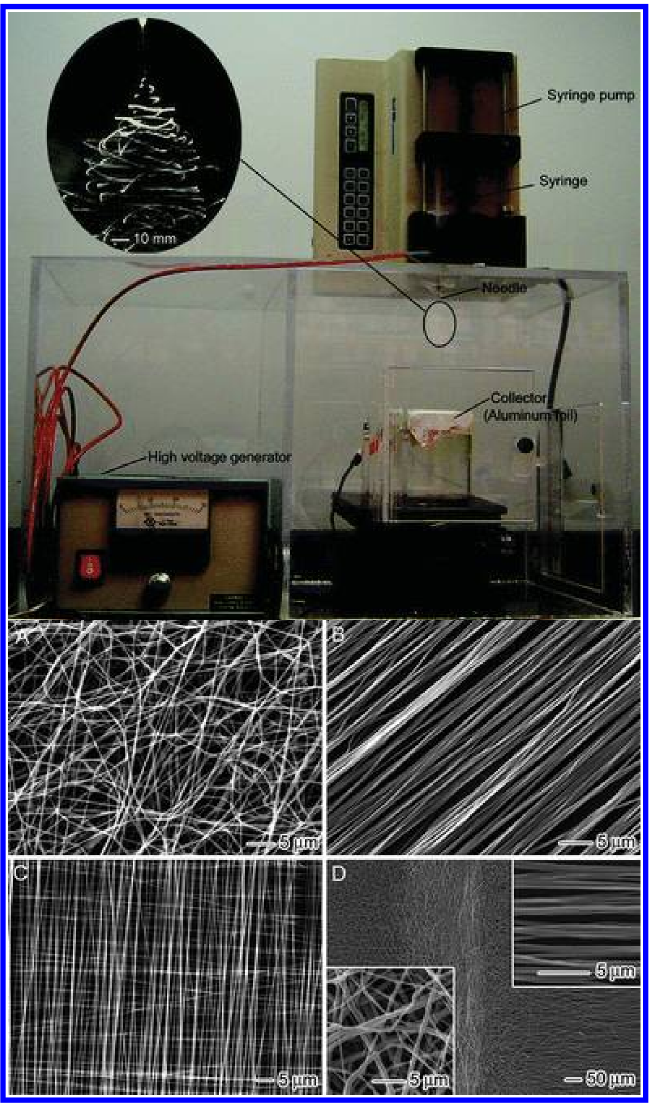


Figure 3.2: (Top) Electrospinning set-up displaying the syringe pump, syringe needle, voltage generator, and stationary collecting plate. Inset shows fiber of the electrospun polymer traveling through the electric field before it reaches the collector. (Bottom) Electrospun meshes can take on a variety of forms (all fibers made from PCL). (A) Random fiber meshes are easy to form with a stationary collecting plate. (B-C) Specifically aligned fibers can be made using rotating mandrels or spinning collectors, and layered scaffolds can be made by repeating the process. (D) Another method used to form aligned fibers involves two electrodes and an insulating gap between. Insets show random fibers on the electrodes (left inset) and parallel fibers formed between the electrodes (right inset). Image reprinted from [104].

designed to provide the reader with a basic understanding of common neural TE biomaterials, with specific examples of past applications and unique features of each material. Reiterated throughout the literature is the commonality that a successful TE construct requires a composite of many stimuli built into the scaffold. Many of the materials discussed here are typically used as copolymers, blends, or in conjunction with other molecules to encourage the appropriate tissue responses both *in vitro* and *in vivo*.

3.3.1 NATURAL MATERIALS

Many researchers have used the body's most abundant ECM component, collagen, to form constructs for neural TE. Collagen is composed of chains of amino acids linked by peptide bonds, or the covalent bond between the amino group of one amino acid and the carboxylic acid of another amino acid. These chains form secondary structures and tertiary structures; combinations of multiple tertiary structures form higher order structures and finally collagen fibrils [74]. Over 20 collagens have been identified; however, collagens I-IV are the most abundant in the body and only not all collagen types are found in the brain [5, 74]. Since collagen is native to the body, it is recognized by cells, promoting adhesion, however, with the potential for immune response. For this reason, allograft or xenograft collagens are especially avoided due to their immunogenicity and possibility of disease transfer [74, 106]. Enzymatic degradation of collagen is carried out by MMPs at specific cleavage sites. As previously noted, collagen I can be cleaved by MMP-1 to allow for matrix remodeling and cell migration. MMP-2 and -9, both gelatinases, can degrade collagen IV, a common basement membrane protein found in the CNS [85]. As an insoluble protein, collagen is most often dissolved in a cold, acidic solution and can be made into a hydrogel by adjusting the pH and temperature to physiological conditions [76]. In this way, collagen can be used as an injectable hydrogel for TE applications. In contrast to physically formed gels, chemical crosslinking of collagen (by aldehydes, carbodiimides, polyepoxides, hexamethylene-diisocyanate, etc.) is often used to control scaffold properties such as the mechanical properties, degradation time, and immune reactivity [74, 106]. Concentration and the degree of crosslinking can be used to adjust the stiffness of collagen gels, as discussed earlier; collagen can also be blended with other materials to increase mechanical compliance or stiffness, depending on the additive. As a native ECM component, collagen has integrin binding sites, and can be further enhanced for neuronal regeneration by incorporating growth factors such as neurotrophins. Neurotrophin-3 (NT-3) was recombinantly functionalized with a collagen binding domain (CBD), allowing it to complex to collagen I scaffolds; these scaffolds were subsequently implanted into the transected spinal cord of rats with positive functional results [107]. The collagen-NT-3 combination yielded significant locomotor improvement over collagen scaffolds with soluble NT-3. NGF was also functionalized with a CBD and used as an injectable scaffold for sciatic nerve regeneration (discussed later in Chapter 5) [108]. Gelatin, a hydrolyzed derivative of collagen, can also be used in neural TE to make gel scaffolds [109, 110]. Collagen can be processed into gels, meshes, and even a powdered form to suit a variety of needs. In addition, collagen can be electrospun

into a fibrous mesh and crosslinked for stability, leading to excellent neural outgrowth responses *in vitro* [111, 112].

Since it is a regular component of the ECM, HA (hyaluronic acid) is being studied as a biomaterial for the CNS [70, 75]. HA is very viscous due to its high molecular weight and can form soft gels via chemical crosslinkers, such as carbodiimide, or modified for photocrosslinking [76, 113]. Natural degradation of HA occurs in the body because of interaction with free radicals, MMPs, and hyaluronidases allowing for native remodeling of these scaffolds [74]. Increased spreading and attachment of hippocampal neurons on HA gels as compared to unmodified HA gels were achieved with immobilized Nogo receptor antibodies (antiNgR) [92, 114]. Further, HA-antiNgR gels were able to support NSC survival and differentiation [92]. HA on its own has poor mechanical properties; thus, it is often blended with polymers and stiffening agents to increase rigidity. HA has been used with collagen and methylcellulose to decrease their compressive elastic moduli closer to $\sim$1 kPa, which is similar to brain tissue [99, 113]. HA may be preformed or injected to form brain and spinal TE constructs.

Agarose is a thermo-setting, linear polysaccharide isolated from red algae, and is often incorporated in the food industry for its gelatinous properties. Agarose can be formed into a hydrogel by dissolution with heat and subsequent cooling until gelation; however, traditional agarose gels must reach a temperature below physiological body temperature to fully set and thus require a cooling system to gel *in situ* [97]. Different types and derivatives of agarose are now commercially available that can gel at many temperatures, including close to body temperature, to alleviate the need for complex cooling systems. The mechanical properties and porosity of agarose can be varied by adjusting its concentration in the initial solvent. Agarose has been shown to exhibit minimal inflammatory response; however, it is undesirably non-biodegradable and does not allow cellular adhesion [115]. Agarose hydrogels can be molded into a variety of shapes in order to treat many different sized and shaped lesions. A channeled agarose scaffold containing bone marrow stromal cells (bMSCs) and NT-3 induced spinal cord axons to penetrate into the channels with good linear alignment due to the construct's honeycomb geometry (Fig. 3.3) [116].

Alginate is an anionic linear polysaccharide extracted from the cell walls of brown algae, which gels in the presence of multivalent cations such a Ca^{2+} [76, 117]. Alginate can be formed into hydrogels, and has recently been used in cryogels [118]. To form a cryogel, the monomers are gelled at subzero temperatures, allowing ice crystals to form large, interconnected pores when the gel is later thawed. Alginate/agarose cryogels show excellent mechanical properties and cellular attachment; however, they were not tested with neuronal or glial cells [118]. Although it cannot be electrospun alone, alginate can be blended with other polymers to create fibrous structures. Alginate/poly(ethylene oxide) (PEO) meshes can support *in vitro* fibroblast attachment, which can be further enhanced by the immobilization of adhesive peptides [119, 120].

Chitosan is derived from chitin, a naturally abundant polysaccharide found in the shells of crabs and other shellfish. Considered biocompatible by most researchers, chitosan may illicit an inflammatory response through macrophage activation with low deacetylation; therefore, it is typically

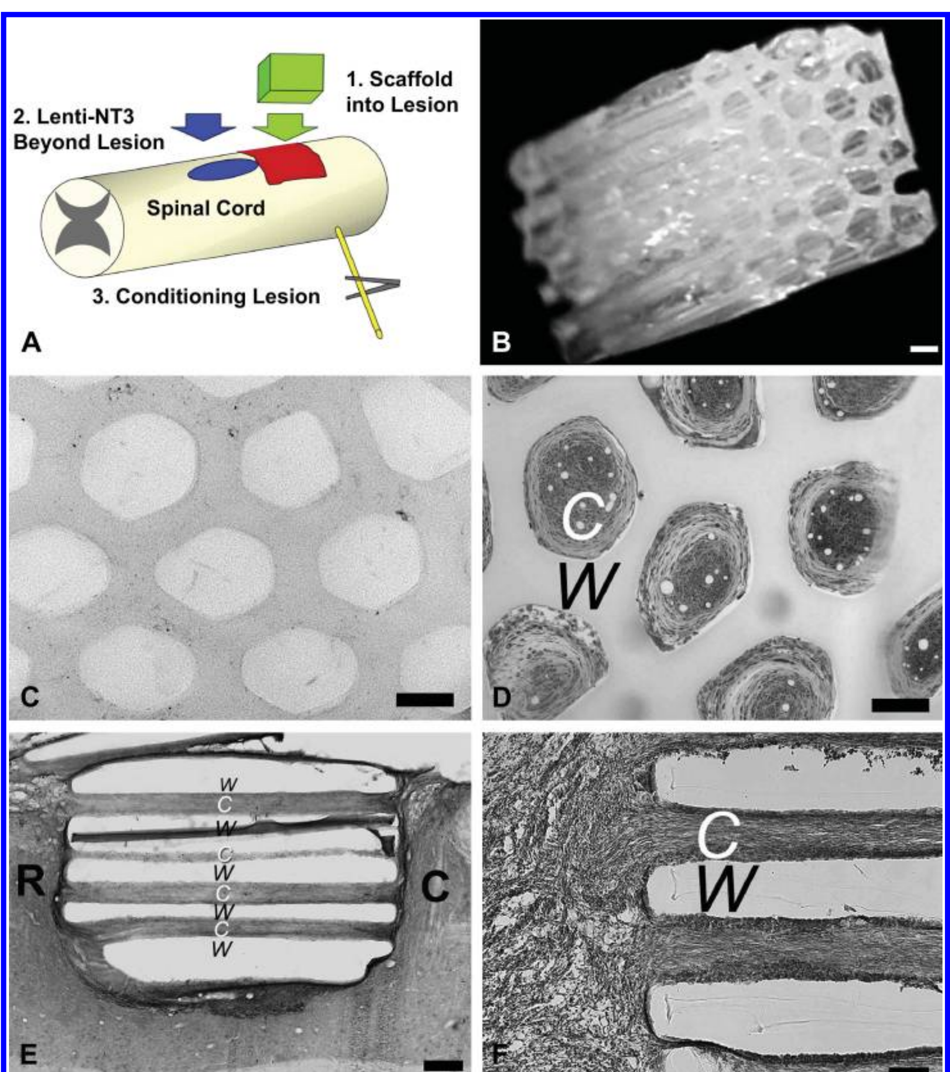


Figure 3.3: Templated agarose scaffolds. (A) Schematic of implantation of agarose scaffold (1) that could be accompanied by lentiviral NT-3 delivery (2), and/or conditioning lesions of the sciatic nerve (3). (B) Macroimage of agarose scaffold showing channels in a columnar configuration. (C) Cross-section of agarose scaffold where honeycomb like pores are seen. (D) Cell infiltration seen in the channels (white C) after four weeks of implantation. Scaffold walls (black W) are still intact. (E, F) Sagittal view of scaffold where tissue integrated into the length of the scaffold; however, there is a slight reactive cell barrier surrounding the implant. (black R, rostral; black C, caudal; white C, channel; black W, wall or scaffold). Scale bars B = 200 μm, C = 100 μm, D = 250 μm, E, F = 100 μm. Image reprinted from [116].

used at deacetylation percentages above 80% [121]. Chitosan is recognized to have antibacterial properties, which are a useful property for biomaterial scaffolds [122]. Chitosan can be covalently bonded or thermogelled in the presence of glycerophosphate salt or blending with a thermogelling polymer. Thermogelled chitosan was able to support comparable mouse cortical neuron survival and neurite outgrowth *in vitro* to cells grown on poly(D-lysine), a common adhesion enhancing agent [123]. Alternatively, a methacrylated form of chitosan has been synthesized that can be formed into hydrogels via exposure to UV light in the presence of a photoinitiator [124]. This methacrylated chitosan can support the survival and differentiation of cultured NSCs and allows for the easy tuning of substrate stiffness via photoinitiator concentration or exposure time [94, 124]. Chitosan can be formed into a cryogel, either alone or blended with other polysaccharides, similar to alginate and agarose [125–128]. In addition, chitosan blends have the capacity to be electrospun, and these scaffolds have been used in PNS TE [129, 130].

Methylcellulose (MC) is an inverse thermogelling polymer that is derived from cellulose (a polysaccharide found in plant cell walls), and has been shown to invoke a low inflammatory response *in vivo* [71, 75]. Due to its hydrophilicity, MC has low cellular adhesion unless modified, but shows biocompatibility *in vitro* and *in vivo* [131, 132]. An injectable hydrogel composite of HA and methylcellulose (HAMC) incorporating synthetic polymer nanoparticles showed very little microglial response when injected into the intrathecal space of rat spinal columns [91]. The HAMC combination allowed for fast gelling at physiological temperature [99]. Blank nanoparticles were included as a test for a drug delivery vehicle that could release therapeutic agents in future studies [91].

Matrigel™ (BD Bioscience) is a thermogelling mixture of ECM proteins isolated from Engelbreth-Hom-Swarm mouse sarcoma. The complex medley of proteins leads to intricate cell interactions and behaviors which have been shown to often be favorable for neural regeneration; however, due to its derivation from mouse tumor cells, it is unlikely that Matrigel™ can be used for human implantation [75, 76]. Similar to use of fetal serums, Matrigel™ is not necessarily applicable to translation to human studies, but successes using both suggest the need for multi-component systems with multiple cues. In addition to creating traditional scaffolds, Matrigel™ has been electrospun in an attempt to create constructs resembling basal lamina for enhanced SC growth [133].

3.3.2 SYNTHETIC MATERIALS

PEG is a hydrophilic polymer that is biocompatible and typically nonfouling and therefore often requires modifications to increase cellular adhesion [75, 76]. One way to adjust the surface interactions of PEG or its degradation characteristics is through polymer blends or composites. Bjugstad *et al.* recently performed a comprehensive assessment of the biocompatibility of PEG hydrogels in the brain. In the study, PEG/lactic acid (PEG/LA) gels were injected into rat cortexes, with the slow degrading (less LA) and non-degradable (no LA) PEG gels demonstrating the lowest microglial and astrocytic response [134]. The non-degradable and slower degrading gels showed less glial activation in a 50-200 μm region surrounding the implant than the sham. While LA is useful for making a PEG based scaffold degradable, it likely leads to observed glial activation by increasing the acidity

in the local environment. In other studies, photopolymerized LA/PEG hydrogels were utilized as a delivery system for the neurotrophic agent NT-3 [135]. When the scaffolds were implanted into hemisectioned rat spinal cords, improved movement and coordination, axon ingrowth, and host NT-3 concentrations were observed.

Poly(caprolactone) (PCL) is a polyester with high solubility, low toxicity, biodegradability, and the capacity to blend with other materials [70]. Ester bonds on the PCL backbone allow it to be hydrolytically degraded. Recently, PCL/PLGA electrospun hollow tubes were filled with self-assembling peptides and implanted into spinal contusion cyst sites, leading to endogenous cell ingrowth but little functional recovery [105]. Results from PCL/PLGA electrospun fiber testing in the CNS have been positive as well, showing the diversity of both polymers.

Methacrylate gels are widely used in biomedical engineering. In particular, poly(hydroxyethyl methacrylate) or pHEMA, which is well known for its use in soft contact lenses, has shown utility for CNS TE. Although pHEMA is non-biodegradable, it has many desirable qualities including its inertness and ability to be heat sterilized; pHEMA is easy to prepare and allows for the tuning of its mechanical properties with the adjustment of crosslinking density [70]. As mentioned in the introduction to Chapter 3, surface chemistry can be modified to adjust cellular interactions with pHEMA gels (Fig. 3.1). Mixed charge and positively charged gels demonstrated the best connective tissue penetration, but the mixed charge functionalized gels had a lower astrocytic response [73]. Yu *et al.* produced a co-polymer scaffold of pHEMA with 2-aminoethyl methacrylate (AEMA) to allow the attachment of laminin-derived peptide sequences to increase neuron adhesion to HEMA scaffolds [136]. Primary sensory neurons adhered and sent out longer processes on the peptide modified gels over unmodified gels; and cell areal cell density was eight times higher as assessed by fixation and immunostaining [136]. Both surface modifications of the methacrylate gels showed improved cellular response, and are common to many scaffold strategies used in CNS TE.

Self-assembling peptides (SAPs) offer an attractive injectable biomaterial scaffold for spinal cord and brain injuries since they form under physiological pH and temperature. Engineered peptides allow a defined linear chain of repeating units of amino acids to be produced synthetically and are easily processed [76, 137]. Scaffold mechanical properties and assembly are dependent on the characteristics of the peptides used. High hydrophobicity and longer amino acid sequences promote scaffold assembly with better mechanical properties [138]. One advantage of utilizing SAPs is that they can be functionalized to promote cellular activities such as migration and differentiation. In a recent study, the self-assembling 16 amino acid peptide RADA16-I was enhanced with a bone marrow homing motif to increase cell survival within the injured spinal cord of rats, and it was shown to significantly improve motor control and coordination after 7 wks of recovery (as evaluated by the BBB scale) [137]. In this study, RADA16-I was delivered by 3 injections into the lesion and gelled via salt induction [137, 138].

Since electrical activity is an important property of neural tissue, conductive polymers are also used in neural TE constructs. Conductive polymers have loosely held electrons in their backbones and use charge transfer from dopant molecules to enter a highly conductive state [139]. Poly(pyrrole)

(PPy) and poly(aniline) (PANI) are the most common conducting polymers and have been used in neural applications. Conducting films of ester functionalized PPy with conjugated NGF supported the culture of PC12 cells *in vitro*, displaying the biocompatibility of PPy as well as its ability to covalently attach growth factors while remaining conductive [140]. Biodegradable electrically conductive composites have been created by mixing PPy nanoparticles into hydrolytically cleavable scaffolds. PPy/polylactide composites are the most popular choice for scaffolds and have been tested with fibroblast and neuronal cell cultures [141–144].

Neural TE scaffolds exhibit strict demands on their bulk biomaterial properties as well as surface characteristics. The materials discussed here all possess strengths and flaws, which should be weighed against another depending on the specific application. In many studies, natural and synthetic materials can be blended or conjugated together for improved properties and to gain additional functionality. For CNS TE, it is important to remember the restrictive environment that can be created by microglial and astrocytic activation, and to make sure any material used in a TE scaffold does not illicit any undesirable response. Subsequent discussions in Chapter 5 will also provide insight into further surface modifications, physical and chemical, that can mask bulk material properties and further enhance cellular responses of the scaffold material.

CHAPTER 4

Cell Sources for CNS TE

When designing CNS tissue constructs, inclusion of encapsulated and surrounding cells are of great importance to achieve tissue regeneration (Fig. 1.1). Biomaterial scaffolds discussed previously are commonly impregnated with cells to expedite recovery by replacing lost tissue or by decreasing the migrational distance of natural cells in the surrounding area. In this chapter, a brief overview of commonly used and promising cell sources follows, and is meant to give the reader an introduction to each one. Table 4.1 presents common cell sources and their acronyms, and offers references for further reading.

Table 4.1: Common cell sources for CNS tissue engineering.

Cell Type	Abbreviation	Potency	Source	Reviews
Embryonic stem cells	ES cells	Pluri	Embryo	[175, 499-500]
Induced pluripotent stem cells	iPS cells	Pluri	Multiple: skin, embryo, SVZ	[201, 501-502]
Mesenchymal stem cells	MSC	Multi	Bone marrow, umbilical cord, adipose tissue	[251-252]
Skin-derived precursors	SKP	Multi	Skin	[263, 503]
Neural stem cells	NSC	Multi	Multiple: SVZ, DG, spinal cord	[221, 504-505]
NSC: Dentate gyrus cells	DG cells	Multi	Hippocampus, specifically the dentate gyrus	[221]
NSC: Spinal cord ependymal cells	Ependymal cells	Multi	Spinal cord	[23, 221, 506]
NSC: Subventricular zone stem cells	SVZ cells	Multi	Cortex, walls of the lateral ventricles	[221, 507-508]
Olfactory ensheathing cells	OEC	None	Nasal cavity	[509-510]
Schwann cells	SC	None	Peripheral nerves	[508, 511]

TE design criteria for cells are similar to those for biomaterials, in that any elicited response that impedes regeneration is unacceptable. For this reason, autologous cells are the gold standard for TE since they rarely evoke an undesired response (there are exceptions, discussed later). However, the price of using the patient's own cells is paid by donor site morbidity, as well as additional time spent on surgical isolation and culture. In addition, care must be taken to correctly choose cells for different injury and disease states. For demyelinating wounds and diseases (e.g., MS), reestablishment of the myelin sheath by glial cells is of utmost importance. In contrast, when neurons or both neurons and glia are lost, typically strategies shift focus to the neuron. Subsequent discussion of specific cell types will show that opinions differ on the optimal therapy in these cases. Some researchers attempt neuronal implantation while others incorporate glia or stem cells to encourage native neurons to regrow through the lesioned area. An overall strategy for cell incorporation or stimulation is not agreed upon, and is usually formulated for the specific application.

Somatic cells of the CNS are rarely used as a cell source for CNS TE. Issues of secondary injury sites, surgical accessibility, and poor mitotic ability restrict the use of primary cells. As an alternative, the use of the peripheral myelinating cells have become popular; this includes protective ensheathing cells within the nasal cavity. During homeostasis and development, glia serve as the supporting cells of the neuron. Most CNS TE strategies incorporating glia focus on maintaining unaffected surrounding tissue while remyelinating damaged tissue.

Stem cells offer an attractive alternative to somatic cells, and provide increased cell expansion and decreased donor site morbidity. Stem cells can be derived from a number of sources, and depending on their location and age, they possess different variations of two intrinsic stem cell characteristics: differentiation (or ability to form multiple cell types) and proliferation (or ability to self-renew). Since they can be readily expanded, less stem cells are needed initially to achieve high cell numbers compared to somatic cells. Totipotent stem cells have the ability to become any cell type of the body, as well as expand indefinitely; from here differentiation potential or plasticity decreases to pluripotent, to multipotent and finally to progenitor stem cells. Pluripotency is possessed by cells very early on in development, namely embryonic cells, but also has been induced in somatic cells by genetic alteration, as will be discussed later this chapter. Multipotent and progenitor stem cells persist throughout the body in stem cell niches into adulthood, and several populations of adult NSCs reside within the CNS.

4.1 PRIMARY CELL TREATMENT OF CNS INJURY

4.1.1 GLIAL CELLS

As mentioned previously, SCs (Schwann Cells) are the myelinating cells of the PNS. SCs wrap around the axon many times allowing multiple SCs to myelinate a single axon. In contrast to oligodendrocytes in the CNS, SCs resorb fragmented myelin and the distal axon from an injured site, recruit macrophages, and then work in synergy to clear debris and encourage axon resynapsis [145, 146]. SCs play an encouraging role in PNS regeneration and serve to augment axon synapse reformation. They do this by transforming to a non-myelinating phenotype, increasing secretion of axon

recruitment factors brain derived neurotrophic factor (BDNF, sometimes referred to as BDGF), ciliary neurotrophic factor (CNTF), and NGF, and forming a tube that guides the growth cone of the proximal axon to its destination [147, 148]. Though not typically found in the CNS, SCs are capable of myelinating CNS neurons; and endogenous SCs have been found to migrate into the injured spinal cord and myelinate CNS axons [149–151]. The mechanism of SC migration into the CNS is still unknown, as is the extent of their participation in CNS nerve regeneration, though it is known that their contribution is minimal [152, 153]. Encouragement of SC migration into the injured spinal cord has not been well researched; rather, the majority of studies to date have injected or transplanted SCs into the injured area in an attempt to encourage regeneration. SC myelination of axons is limited to regions with low astrocyte numbers. *In vivo* and *in vitro,* astrocytes and SCs are known to inhabit mutually exclusive areas. Early studies by Blakemore *et al.* created areas of demyelination in the cortex or spinal cord with ethidium bromide, which resulted in low or compromised astrocyte populations [154, 155]. SCs were able to myelinate endogenous axons in the areas with decreased astrocytic inhabitance. Modifications to SCs using exogenous proteins or transcription factors increase SC interactions with astrocytes and improve their migration and integration into the CNS. Recent work has focused on altering SC neural cell adhesion molecules (NCAMs) by inducing SC expression of polysialic acid (PSA) through viral delivery of sialyltransferase X (STX) [156, 157]. PSA associates with the fourth domain (extracellular portion) of NCAM and decreases adhesiveness so that the cells can more easily separate. Expression of PSA-NCAM is found on oligodendrocyte precursors during development and regeneration, and has been found to increase SC migration into astrocyte territory without adversely affecting their myelination capabilities [156]. Adult primate SCs were transplanted near experimentally demyelinated areas of the spinal column and demonstrated faster and more efficient migration when transfected with STX viral vectors [157]. In this study, remyelination was enhanced in transfected SCs; however, no functional analyses were conducted. One significant advantage of SCs is their ease of isolation and expansion capabilities. Obtaining SCs by biopsy, expanding them in culture with mitogenic agents, and purifying them from fibroblasts provides a source for autologous cells [158–160]. Glial growth factor (GGF) and the cAMP activator forskolin are two popular mitogenic agents, but as with any mitotic factor, their use must be closely monitored and inhibited before implantation to avoid tumorigenesis [158, 159, 161, 162].

Olfactory ensheathing cells (OECs) are radial glia of the olfactory bulb and nasal mucosa, located in the upper region of the nasal cavity. OECs are similar to SCs in their CNS regenerative ability, with the exception that they are less inhibited by the presence of astrocytes [163, 164]. OECs have been used in sensory and motor tract regeneration studies, but results are mixed and it is unclear whether they enhance tract regeneration or simply increase axon sparing [164, 165]. The corticospinal tract is the group of motor fibers in the brainstem connecting the cortex with the spinal cord, which are responsible for transmitting signals required for skilled movements. In the corticospinal tract, transplanted OECs have facilitated positive recovery of forepaw motor skills in animal models [166, 167]. The motor tract responsible for large movements, i.e., the rubrospinal

tract, has shown limited regenerative success using OECs, even with the addition of growth factors such as BDNF or glial cell-derived neurotrophic factor (GDNF) [168, 169]. Age of OECs is an important factor to consider for remyelination and functional recovery. OECs isolated from juvenile rats were able to migrate to post-transplantation lesions created with ethidium-bromide, which is not observed with the use of adult OECs [170–172]. Age is not the only factor affecting the efficacy of OECs. Source, location, and delay time between injury and therapeutic administration often play a significant role in effectiveness [165, 170–172].

In an attempt to utilize positive attributes of both cell types, OECs have been implanted in combination with SCs. OECs have been shown to enhance SC myelination capabilities as well as decrease astrocytic segregation of SCs [173]. Axon growth into SC bridges containing chondroitinase ABC enhanced by OECs at the interface between construct and endogenous cells [174]. This three-part combination was used in adult rat spinal cord lesions by Fouad *et al.* in 2005 and resulted in increased myelinated axons within the construct as well as serotonergic fibers re-entering the caudal spinal cord from the graft [174]. Increased myelinated axons correlated to increased functional recovery, as assessed by BBB scoring.

4.2 PLURIPOTENT STEM CELLS

4.2.1 EMBRYONIC STEM CELLS

Pluripotent stem cells, namely embryonic stem (ES) cells, have the ability to become neurons or glia for use in CNS TE when provided the correct cues. ES cells have also been proposed as an abundant source for NSCs, not only for use in cell based therapies but for screening assays and disease progression studies. It has even been argued that NSCs derived from ES cells retain better differentiation capabilities or 'developmental competence' [175].

One attractive property of ES cells for neural TE, other than their excellent proliferative capabilities, is their predictable behavior in response to developmental cues. Embryonic cells are derived from the inner cell mass of the blastula, and neuronal cell induction begins once the blastula enters gastrulation. Development of the neural system begins with induction of neural precursors which are differentiated to neurons and glia, proceeded by axon journey and preliminary synapse formation to their targets. Neural functionality is made possible by final remodeling of the neural network. Neural cells arise from the ectoderm, or the outermost layer of the gastrula, on the dorsal side where the inhibitory bone morphogenetic proteins (BMPs) are suppressed by the organizer molecules noggin, chordin, follistatin, cerberus and Xenopus nodal-related 3 (XNr3) protein (Fig. 4.1) [4, 176]. After the inhibition of BMPs, ectodermal cells are permitted to become neural precursor cells and are directed further by neural promoting molecules. Once the neural tube is formed in the embryo, spatial patterning occurs to direct cell fate. Caudal formation is led by Wnt-8, basic fibroblast growth factor (bFGF), and retinoic acid (RA) [177, 178]. The spinal cord is patterned ventrally by sonic hedgehog (Shh) protein and RA, and dorsally mainly by BMPs [179, 180]. The PNS is derived from neural crest cells that migrate from the neural tube; neural crest cells may become PNS neurons and glia, smooth muscle cells, or pigment cells [181–183]. The cell type of neural crest cells is determined

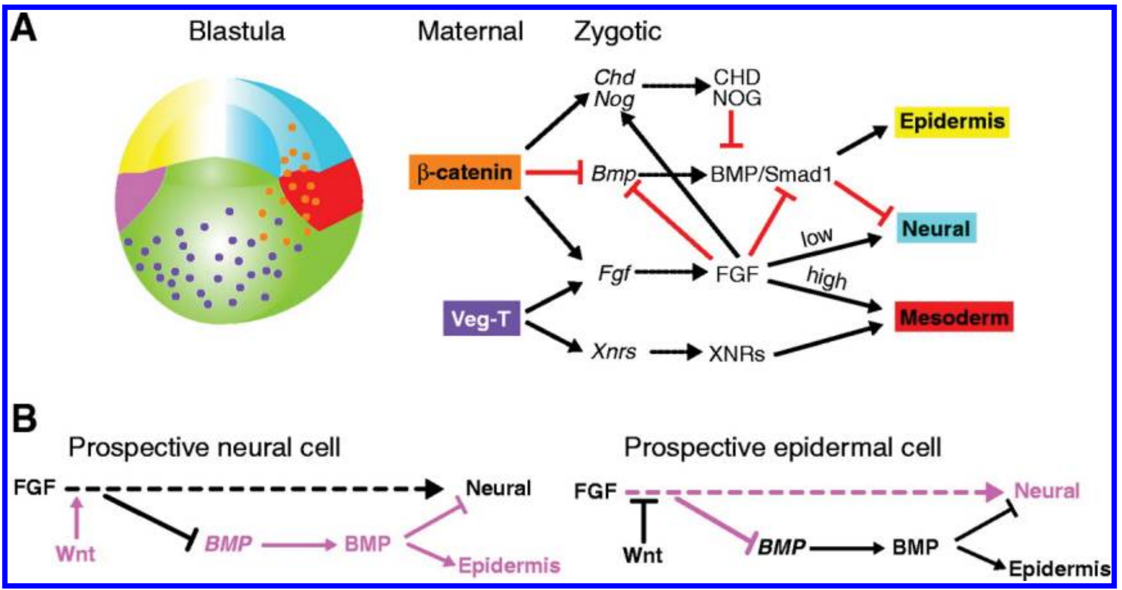


Figure 4.1: Diagram of neuronal development from the blastula in Xenopus (A) and chick (B), illustrating the roles of BMP, FGF and Wnt. For both species, FGF promotes neuronal induction by blocking BMP activity. Chd, chordin; Nog, noggin; NXR, nodal-related factors. Image reprinted from [494].

by migration route and environmental cues encountered. Events directing development have been well studied and are often mimicked by applying certain factors in a sequential manner to ES cells in order to derive NSCs and even specific somatic cell types [184–186].

Defined differentiation protocols eliminating the use of serum are in development to improve the clinical relevance of ES cells. In many cases, neural differentiation is initiated by culturing ES cells on feeder cells (typically embryonic mouse fibroblasts), followed by culture as suspended embryoid bodies with RA (Fig. 4.2). This culture regime has been found to promote a neuronal phenotype and suppress mesodermal cell types [187, 188]. Embryoid bodies are subsequently dissociated and plated on laminin coated surfaces with N2, a serum free neuronal supplement, in media for the neuronal phenotype [188]. Motor neuron specification has been achieved by activating the Shh pathway subsequent to RA; RA is necessary for early neural induction but should be followed by other factors if further differentiation is desired [184–186]. This differentiation regime mimics development closely, as ES cells are given caudalization signaling from RA and then sequential ventralization signaling from Shh. Testing of *in vitro* differentiated ES cells by most groups involves immunostaining or electrophysiology testing; however, Wichterle *et al.* alternatively implanted differentiated motor neurons into developing chick embryos to test functionality [186]. This group observed that ES derived cells localized into the correct area of the spinal cord and projected axons to the appropriate peripheral regions. Directing ES cell differentiation has not been limited to development-linked

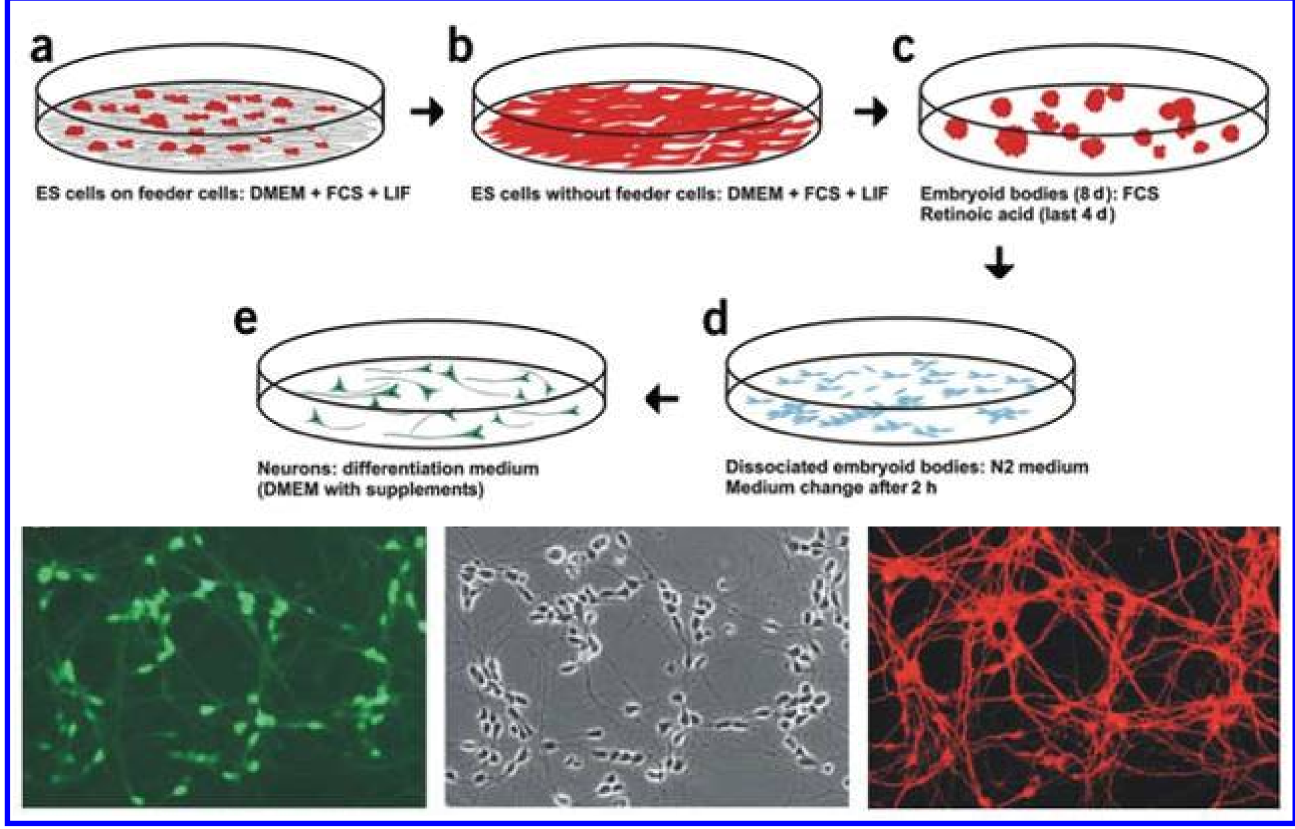


Figure 4.2: (Top) Schematic representation of neuronal differentiation of ES cells accompanied by actual images of the differentiated cells (Bottom). Left image shows endogenous GFP expression, center image shows the same field in phase contrast, and the right image shows βIII tubulin expression for the region. Image reprinted from [188].

factors and molecules. A recent study found that soluble amyloid precursor protein (APP) not only induced neural progenitor differentiation but also led to ES cell expression of βIII tubulin, a common neuronal marker, in only 5 d [189]. APP, a protein thought to be a synapse regulator, is more well known for its transformation into amyloid plaques in Alzheimer's disease [51, 52].

Since success has been achieved in directing ES cell fate into neural cell types, focus has shifted on incorporating ES cells or ES-derived cells into functional neural TE constructs. In an attempt to enhance serotonergic fibers two weeks after a hemisectioned SCI, embryonic neural precursor cells (NPCs) from the neural tube were injected caudal to the injury with generally positive results [190]. Even though the NPCs showed superior cell morphology and axon extension in tissue sections, functional improvement of coordination and maneuverability were comparable to injected mesenchymal stem cells (MSCs) and both showed significant improvement over control mice. Similarly, successful treatment of a simulated crush injury demonstrated the regenerative efficacy of human NPCs transfected to express Neurogenin 2 (Ngn2), a proneural factor that inhibits astrocytic differentiation, injected a week post-injury in adult rats [191]. NPCs grafted without Ngn2 did not result in any functional improvement; however, NPCs that expressed Ngn2 resulted in significantly higher scores in functional motor skill tests. This study by Perrin *et al.* showed the utility of human embryonic NPCs in a more realistic model where induced cells were administered a week after injury. Outside of the research lab, injury to CNS tissue is not treated immediately following insult due to delays in diagnosis, treatment availability, not to mention many other obstacles. The ability of human cells to perform well in animal models coupled with a more realistic delay of treatment makes this work very exciting for future CNS regeneration treatments.

4.2.2 INDUCED STEM CELLS

The ethical considerations surrounding ES cells and the associated controversy have dampened the enthusiasm for incorporating them as a cell source into TE strategies. In recent years, an alternative source of pluripotent stem cells is under active development. In 2006, Shinya Yamanaka's group demonstrated the creation of pluripotent stem cells from adult fibroblasts using gene therapy to introduce Oct3/4, Sox2, c-Myc, and Klf4 expression [192]. Yamanaka's group termed these cells, 'induced pluripotent stem (iPS) cells', and observed multiple lineage formation after injection into adult nude mice or blastocysts. Development of two factor iPS production (only Oct4 and Klf4) from mouse NSCs may offer better clinical relevance since it eliminates the c-Myc gene that is a well know oncogene implicated in tumorigenicity [193]. iPS cells behave similarly to ES cells and can be induced to form neurons in a similar manner (Fig. 4.3).

Despite the attractiveness of using the patient's own cells and avoiding embryonic cell use, iPS cells still have many problems. Transfection of genes has inherent difficulties. Viruses are typically used to delivery genetic material to generate iPS cells since they have natural mechanisms that can penetrate the cell wall and release nucleotides into the tight security of the cell nucleus. The downside is that viral gene delivery by retroviral and even lentiviral vectors causes random insertion into the host genome and could lead to unwanted mutations [194]. Adenovirus vectors, as well as non-viral

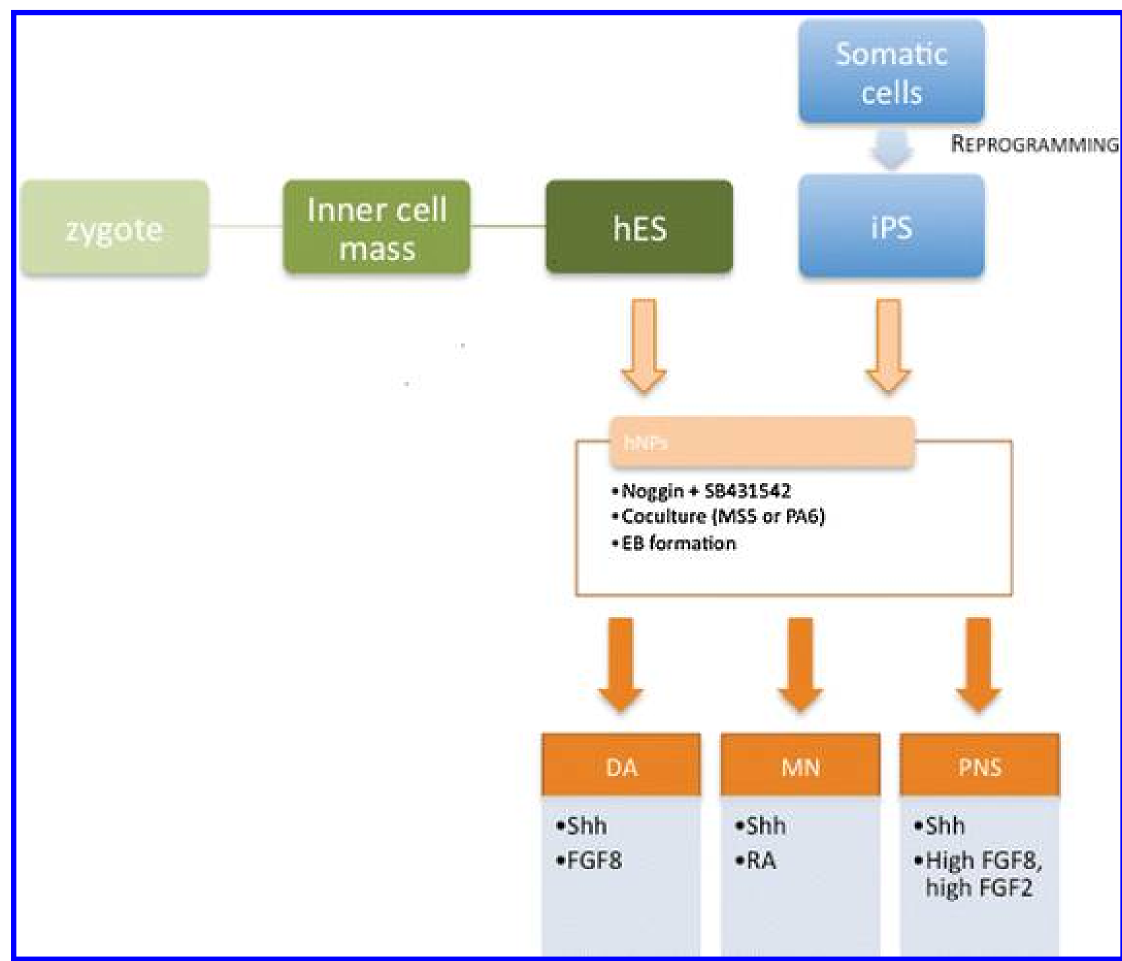


Figure 4.3: Diagram of differentiation paths for ES and iPS cells into specific populations of neurons. hESCs, human embryonic stem cells; hips, human induced pluripotent stem cells; hNPs, human neural precursor cells; DA, dopaminergic neurons; MN motor neurons; PNS, peripheral nervous system derivatives; EB, embryoid body. Image reprinted from [201].

methods, do not carry the same threat of random genome insertion but are often less efficient [194]. Alternative methods to viral gene delivery have been developed that are more effective, involving nanoparticle delivery or the use of commercial transfection kits [195–197]. Electroporation using commercially available systems allows increased permeability of the cell and nuclear membrane and has been used to deliver a single plasmid containing all four genes for induction of iPS cells [195]. Other commonly used methods include complexation with a cationic polymer or lipid to stabi-

lize DNA and cross the cell membrane [198]. In depth discussion of other nonviral systems and subsequent uptake into the cell and nucleus can be found in other reviews [199, 200].

Although iPS cells are still far from clinical studies in CNS TE, they are being actively developed for diagnostic uses. It is proposed that patient specific cells could be used to screen drugs and therapeutic treatments, or conversely to study genetic and contracted diseases using patient iPS derived neurons or glia [201]. Disease specific iPS cells are currently being used to investigate neurodegenerative disorders including ALS, Parkinson's, and Huntington's disease, among others [202–204]. Continued work with iPS cells may prove them valuable in CNS TE approaches in the future.

4.3 ADULT STEM CELLS

4.3.1 ENDOGENOUS STEM CELLS IN THE BRAIN AND SPINAL CORD

Stem cells niches throughout the postnatal body retain populations of multipotent cells into adulthood (Fig. 4.4). In contrast to ES or iPS cells, NSCs within the brain and spinal cord closely interact with their surrounding cells and are committed to the neural lineage. Adult NSCs may also offer a more popularly acceptable source of stem cells because they lack the stigma that surrounds embryonic research and possess decreased tumorigenicity. Defined populations of multipotent adult NSCs have been identified in the subventricular zone (SVZ) (Fig. 4.4B) of the lateral ventricles, the dentate gyrus (DG) of the hippocampus, and around the central canal of the spinal cord (Table 4.1) [205].

SVZ and DG cells can differentiate into neurons, oligodendrocytes, or astrocytes [206, 207]. *In vitro*, these two stem cell populations behave very similarly. Both areas in the brain produce neurons under non-pathological conditions. Physiological neurogenesis in the DG takes place in the innermost region of the subgranular zone of the hippocampus, quickly becoming neurons with mature phenotype that incorporate into the granule layer [208–211]. Cells from the SVZ naturally migrate along a track lined by astrocytes to the olfactory bulb where they differentiate into granule cells [212–215]. The rostral migratory pathway is specific for these migratory cells, and transplantation of other neurons into the SVZ does not result in migration to the olfactory bulb [212]. On the contrary, NSCs from the DG of the adult hippocampus can be transplanted into the SVZ migration pathway and will differentiate into neurons typical of the olfactory bulb [216]. Adult NSCs are of interest for endogenous activation or exogenous transplantation for TBI or brain diseases owing to their multipotency and natural brain origin. Their implantation has been shown to decrease recovery time in SCI and enhance tissue bridging when used in conjunction with a nerve guidance conduit (NGC) (Fig. 4.5) [217].

Due to the delicacy of brain surgery and implantation, endogenous activation of the SVZ or DG populations is a motivating interest for many researchers. Multiple injury models have been used to stimulate cell activity in the NSC niches of the brain. In a study by Rice *et al.*, TBI was induced in adult rats and proliferating cells were labeled and counted in tissue sections, which revealed increased proliferation and migration of cells from both the SVZ and DG post injury [218]. Interestingly, results from this study showed significantly timed 'waves' of increased mitotic activity

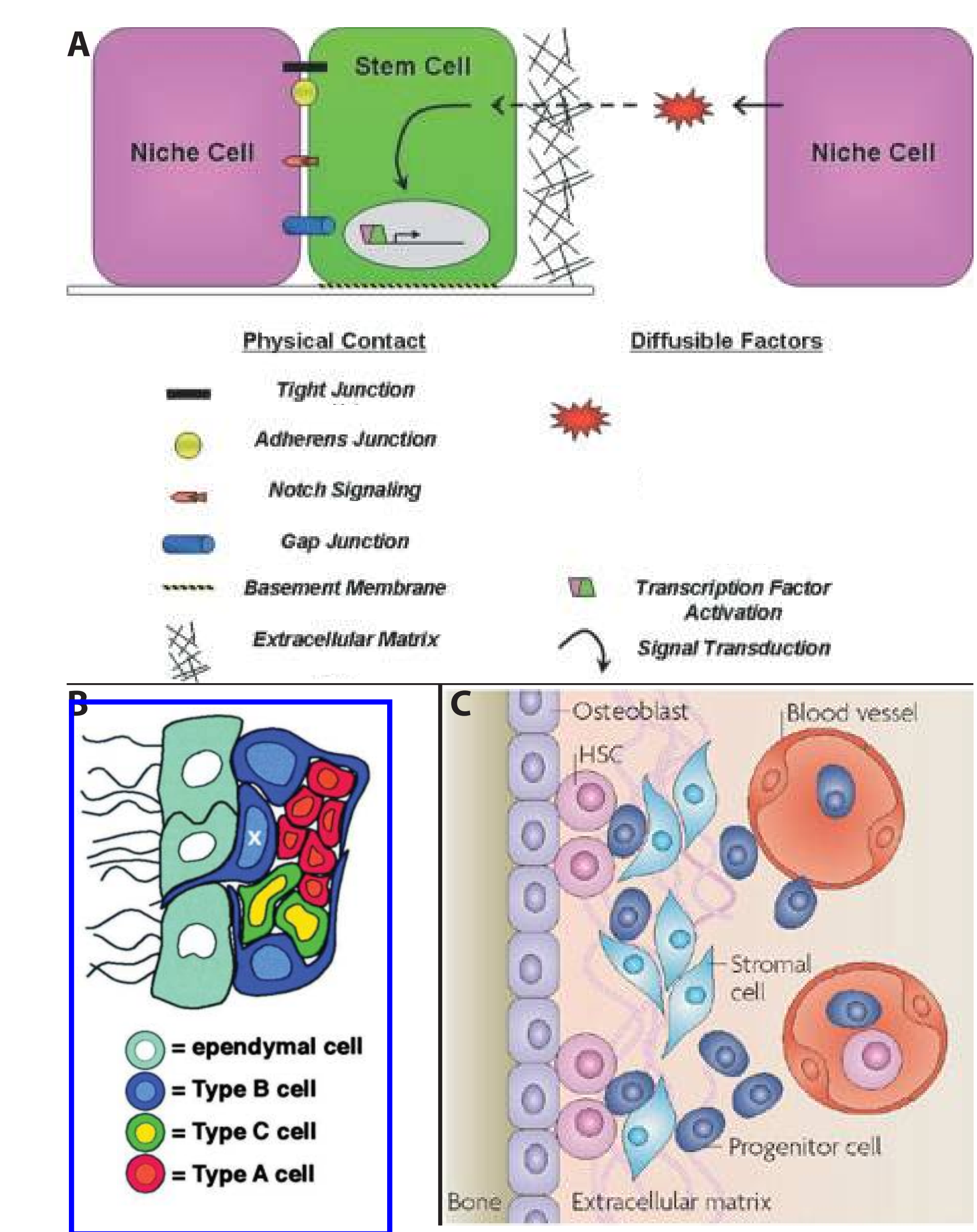


Figure 4.4: (A) General stem cell niche illustrating the multitude of cues that the niche environment encompasses. (B) SVZ niche containing astrocytes (type B cells) in close proximity with neuroblasts (type A cells). (C) Bone marrow niche. Images reprinted from [495–497].

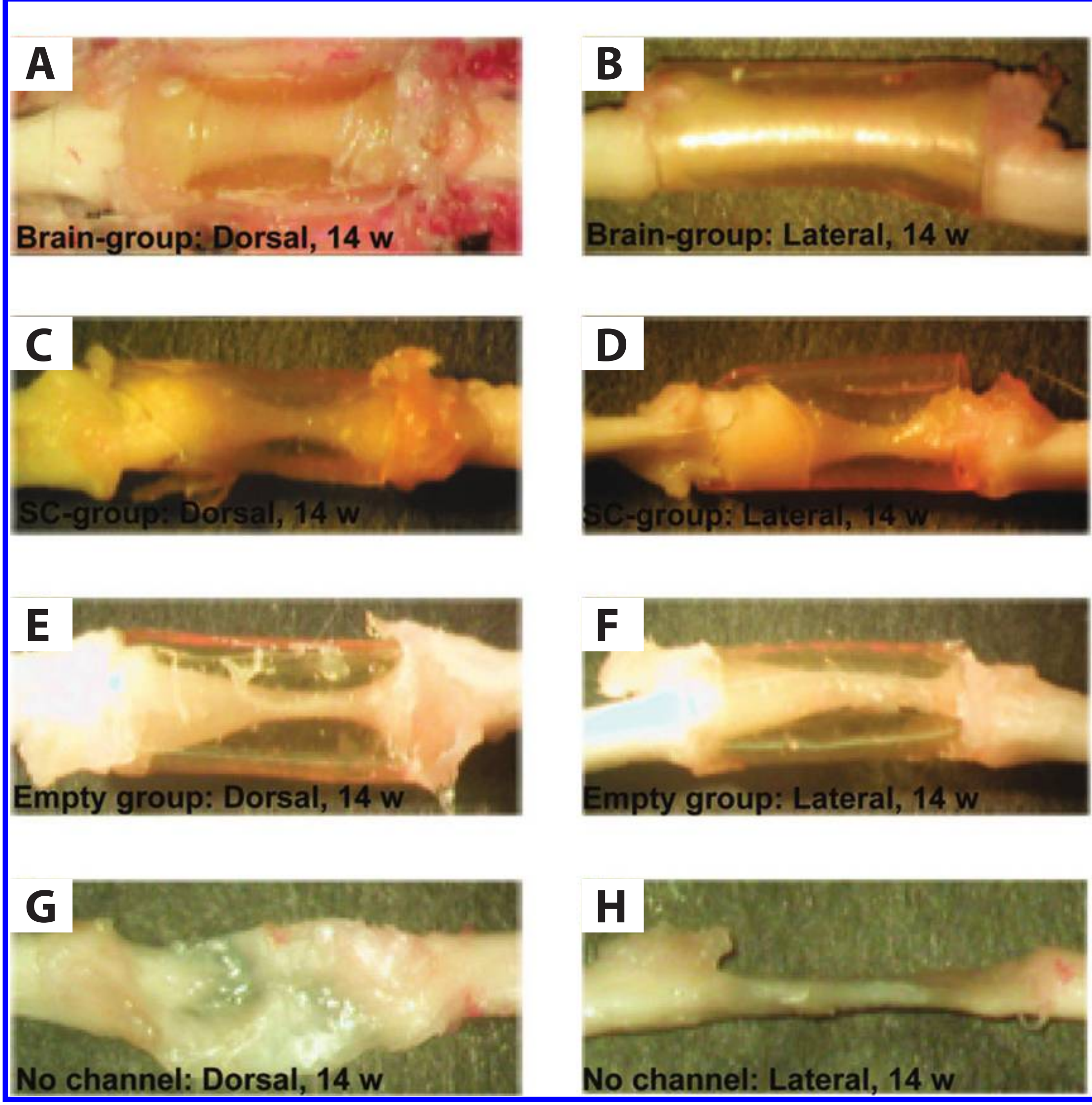


Figure 4.5: Spinal cord full transection injuries were treated with NSCs from the SVZ (Brain-group) or the spinal cord (SC-group) coated inside chitosan nerve guidance conduits. Analysis at 14 d showed improved tissue bridging in chitosan tubes containing NSCs from the brain (A, B) and the spinal cord (C, D) over acellular chitosan tubes (E, F) and the control (G, H). Image reprinted from [217].

from the DG. Similarly, after traumatic axonal injury, proliferation and migration of SVZ and DG cells, as well as an increased gliogenesis, persisted even eight weeks post-injury [219]. After inducing demyelinated lesions in the mouse brain, SVZ cells were observed to expand and migrate towards the lesion [220]. Observations show that NSCs in the adult brain respond differently to different injury models, presenting astrocytic preference in response to TBI and oligodendrocyte preference after demyelination [221]. Alternatively to injury-induced activation, injections of different neurotrophic agents have been studied for stimulation of growth, migration, and differentiation of NSCs in the brain. System perfusion of insulin-like growth factor I (IGF-I) in rats produced marked proliferation and neuronal differentiation in the DG without any increase in astrocyte production [222]. In the rat SVZ and olfactory bulb, proliferation and neuronal differentiation was induced by viral delivery of BDNF [223]. New neurons persisted 5-8 wks after the adenoviral injection into the lateral ventricles. It is clear that NSCs in the brain will respond to injury and a local or systemic delivery of neurotrophic agents. A properly designed CNS TE system has the potential to augment the stimulation of large populations of new neurons and glia from the SVZ and DG.

In vitro, SVZ derived adult NSCs can be induced to differentiate into neurons and glia by a variety of factors including IGF-I, NGF, BDNF, angiopoietin-1, cAMP, BMP-2, platelet derived growth factor (PDGF-AA), and the cytokine interferon gamma (IFN-γ), among others [224–228]. IGF-I has similar action *in vitro* to *in vivo*, and is mitogenic and neurogenic on NSCs [229, 230]. The combinatorial administration of IFN-γ in combination with an analog of cAMP to SVZ NSCs leads to a significantly higher population of neurons than soluble administration of neurotrophins, NGF, and BDNF, or tumor necrosis factor alpha (TNF-α) [227, 228, 231]. Even though biochemical treatments have been found to induce neuronal and glial differentiation for transplantation into the injured CNS, the question still remains whether these cells will integrate into the existing network of tissue, resulting in functional recovery.

Ependymal cells located near the central canal of the spinal cord have been identified that possess high proliferation rates and multipotency under specific conditions [22, 23, 205, 232–235]. Similar to NSC responses in the brain, injury of the spinal cord results in a protective response of the spinal cord ependymal cells [232, 233, 236]. Additionally, introduction of EGF and bFGF into the spinal column leads to increased proliferation of ependymal cells [237]. These results are expected, since EGF and bFGF are mitogenic agents, well known for their use in the *in vitro* expansion of NSCs. Ependymal NSCs were less effective than SVZ NSCs as a direct cell treatment for SCI in rats (Fig. 4.5) [217].

Obtaining adult NSCs from the brain and spinal cord is difficult and risky to the patient, which is the biggest drawback of these cells. However, isolating these cells has been shown possible and further advances in surgical techniques will increase their possibility for clinical use [238]. Another drawback of adult NSCs, in contrast to pluripotent cells such as iPS and ES cells, is that these cells show decreased proliferation with age [239]. Research with NSCs is still extremely useful as it could be applied directly to NSCs derived from ES or iPS cells. TE strategies that target endogenous activation of stem cell populations in the brain and spinal cord would be useful cues to include in

CNS constructs. In this respect, incorporation of these signals into a degradable scaffold could allow for a long release time to facilitate long-term regeneration by native tissue stimulation.

4.3.2 MESENCHYMAL STEM CELLS

MSCs have also been utilized for the purpose of CNS therapies. These multipotent stem cells are derived from a number of locations in the adult body and have the capability to differentiate into many different types of tissues with varying efficiency [240]. One of the main sources of MSCs is the bone marrow (Fig. 4.4C); these cells can be differentiated into smooth muscle cells, osteoblasts, chondrocytes, cardiomyocytes, liver cells, SCs, and to some degree, neurons [241–246]. In addition, MSCs isolated from the umbilical cord have similar characteristics and can express neuronal phenotypes following neural induction [247–249].

MSCs are able to promote regeneration in neural TE; however, experts are unsure of the therapeutic mechanism by which they accomplish this. MSCs may transdifferentiate into neurons and glia to augment tissue regeneration; alternately, inflammatory and immunological agents may be recruited to the damaged site by way of MSCs [250–252]. A secondary protection mode of MSCs is their secretion of neuroprotective factors that provide neural sparing and encourage endogenous axon regeneration. Crigler *et al.* studied MSCs for gene coding and expression of neurotrophic factors and found cultured MSCs could express BDNF and NGF [253]. Further testing showed that neuronal blastoma cells as well as explanted DRG both co-cultured with MSCs demonstrated significantly increased neurogenesis and neurite outgrowth. In these studies, inhibition of BDNF activity resulted in small decreases in outgrowth and proliferation, but the effects of MSCs were not completely negated, suggesting that other neurogenic factors are also secreted by MSCs.

Transplantation of undifferentiated MSCs into SCIs has shown positive therapeutic effects. Adult bone marrow MSCs transplanted two days after a spinal contusion injury showed neuroprotective effects that led to increased myelination at the injury site over the control group [254]. Histological analysis revealed increased laminin expression with MSCs along with neurite extension and alignment with spinal cord direction, but functional analysis using BBB scoring showed no significant difference between MSC treated injuries and the controls. Results suggested that MSC expression of laminin was responsible for aiding neurite alignment. As mentioned above, MSCs are also known to differentiate into myelinating cells. Three days after a focal demyelinated lesion was made in adult rats, undifferentiated MSCs delivered to the site helped to improve electrical conduction velocity across the lesion [255]. Spinal cord axons treated with MSCs contained myelination typical of the PNS, suggesting MSC differentiation and myelination within the wound. Direct injection and intravenous administration of MSCs in response to a demyelinated lesion in the rat spinal cord revealed that remyelination occurred in a dose-dependent fashion, as shown by histological examination [256]. This work also demonstrated the effectiveness of intravenous delivery of MSCs; the treatment is less invasive, although, almost 100 times more cells had to be used. MSCs also have aided healing in brain injury models. In an *in situ* environment, co-transplantation of MSCs with NSCs into hippocampal slice cultures led to the majority of NSCs differentiating into

oligodendrocytes [257]. In contrast, when the NSCs were transplanted in the tissue slices alone, the majority became astrocytes. This effect translates to *in vivo* studies as well. A recent study involving MRI tracking of tissue and cortical blood flow (CBF) showed that administration of MSCs in the brain significantly reduced ventricle expansion and helped maintain CBF in regions adjacent to injury [258]. Compared to saline injections, the MSC-treated rats demonstrated significant functional improvements as assessed by neurological severity score and the Morris water maze test.

From a TE standpoint, MSCs could provide an efficient and elegant way of administering therapeutic agents in multi-cued constructs for CNS injury and disease. They provide neuroprotection, can be manipulated to secrete many growth factors, and can differentiate into myelinating cells. Ongoing research suggests MSCs could be used as a source of growth factors as well as for inflammatory and immune agents. Thus, incorporation of MSCs may address several issues at once. Isolation of cells from a non-invasive, although painful, bone marrow biopsy procedure is also very attractive to limit extra surgical procedures outside of treatment. In depth investigation of the long term effects of MSC transplantation are underway, and based on the above findings, MSCs present a promising choice for cell therapy for CNS regeneration applications.

4.3.3 NEURAL CREST-LIKE STEM CELLS

Skin-derived progenitor cells (SKPs) are a population of cells within the skin that have neural-crest cell capabilities [259–263]. These multipotent SKPs were first described in 2001, and have since been shown to possess high proliferative ability and the capability to differentiate into neuronal and glial lineages [261, 262]. During development, the neural tube forms from the ectodermal germ layer and neural crest cells migrate from here to become a variety of cell types, including peripheral neurons, SCs, melanocytes, smooth muscle cells, connective tissue cells, cartilage, etc. [181–183]. SKPs can be cultured similar to adult NSCs in substrate free media with the mitogenic agents EGF and bFGF to produce neurospheres [261, 262]. SKPs have been shown to express nestin, as discussed earlier, a marker for NSCs [264–266].

The transplantation of SKP stem cells into SCIs has afforded functional recovery in walking tests; however, in the study SKPs were not compared to any other cellular treatments (Fig. 4.6) [267]. Neuronal differentiation of SKPs has had limited success so far, and very little success in serum free media [265]. Low concentrations of serum combined with neurotrophins, results in β-III tubulin positive cells co-expressing neurofilaments; however, after patch-clamping analysis, the cells were found to have weak electrophysiological profiles [265]. The development of a more robust differentiation protocol is required to yield significant populations of functioning neurons from SKPs. To date, better success has been achieved in directing SKP differentiation to a glial-lineage. Over 80% pure populations of SCs from SKPs are possible utilizing efficient protocols with the differentiation cue Neuregulin-1β [268, 269]. As mentioned previously, SCs have been shown to remyelinate CNS axons in the spinal cord even though they are typically found in the PNS. In fact, SKP derived SCs were shown to have excellent morphology and myelinating capabilities,

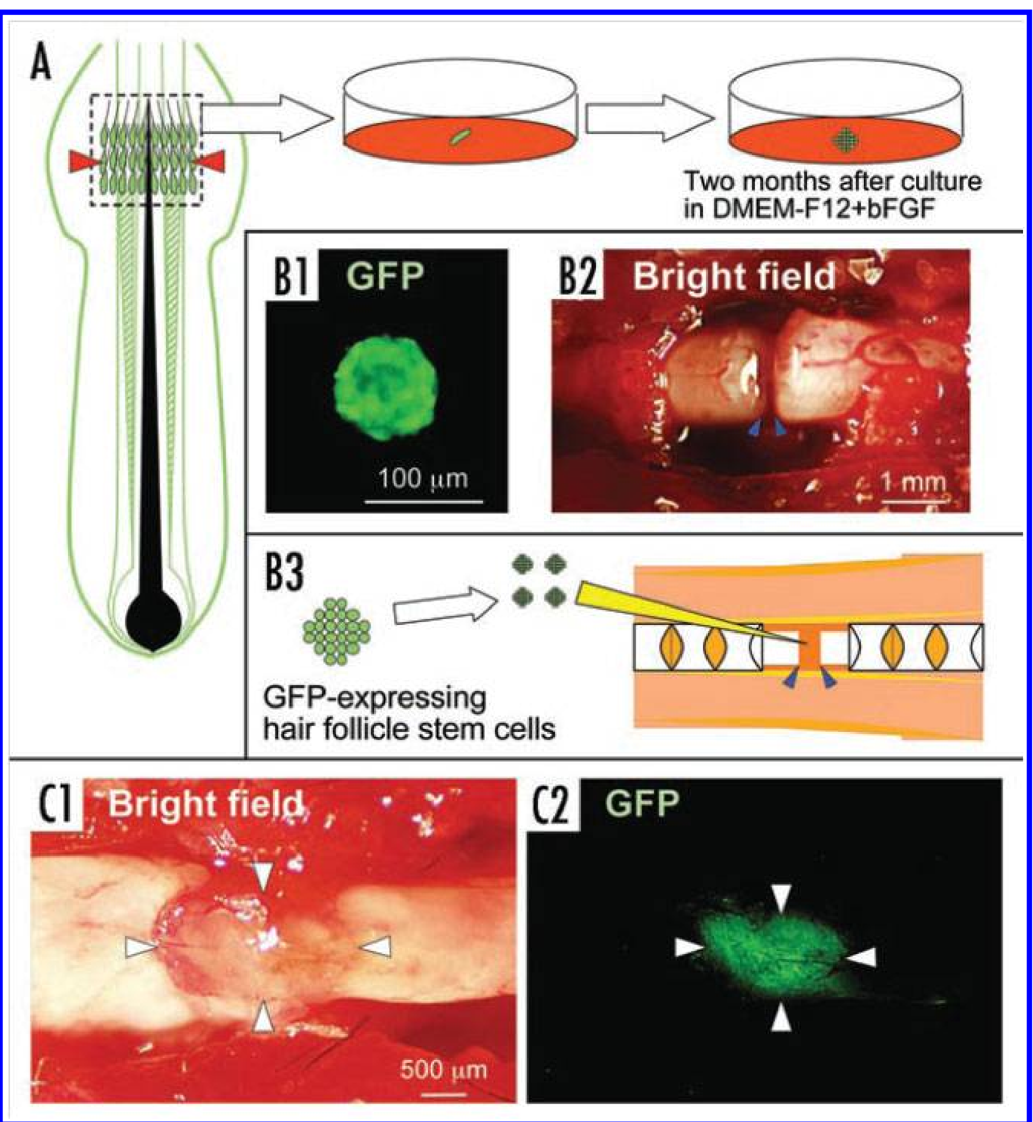


Figure 4.6: (A) Schematic representation of SKPs being isolated from the hair follicle bulge and cultured in neurosphere forming media. (B1) Example of GFP staining in a SKP neurosphere. (B2) Transection injury in the thoracic region of 6-8 week old mouse. (B3) Schematic representation of transplantation into the transected spinal cord. Two months post-transplantation the SKPs have rejoined the spinal cord, shown in brightfield (C1) and GFP (C2). Image reprinted from [267].

significantly higher than undifferentiated SKPs, when transplanted into a contusion injury in the rat spinal cord [268].

One flaw that is redundant throughout adult cell therapy techniques is the decreased plasticity and activity of aged cells. A comparison of SKPs derived from different regions of skin from patients 8 months to 85 years old revealed that proliferation and differentiation capabilities drastically declined in cells from the elderly [270]. SKPs still have many advantages and continue to be developed for additional CNS TE applications.

Many cell sources exist to choose from for treatment of CNS injuries and diseases. The specific needs of TBI, SCI, and neurological diseases call for tailored approaches when developing and optimizing neural regenerative methods. TE constructs that incorporate cells and/or encourage ingrowth have the capability to augment and accelerate the restoration of normal CNS function, improving quality of life.

CHAPTER 5

Stimulation and Guidance

Multi-cued CNS TE constructs are made possible through incorporation of surface and soluble prompts combined with scaffolding (Chapter 3) and cell components (Chapter 4). Cell migration in combination with process extension are essential early steps in nervous system development; thus, developmental signals are often the source for stimulation schemes aimed at controlling cell behavior and reinnervation after injury Immature cells are partitioned and guided to the appropriate targets by a complex mixture of chemical and physical cues. During nervous system development, neurons rely on the surrounding environment for guidance in order to innervate their targets and create a functional neural network system. Initially, neurites extend from the soma in all directions, and some gradually unite to become the primary axon while remaining neurites form dendrites [271, 272]. The growth cone, located at the tip of the axon (Fig. 2.2A), serves to decipher chemical and physical cues, both in space and time, determining the axon's trajectory [273, 274]. As it senses these signals, the growth cone pauses and enlarges as the cytoskeleton reorganizes, preparing itself for the next move [275]. As highlighted in Chapter 2, in the periphery of the growth cone are located lamellipodia that contain a web of actin. From this region, finger-like projections called filopodia protrude and sense the environment. Microtubules are responsible for transmitting this information from the growth cone to the soma and back to the tip. Axon branching can occur where the growth cone will split or interstitial branches will form from actin remnants as a result of cytoskeletal changes [275]. Guidance of the growth cone is vital to properly guide axons to their targets. Several strategies for encouraging and directing growth cones to specific targets will be introduced including physical, chemical, and electrical applications. Most can be used in conjunction with each other for complex control over neuronal behavior.

5.1 PHYSICAL CUES

Guidance and stimulation of cells can result from physical connection with the environment around them. Cells are sensitive to changes in substrate stiffness, mechanical stresses as well as topographical changes. Analyzing how physical cues modulate cellular adhesion, differentiation and guidance will provide a framework for developing optimal biomaterials and bioreactors to optimize translation of neural TE constructs *in vivo* (Fig. 1.1). Therefore, material stiffness, physical elongation and topographical guidance methods used for neural regenerative strategies will be outlined in addition to a brief review of topographical fabrication techniques.

5.1.1 PHYSICAL STIMULATION

Material Stiffness

As mentioned in Chapter 3, many biomaterials used in TE have tunable mechanical properties that can be adjusted by material blending and degree of crosslinking. This is important for biological applications because cells tend to favor their native tissue moduli. Additionally, during development and in certain disorders, changes as well as gradients in ECM stiffness are important for proper development and healing responses. Cell interpretation of mechanical stimuli and the resulting response, including the activation of downstream pathways, is known as mechanotransduction. Vascular tissue and cell migration mechanotransduction is better understood compared to responses in the nervous system; thus, there are still many questions concerning the pathways activated in response to mechanical forces in the nervous system [276]. Cells adhere to surfaces and exert contractile forces in order to migrate to different areas. In CNS and PNS TE, low substrate elastic modulus (E) is important for neuronal outgrowth [277, 278]. Several studies using DRG have shown that gels with lower stiffness result in more branching and longer neurites than stiffer gels [279–281]. In a study by Flanagan *et al.*, mouse spinal cord neurons and glia were grown on polyacrylamide gels with elastic moduli ranging from 50 to 550 Pa [277]. This group found that on the softer acrylamide, neuron branching was three times higher and glial survival decreased. Gradient stiffness gels can induce neuronal durotaxis, as neurites have grown significantly longer down a decreasing stiffness gradient [280]. Interestingly, a threshold effect has also been observed when stiffnesses were higher than a shear modulus (G) of 100 Pa (E $\cong$ 600 Pa); neurite outgrowth was still present at higher moduli but was not as pronounced as in the range of G = 10-100 Pa (E $\cong$ 60-600 Pa) [282]. Differentiation into a specific lineage or cell type can be specified by culture substrate compliance. Leipzig *et al.* found that extremely soft gels with stiffness, E around 800 Pa, will elicit neuronal differentiation from NSCs, while slightly stiffer gels with an elastic modulus around 7,000 Pa yields an oligodendrocyte phenotype [94].

Physical Elongation

The neuronal growth cone is indeed an important way that axons extend towards a target, but it is by no means the only method of elongation in the axon. During development, growth of the organism continues after synapses are formed. To maintain the neuronal network, the axon must continue to grow in response to the continued tension placed on them. This process has been exploited in a number of experimental settings to elongate axons to great distances [12, 283–285].

Axons can be elongated by micropipette towing of the terminus [286–288]. This pioneering method of axon stretching has been used to investigate mechanical properties of axons. Alternatively, the Smith lab has been very active in extreme axon elongation [12, 283–285, 289–291]. They have developed a device that can stretch millions of axons at a time and accelerate axonal stretch rates up to 1 cm/d without breakage or thinning. Embryonic rat DRG cells were seeded across two substrates to allow neural cell bodies to adhere to each; the substrates are then separated in a step-rest pattern causing the axons between the two populations of cell bodies to be elongated. Stretched constructs

have been shown to retain the same electrophysiological competence as control cultured neurons by displaying similar voltage channel density and patch clamping [289]. When stretch-generated constructs were implanted into rat sciatic nerve lesions, they showed promising integration into host tissue and axons within the tubular graft displayed signs of host myelination [292]. The promise of tension-induced axonal elongation for use in spinal injuries is enticing. Damaged tissue could be excised and bridged by pre-grown neurons stretched to the correct length, cutting down on healing time since large gaps would already be filled with existing axons. Iwata *et al.* teamed up with Smith to create stretch grown constructs and to test their ability to repair a rat hemisection SCI [293]. Embryonic DRG were elongated to 10 mm using tensile elongation, encapsulated in collagen and implanted 10 days after injury. Tissue bridging was observed after four weeks, but the functional benefits were not significant over collagen alone. Thus, proper interfacing and reconnection with host tissue is still a concern, especially in the spinal cord where there are complex organizations of axon tracts.

5.1.2 PHYSICAL GUIDANCE

Physical cues can be used to directionally stimulate cells for guidance strategies. Advancements in microfabrication techniques have allowed for new methods of surface patterning to be generated with enhanced resolution. A few common surface manufacturing approaches will be discussed briefly followed by specific patterns attempted both *in vitro* and *in vivo* for neural guidance (Fig. 5.1). Although the specific cellular effects and changes in signaling due to topography have yet to be elucidated, some theories on cytoskeletal and functional mechanisms will be mentioned. Further review of topographical and surface guidance can be found in the following papers [294–296].

Fabrication Methods

Many of the microfabrication techniques used to make topographical surfaces for cell behavioral control utilize lithographic methods. Very often during the fabrication of neural culture surfaces, soft lithography is used to transfer a substance or molecule from the raised pattern of a soft rubber mold to another substrate. The molecule can be a polymer, bioactive factor, or a chemically reactive compound for further conjugation. Microcontact printing is a form of soft lithography that typically uses a polydimethylsiloxane (PDMS) stamp to transfer a self-assembled monolayer onto a substrate. Another lithographical technique, which is often used to make the original template for soft lithography, is photolithography. This technique is similar to chemical etching in many ways. A photomask is used to selectively protect and expose specific regions of a surface to light. A photoresist material coating the desired surface is polymerized in the lit regions beneath the photomask. After light exposure, any unpolymerized photoresist from masked regions is removed. From here, a PDMS mold can be cured on the pattern, or further processing can be performed to use the original substrate for cell culture (including deposition of a coating or polymer).

The production of biomimetic substrates has become increasingly popular for topographical cell response studies. Research has demonstrated that during neural development neurons can often

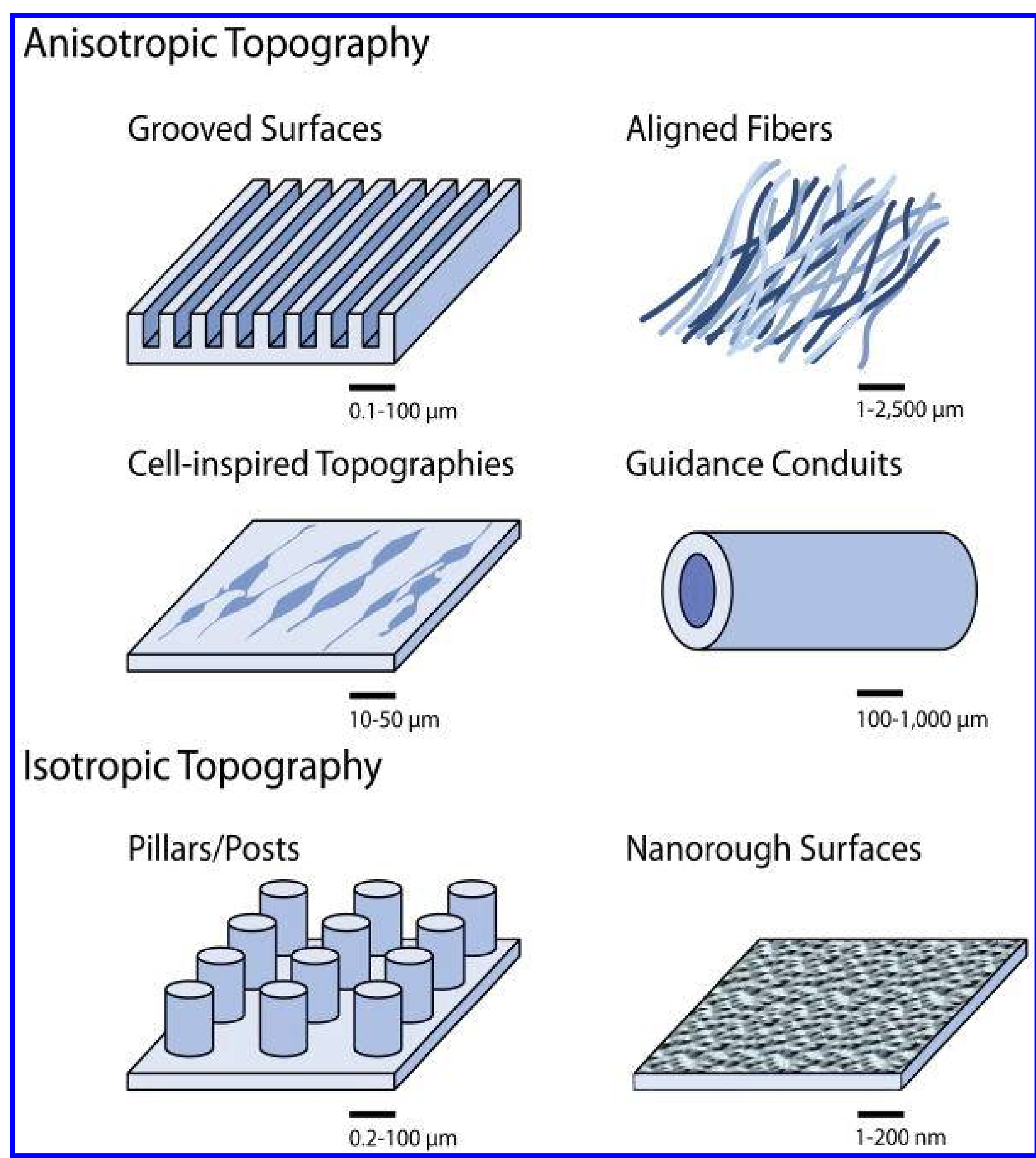


Figure 5.1: Illustration of topographies that can be used in cell guidance, on the nano or micro scale. Image reprinted from [294].

be guided by direct interaction with glia. In response, patterning of live cells is being developed in an attempt to create guidance strategies for neural TE. Recently, a cell printing method has been developed (similar to an inkjet printer for paper) to deposit bioadhesive factors or live cells themselves [297–302]. Alternately, lithography methods have been used to pattern or grow a cell substrate with particular alignment to subsequently grow neuronal cells on the surface [303–305].

Electrospinning, as covered in Chapter 3, is a method that allows imitation of portions of the ECM nanoenvironment. To briefly recap, a polymer is dissolved into a solvent prior to electrospinning, and pumped through a needle through an electric field. The fiber travels across the electric field to a substrate that can be stationary or in motion to allow for specific fiber orientation. Random and aligned fiber meshes can be created with electrospinning with fiber diameters ranging from nano to micro scale. Polymer concentration, flow rate, electric field strength, collection gap, and even collector speed can be adjusted to tune the fiber size [103, 104]. Cell encapsulated electrospinning has been developed using coaxial needles (cell core with polymer shell), but to date this has not been applied to neural cells [306, 307].

Cellular Response to Topographical Designs

One of the most popular anisotropic patterns for cellular alignment is grooved or ridged substrates. These can be made in a large range of feature sizes by adjusting the height, width, and spacing of the grooves in the nano and micro scale. Micron scale grooves have been used with different cell types to induce specific guidance, but with mixed results. With neurons, smaller grooves ($<$10-20 μm) tend to cause a higher occurrence of perpendicular alignment (across grooves), but also include parallel alignment of neurites to grooves [308–310]. Recently, a phenomenon of cell bridging across micron sized grooves was observed with DRG neurons, hippocampal neurons, SCs, and neuroblastoma cells (B104) [311]. Each cell type was seen to extend processes across adjacent plateaus (spaced 30-100 μm), especially with increasing cell number and plateau width (30-100 μm width). In addition to directional orientation, microgrooves have been shown to induce neuronal polarization (axon establishment). Embryonic hippocampal neurons on 1 to 2 μm wide grooves were shown to have significantly higher occurrence of polarization than on flat PDMS, especially when groove depth was increased from 400 μm to 800 μm [308]. From a TE standpoint, this work could be useful in inducing polarization of differentiating stem cells for CNS work. It is also important to note that the current trend is toward the development of degradable, as well as, surface patterned materials. Traditionally, most topographical studies have been performed on PDMS or other non-degradable materials due to their ease of fabrication with current techniques. In order to transition to implantable materials that are biodegradable (for reasons discussed in Chapter 3), some researchers have begun utilizing PLA for grooved substrates [310, 312].

Another common physical pattern, especially for neural TE, is channels. Large tubular NGCs (nerve guidance conduits) and smaller channels imitate white matter tracts found in the CNS and are therefore often used to lead spinal cord axons parallel to the spinal column during regeneration (Figs. 3.3 and 4.5). NGCs are the current preferred bioengineering strategy for regenerating the

PNS. Yu and Shoichet synthesized longitudinally oriented multichannel NGC that increased DRG adhesion and outgrowth especially in peptide modified channels [313]. However, NGC applications can be utilized in the CNS, as mentioned previously in the materials section, where agarose channels were implanted into a rat spinal cord (Fig. 3.3) [116]. Also, NGCs incorporating additional components such as neurotrophins or supporting cells were able to generate enhanced axon growth, suggesting that NGCs could be useful for SCIs [314, 315]. Recently, one study synthesized semiconductor nanotubes (1 to 10 μm in diameter) out of silicon and germanium and formed them into arrays that encourages single axon penetration and outgrowth into the nanostructure [316]. This model electrically and physically mimicked the native myelin and therefore could be utilized for future neural network applications.

The ECM-like morphology of fibers with small geometries has been used to stimulate and guide cells. At a cell and structural level, the ECM ranges in size from 50 to 500 nm [317, 318]. Nanofibrous scaffolds have been created using three main techniques: self-assembly, phase separation, and electrospinning (see review [319]). Aligned fibers have been used in neural cell culture to induce neurite sprouting and guidance [320, 321]. Fiber diameter is also an important factor to consider in optimizing neural outgrowth and guidance [322, 323]. Although not a true fiber scaffold, the nano surface topography of nerve basal lamina was recently replicated using a PDMS stamp on a polystyrene substrate [324]. The resulting topography resembled nanofibrous laminin structures, and supported the growth and alignment of DRG neurons. This study illustrates that cell reaction to fibrous scaffolds is very much tied to the surface structure and the feature size must be small enough for cells to sense and interact with it.

As a substitute to using actual cell topographies in co-culture, biomimetic surfaces aimed at reproducing these structural cues have been fabricated. Microcontact printing has been used to align SCs, which in turn were used for neural guidance [325, 326]. In a creative study by Kofron *et al.*, PDMS was used to make both aligned cell topography (taken from live cultures), and mimetic cell topography from a computer assisted design (CAD) program [303]. The CAD generated topographies were tested against live cultures of astrocytes, endothelial cells, and SCs for DRG neurite alignment and length and displayed comparable or improved results in the CAD topographies as compared to the live topographies. Kofron and Hoffman-Kim also optimized and quantitatively analyzed cellular monolayers of astrocytes, endothelial cells and SCs using Design of Experiment (DOS) and Response Surface Methodology (RSM) [327]. The statistical experimental design of this study afforded insights into the micropatterned feature sizes that affect cellular adhesion, alignment and confluence. These powerful tools, along with other modeling programs, take into account the multiple and complex biological variables and are meant to alleviate time and resources spent on bench top experiments.

5.2 CHEMICAL CUES

Several biochemical guidance signals have been identified including: chemoattractive factors such as neurotrophins and netrins, chemorepulsive agents like semaphorins and slits, or contact-mediated

molecules such as ephrins and those located in the ECM (Fig. 5.2). Deciphering biomolecular guidance activity during nervous system development as well as injury is key to generating new techniques and tactics for improving and restoring function to the nervous system after injury. For this reason, general immobilization techniques and specific PNS and CNS chemical guidance strategies will be preceded by an overview of promising molecules commonly used in these studies.

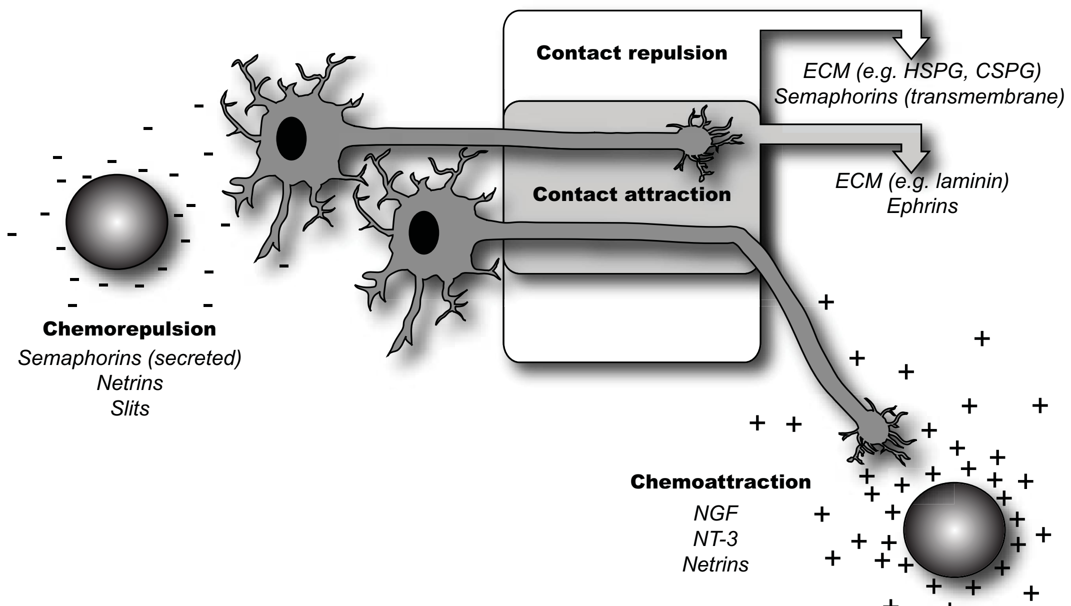


Figure 5.2: The extracellular matrix (ECM) alongside a combination of contact-mediated and soluble factors guide the axons of maturing neurons to their innervating target during development. While the attractive cues pull on axons, the repulsive cues push axons to the path towards their proper targets. Images reprinted from [6].

5.2.1 EXTRACELLULAR MATRIX

Proteins and polysaccharides primarily make up the complex structural framework of the ECM, which is secreted and organized into specific tissues by cellular architects. In turn, the assembled ECM plays a vital role in regulating cell behavior throughout development and adulthood, providing anchorage, mechanical buffering, segregating different tissues and aiding cell-cell communication.

The ECM is paramount to progenitor cell migration and differentiation, as well as to axonal genesis and extension.

Throughout the body, laminins serve as a key component of the ECM, providing binding sites for self polymerization, other ECM macromolecules and cells. They are key contact mediated cell adhesion promoters for neural cells. To date, fifteen laminins have been characterized and all share a similar trimer structure that is generated from five α, four β and three γ chains [328]. Within the nervous system, laminins make up basement membranes that are essential to interactions between neurons and glia. Research has demonstrated that laminin is required for neuronal migration in the developing cerebellum [329, 330]. Laminin contains several binding motifs that interact predominantly with cell-surface integrins (primarily $\alpha 1\beta 1$ and $\alpha 6\beta 1$) and secondarily with α-distroglycan [331, 332]. Interactions between integrin receptors to their ligands contained in ECM proteins are central for anchoring and directing the growth cone in order to initiate guidance [333, 334]. More detailed information regarding integrin interaction and signaling is reviewed elsewhere [335–337]. Jacques *et al.* [338] have showed that neural precursor migration on laminin can be significantly halted by inhibition of $\alpha 6\beta 1$ integrin subunits. Developmental studies have observed that knocking-out $\beta 1$ integrin expression blocks basement membrane formation and the expression of laminin protein [339]. Laminin provides essential attachment points enabling axons to extend and exert forces on the ECM [340–342]. Collagens and fibronectin are also important in the nervous system and are generally supportive to neuronal cell migration and neurite extension [343, 344]. Fibronectin, laminins, and collagens activate similar cell integrin receptors; thus, all three are important in cell substrate adhesion.

Proteoglycans are important ECM molecules that have been implicated in neuronal and axonal guidance. To review their introduction in Chapter 2 , these highly negatively charged molecules consist of a core protein with numerous GAG side chains and are grouped into two major classes: HSPGs (heparan sulfate) and CSPGs (chondroitin sulfate). Several studies have demonstrated that either exogenous addition of HSPG or enzymatic removal of HSPG leads to axonal guidance defects during development [345–347]. This has led to findings that demonstrate a functional association between HSPGs and several secreted and transmembrane proteins. As a result, the role of HSPGs in guidance appears to be tied to sequestering slit, netrin and semaphorin proteins (thoroughly reviewed in [348]). The influence of CSPG on guidance is not as well understood as that of HSPG; however, it is clear that CSPG also has a potent inhibitory effect on neuron guidance. Studies have demonstrated that CSPG makes up part of the glial scar that effectively halts CNS axonal regeneration, and leads to the generation of abnormal axonal growth cones [349]. Work has shown that, similar to HSPGs, CSPGs also functionally associate with semaphorins and inhibit axonal extension [350]. Li *et al.* utilized microfluidic techniques to create parallel and opposing gradients of laminin and CSPG [351]. They found that cultured DRG neurons show preference for higher laminin and lower CSPG in opposing gradients by exhibiting strong axon directionality.

Tenascins are ECM glycoproteins primarily involved in development as well as wound healing. The tenascin (TN) subtypes, TN-C and TN-R, are two of the four TN family members found in the

nervous system and are generally repulsive to axons. However, TN-C and TN-R contain multiple domains that can be either repulsive or attractive depending on presentation, and may provide more precise control of neurite extension, migration and guidance [352]. The inhibitory effects of TN-R on neurite outgrowth can be overcome by laminin and fibronectin used in combination, as shown in a RGC outgrowth assay [353].

5.2.2 NEURAL GUIDANCE MOLECULES

Besides the neurotrophins, the molecules and receptors described below have largely been studied for their guidance and signaling importance in development and injury. A better understanding of these molecules and their interactions will help in the future formulation of approaches for enhanced control of axonal guidance, branching, pruning and synapse formation for the purposes of regenerative medicine and TE. The following discussion is by no means exhaustive, especially in regard to developmental findings and signaling pathways. For more complete reviews please see [14, 354–356].

Neurotrophins

The neurotrophin family is composed of secreted chemoattractant proteins that are integral to nervous system development and maintenance [357]. NGF, NT-3, BDNF, and neurotrophin-4/5 (NT-4/5) are the four major neurotrophins and are similar in structure and sequence [358]. Tropomyosin receptor kinases (Trks) and p75 neurotrophin receptors (p75NTRs), located primarily on the terminal end of axons, are vital for the initiation of neurotrophin signaling [357, 359]. Three Trk receptors have been identified to date. NGF binds specifically to TrkA [360–362], whereas, BDNF and NT-4/5 prefer TrkB receptors [363, 364]. NT-3 complexes with TrkC with high affinity but can also interact with the other Trk receptors [365, 366]. P75NTR shows high affinity for NGF and serves as a low-affinity receptor for all the neurotrophins [367].

The first neurotrophin to be discovered was NGF, which was shown to encourage neurite extension from sensory ganglia *in vitro* [359]. Subsequent work found that NGF exists at significant concentrations in adult tissue showing neuronal specificity; however, NGF has been shown to also elicit responses from other tissues and cell types [359]. The existence of other neurotrophic factors was postulated early on since neurons depend on specific targeting for proper innervation during development. BDNF was the next neurotrophin identified from mammalian brain isolates and has been shown to significantly act on CNS and directly associated neuronal populations [368, 369]. BDNF has been shown to play an important role in the regulation of synapse structure and function, especially in glutamatergic synapses [370]. NT-3 shares significant homology to NGF and BDNF; however, NT-3's primary role occurs during the development of many tissues as indicated by the broad distribution of its messenger RNA [371–373]. Both BDNF and NT-3 have been shown to significantly influence axon path-finding, as well as aiding axonal regeneration in rats following SCI [374, 375]. Moreover, during development both neurotrophins direct the path-finding of maturing axons to their targets by a combination of long range attractive and repulsive cues [376].

NT-4/5, also known as NT-4 and NT-5, influences the survival and outgrowth of sensory and sympathetic neurons [363, 377, 378]. The importance of these neurotrophins in the nervous system have been reviewed in [379]. New experimental evidence has determined that bFGF and GDNF may also be important for nerve regeneration in the PNS and CNS. Both growth factors have been shown to influence neurons, SCs, and oligodendrocytes towards axonal growth and remyelination following injury [380, 381]. Finally, CNTF has been shown to act primarily on neurons as a survival factor following injury in the PNS [382]. To date, CNTF has not demonstrated any functional or regenerative benefits for nerve repair [383]; however, it may show synergistic effects in the presence of other neurotrophins [384].

Ephrins

Ephrin receptor tyrosine kinases (RTKs) are a large family of membrane bound proteins that are largely known for their role in the regulation of axon guidance as well as cell migration, vascularization, tissue segregation and synaptic plasticity [14]. Ephrin ligands are classified based on their extracellular domain sequence, where A-ephrin subclasses are glycosylphosphatidylinositol (GPI) membrane anchored and B-ephrin subclasses are transmembrane proteins [385]. Even though they show preferential binding to their specific ephrin protein, some Eph receptors can be activated by ephrin ligands from the opposite subclass [386, 387]. Ephrin/Eph complexes are remarkable for their ability to transduce signals bidirectionally into the receptor (Eph) expressing cells as well as the ligand (ephrin) expressing cells in what is termed 'forward' and 'reverse' signaling [14]. Both directionalities have been shown to be important in developmental axon guidance. Work has shown that ephrin/Eph signaling can be modulated to control targeting. It was shown that ephrinA2-EphA3 binding in axons initiates protease cleavage of ephrinA2, resulting in axon detachment and termination of contact repulsion [388].

Ephrins play an important role in development as well as regeneration. EprinA2 expression has been shown to decrease after injury to the superior colliculus in the neonatal rat brain leading to aberrant projections of ephrinA2 sensitive RGCs and cortical-tectal axons [389]. It is believed that these new projections most likely result from the removal of ephrin's repulsive cues [390, 391] as well as the possible attractive signaling provided by cortical-tectal axons [390]. Artificial upregulation of EphA4 does not aid in regeneration after SCI; however, it may stabilize synapse formation by reducing deviating projections [392]. Recently, it was shown that EphB/ephrinB contact between fibroblasts and SCs in a wounded peripheral nerve bed activates the Sox2 signaling cascade in SCs, stimulating peripheral nerve regeneration [393, 394]. For a thorough review on ephrin/Eph interaction during development and neurogenesis please see [395].

Semaphorins

The Semaphorins are a large family of proteins that contain approximately 500 amino acids each and are broken down into eight subclasses based on characteristics such as membrane anchorage (transmembrane or GPI-linked) or secretion [396] with the vertebrate semaphorins making up subclasses

3-7 [397]. Semaphorins and their receptors have recently been reviewed in more detail in [398]. Semaphorins are largely known for their growth cone collapsing properties [399]; however, recent work has revealed sub-types that attract extending axons [400]. Neuropilin and plexin have been identified as the two primary semaphorin receptors that transduce semaphorin signals [401–404]. The majority of semaphorins utilize class specific domains to bind directly to plexin; however, class 3 semaphorins cannot directly bind to plexin [403, 404]. Instead, Sema3A binds to first to neuropilin, and this complex can then bind and activate plexin receptors. Plexin activation in turn activates the Rac1 (Rho GTPase) signaling cascade, leading to growth cone collapse and retraction [403, 404]. Class 3 semaphorins and their receptors are important in the development of many nervous system regions including the hippocampus, cortex, olfactory system as well as cranial and spinal nerves [405].

Semaphorins emit long or short range repulsive cues by presenting as either soluble or membrane bound molecules, respectively, and it is important to understand how they interact with their environment during injury to exploit their potential role in regeneration. Following adult sciatic injury, it was shown that the expression of five out of the six class 3 semaphorins (Sema3A, Sema3B, Sema3C, Sema3E, and Sema3F) as well as their receptors neuropilin-1 (NRP1) and neuropilin-2 (NRP2), were upregulated [406]. Class 3 semaphorins can act either in an attractive or repulsive manner following nerve injury; thus, at this time their exact role is not fully understood. For example, class 3 semaphorins were found to contribute to the inhibitory nature of the glial scar after SCI [407]. Conversely, NRP2 upregulation was directly associated with peripheral nerve regeneration following sciatic nerve injury in mice [408]. It was postulated that NRP2 signaling could lead to SC recruitment, which has been shown to offer vital cell support during axonal regeneration in the PNS [406, 408, 409]. Class 3 semaphorins dual nature has also been examined in other studies [410, 411] Semaphorins are typically not utilized for neural regenerative applications when attractive guidance biomolecules are more readily available; however, their repulsive and attractive signals could prove beneficial in optimizing TE strategies.

Slit and Roundabout

Slits are a class of secreted proteins that bind to roundabout (robo) transmembrane receptors. The ligand-receptor couple controls many processes in neuronal development including neuron migration, axon pathfinding, and axonal and dendritic branching [14]. Slit-1, slit-2 and slit-3 are expressed during development in the mammalian brain, spinal cord and thyroid, respectively [412]. Slit proteins act as a repellent cue to neurons during development [413, 414] and bind to four known receptors: robo-1-4 [415–417]. Interestingly, slit-2 is unique in that it can be proteolytically cleaved into a smaller, diffusible C-terminal fragment and a larger N-terminal fragment [418]. Each part of slit-2 has contrasting effects on different populations of cells; both the full slit-2 protein and the N-terminal fragment repel olfactory bulb neurons. Only N-terminal slit-2 fragments are able to induce DRG branching and extension [419]. The purpose of the C-terminal slit-2 fragment is not fully understood and cleavage of this end terminus may activate the N-terminal fragment for certain signaling pathways [419]. The robo-slit interaction plays a major role in commissural axon

guidance at the midline during development [420, 421], assists in spatiotemporal patterning and neurite projections during brain development [422–424], as well as in properly directing axonal extensions in the optic system [425, 426].

Slit activity is vital to proper development, homeostasis and nerve regeneration. Slits and their receptors have been tied to CNS injuries. Slit-1 expression is upregulated in the cerebellum following injury, slit-3 upregulation occurs after SCI and microglial cells express both slit-1 and slit-3 in response to injuries in both regions [427]. Astrocytes have been shown to express slit-2 after brain injury in the hippocampus [428]. These findings suggest that slits and their repulsive nature contribute to regenerative failure after CNS injury. On the other hand, another study suggests that slit-1 supports axon elongation, whereas slit-2 is responsible for the remodeling of dendritic branches around the soma [429]. The HSPG, glypican-1 works in combination with slit-1 and robo-2 to directionally guide DRGs via repulsive cues as well as to prevent random, uncontrollable axonal elongation following both sciatic nerve injury and SCI [430]. This finding is surprising since these proteins were previously thought to only be present at development and not in both types of injuries. The existence of these molecules suggests that pruning and maintenance of the nervous system occurs after injury to the PNS and spinal cord.

Netrins

Netrins play a central role during spinal cord development, providing cues to guide commissural axons across the two halves of the spinal cord [431, 432]. They are a small family of secreted proteins with protein sequence homology to laminins. Netrins have complex interactions that can be attractive and repulsive depending on cell type and age of the host. Netrins interact with the transmembrane receptors neogenin and the receptor deleted in colorectal cancer (DCC); ligand receptor interactions initiate signaling pathways in commissural axons allowing for CNS interconnections [433–435]. Src family kinases and focal adhesion kinases act downstream of DCC and regulate growth cone adhesion and actin dynamics [436]. Since the subdomains of neogenin have similar homology to DCC, it is believed that netrin-neogenin binding activates similar pathways, leading to cell adhesion and axon guidance [437]. Uncoordinate-5 (UNC-5) is a family of netrin receptors that mediate axon repulsion by forming a receptor complex with DCC [438–440]. Netrin attraction can be silenced by slit-robo interactions via the formation of heteromultimeric receptor complexes [441]. Silencing of axons that cross the midline during development is extremely important, since these axons must quickly overcome their attraction or forever remain at the midline [14]. Following CNS injury, several netrin homologues play an important role in the regeneration of brain neurons and ventral nerve cord cells [442]. After rat RGC axotomy, DCC receptor expression is down-regulated, suggesting that if netrin-1 expression is targeted for nerve regeneration, its co-receptors must be targeted as well [443]. Therefore, it is important to consider the interrelationship netrins have with their receptors when modeling reinnervation techniques because they can be chemoattractive or chemorepulsive depending on what receptor is active.

5.2.3 TETHERING OR COVALENT IMMOBILIZATION OF NEURAL GUIDANCE FACTORS

Protein Immobilization Strategies

Protein patterning has gained significant attention with advances in techniques such as lithography, microfluidics, microcontact printing, and biosensors. Protein patterning has enabled high-throughput measurements of biological responses using micro and nano scale protein assays. There are three primary methods used to immobilize protein: physisorption, chemisorption and bioaffinity (Fig. 5.3). Physisorption is the physical adsorption of proteins to the surface via intermolecular

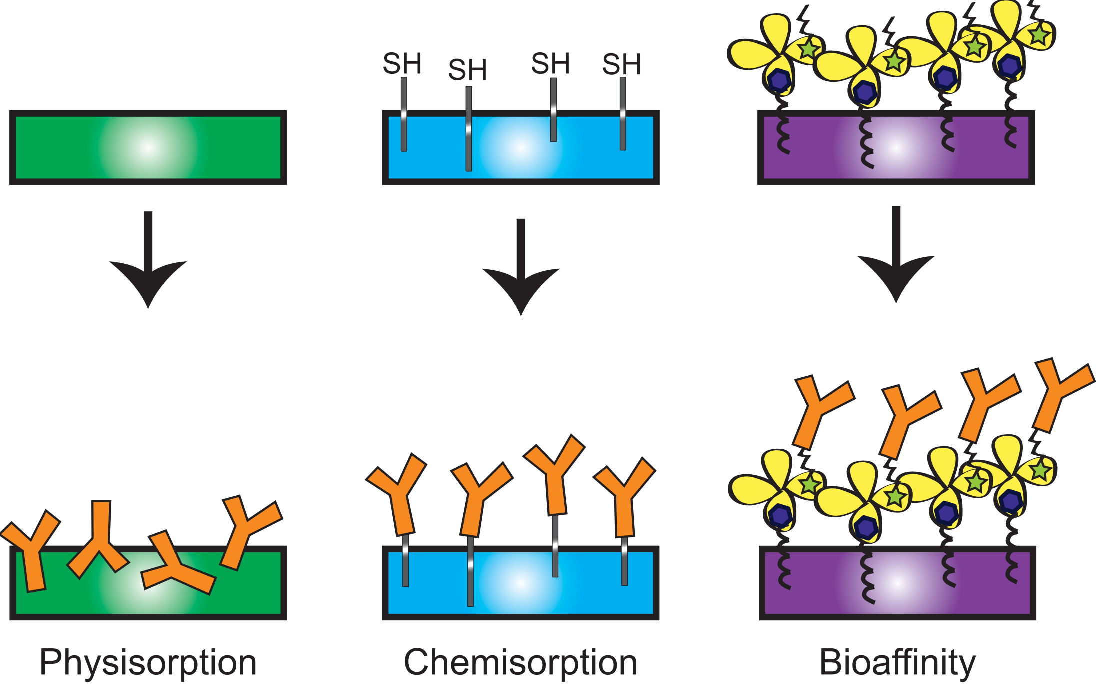


Figure 5.3: Common methods of protein surface attachment. Physisorption is the physical nonspecific adsorption of proteins to the surface. Chemisorption utilizes covalent bonding between protein functional groups and a modified substrate. This figure depicts thiol-maleimide interaction. Bioaffinity depicts the strong noncovalent affinity steptavidin (tetrameric protein) has with biotin (small molecule on the protein). This type of immobilization shows specific linkage of a protein to modified surface.

forces such as hydrophobic and polar interactions. This type of immobilization is nonspecific, such that proteins are adsorbed heterogeneously across the surface and are oriented to minimize repulsive forces with other molecules and the substrate. Adsorption is cheaper and easier than chemical means; however, the binding affinities are low and proteins can desorb easily, quickly leaving

the original surface exposed. Chemisorption, on the other hand, is a type of immobilization that results in covalent bonding between exposed side-chain functional groups and modified surfaces. *N*-Hydroxysuccinimide (NHS)-amine, carboxyl, thiol-maleimide, epoxy, and photoactive chemistry are all common strategies used for covalent attachment. Often with these techniques, nonspecific chemisorption takes place as exterior residues containing the reactive groups on the protein attach to the surface. Nonspecific attachment could block active regions of the protein of interest or inhibit important conformational changes. Therefore, site-specific protein immobilization is desired to reduce unwanted attachments. This requires the insertion of functional moieties into proteins specifically for immobilization to surfaces with the corresponding coupling molecule. The biotin-avidin system is a well known biochemical immobilization strategy largely because it exhibits one of the strongest noncovalent bonds known ($K_d=10^{15}$ M^{-1}) [444]. Streptavidin is a tetrameric protein that has a comparable affinity to biotin because it is structurally similar to avidin. Alternatively, histidine/nickel interaction, that allow tagged histidine regions on proteins to bind to nickel chelated complexes such as Ni-nitiloacetic acid (NTA), and DNA-directed systems, such as DNA microarray technology and DNA-protein bioconjugation, are two other types of bioaffinity immobilization approaches commonly used for protein immobilization [445–447]. These methods of immobilization are attractive due to the specificity and homogeneity of the oriented molecules. For more information on immobilization strategies, please see [448, 449].

Utilization of Immobilized Neural Biomolecules

Recent work has begun to reveal how tethering or immobilization of growth factors and guidance molecules modifies cell and stem cell function [450–459]. Immobilization of bioactive factors to biomaterial substrates allows for not only migrational stimulation, but spatial control of differentiation with sustained dosing, which is not possible with soluble factors [451, 455]. At the same time, cytokine immobilization allows for the study of the dynamics and the necessity of cellular internalization for activation of signal transduction [458]. Research has recently shown that IFN-γ as well as PDGF-AA can be immobilized to hydrogel scaffolds to spatially guide the differentiation of NSCs [450, 456, 459]. Immobilization offers a number of advantages over soluble dosing. Immobilized molecules do not diffuse away from a scaffold like they would in soluble form and require smaller amounts of bioactive molecules over the course of treatment maintaining local concentrations, potentially maximizing cell interactions while reducing cost. Immobilization also allows for the creation of permanent gradients and this strategy has been utilized for small adhesion peptides [460–463] and more recently for growth factors [464, 465]. Consequently, it has become a preferred strategy employed to modify cell guidance and migration [460–463]. In achieving these effects, the presentation of proteins in gradients and/or 3D patterns can be highly beneficial since it more closely mimics how cells experience many proteins *in vivo*. Control of spatial distributions of proteins is key to achieving greater control of cell and tissue functions.

Attachment peptides derived from ECM proteins have been immobilized to culture substrates for neural guidance to mimic ligand presentation during development and injury. A RGDC peptide

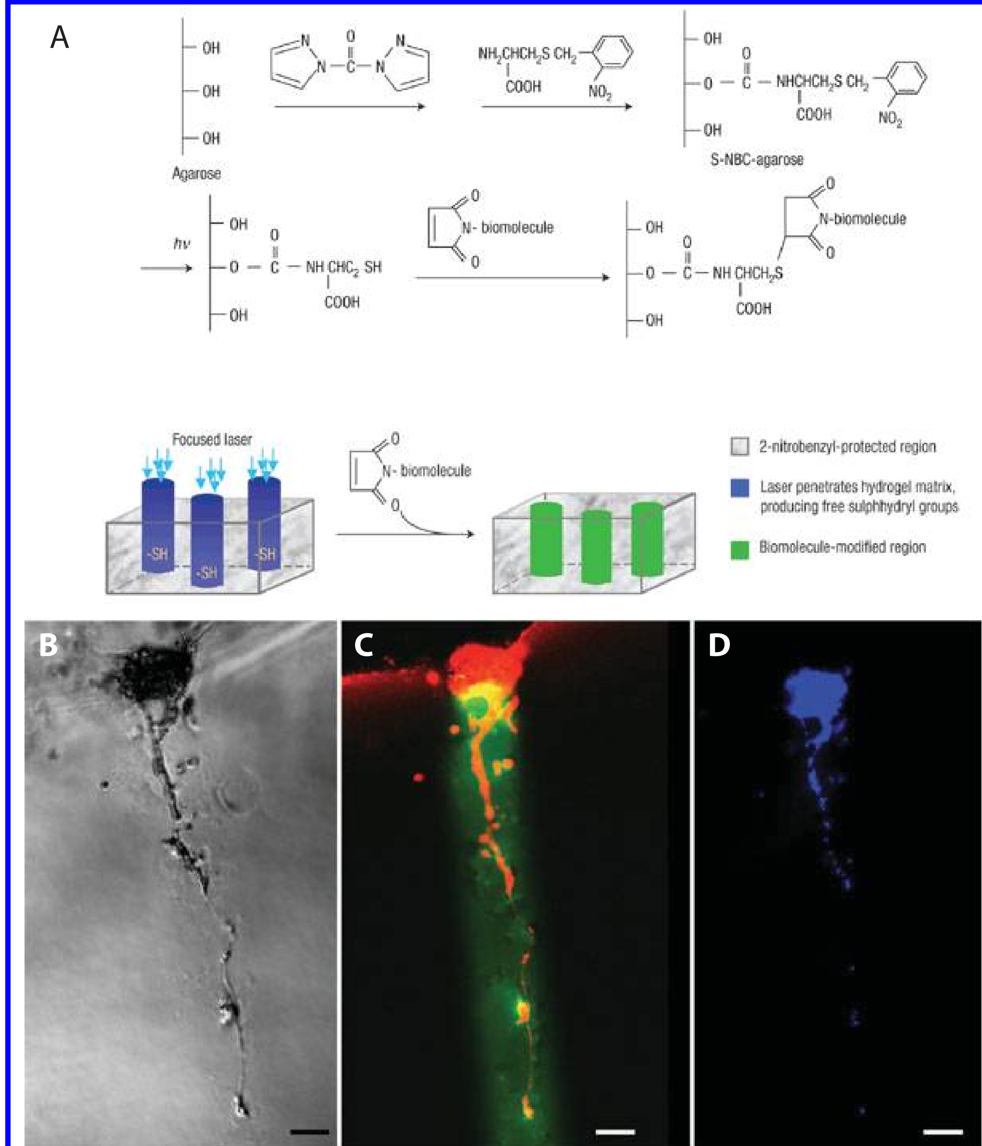


Figure 5.4: Chemically modified agarose hydrogels containing free sulfhydryl groups after exposure to a laser reacted with maleimido-terminated biomolecules (A). DRG cells extended into GRGDS-modified channels and not into the surrounding agarose matrix. Typical cell migration and axon extension is shown with: (B) optical microscopy; (C) confocal microscopy with cells stained red and the channel green; (D) fluorescent microscopy indicating cell migration into the GRGDS-modified channel via nuclear DAPI stain (blue). Images reprinted from [467].

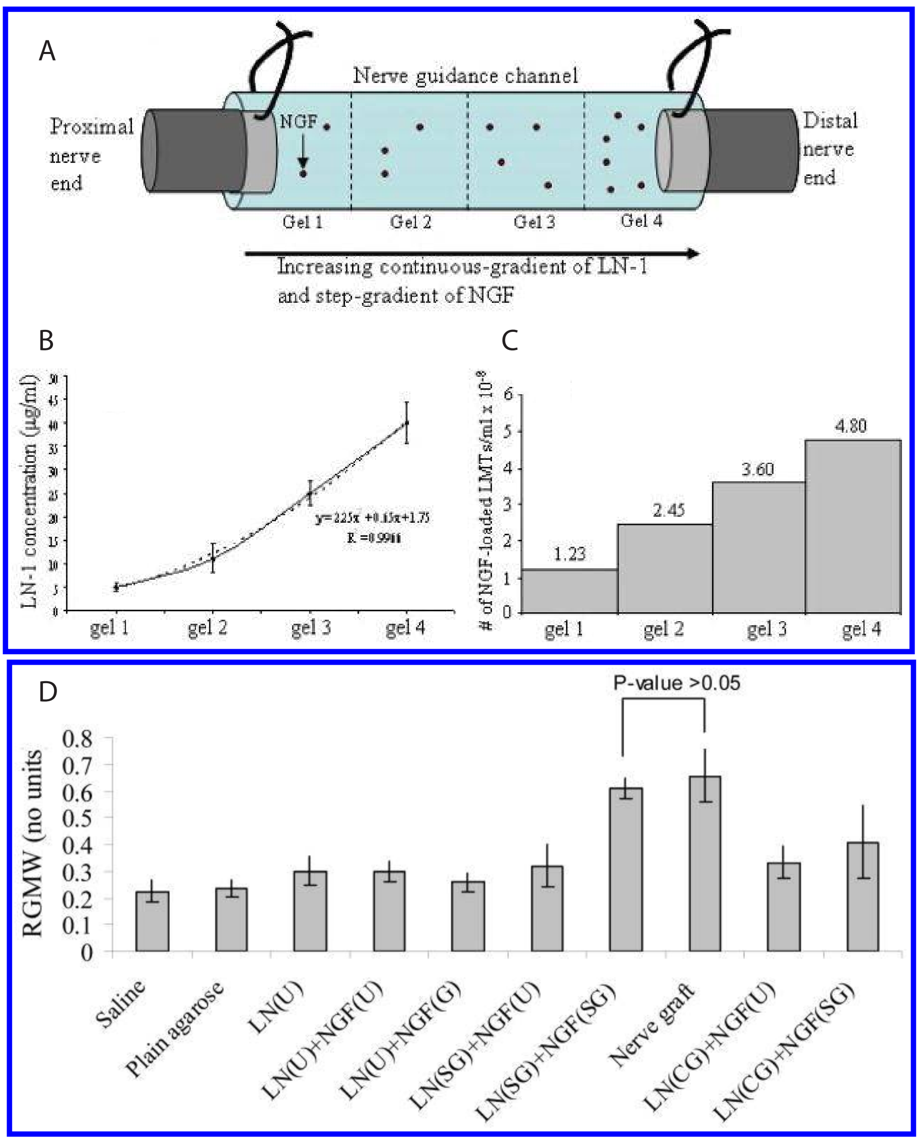


Figure 5.5: A nerve guidance channel scaffold for peripheral nerve repair made up of agarose with laminin (LN-1) and NGF (A). Within this scaffold the LN-1 gradient is continuous, as determined by ELISA (B), while NGF-loaded lipid microtubules (LMTs) are distributed in a step gradient (C). Implants with step gradients resulted in similar relative gastrocnemius muscle weight (RGMW) as nerve grafts and both treatments were significantly better than single and combinatorial treatments of LN-1 and NGF in uniform (U), step gradient (SG) and continuous gradient (CG) agarose scaffolds (D). Images reprinted from [100].

(found in laminin, collagen and fibronectin) and axonin-1, a cell adhesion protein, were patterned to a culture surface using photolithography techniques; this allowed *in vitro* neurite extension and network formation [466]. Similar studies by Luo *et al.* demonstrated that 2D photo-immobilized GRGDS enhanced local neurite density and elongation, as well as DRG extension, into immobilized GRGDS in 3D (Fig. 5.4) [467]. Photolithography paired with the development of multiphoton scanning microscopes [468–470], allows for even tighter control of immobilized factor concentration and spatial location, potentially providing axon guidance with submicron precision. Recent work from Yu *et al.* [464, 465] has utilized two-photon confocal patterning to tether NGF to chitosan surfaces. They demonstrated that immobilized NGF does encourage DRG axon extension *in vitro* and that axons can be guided by gradients of immobilized NGF.

Recombinant fusion proteins of growth factors and binding domains have been created previously to control immobilization to specific ECMs, biomaterials and cells. Fibronectin cell-binding domains have been incorporated in fusion proteins along with bFGF and EGF to stimulate vascularization and wound healing [471]. Collagen binding domains have also been incorporated into fusion proteins of bFGF [472], EGF [473, 474], PDGF [475], hepatocyte growth factor (HGF) [476] and NGF [108, 477] for targeted wound regeneration. Recently Sun *et al*. compared collagen binding NGF (CBD-NGF) to native NGF (NAT-NGF) in a crushed rat sciatic nerve model [108]. They found that CBD-NGF injection treatment enhanced remyelination of axons after crush injury. CBD-NGF resulted in better myelinated axons when compared to NAT-NGF and PBS sham treatments at 8 and 12 wks. Dodla and Ballamkonda [100] created nerve guidance scaffolds containing gradients of immobilized laminin and NGF in agarose hydrogels (Fig. 5.5) and showed that axons were able to bridge a 20 mm nerve gap after sciatic nerve injury using scaffolds with a continuous gradient of laminin and a step gradient of NGF in the same direction.

5.3 ELECTRICAL STIMULATION

Natural electrical activity of the nervous system has led to investigation on the effects of electrical stimulation, especially on neurons and subcellular behavior. The application of electric stimulants to neural cells is not a new idea by any means, and has been studied for over 30 years [478]. Despite its long history, mixed effects of applied electric fields still leave questions about their control over cell behavior. Neural stimulation *in vitro* could be used to promote desired cell behaviors such as alignment and outgrowth of processes. *In vivo*, electrical activity could be promoted by incorporating a conductive polymer mentioned in Chapter 3, such as PPy or poly(aniline).

Electric fields have been used to stimulate neurite outgrowth and have been found to align cells, giving them polarity. The presence of a direct current (DC) electric field causes growing axons *in vitro* to align, extend, and accelerate toward the cathode and to increase branching [479–483]. DRG in particular have been used extensively in studies of applied electric fields to study neurite sprouting, length, and alignment [484–486]. Neurites from DRG were observed to align in the direction of the field and extend significantly farther than control treatments, but these results were governed by surface properties [486]. Laminin coated surfaces induced the significant neurite lengthening,

while collagen surfaces did not. In addition, substrate surfaces have shown variable results in neurite alignment and directionality in applied electric fields [296, 487].

Electrical stimulation has been investigated as a differentiation cue in mammalian neural stem cells. Ariza *et al.* recently found that DC electric fields of around 500 mV/mm applied across NSCs from the DG (dentate gyrus) induced significantly higher neuronal differentiation than alternating current (AC) electric fields or no electric field application [488]. In the experiments, DC voltage was applied *in vitro* for the first three days and the last day of culture but AC was applied the full 6 days. DC voltage did appear to cause a lower cell density and some cell death. Results suggest that DC electric fields might be selective for the neuronal phenotype and cause alignment of neurons from NSCs of the DG [488]. NSC migration in the presence of electric fields is of interest in inducing or guiding endogenous stem cells to injury sites. Adult rat NSCs did have directional migration toward the cathode over cells on which no electric field was applied [489].

Animal studies have shown that brief electric field stimulation (1 h/d) works as well or better than long stimulation (4 h/d to continuous) [490, 491]. Electric field stimulation has been shown to greatly enhance nerve regeneration in animal models, resulting in significant decreases in regeneration time. One hour a day of electrical stimulation has reduced healing time from 9 wk to only 3 wk in rat models [490].

Electric fields have shown considerable promise for enhanced neuronal development and regeneration; however, further work is needed to understand the optimal way to utilize electric fields for TE approaches, especially for methodologies involving stem cells.

CHAPTER 6

Concluding Remarks

Tissue engineering is poised to generate new techniques to replace or restore tissue damaged from injury or disease. The concept itself is not new, as was contemplated by several visionary scientists speculating on where basic biological knowledge gained decades ago would take future researchers [492]. Though significant progress has been made toward neural TE, especially in the PNS, there is still a long road ahead to formulate clinically relevant CNS solutions that achieve significant long-term functional benefits. Major issues in the area remain unresolved including global injury and disease models, functional assessment, complete eradication of unwanted immune response, an absolute cell source, and concrete mechanisms and techniques for physical, chemical, and electrical cues.

The continuing advancement of new technology and techniques coupled with the uncovering of basic knowledge brightens the CNS TE outlook and the creation of restorative therapies for devastating injuries such as SCI or TBI. Design of biomaterials from the ground up, including proper cellular and tissue functionalities, will allow for the creation of ideal brain and spinal cord regenerative constructs. In any TE strategy it is important to incorporate cues (chemical, physical, electrical, etc.) derived from native cellular microenvironment (Fig. 1.1) to instruct cells and tissues to predictably regenerate. The incorporation of multiple cues experienced during development and regeneration into CNS treatments has propelled neural regeneration and TE into new exciting frontiers. Neural TE researchers are progressing toward the hallmark milestones of enabling the restoration of speech after a TBI and empowering a paralyzed SCI patient to walk again.

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Authors' Biographies

ASHLEY E. WILKINSON

Ashley E. Wilkinson is currently pursuing her PhD in Chemical and Biomolecular Engineering at the University of Akron (Akron, Ohio). She received her Bachelor of Science in Biomedical Engineering in 2010 from the University of Akron with a specialization in drug delivery and tissue engineering. During her undergraduate studies, Ashley participated in cooperative education at DePuy Orthopaedics as a product development engineer. Her current research interests include neuroregenerative strategies in the brain and spinal cord, specifically stem cell differentiation via specific chemical and mechanical stimulation.

ALEESHA M. MCCORMICK

Aleesha M. McCormick obtained her Bachelor of Science at Kent State University (Kent, Ohio) in Integrated Science Education and taught high school chemistry and physics for two years. She is currently pursuing a PhD at the University of Akron (Akron, OH) in Chemical and Biomolecular Engineering. Her research area focuses on recombinant protein analysis and production for utilization in axon guidance and neural regenerative applications as well as examining potential cell sources for neuronal differentiation.

NIC D. LEIPZIG

Nic D. Leipzig is the Iredell Chair Assistant Professor in Chemical and Biomolecular Engineering at the University of Akron (Akron, OH). He received a Bachelor of Engineering in Chemical Engineering from McGill University (Montreal, Quebec) in 2001 and a PhD in Bioengineering from Rice University (Houston, Texas) in 2006. During his PhD he studied the biomechanics of single chondrocytes, or cartilage cells, explored how growth factors change both the cytoskeleton and the material properties of chondrocytes, developed new methods for measuring gene expression in single cells and utilized these techniques to be the first to successfully demonstrate gene expression changes by mechanotransduction in single chondrocytes. He was a postdoctoral fellow at the University of Toronto (Toronto, Ontario) in the department of Chemical Engineering and Applied Chemistry from 2006 to 2009, where he developed hydrogel systems to enable precise control of the cell microenvironment, or niche, in order to guide the differentiation of adult stem cells. He has also revealed that substrate stiffness can influence neural stem cell proliferation and differentiation and demonstrated the advantages of covalently attaching growth factors for precisely guiding stem cell

differentiation. Dr. Leipzig's current research is pioneering approaches for tissue engineering of the central nervous system utilizing engineered biomaterials, incorporating niche level stimuli and new stem cell sources.